

BBRC

Bioscience Biotechnology
Research Communications

Volume-14 Number (4) Oct-Nov-Dec 2021

Print ISSN: 0974-6455

Online ISSN: 2321-4007

CODEN: BBRCBA

www.bbrc.in

University Grants Commission (UGC)

New Delhi, India Approved Journal

An International Peer
Reviewed Open Access Journal

Published By:

Society for Science & Nature (SSN) Bhopal India

website: www.ssnb.org.in

Online Content Available: Every 3 Months at

www.bbrc.in



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Editors Communique

Stay Protected, Stay Safe in the Cradle of Nature

On behalf of Bioscience Biotechnology Research Communications we falter at words to express our deep sense of solitude and grief on the catastrophic events of the world wide pandemic, spanning over a year now, with no signs of relief. We pray to Almighty to give us the strength to bear this universal calamity and come up with long lasting fortitude to eradicate it soon.

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On behalf of Biosc. Biotech. Res.Comm. its my privilege to thank its reverend readers, contributors, reviewers and well-wishers who have helped it to achieve the distinction of entering the 14th year of successful publication, carving a niche of its own.

Quality publication is one of the ways to keep science alive, and good journals have a leading role to play in shaping science for humanity! As teachers, we have great responsibilities, we have to advocate our students to accomplish and show them the path to test their mettle in hard times to excel, especially in the post COVID 19 era. Science and its advocates will rise to the occasion and will soon provide succor to the already grief stricken humanity.

We have to fuel our science students with a never say die attitude to let humanity survive!

Amicably yours

Sharique A. Ali, PhD

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Living the Challenges of a Pandemic Through the Succour and Strength of Science

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At the outset, on behalf of the editorial team, we falter at words to express our deep sense of solitude and grief on the catastrophic events of the world-wide pandemic. Millions of precious lives have been lost, maiming a larger grim number, with no signs of relief since the last two years. We pray to the Almighty, to give us the strength to bear this universal calamity and come up with long lasting fortitude to eradicate it soon.

As all pandemics in the past have shown signs of wearing off in due course of time, we expect and pray that the COVID-19 will also wane, if not disappear totally. Since the virus is asymptomatic, and has the capability to cross barriers of animal and human species, it will not be reasonable to label its death so soon. We will have to live with it, at least for a few years, till our immune systems acclimatize the emerging viral mutations, as nothing but bouts of colds and flus. Science and Technology has the power to do it, and God willing, soon this great news will greet us.

The preceding year has been much brighter than its predecessor, we have seen a surge of scientific response beyond comparison by any means. Since the beginning of the pandemic till 2021, we have seen unprecedented scientific innovations, researches and developments. As many as 26 effective vaccines are in use, and several hundred in the pipeline, these outstanding fast track developments have made it a year of vaccines, chasing the equally populous mutations, beta, the delta and now the omicron, trying to have a date with almost every one of us. The Lancet in its 18th December 2021 editorial, for all its peaks and troughs, has rightly heralded it as a year of extraordinary COVID-19 science, (The Lancet, 18th Dec 2021).

Biosc.Biotech. Res. Comm. is in the 15th year of successful publication, and it has been indeed a privilege for us to be associated with it. The journal amidst the waves of pandemic, since its issue of Jan-Feb-March (2020), has also published several interesting articles on various aspects of COVID-2019 since the dawn of the viral onslaught.

A large number of well written contributions were received from distinguished authors, few to be mentioned here: Miraj (2020) described the Coronavirus Disease-2019 and its Public Health Challenges and Preparedness, an interesting write up, which has figured in WHO's data-base on COVID 19 Literature. Miraj (2020) stressed on how to be prepared for such catastrophic events at all times and especially for huge country populations, where it is necessary for the respective governments to allocate special funds for such crisis management. This must be without the maladies and stigma of corruption, dishonesty, procrastination and irresponsibility, which are new obstacles in saving humanity. She focused on the fact that if we have to protect ourselves, it will be the out-of-pocket expenses, which as usual will give succor from such health-related emergencies, including the recent pandemic. Global collaboration devoid of politics, seems to be another plausible solution to repair the world's biggest damage. There is no dearth of funds for the humanity, but effective management is, (Miraj 2020).

Yunus (2020) showed a significant growing attention on the topics of novel coronavirus outbreak (COVID-19) by way of large number of international publications on the pandemic. Her analysis showed that, authors from USA and China are more active than other countries, followed by England, Italy, Canada and India. The number of publications on novel coronavirus (COVID-19) increased rapidly in April-May, 2020 as the threatening disease spread throughout the world. The analysis also revealed information on the best keywords used, co-authorships, co-occurrence, citations and the journals, among

Article Information: Correspondence at editor@bbrc.in
Published: 31st December 2021 Pp- 1391-1392

This is an open access article under Creative Commons License,
Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.1>

others. The study has included number of publications on novel coronavirus from around the world along with keyword analyses, co-authorship analyses, co-occurrence, and citation analyses. The analysis has also recognized the best writers and journals in the field, and it nicely attempted to determine the future proposals for monitoring such vital areas of research.

John (2020) highlighted how the virus staggered the public health aspect of human life. With its far-reaching repercussions having overwhelmed the health care systems due to increased demand and grave under-preparedness, how the pandemic disrupted the global economy causing millions of enterprises to face an existential threat. The article summarized a careful review of literature focusing on how the pandemic has impacted public health, hindered health care delivery and imposed new behavioral and business mandates upon us in the future. As the tragic scenario unfolds, it has become imperative for agencies and stakeholders, worldwide to come forward and take initiatives to restore the health systems and formulate well-thought-out and inclusive strategies.

Alshrari (2020) reported about the preparedness in containment of Coronavirus Disease-19 in the African Continent, observing that most infection spread is attributed to improper hygiene / protective measures, for example, hand washing and social distancing. Accordingly, the large-scale advertisement and conduction of the COVID-19 educational programs are highly recommended. The African countries lack appropriate numbers of biosafety levels, 3 and 4 research facilities, trained personnel / emergency units, and funding resources to combat COVID-19 and, similar pandemics. It is advisable to build up more biosafety research facilities, trained emergency response units, isolation units, and substantial funding agencies in every African country with clear rules to combat outbreaks like the COVID-19. The African countries may also ask support from other countries with successful experience against COVID-19. The implementation of the suggested strategies will be helpful to African countries against COVID-19.

Similarly, Das (2020) described how human existence in this pandemic situation reminds mankind of early preparedness and speculation of the invisible threat beforehand which needs an attention over improving daily behavioral practices, health care facilities and insurances, economic alternatives and boosting of science and technology in the field of research and medicine. Various insights such as online education, working from home, adequate importance for research and scientific temperament, behavioral changes in terms of social distancing, frequently washing hand, change and greeting habits, rejuvenation of ecology and environment becomes a positive factor for a new normal in today's tragic times.

Another article of Swaminathan (2021) mentioned the environmental effects particularly, on air and water by assessing the conditions before and after the outbreak of the pandemic COVID-19. The study showed that the purity of air and water improved during the pandemic period when compared with

the period before the outbreak of COVID-19 virus. As, waste generated from self-quarantine houses, hospitals and self-hygiene practices followed by people posed an enormous effect on waste management sector, its effective disposal along with municipal solid waste has created a warning to people for effective handling of the waste and the environment. Based on the environmental analysis performed on air, water and waste management, solid guidelines have been provided in treating the waste. This paper recommended the need for improving the waste-treatment using better methodologies in developing countries and the significance of related policy framework to face such pandemics in future. Indeed it is the sanitary condition which plays a big role in any disease containment.

On an endnote, we in these two years had a hard time handling a large number of good manuscripts, regrettably many of them had to be returned, owing to space and priorities. A 'big thank you' to all of our reviewers who with their magnanimous co-operation and help, carried out their job so efficiently, we are immensely grateful to them. Last, but not the least, our valuable readers, contributors and of course the indexing agencies, need a word of appreciation for their consideration of the journal, to enable it carve a niche of its own, despite the odds. We got listed in the Journal Citation Reports (2021) (JCR, 2021) as a Q4 publication of Web of Science, Clarivate Analytics, USA.

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Dental Communication

Perception, Awareness and Knowledge of Dental Professionals About Teledentistry in Saudi Arabia - A Literature Review

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ABSTRACT

Access to dental care in Saudi Arabia is a challenge due to the lack of proper distribution of the dental workforce. Teledentistry is the field of dentistry that helps to receive and provide dental service remotely using digital platforms. It can facilitate access to dental care, especially in underserved regions. The implication of teledentistry needs to address the concerns of dental professionals to ensure effective teledentistry application in dental practice. This review aimed to explore the literature about the perception, awareness, and knowledge of dental professionals about teledentistry in Saudi Arabia. Peer-reviewed literature was searched in databases including Web of Science, Scopus, PubMed, and Google Scholar with no time limits using the keywords: "Teledentistry", "Dental Telemedicine", "Saudi Arabia", "Perception", "Knowledge", "Awareness". We identified 7 relevant studies. All of them were after the breakthrough of the COVID-19 pandemic. Two studies targeted all dental professionals while another two studies investigated the dentists. Undergraduate dental students were investigated by one study as well as postgraduates. One study focused on both undergraduate and postgraduate dental students. Our review found that the perception, knowledge, awareness, and attitude of Saudi dental professionals are positive. Many studies pointed out that the dental professionals in Saudi Arabia are practicing teledentistry and are open to learn and get more knowledge about it. Teledentistry got more attention during the post-COVID19 time by Saudi dental professionals. Structured and sustainable models and education programs are recommended to utilize the benefits of teledentistry in Saudi Arabia.

KEY WORDS: TELEDENTISTRY, SAUDI ARABIA, PERCEPTION, KNOWLEDGE, AWARENESS REVIEW.

INTRODUCTION

Lack of access to dental care and regular oral health surveillance are the most common barriers to have dental health (Morgano et al., 2010) (Almutlaqah et al., 2018). Access to dental care is highly affected by the presence of dental professionals who are lacking especially in rural and disadvantaged areas (AlShammery, 2016) (Alabdullah et al., 2020). The problem of difficult access to dental care has been aggravated during the COVID-19 pandemic due to the shutdown of dental clinics and the protocols of the physical distancing (Falahchai et al., 2020).

Jannadi et al. (2008) reported that the healthcare system in Saudi Arabia has workforce distribution challenges (Jannadi et al., 2008). Recently, the Saudi healthcare system has

undergone rapid expansion. But providing high-quality services is faced by range of complex challenges such as workforce planning, sustainability, healthcare professionals training, changing demographic and disease patterns. Moreover, in the past decade, Saudi Arabia has shown a boom in dental education. The number of dental schools has grown from four dental schools in 2005 to 24 dental schools in 2015.

However, there is a skewed distribution of the dental workforce because of the vast geographic extent of the country, which makes the proper distribution and utilization of dental labor critical (AlBaker et al., 2017). The increasing population of the country and shortage of local dentists justify the need for this increased workforce need and necessitate looking for alternative ways to overcome the shortage of dental professionals that are associated with the increased demand for dental care. Due to the lack of dental specialists and consultants in the rural and border areas, this workforce must be utilized most effectively and efficiently possible.

Article Information:*Corresponding Author: dr_shaya@hotmail.com

Received 25/09/2021 Accepted after revision 30/11/2021

Published: 31st December 2021 Pp- 1393-1397

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.2>

Wancheck and Rephann assessed the effect of the presence of a dental school in a rural area on the access to dental care by people living there. They found that the initiation of a new dental school in every rural area is not cost-effective and recommended more effective alternatives. They suggested the inclusion of virtual dental clinics to improve oral health in rural areas (Wancheck and Rephann, 2013).

Teledentistry is the field of dentistry that helps to receive and provide dental service remotely using digital platforms. Its development began with the US Army which conducted the first study at Fort Gordon, Georgia in July 1994 (Rocca et al., 1999). The introduction of teledentistry to the dental practice was suggested to help the utilization of the time and efforts of the specialists and consultants (AlShammery, 2016) (AlBaker et al., 2017). (Eraso et al., 1996). It showed a useful alternative cost-effective approach to increase access to dental services. Irving et al. in 2017 found that the teledentistry framework has a positive effect on the delivery and sustainability of dental care (Irving et al., 2018).

Moreover, Torres-Pereira et al. examined the feasibility of emailing the clinical images to diagnose oral diseases remotely. They found this process helped the primary healthcare providers in rural areas where specialists are not available (Torres-Pereira et al., 2008) (Torres-Pereira et al., 2013). Based on the increase in cloud services, Estai et al. (2016) established a cloud-based teledentistry system that helps to increase access to dental care (Estai et al., 2016). In Saudi Arabia, although teledentistry is practiced daily, especially with the spread of COVID-19, via different approaches however the literature showed no established projects dedicated to teledentistry applications (Alawwad et al., 2019) (Aboalshamat, 2020).

Dental professionals can provide these tele-dental services from different situations such as undergraduates, postgraduates, board residents, general dentists, and consultants. Therefore, it is important to know the current state regarding the perception and knowledge of all these dental professionals about teledentistry application before attempting to establish teledentistry projects. Different studies investigated awareness, perception, and knowledge of teledentistry, and each was with a different type of dental professional. Therefore, there is a need to collect the whole picture of all different dental professionals. This review aimed to explore the literature about the perception, awareness, and knowledge of all dental professionals about teledentistry in Saudi Arabia.

METHODS

Peer-reviewed literature was searched in databases including Web of Science, Scopus, PubMed, and Google Scholar with no time limits using the keywords: “Teledentistry”, “dental telemedicine”, “Saudi Arabia”, “Perception”, “Knowledge”, “Awareness”.

RESULTS AND DISCUSSION

There were seven relevant studies published between 2019

and 2021. Two studies targeted all dental professionals while another two studies investigated the dentists. Undergraduate dental students were investigated by one study as well as postgraduates. One study focused on both undergraduate and postgraduate dental students (Table 1).

Perception, awareness of teledentistry among dentists:

Alawwad et al. (2019) conducted a cross-sectional study among dentists in Abha, Saudi Arabia to assess their knowledge and awareness regarding teledentistry. They found that 66% of them were not familiar with teledentistry. Interestingly, more than half of the participants were willing to practice teledentistry after it has been explained to them. Moreover, around 48% of the dental professionals answered; yes, when they were asked if they thought teledentistry would help in health education. The study concluded that the level of knowledge among the participants was low while attitude towards it was good which in accordance with dental professionals perception and knowledge from Rwanda and Pakistan (Latif et al., 2016) (Murererehe et al., 2017) (Alawwad et al., 2019) (Abbas et al., 2020).

Al-Kalifah and AlSheikh asked the Saudi dental professionals regarding their beliefs about the usefulness of teledentistry for the patients as well as the dental practice. They also explored their perception of the potential and any concerns about teledentistry. The authors found that 70% of the participants are strongly believing that teledentistry would improve dental practice. Therefore, they concluded that the Saudi dental professionals are ready to participate in teledentistry projects which are different from the Brazilian dentists who showed inadequate preparedness to implement teledentistry (Al-Khalifa and AlSheikh, 2020) (Raucci-Neto et al., 2021)

Another cross-sectional study examined the perception of teledentistry among a convenient sample of Saudi dentists between January and December 2017 (Alsharif and Al-harbi, 2020). They used the Teledentistry Survey which is a questionnaire developed by Mandall et al. (2005) for assessing dental practitioners' self-perceptions of teledentistry. It measures the teledentistry perception by asking about efficiency in patient care, cost reduction, capabilities for improving practice, and security and confidentiality (Mandall et al., 2005). Results of Alsharif and Al-harbi showed that 65% agreed that it could shorten waiting lists. Moreover, half of the participants could see teledentistry reducing the potential cost of dental care (Alsharif and Al-harbi, 2020). This contrasts with the perception of dentists from the UK who questioned the effect of teledentistry in terms of the dental care cost and the time of dental visits (Benson, 2005).

More recently, Almazrooa and her colleagues explored the benefits and concerns of Saudi dentists regarding teledentistry. Through a validated 40-item questionnaire, they investigated 148 dentists from different specialties. Remarkably, eighty percent were confident about the positive impact of teledentistry on their daily dental practice. They found that half of the participants had a previous clinical experience with teledentistry. Of those

who practiced teledentistry before, around 74 % were using their smartphones to take clinical photographs (Almazrooa et al., 2021).

On the other hand, Zahara et al. found that 80% of their Pakistani dentists' sample never used teledentistry although

they were believing in its benefits and applicability especially during the COVID-19 pandemic (Zahra et al., 2020). Almazrooa and her team recommended that a nationwide educational program be implemented to enhance the awareness about teledentistry among Saudi dental practitioners since teledentistry has the potential to improve the diagnostic dental service (Almazrooa et al., 2021).

Table 1. Perception, Awareness, and Knowledge of Dental Professionals About Teledentistry in Saudi Arabia

Author, Year	Participants	Outcomes	Results	Recommendations
Alawwad et al 2019	Dental Professionals	Knowledge	Low	1- Structured education programs and courses. 2- Central government legislation of teledentistry.
Al-Khalifa and AlSheikh 2020	Dental Professionals	Perception of usefulness of the teledentistry Perception of the capability of the teledentistry to improve practice	70% agree or strongly agree 60 % uncertain with technical reliability, privacy, and diagnostic accuracy.	1- Further investigation of the teledentistry models is needed to understand more. 2- Educate dentists and the public on teledentistry and its potential.
Alsharif and Al-harbi 2020	Dentists	Attitude	Good	1- make evidence-based policy decisions. 2- Further research to examine patients' acceptance of teledentistry.
Almazrooa et al. 2020	Dentists	Experience Attitude Experience	50% of the participants had experience 83% believe it can improve daily dental practice 70.5% never used teledentistry before the COVID-19 pandemic	Development of national programs to educate the public and promote teledentistry among dental practitioners are warranted.
Nishath et al 2020	Undergraduates and Postgraduate Dental Students	Knowledge Attitude	33.3 % were aware that teledentistry is practiced in Saudi Arabia Above 75% have interest in teledentistry training	Implement of teledentistry-related continuous dental education programs and workshops in the dental schools of Saudi Arabia
Khalid T. Aboalshamat 2020	Undergraduates	Knowledge	Low	1- Greater efforts are needed to educate dental students on teledentistry 2- There are barriers to teledentistry that need to be addressed
AlAssad et al. 2021	Dental Students Postgraduate Dental Students	Attitude Awareness Experience	Acceptable 69% were aware of teledentistry 70.5% never used teledentistry before the COVID-19 pandemic	1-Further awareness among postgraduate dental students in Saudi Arabia should be encouraged. 2- Conducting continuous dental education programs and awareness campaigns.

Perception, awareness of teledentistry among dental students: At the time of the COVID-19 pandemic, Aboalshamat et al. investigated, through a validated self-reported questionnaire, the knowledge, attitudes, practices, and barriers to teledentistry use among dental students. Only 17% of the participants were familiar with the term “teledentistry”. However, when it was explained to them, more than 65% stated that they would like to practice teledentistry and about 70% thought that the adoption of teledentistry is compatible with the national Saudi Vision 2030 (Council of Economic and Development Affairs, 2016). The perception of these students is similar to what George et al found where younger Indian dental professionals are more positive about teledentistry than older dentists (George et al., 2021). Aboalshamat et al. pointed out that patient satisfaction and privacy issues were the main concerns of the participants about teledentistry (Aboalshamat, 2020).

Moreover, Nishath et al. in 2020 assessed the knowledge, awareness, and attitude of teledentistry among undergraduate, graduate, and postgraduate dental students in Riyadh City through a structured, self-administered, and close-ended questionnaire. They found that out of 240 dental students who completed the survey around one-third of dental students knew about teledentistry. Significantly, most of the students (postgraduates and graduates) were willing to have teledentistry training. The authors concluded that postgraduate and graduate dental students have enough knowledge, awareness, and attitudes towards teledentistry particularly after the COVID-19 experience (Nishath Abdullah et al., 2020).

This impact of COVID-19 on the perception, knowledge, and attitude of Saudi dental students towards teledentistry is similar to that of Colombian one (Plaza-Ruiz et al., 2021). Recently, AlAssad et al. carried out a cross-sectional study to measure the knowledge, awareness, and attitude of teledentistry among 102 postgraduate dental students. Sixty-nine percent of the participants were aware of teledentistry. However, interestingly, seventy percent of the participants practiced teledentistry only after the COVID-19 pandemic (AlAssad et al., 2021). The authors pointed out that there was no age or gender statistical difference with regard to teledentistry questions which is opposite to the findings of George et al. (George et al., 2021). This can be explained as the sample of AlAssad was confined to postgraduate students who are most likely similar in their age.

CONCLUSION

The perception, awareness, knowledge, and attitude of dental students and dental professionals in Saudi Arabia about teledentistry are similar to those of their colleagues worldwide. When compared to other different countries, dental students and professionals in Saudi Arabia have a more positive attitude which could be due to the young age of majority of Saudi people. Sustainable and structured teledentistry models and formal education programs are recommended to utilize the benefits of teledentistry.

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Biomedical Communication

Clear Cell Odontogenic Carcinoma: A Rare Case Report

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ABSTRACT

Clear Cell Odontogenic Carcinoma (CCOC) is a slow-growing, locally invasive odontogenic tumor affecting the jaws. It usually has confusing clinical characteristics, radiographical and histological features, making its recognition more challenging. In (2005), the WHO has reclassified CCOC as a malignant odontogenic tumor due to its aggressive behavior. This case report is about a woman who aged 42 years old, experienced swelling of the lower jaw and complained of paresthesia of the lips for two years. Radiographic findings showed an extensive, large, multilocular radiolucency lesion associated with scalloping, non-sclerotic border, and crossing the midline of the mandible. Histopathologically, the high-power view illustrated lobules of clear epithelial cells with clear cytoplasm. A review of English literature in PubMed Medline revealed few similar cases of CCOC affecting the mandible. The definitive diagnosis was consistent with CCOC; therefore, the patient was admitted to the hospital and surgical resection of the mandibular tumor was performed under general anesthesia. Long-term follow-up visits showed no signs of recurrence or post-surgical complications. We explain the signs and symptoms of CCOC such as symptomatic or asymptomatic jaw swelling, teeth loosening, displacement and mobility of teeth, and thinning of the mandible. Also, differential diagnosis and the nature of CCOC under the microscope were discussed and explained. The presentation of clear cell tumors is challenging, and it needs a meticulous investigation to determine the type of the tumor for proper diagnosis and management. CCOC should be included in the differential diagnosis list of jaw swelling that associated with slow-growing mass and paresthesia in the affected jaw.

KEY WORDS: CASE REPORT, CLEAR CELL ODONTOGENIC CARCINOMA, MANDIBLE, ORAL TUMOR, PARESTHESIA.

INTRODUCTION

Clear cell odontogenic carcinoma (CCOC) was first reported in (1985) still the existing reported cases of CCOC are very few due to which the behavior and pathology pattern of this tumor is still considered mysterious and not fully understood. It is a rare, aggressive, odontogenic malignant tumor (Hansen, Eversole and Green 1985). Approximately 74% of CCOC reported cases to affect the mandible with high incidence in females (Guastaldi and Faquin 2019). Previously, CCOC was classified as a benign tumor, and it was also known as clear cell ameloblastoma. CCOC owns aggressive features such as destructive growth patterns, regional lymphadenopathy, distant metastasis, and a high recurrence rate. Therefore, in (2005), the WHO reclassified it as a malignant odontogenic tumor (Guastaldi and Faquin 2019). A thorough review of literature in PubMed Medline

revealed few similar cases of CCOC in the mandible. The reported case of CCOC in a 42-year-old, female patient with a swelling in the mandibular region. The work has been reported in line with the SCARE criteria (Agha et al. 2020).

Case Presentation: Forty-two years old women presented with paresthesia of the lower lip and large swelling in the mandible for more than a year and a half. The patient reported no previous history of trauma and no abnormal discharges such as pus or blood coming out of the mass. However, paresthesia and slight mobility were noted. She was diabetic and on medications for two years and she was allergic to pineapple which is associated with urticaria. Moreover, family medical history and genetic conditions were not remarkable. Moreover, the patient had a clean social record without smoking or abuse alcohol drinking. She had updated routine blood tests which were within normal limits. Extraoral examination revealed a significant enlargement of the chin with intact, and normal colored skin.

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Received 20/10/2021 Accepted after revision 20/12/2021

Published: 31st December 2021 Pp- 1398-1401

This is an open access article under Creative Commons License,

Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.3>

The examination illustrated no regional lymphadenopathy was noted. Remarkably, the extraoral examination confirmed the loss of sensation in the lower lip.

Informed written consent was obtained from the patient for publication of this case report and accompanying images. Intraoral examination showed a large, painless intra-bony swelling with a firm consistency and extending from the right lower first molar to the left lower first molar crossing the midline; area of teeth No. 20 to 30 (according to the universal numbering system). Notably, the patient had multiple missing teeth in the mandibular swelling area as follows tooth No.20, and No. 28. Vitality testing of the involved non-carious mandibular teeth was performed which reflected no abnormal responses. Radiographic analysis including orthopantomogram radiograph depicted an extensive, large, multilocular radiolucency associated with scalloping, non-sclerotic border lesion extending from the right lower first molar to the left lower first molar crossing the midline from tooth No. 19 to 30 without root resorption of the aforementioned teeth. Additionally, the radiographic film showed thinning of the inferior alveolar border in correspondence with the anterior mandible. Also, shifting of tooth No. 29 distally (Figure 1a and 1b).

Figure 1: Panoramic radiograph shows an extensive, large, multilocular radiolucency associated with scalloping, non-sclerotic border lesion extending from the right lower first molar to the left lower first molar crossing the midline from tooth No. 19 to 30.

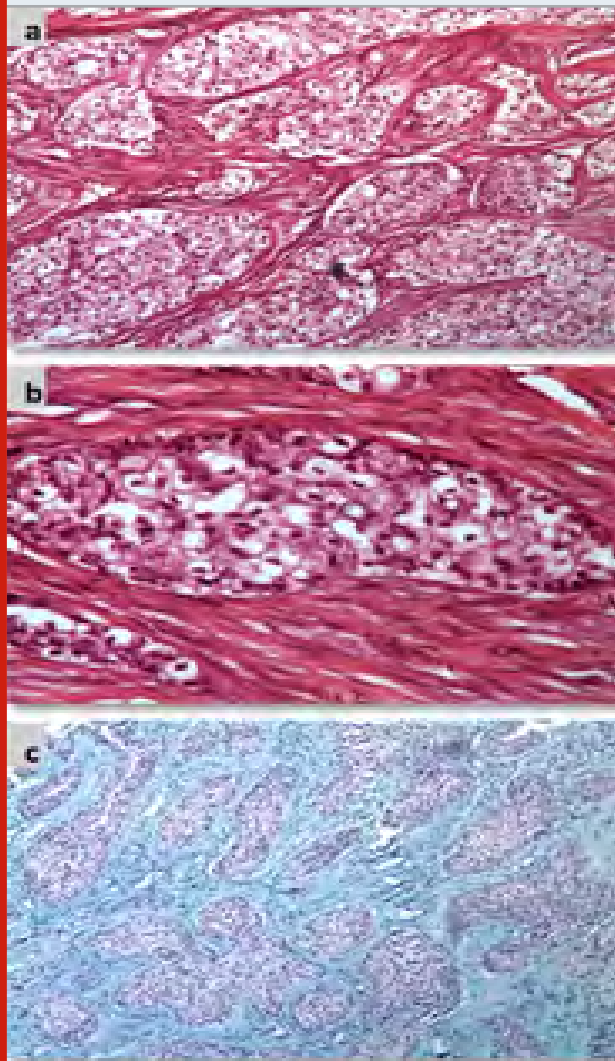


The patient medical history, the clinical and radiographic findings were all indicating the odontogenic tumors. The differential diagnosis of the mandibular mass included clear cell variant of ameloblastoma, clear cell odontogenic carcinoma, clear cell variant of a calcifying epithelial odontogenic tumor, mucoepidermoid carcinoma, acinic cell carcinoma, epithelial myoepithelial carcinoma, melanocytic tumors. She was educated about the nature of the tumor, different treatment plan options, possible results, and prognosis. An incisional biopsy was considered as the first line of management to determine the type of the tumor. The biopsy was performed under local anesthesia, an incisional biopsy was obtained and sent for histopathology analysis.

Two different stains were used for histopathologic analysis to provide an accurate diagnosis. Hematoxylin and eosin

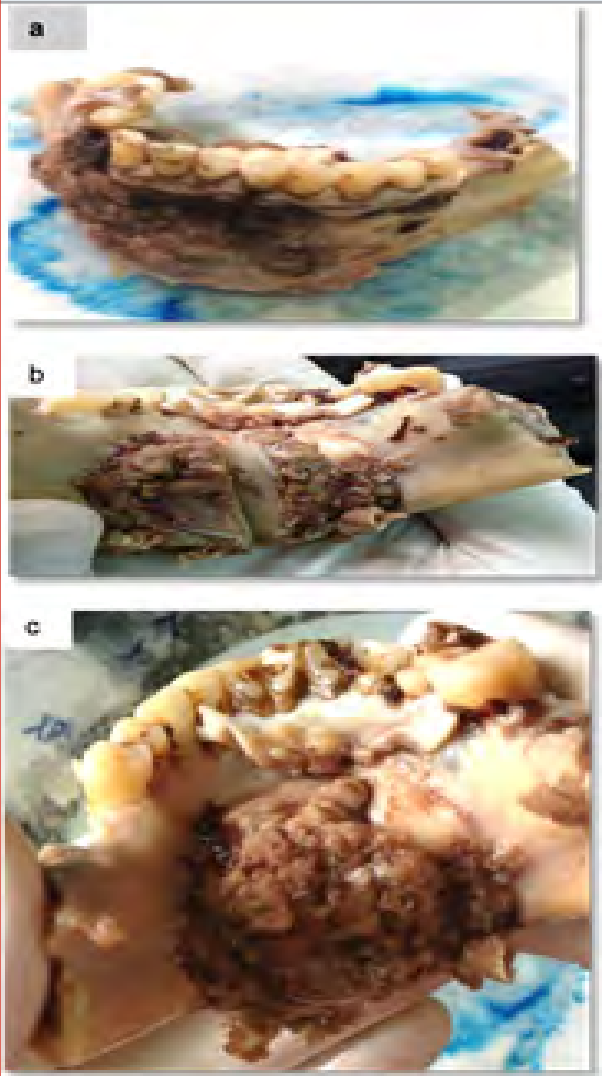
(H&E) stain; an immunohistochemistry stain, and Alcian blue stain; a non-immunohistochemistry stain. Under the microscope, the lesion appeared to have notable stromal hyalinization with small multiple nests and strands. Also, it showed lobules of epithelial cells with clear to eosinophilic cytoplasm (H&E) (Figure 2a, and 2b). Due to the overlapping and the similarity between odontogenic and salivary clear cell tumors, an Alcian blue stain was used to distinguish between them. The Alcian blue stain was negative for cellular mucin; therefore, salivary clear cell tumors and metastatic tumors were ruled out (Figure 2c).

Figure 2: (a) photomicrograph (H&E stain) shows stromal hyalinization with small multiple nests and strands; (b) a high-power view shows lobules of clear epithelial cells with clear cytoplasm to eosinophilic cytoplasm; (c) Alcian blue staining negative for cellular mucin.



The diagnosis was made based on the histopathologic features under the microscope which were strongly consistent with clear cell odontogenic carcinoma. Consequently, the patient was admitted to the hospital and surgical resection of the mandibular tumor with free margins was performed under general anesthesia (Figure 3a, 3b, and 3c).

Figure 3: Gross specimen shows CCOC in (a) the labial and (b & c) lingual aspects of the anterior mandible.



Then, the mandible was reconstructed to cope with the issues of functions and esthetic. The diagnosis of CCOC was, also, confirmed by the histopathological analysis of the resected portion of the mandible. Surgical and post-surgical instructions were delivered verbally and in written form to the patient. Also, all patient's concerns and inquiries were taking care of. The patient was monitored at the hospital for five days post-surgically. A two-week, one-month, and two-month, three-month, four-month, six-month, and one-year follow-up visits, she showed no recurrence or complications.

Analysis on the case presentation: CCOC is a slow-growing, and locally invasive odontogenic tumor affecting the jaws. A rare tumor that mostly affects females more than males. It usually has confusing clinical characteristics, radiographical features and poses benign first glance histological features, making its identifications even

more difficult. Clinically, most cases of CCOC show pain, however, in this case, the patient had paresthesia of the lower lip with no sign of pain. Moreover, swelling of the jaw, teeth loosening, displacement and mobility, thinning of the mandible are other signs of the tumor. Histopathologically, the name of the clear cells came from the special appearance of the cytoplasm under the microscope which shows a clear cytoplasm with H&E stain. The accumulation of intracellular components such as lipid, mucin, and glycogen resulted in the special appearance of the clear cell (Upadhyay et al. 2019).

The picture of the tumor cells under the microscope varies in each case and it is hard to standardize. It includes different figures of stroma containing clusters and sheets of epithelial cells with color variations of cytoplasm, ranging from colorless to eosinophilic hue. In addition, in some cases, the epithelial cells could be arranged in a palisaded pattern which is similar to ameloblastoma. Alcian blue stain to rule out mucous cells containing tumors such as mucoepidermoid carcinoma and clear cell tumor of salivary glands. Clear cells are, also, seen in odontogenic and non-odontogenic tumors. Clear cells of odontogenic origin such as odontogenic cysts, clear cell ameloblastoma, mucoepidermoid carcinoma, clear cell tumor of salivary glands, calcifying epithelial odontogenic tumor, acinic cell carcinoma, myoepithelial carcinoma, and squamous cell carcinoma. On the other hand, in patients with a history of cancer, distant metastasis is possible. Therefore, undifferentiated clear cell tumors of non-odontogenic origin from kidneys, thyroid, breast, lungs, and colon could be metastasis to the mandible (Swain, Dhariwal and Ray 2013; Liu et al. 2020).

Consequently, a meticulous examination should be done for patients with a possible diagnosis of CCOC to rule out clear cell metastasis from distant organs (Werle et al. 2009). Diagnosis of CCOC depends on the detailed medical history, thorough physical examination, radiographic imaging, and histological analysis of the provided incisional specimen. CCOC ideal treatment is not clear yet due to the rarity of the documented and reported case. Most cases undergo radical surgical resection of the tumor with safe bone tissue margins. However, when palpable lymph nodes or extensive destruction of the bone with tissue invasion are involved, the management would be twisted and shifted to be more aggressive; to reduce the chance of recurrence. It would indicate lymph node removal, radiotherapy, or/and chemotherapy. In our report, the patient did not show signs of lymphadenopathy or metastasis, and the resection of the mandibular tumor was performed with safety margins. Therefore, in this case, the patient did not go through lymph node removal, radiotherapy, or/and chemotherapy. Long-term follow-up of CCOC cases is highly recommended to exclude recurrence and metastasis of the tumor (Upadhyay et al. 2019; Liu et al. 2020).

CONCLUSION

The findings of the present case showed confusing clinical features, radiographical patterns, and poses benign first glance histological forms, making its recognition and diagnosis more difficult. A detailed investigation is essential to reach the definitive diagnosis and to manage the tumor properly. The presentation of clear cell tumors is challenging, and it needs a meticulous investigation to determine the type of the tumor for proper diagnosis and management.

Conflict of Interests: Authors declare no conflicts of interest to disclose.

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Biomedical Communication

An Instrumented Assessment of Cerebral Palsy: A Systematic Review

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ABSTRACT

Cerebral palsy is a neurological problem which mainly affects the children and is a frequent reason of physical inability. It affects both motor as well as sensory system. Due to physical disability quality treatment is required for CP children. For an effective treatment proper assessment and selection of correct assessment tool is necessary. This review was aimed to recognize the various assessment scales which are available to assess different domains in person of cerebral palsy patient along with their psychometric properties. We had done a review of literature through Cochrane library, Ovid MEDLINE and GOOGLE SCHOLAR, CINAHL till March 2020. The studies in English-language related to the assessment scales for the cerebral palsy were reviewed. Many scales were evaluated for different symptoms and good psychometric properties in CP child. We reviewed many research and review article related to assessment for CP. Various titles, abstracts, and references were checked for the relevancy. Review was done for the assessment of alteration in muscle tone, impaired voluntary movement, pain, gross motor function, balance, cognition and gait. Cerebral palsy was the primary goal of findings in 198 studies. Out of these, 58 studies were not proved the definition of cerebral palsy. 96 studies reported assessment of cerebral palsy for different domain, 25 studies used etiology and incidence and 19 studies for other domain which are related to CP. Many cerebral palsy scales are available, but only a very small number of scales were thoroughly validated for use in clinical practice in India. This review will help therapist in selection of appropriate tool and study of various symptoms in CP child before starting the treatment..

KEY WORDS: ASSESSMENT SCALES, CEREBRAL PALSY, PSYCHOMETRIC PROPERTIES.

INTRODUCTION

CP is the mainly widespread reason of physical inability in children. Developmental delay and motor deficits are usually main presentations of CP child. It was first described in 1862 by, William James Little an orthopedic surgeon. The prevalence of CP globally is around 2 to 2.5/1000 live births (Jan 2006). In this disorder a permanent non progressive changes were occur in fetal brain (Ghasia et al. 2008). It is a neurological problem which mainly affects the children (krigger 2006a; Ghasia et al. 2008). The prevalence of CP ranges from 1.5-4 per 1,000 live births; this range is for children of 10 to 14 years. The overall birth prevalence of CP is approx. 2 per 1,000 live births (Donald et al. 2014; Stavsky et al. 2017). CP is neither a diagnosis nor an illness but it is an umbrella term for so many conditions (Garfinkle et al. 2020).

The etiology of 70% of CP cases leftovers unidentified. In 20% of children, it may be associated with prematurity, perinatal trauma, or brain hypoxia (Blair and Cans 2018; Boruczkowski et al. 2019). Some other factors like fetal infections and birth defects also can cause CP. CP causes impairment in the posture, movement, intelligence, vision and hearing by damaging the fetal brain. On the basis of severity, CP can be classified as mild, moderate, severe or no CP but not any set of criteria present. Inflammation and coagulation abnormalities also cause CP along with hypoxic ischemic encephalopathy (Beckung and Hagberg 2007; Beckung and Hagberg 2007; Horber et al. 2020; Garfinkle et al. 2020).

CP child exhibits various symptoms like bladder dysfunctions, bowel dysfunction, drooling, sleep disturbances, hearing loss, visual abnormalities, orthopedic associated sensory impairment which are not properly understood (Wingert et al. 2008). CP can also be classified on the basis of both Pathophysiology and area of brain involve. The cortical involvement results in choreoathetosis CP, cerebellar involvement results in abnormal movements, pyramidal

Article Information:*Corresponding Author: shikhasinghmpt@gmail.com

Received 15/08/2021 Accepted after revision 25/12/2021

Published: 31st December 2021 Pp- 1402-1408

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.4>

involvement results in spasticity and involvement of basal ganglia result in hypotonic movement. It can also be classified on the basis of nature of the motor disability and part of the body involve. Examples of motor impairments are Paresis, Hypertonia, Hypotonia, Dystonia, Dyskinesia, and ataxia (Rethlefsen et al. 2010; Horber et al. 2020). Causes of CP are placental insufficiency, uterine infection, metabolic disorder, placenta previa and neonatal asphyxia; and intraventricular hemorrhage of the newborn, Periventricular Leukomalacia (PVL), blood infection, and perinatal stroke (Compagnone et al. 2014; Veldeet et al. 2019). Symptoms of CP shows some negative sign like weakness of skeletal muscles and delayed milestones and some positive signs like a velocity dependent increased muscle tone with spasticity, clonus, rigidity, spasms and hyper reflexia. Some children with CP show other symptoms as abnormal movements like athetosis, chorea, and dystonia and some are mixed CP those who shows a combination of features such as epilepsy, feeding, nutrition, growth problem, mental retardation (Garfinkle et al. 2020).

Topographic classification shows how many structures are involved and then classified as Hemiplegics, Paraplegics, Tetraplegics, Diplegic, or Monoplegics. Since the (1800s), the clinicians try for the timely identification of CP but still identification is not made until 12 to 24 months in high earning countries and as late as 5 years in low source settings. Children with spastic CP have restrictions to execute numerous daily actions including sitting, standing and walking (Bayon et al. 2016; Veldeet et al. 2019). Sitting is an essential aptitude for many practical action, mainly because it facilitates transfers and allows the self-governing use of upper limbs to control objects (Salazar et al. 2019). A diagnosis of CP made if there is Persistence of primitive reflexes delay of motor development. It is first assumed when there is a disappointment to achieve positive key milestones at predictable age (Patel et al. 2020).

METHODOLOGY

We reviewed various research articles by going through different search engines like Ovid MEDLINE Cochrane library, and EBSCO CINAHL. The following search vocabulary were used: cerebral palsy, assessment scales for CP, different assessment tools for CP, psychometric properties of different assessment scale of CP. Research studies (review and research) that reported assessment of cerebral palsy for both children and adults were included in the literature. Articles published till March (2021) was included without restriction of date. Relevant articles were screened by title and abstract. The most relevant articles were downloaded and evaluated for inclusion in the review. The lists of reference of these articles were also checked for other potentially relevant researches, and these studies were also retrieved. All articles that examined CP in children and used CP assessment scales were incorporated in the review. Each symptom was examined for assessment. Information regarding number of scales available and number of scale having good psychometric properties, were also recorded. The chosen scales for the Assessment of CP were Spasticity, Dyskinesia, Dystonia, Motor abilities; Gait, Cognition, Balance and Pain were used.

Scales for the Assessment of CP: Spasticity: Apart from so many disorder Spasticity is most important reason of disability in children and adults suffering from CP. It occurs due to upper motor neuron syndrome which causes difficulty in daily routine and compromises the excellence of life in CP children (Verrotti et al. 2006). The measurement of muscle tone is very essential to find out usefulness of treatment on spasticity and also to plan further medical or surgical treatment and decide physiotherapy goals. For the assessment of spasticity there are so many scales are available such as Ashworth, Modified Ashworth and Tardieu and MTS. Two scales AS and MAS are used to check tightness to inactive movements and therefore measured tone of muscles in CP children. Literature reveal that there were high inter rater reliability of the Ashworth and modified Ashworth scales but this is not true for all conditions. However, data related to the reliability was missing for most of the scales. Particularly this was seen for test retest reliability (Platz et al. 2005; Mutlu et al. 2008).

Dyskinesia: It is also known as Dyskinetic Cerebral Palsy (DCP). After spasticity Dyskinesia is another most frequent type of symptoms of CP. Dystonia is a neurological movement disorder characterized by involuntary muscle contractions, slow repetitive movements, and abnormal postures of the trunk, neck, face, or arms and legs. Sometimes spasticity is misunderstood with dystonia, which can be created because of psychogenic reason (Jankovic 2006; Verrotti et al. 2006). DCP is classified in two subgroups like dystonic and choreoathetotic. In Dyskinetic CP, sudden tight involuntary contraction and involuntary twitching and writhing are present simultaneously (Stewart et al. 2017). The six scales for the assessment of dyskinesia were present. These were the Burke–Fahn–Marsden Dystonia Rating Scale (BFMDRS), Barry–Albright Dystonia Scale (BADS), Unified Dystonia Rating Scale (UDRS), Movement Disorder-Childhood Rating Scale (MD-CRS), Movement Disorder-Childhood Rating Scale 0–3 Years (MD-CRS 0–3), and the Dyskinesia Impairment Scale (DIS) (Stewart et al. 2017).

Dystonia: Dystonia is diagnosed on the bases of its clinical features. Dystonia is classified in to primary and secondary dystonia. Primary dystonia is different in the form of the deficiency of extra neurological abnormalities and the short of probable acquired causes. The assessment of dystonia is still not well understood due to the complexity of the symptoms and lack of researches (Jethwa et al. 2009; Donald et al. 2014).

For diagnosis of muscle tone 21 tools were available but not specific for the CP child. Some literature identified 21 tools for the assessment of tone of the muscle in the children between the ages of 0 to 12 years. Most of the tools were planned for children up to 5 years of age and were used for the examination of development pattern along with the muscle tone examination or as subscale. Four tool were considered as the assessment tool of active and passive tone of muscle but not having the good psychometric properties. The Amiel- Tison Neurological Assessment (ATNA) at term, Neonatal Intensive Care Unit Network Neurobehavioral Scale (NNNS), Premie-Neuro for newborn infants, and the

Hammersmith Infant Neurological Examination (HINE) for infants aged 2 months to 2 years. The Neurological Sensory Motor Developmental Assessment (NSMDA) has been developed for the assessment of passive and active muscle tone (of the infant) with some degree of validity (Goo et al. 2018). Early assessment is essential for the appropriate treatment of dystonia (Frucht et al. 2021).

Motor abilities: For the assessment of the motor abilities in CP child 17 instruments are present (Beckung and Hagberg 2007). Functional limitation of CP child not only depends on the age of the child but also according to Gross motor function classification. The Gross Motor Function Classification System (GMFCS) is used to explain motor function in children with CP (Carnahan et al. 2007; Curtis et al. 2015). The gross motor functioning is mainly assessed by two tools: Gross Motor Function Classification System (GMFCS) and Functional Mobility Scale (FMS). GMFCS describe about five types of CP which depends on functional limitations, the need for hand-held mobility devices (such as walkers, crutches or canes) or wheeled mobility, and to a much lesser extent, quality of movement. Literature suggest that it also depends on year of the child (Wiedemann et al. 2019).

Along with GMFCS the Sarah scale also used for assessment of the motor skills and it is a suitable tool for the assessment of the functional performance and the motor skills of children and adolescents with CP (Pinto et al. 2016). Gross Motor Function Measure (GMFM-88) was also used for the assessment of gross motor functioning (Bayon et al. 2016). Gross Motor Function Measure (GMFM-88) and (GMFM-66) were used as outcome measures to determine the changes in gross motor function in children with CP undergoing treatment (Alotaibi et al. 2014; Wiedemann et al. 2019).

Literature suggests that 17 instruments are used to assess the functional motor abilities in children with CP. Due to lackness for evaluation in change of functional abilities, it was found that most measures are developed and validated for discriminative purposes. The tool developed in last decade have good psychometric properties and only two evaluative assessment measures, the Gross Motor Function Measure (GMFM) and the Pediatric Evaluation of Disability Inventory (PEDI), fulfill the criteria of reliability and validity with respect to responsiveness to change (Ketelaaret al. 1998; Bodkin et al. 2003). The gold standard tool for the assessment of motor abilities is The Gross Motor Function Measure (GMFM). It have simple language and easy to understand by the parents (Rachel 2020).

Gait: For Gait, CP children are suffering from so many motor problems these causes gait deviations. So assessment of gait in these child is essential. There are 14 specific abnormalities are seen in CP child which are of different types (Paralysis on one side of body, paralysis of lower half of body or paralysis of whole body), age, and history along with previous surgery (orthopedic surgery in lower extremity). There are diminished knee flexion in swing, limiting upward bending motion of ankle joint, and feet turn inward while walking or running, restricted hip

mobility and crouch hip adduction, out-toeing, deformity of calcaneus bone, and crouch increased with prior surgery, rotational misalignment of the leg (internal hip rotation without-toeing).

Varus and valgus foot deformities, and hip internal rotation (Maathuis et al. 2005). Spastic CP most commonly suffering from disorder of gait. Observational tools are found from the available literature from which the Edinburgh Visual Gait Scale (EVGS), the Visual Gait Assessment Scale (VGAS) and the Observational Gait Scale (OGS) are commonly used for the assessment of gait in CP child (Brown et al. 2008). Edinburgh Visual Gait Score have good psychometric properties than the other tools. Physician Rating Scale in children with hemiplegic cerebral palsy also shows good psychometric properties (Dickens and Smith 2006; McIntyre et al. 2013; Rathinam et al. 2014; Armand et al. 2016c). For correction of gait deviations objective assessment is important. For intent analysis of gait Clinical gait analysis (CGA) should be done (Mazure et al. 2020).

Balance: Balance is also an important component of gait disorder in CP child Apart from other problems postural control (Woollacott et al. 2007). Balance is a combination of multiple body systems including vestibular, visual, auditive, Proprioceptive and higher level pre motor systems. The functional goal of the balance system includes: (1) conservation of postural alignment like sitting or standing; (2) assisted voluntary movement and (3) re-establish equilibrium after peripheral disturbances, such as a trip, slip or push. From the available literature we found 22 tools for the assessment of the balance.

Two tools were also validated in adults with CP. A strong level of evidence was present for content validity for the majority of the tools but limited evidence was present for their Intrarater, Interrater, and test-retest reliability. In addition, no evidence was present for internal consistency, measurement error, criterion validity, and responsiveness for the majority of the tools. These are the Berg Balance Scale (BBS), the Functional Reach Test (FRT), the Functional Walking Test (FWT), the Heel-to-Toe Stand (HTS), the Level of Sitting Ability (LSA), the Level of Sitting Scale (LSS), the Modified Posture Assessment Scale (MPAS), the Pediatric Balance Scale (PBS), the Pediatric Reach Test (PRT).

The Pediatric Clinical Test of Sensory Interaction for Balance (P-CTSIB), the Posture Assessment Scale (PAS), the Posture and Posture Ability Scale (PPAS), the Seated Posture Control Measurement (SPCM), the Segmental Assessment of Trunk Control (SATCO), the Sitting Assessment for Children with Neuromotor Dysfunction (SACND), the Sitting Assessment Scale (SAS), the Spinal Alignment for Range of Motion Measure (SAROMM), the Timed One-Leg Stance (TOLS), the Timed Up and Down Stairs (TUDS), the Timed Up and Go (TUG), the Trunk Control Measurement Scale (TCMS), and Trunk Impairment Scale (TIS). Two of the clinical balance tools, PPAS and SPCM, were evaluated for adults (Saether et al. 2013). As all the CP's are not having the same symptoms so there is need of many assessment instruments with different

options to adapt to CP's mixed inhabitants (Apolo-Arenas 2021).

Cognition: Cognition impairment is most commonly seen in CP child. Instead of having difficulty in standardized IQ tests designed for the general population, children with developmental disabilities will earn low IQ score. Nine give psychometric result for CP children. The included tests were The Columbia Mental Maturity Scale, The Leiter International Performance Scale, The Peabody Picture Vocabulary Test, The Pictorial Test of Intelligence, The Raven's Colored Progressive Matrices, The Stanford-Binet Intelligence Scales, The Wechsler Adult Intelligence Scale, the Wechsler Intelligence Scale for Children, and The Wechsler Preschool and Primary Scale of Intelligence (Foo et al. 2013). Without proper evaluation and treatment of cognitive functioning intellectual disability may occur (Stadskleiv 2020).

Pain: In CP children pain is also a significant factor that impacts the quality of life of these children. Pain in children with CP is not commonly known and left untreated so it becomes chronic (Houlihan et al. 2007; Penner et al. 2013). Chronic pain affects health both emotionally and psychologically. For more than 3 months, pain remains known as chronic pain. The literature includes a total of 54 chronic pain assessment tools which have strong psychometric properties from which 15 chronic pain assessment tools were chosen for inclusion in the best practice toolbox. The tools are Body Diagram, Non communicating Children's Pain Checklist-Revised [NCCPC-R], Pediatric Pain Questionnaire [PPQ], track pain over time (Bath Adolescent Pain Questionnaire [BAPQ], Child Activity Limitations Interview [CALI], Pediatric Pain Interference Scale [PPIS]), or both (Pediatric Pain Profile [PPP]).

They relied on observational (NCCPC-R, PPP), self-report (Body Diagram), or combination reporting styles (BAPQ, CALI, PPIS, PPQ). All tools had been validated with a pediatric population and a diverse variety of medical conditions; note that the NCCPC-R, PPIS, PPP and PPQ were primarily used to assess pain in CP children. It provides scores of psychometric properties, therapeutic usefulness and method use guidelines according to GMFCS standards. We accept that 54 chronic pain evaluation methods were officially evaluated for psychometric features, therapeutic effectiveness and expert opinion from which 15 chronic pain evaluation tool were chosen for use in the best practice toolbox (Kingsnorth et al. 2015; Pelrine et al. 2020).

RESULTS AND DISCUSSION

In this systematic analysis of clinical studies using cerebral palsy as the primary endpoint, we found various descriptions of cerebral palsy, 167 separate assessment scales for 8 context, a large variance in evaluation methods and CP rates recorded, and very little psychometric examination of the current scales. We found a wide range of meanings among the 198 studies which clarified how they defined CP, either by scale or meaning alone. There are broad range of symptoms found in cerebral palsy from them spasticity,

dystonia, dyskinesia, gait abnormalities, balance disorders, cognition abnormalities and chronic pain were the problem which were commonly faced by person suffering from CP (Gupta and Appleton 2001).

Numerous incremental measures of symptoms severity gradation have been developed over the past 40 years, but recurrent drawbacks include: (1) not all of the necessary symptoms may be present; and (2) a patient might not show the sign in the specific sequence specified by the measure, thereby may not meet the cerebral paralysis threshold (Knox and Evans 2002). Dysphagia, vomiting, and chronic constipation have been also reported in children with neurologic impairment like CP and also to be evaluated but none scale is found which have good psychometric property (Elbasan and Bezgin, 2018). None scale was found with good psychometric property for sleep related domain. Some questionnaire and tools for the assessment of CP are widely used such as The Gross Motor Function Classification System (GMFCS), for the assessment of gross motor function in cerebral palsy but some were neglected because they were not having the good psychometric properties (Rosenbaum et al. 2018). CP children present complex and heterogeneous motor disorders so there was a need of a measuring tool which can assesses all the symptoms related to CP (Armand et al. 2019).

Assessments of motor-functional aspects in CP were crucial to rehabilitation programs so it was necessary that it should be assess on priority bases (Pinto et al. 2016). However, for many scales reliability data was missing. This was particularly true for test retest reliability (Platz et al. 2019; Hutson and Snow 2020). There was no uniformity of composite measures as to how many symptoms were available to apply for CP. Numerous incremental measures of symptoms severity gradation have been developed over the past 40 years, but recurrent drawbacks include: (1) not all of the necessary symptoms may be present; and (2) a patient might not show the sign in the specific sequence specified by the measure, thereby might not meet the cerebral paralysis threshold (Knox and Evans 2020).

There were numerous shortcomings to our analysis. Like, we only collected researches that were conducted in English, analyzing various CP domains. Some domains were still not reviewed like scales for fine motor skills, hand functions and sensory assessment. We did not approach authors of the analysis to inquire for possibly undisclosed psychometric results. We were unable to find any older paper (pre-1985) that mentioned CP, so some earlier CP resources could have been missing. It was also likely that newer and undisclosed CP scales were in use and yet not published.

CONCLUSION

The findings of the present study found for 8 impairments there were total 167 scales available but only 53 had psychometric properties and none of the scale could assess all impairments of CP. The selection of appropriate measurement tools is essential for effective clinical practice yet, it is unclear how best to assess CP because no existing scale has undergone for all impairments of CP. So many

scales are available but most of them assess only one or two limitations of CP. There is still need to develop an assessment tool which cover the all domains of CP.

ACKNOWLEDGEMENTS

This study was financially supported by the Maharishi Markandeshwar Institute of Physiotherapy and Rehabilitation, Mullana, Ambala. Authors acknowledge the staff for their constant support and encouragement.

Conflict of Interests: Authors declare no conflicts of interests to disclose.

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Dental Communication

Noninvasive Multi-Disciplinary Management of Ectodermal Dysplasia with Hyposalivation – A Case Report

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ABSTRACT

Ectodermal dysplasia (ED) is a rare genetic condition with nearly 200 documented traits. As the name states, ED targets tissues derived from ectoderms, such as hair, skin, nails, sweat glands, and teeth. Other orofacial structures might be affected, such as salivary glands and hard palate. Lack of teeth and diminished facial height can impact negatively on child growth and psychological well-being. Therefore, assessment and an interdisciplinary management plan of orofacial components of ED children should be installed as early as possible. Here we report an early assessment and multi-disciplinary management of ED child's orofacial structures, which allow restoration of facial height and dental function and saliva reduction by the least invasive restorative approach in the form of the composite build-up of microdont and overdentures. It also highlights the importance of periodic evaluation of growth and treatment plan adjustment as an integral part of the transitional management until the age of a definite dental treatment.

KEY WORDS: ECTODERMAL DYSPLASIA, SALIVARY GLANDS, HYPOSALIVATION, MAXILLOFACIAL REHABILITATION, HYPODONTIA.

INTRODUCTION

Ectodermal dysplasia (ED) consists of an assembly of infrequent congenital disorders with a prevalence of one per 10,000 to 100,000 live births with no known geographical predilection. The condition targets all or some ectodermal-derived tissues, such as hair, skin, nails, sweat glands, and teeth. The most common traits are hypohidrosis and hydrotic, with the main difference being the number and function of sweat glands (Wright et al., 2019, Blattner, 1968).

The faces of these children are often similar due to unique maxillofacial features, including a prominent forehead, flat nasal bridge, thick lips, and periorbital and perioral hyperpigmentation. Oral findings of ED include underdeveloped palate and alveolar ridges, dental anomalies that vary from complete to partial dental agenesis, and teeth malformation, such as microdontia (conical teeth) and enamel hypoplasia (Blattner, 1968, Halai and Stevens, 2015, Wright et al., 2019, Gill et al., 2008). Teeth deficiency and malformation have a direct impact on the child's growth and general well-being. ED management should commence as early as possible through multi-disciplinary care involving

medical and dental teams. Dental rehabilitation for these children is crucial to restoring the required functional, esthetic, and psychosocial aims (Gill et al., 2008, Halai and Stevens, 2015).

Case Report: This is the case of an 8-year-old girl who attended with her parents after a referral for treatment. The child was diagnosed with autosomal recessive hidrotic ED at 3 years old due to delayed teeth eruption. No genetic mapping was done. The main complaint was the appearance of her small spiky front teeth, which result in bullying at school. The patient mentioned that she experiences dryness in her mouth, especially during eating, which improves by drinking sips of water frequently. A detailed dental and medical history was obtained from the child as well as the parents. Family history revealed that HED was affecting family members on her mother's side.

The child presented with some maxillofacial features, such as a prominent forehead, protruding low-set ears, saddle nose deformity, and decreased vertical height with thick everted lips. All primary teeth were present upon intraoral clinical examination, except the upper right and left primary lateral incisors. A dental panoramic tomogram was requested to assess permanent teeth development (Figure 1). Severe hypodontia was diagnosed with 22 missing permanent teeth

Article Information:*Corresponding Author: salhamed@kau.edu.sa

Received 15/10/2021 Accepted after revision 20/12/2021

Published: 31st December 2021 Pp- 1409-1412

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.5>

excluding third molars, with microdontia of the lower anterior teeth.

Skeletal jaw relation assessment showed severe maxillary hypoplasia and reduced lower facial height. We detected a dental class III anterior relationship with severe reverse overjet of 3 mm and full arch crossbite. Orthodontic consultation was recommended to delay orthodontic treatment and reevaluate the case annually to determine the proper approach. Oral medicine consultation was obtained, and the stimulated saliva flow rate was evaluated to be 1 ml per min, which is low. The patient was diagnosed with hyposalivation and present some signs of mucosal dryness such as lobulated tongue and frothy saliva. Artificial saliva gel was prescribed along with instructions to continue water drinking and lubrications using olive oil and petroleum jelly. The periodic revaluation was planned.

Figure 1: Preoperative dental panoramic tomogram showing severe hypodontia.



Figure 2: Preoperative clinical pictures.



Dental management was based on noninvasive preservation of the remaining dentition with the aesthetic restorative treatment of teeth morphology and function. Continuous follow-up and adjustment were scheduled until the patient reaches an age at which she can receive definitive treatment. An osseointegrated implant option was not considered for several reasons. First, the patient was anxious, and her parents wanted all the treatment to be atraumatic and conservative. Second, the patient presented with an adequate set of primary dentitions, which allows overdentures as a sufficient alternative plan. Moreover, implant positioning in a growing child has many drawbacks and should be planned only in selective cases.

The treatment plan was divided into preventive, rehabilitation, and maintenance phases. The rehabilitation phase was planned to cosmetically improve the appearance of microdont primary incisors followed by maxillary and mandibular overdenture to increase lower facial height. The patient presented for 6 months follow-up visit with good oral hygiene and intact restorations. However, the upper right central incisor showed grade 3 with complete root resorption. For that, an immediate denture was constructed and inserted after extraction. Prosthetic consultation stated that no need for facebow registration at the current stage and anterior teeth should be aligned in edge-to-edge relation (Figure 3,4).

Figure 3: Six months follow-up.



Figure 4: Immediate denture inserted after extraction of URA with an edge to edge occlusion.



A maintenance phase through periodic recalls was arranged for every 6 months for prevention, evaluation of oral hygiene, teeth development, and eruption. Moreover, this would include evaluation of dentures and planning for adjustment or remaking, when required. The last demonstrated review was 3 years post-treatment (Figure 5).

RESULTS AND DISCUSSION

Hypodontia is the term used to describe the congenital or developmental absence of one or more primary or secondary teeth (excluding third molars) (Gill et al., 2008). In Saudi children, the prevalence of hypodontia was reported to be 2.6%, excluding the third molar (Salama and Abdel-Megid, 1994). Severe hypodontia in children is a very rare condition, most often associated with congenital syndromes,

such as Down syndrome or ED (Crawford et al., 1991). Lack of teeth can significantly impact the child's physical and emotional well-being, especially during adolescence. As in this case, hypodontia could be a reason for bullying, leading to social isolation, loneliness, and underachievement, (Crawford et al., 1991 Bohner et al, 2019).

Figure 5: Three years follow-up with upper and lower overdentures.



Prosthetic rehabilitation using an osseointegrated implant in growing children as young as 3 years old was documented in the literature (Bohner et al., 2019, Wang et al., 2016). However, a recent systematic review investigating long-term implant outcomes in children stated that infraocclusion and rotation are common complications in these patients. Moreover, regular adjustments are essential in growing jaws (Bohner et al., 2019). Another systematic review stated that although implants are commonly used in ED children for dental rehabilitation, there is a lack of long-term data on bone augmentation and implant success in the literature (Wang et al., 2016). As osseointegrated implants are considered only in special circumstances, other noninvasive approaches, such as overdentures, are good options to reduce psychological trauma, continuous adjustments, and traumatic treatment (Bohner et al., 2019, Wang et al., 2016).

Hyposalivation is an objective term used to describe an actual reduction in salivary flow (Turner, 2016). There are many reasons for hyposalivation which range from temporary medication Side effects to degenerative exocrine autoimmune conditions such as Sjogren syndrome. As ectodermal dysplasia affects ectoderm-derived tissue, there has been an association with xerostomia and hyposalivation (Lexner et al., 2007, Nordgarden et al., 2003). In addition, qualitative changes have been demonstrated in ED patients such as reduced amylase activity and concentration (Nordgarden et al., 2003). Management of chronic hyposalivation aims to reduce symptoms and improves patient's daily activities such as speaking and eating (Lapiedra et al., 2015, Martín et al., 2017). Those patients such as this case learn to continuously lubricate oral mucosa using natural sources such as olive oil and rely on sips of water throughout the day to facilitate oral functions including denture use (Nordgarden et al., 2003, Ship et al., 2007, Lapiedra et al., 2015, Martín et al., 2017).

CONCLUSION

Instead of invasive procedures with implants, more conservative alternative approaches include using overdentures for dental rehabilitation until completion of growth (Bohner et al., 2019, Wang et al., 2016). The patient was gradually introduced to wearing dentures that satisfactorily restored function, aesthetic, and facial appearance by increasing lower facial height. However, the patient and parents were warned about the problems related to dentures, including limited retention, initial speaking difficulty, dietary limitations, loss of appliance, and the need for adjustments, relining, and replacement.

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Dental Communication

Over-The-Counter Oral Hygiene Products Misuse: A Case Report

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ABSTRACT

Oral hygiene products (OHP) are recommended to maintain the teeth and health of the oral cavity tissues by assisting in lowering the plaque level as well as the opportunistic flora. Oral hygiene products could cause oral health problems that may vary in severity. Due to the availability of over-the-counter (OTC) medications and lack of knowledge, customers may misuse these products. An oral hygiene product is a double-edged sword; a customer would develop side effects from it without even knowing or correlating the source. Many signs and symptoms may appear on the patients such as oral and perioral burning sensations, swelling, erythema, dysgeusia, and dysphagia. To our knowledge and based on the review of English literature through PubMed Midline, this is the first case report of misuse because of an over-the-counter oral hygiene product in which the patient used a mouthwash continuously and aggressively for 15 days without knowing of its side effects. This case report presents an eighty-two-year-old, male patient who complained of pain, burning, swollen, and dry mouth and lips. The author discussed the most common ingredients of oral mouthwashes. Some active ingredients may irritate the oral mucosa and lips. Also, the author discussed the possible differential diagnosis of painful, sensitive, burning, swollen, and dry mouth and lips. This report is intended as an overview of a rare and unusual oral condition associated with the misuse of OTC oral hygiene products. The findings of the present study necessitated the need for clear verbal and written instructions to be provided to the patients before recommending or prescribing any type of medication.

KEY WORDS: ATROPHIC GLOSSITIS; CHEILITIS; DRY BURNING MOUTH; MUCOSAL ERYTHEMA; MOUTHWASH.

INTRODUCTION

Oral care has been an important part of human social life as well as cultural development for hundreds of years. Many OHPs have been invented and produced for the purpose of maintaining good oral hygiene levels. OHPs have been used to control halitosis, dental caries, gingival diseases by lowering the dental plaque and oral bacteria. The accessibility and affordability of OTC oral hygiene products as well as lack of education, explain the misuse of these products (Bourgeois and Llodra 2009). Notwithstanding, OTC oral hygiene products most of the time are safe if used as instructed; however, they have many ingredients that

could cause oral irritation if not used properly (Aspinall, Parker and Khutoryanskiy 2021).

The sign and symptoms of acute oral burning, swelling, and erythema are not commonly reported in the literature as side effects of oral hygiene products. Based on a careful review of English literature through PubMed Midline, this is the first case report of misuse because of oral mouthwash in which the patient used the mouthwash continuously and aggressively for 15 days without knowing of its side effects. The lack of knowledge of using OTC products led to acute oral irritation. In this report, the author discusses a case of an eighty-two-year-old man complaining of constant burning and pain in his mouth and lips. After precise exclusion of the diseases and the causative factors, the diagnosis was consistent with acute chemical burn due to misuse of OTC oral hygiene products. "The work has been reported in line with the SCARE criteria" (Agha et al. 2020).

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Received 15/09/2021 Accepted after revision 21/12/2021

Published: 31st December 2021 Pp- 1413-1416

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbr.in/> DOI: <http://dx.doi.org/10.21786/bbr/14.4.6>

Case Presentation: This case report is about an eighty-two-year-old man, who was referred to an oral medicine clinic to diagnose and manage the patient's oral condition. He reported a twelve-day history of dry, painful, burning sensations all over the mouth including the lips. He also, mentioned that he felt his lips are chapped and swollen. He could not eat or drink comfortably since he had got that complaint. Also, he mentioned that spicy and sour food had aggravated the burning and pain. It all started after removing the lower anterior four-unit bridge, he visited a dentist who recommended rinsing with an OTC mouthwash to reduce the intensity and sensitivity of the patient's exposed lower anterior teeth. The patient reported that he used to swish with 20 mL of an undiluted, mint-flavored mouthwash for four minutes, eight times a day for 15 days. He had diabetes, hypertension, glaucoma, hearing difficulty, and was wheelchair bonded. He was taking metformin 500 mg once per day, lisinopril 10 mg one time per day, and latanoprost 0.005 % once a day before bedtime.

He also denied any changes in his medication list in the last two years. Moreover, he denied a previous history of herpes infection or skin lesions. This also includes a negative history of smoking, alcohol drinking, and drug abuse. The patient revealed that his father had diabetes and passed away due to liver cancer. His mother was healthy, and she died due to a car accident. The oral mucosa and lips were very sensitive to touch that even the slight stretching of the mucosa during examination irritated the patient. Cheilitis was obvious during the extraoral examination which displayed scaly, dry, swollen, and chapped lips (Figure 1).

Figure 1: Extra-oral examination shows scaly, dry, swollen, and chapped lips.



Figure 2: Intraoral examination shows generalized, smooth, bright red, and shiny gingiva.



Intraoral examination displayed generalized, diffuse, smooth, bright red, shiny, and edematous buccal and labial mucosae. Additionally, he had gingival and palatal erythema (Figure 2) and atrophic glossitis (Figure 3).

Figure 3: Intraoral examination shows generalized, smooth, bright red, shiny, and tongue "atrophic glossitis".



No intraoral vesicles were detected and the Nikolsky test was negative. Regarding the dental concerns, he had multiple missing and carious teeth. Moreover, an old, ill-fitting, porcelain fused to metal (PFM), a 3-unit bridge covering the maxillary edentulous area from tooth number 3 to 5, and a 6-unit bridge covering the maxillary edentulous area from tooth number 6 to 11 (according to the FDI World Dental Federation Notation), and a PFM crown on tooth number 30. A blood sample was collected to detect any systemic problems such as hormonal or nutritional issues; luckily, all blood values showed normal readings. The differential diagnosis included chemical burns, contact stomatitis, cheilitis, microbial infections mainly oral candidiasis, immune-induced lesions including erythema multiforme, nutritional deficiency, and drug-induced oral lesion were all considered. The diagnosis of this condition was made based on the medical and dental history alongside the exclusion of all possible causative factors.

The patient was diagnosed with acute oral chemical burns and cheilitis due to the OTC misuse of the mouthwash. In management, the patient was asked to discontinue the use of OTC mouth rinse instantly. Additionally, he was recommended to avoid spicy and sour food and all flavored oral hygiene products. He was, also, recommended to brush and clean his teeth with baking soda toothpaste gently and to keep away from the gingiva. Therapeutically, topical magic mouthwash was prescribed including viscous lidocaine 2%, diphenhydramine 12.5 mg/5mL, nystatin 100,000 IU, prednisolone 5mg/5mL. The magic mouthwash is to be

used as follows, 5 mL of the solution, swish for two minutes then spit it out, four times per day for 10 days. In addition, for treating the painful cheilitis, the dentist prescribed a mix of clobetasol 0.05% ointment and miconazole 2% to be applied on the lips three times per day for 10 days. The patient came after two weeks for re-evaluation of the oral lesions, the oral mucosae and lips recovered very well with no other complications. Also, he was re-evaluated after one month then two months since the initial visit, he was well and satisfied with the therapy.

Analysis on the case presentation: Oral hygiene products (OHP) are intended to maintain the oral cavity clean and fresh by helping in lowering the plaque level and maintaining healthy gingiva and teeth. Broadly speaking, oral hygiene is ideally performed by mechanical and chemical methods. Mechanical cleaning by using toothbrushes, interdental brushes, dental floss, and dental water flosser. Chemical cleaning by using kinds of toothpaste and mouthwashes. The overuse of OHP products could cause oral mucosal damage that ranges from mild oral irritation and peeling to levels of severe oral mucosal inflammation, ulcerations, and allergic reactions. Ignorance of reading the printed instructions on the OHP packages is the most common cause of OTC-OHP misuse. Also, the availability and accessibility of the OHP on the market/pharmacy shelves make patients think that these products are safe to use regardless of the quantity and frequency (Filipović et al. 2020; Langa et al. 2021).

Mouthwash is a mix of active and inactive ingredients. Active ingredients such as sodium lauryl sulphate (SLS), eucalyptol, menthol, methyl salicylate, fluoride, thymol, peroxide, essential oils, and cetylpyridinium chloride. Inactive ingredients such as alcohol, and benzoic acid. SLS is an active element in many cosmetics and OHPs that works by blending the ingredients. Also, it is considered a foaming agent. Unfortunately, SLS is highly linked to mucosal sensitivity. It was reported that SLS has been associated with oral mucosal inflammation, sloughing, increase chemical-tissue permeability, and alteration of protein structures (Rubright et al. 1978). Moreover, menthol, eucalyptol, methyl salicylate, cetylpyridinium chloride, and thymol are antimicrobial and antiseptic agents that are widely used in OHP including mouthwashes. In addition, most OHP companies add fluoride to OHP due to its proven effectiveness in reducing dental caries (de Araujo et al. 2012). Peroxide is also used in OHP, specifically in some mouthwashes and kinds of toothpaste that are intended to give teeth a brighter and lighter look (Filipović et al. 2020; Langa et al. 2021).

For more information about peroxide, it is an active content that is also linked to oral irritation and mucosal pigmentation (Aloyouny, Albagieh and Alharthi 2020). Benzoic acid or sodium benzoate is used to increase the lifetime of the OHPs. The differential diagnosis of burning and painful mouth is listed as microbial infection, immunological causes, medications, nutritional factors, and blood disorders. Oral microbial infections are divided into viral, bacterial, and fungal. Viral infections such as herpes stomatitis, herpangina, acute pharyngitis, and cytomegalovirus. Bacterial infections such as streptococcal

pharyngitis or strep throat, and acute necrotizing ulcerative gingivitis or periodontitis (Prieto-Prieto and Calvo 2004; de Araujo et al. 2012). However, the patient in this report did not present with intraoral ulcers, therefore viral infections were excluded. The most common oropharyngeal fungal infection is oral candidiasis that is mainly caused by *Candida albicans* (Vila et al. 2020).

Painful oral mucosa could be caused by different types of irritations, for instance, traumatic, thermal, and chemical oral irritations. Nutritional deficiency and allergic reactions are proven to cause burning sensations, erythematous and sloughing mucosa, and even ulcerations. Many immunological factors and diseases could induce oral irritation, inflammation, and ulcerations such as lichen planus, erythema multiforme, or in more severe form Steven-Johnson syndrome. Additionally, vesiculobullous diseases such as oral mucous membrane pemphigoid, pemphigus Vulgaris, and paraneoplastic pemphigoid. However, the patient's physical examination, in this case, revealed no extraoral or intraoral vesicles, therefore vesiculobullous diseases were not considered. Blood diseases such as acute leukemia could harm the oral tissue appearance and nature (Mortazavi et al. 2016). Luckily, the blood test values in this patient were all within normal limits. In this report, the patient misused the mouthwash as he used it undiluted four times a day for 15 days which caused sloughing of the oral epithelium, dryness, burning, and edema. Mostly, SLS and peroxide, the mouthwash active ingredients caused mucosal tissue irritation (Vila et al. 2020).

On the other hand, with time, all contents of the mouthwash aggravated the oral tissue condition. In case of drug adverse effects or drug misuse, the first line of management is to discontinue the use of the causative agent. It is worth mentioning that in this case, the patient is elderly and had hearing issues. Therefore, the dentist should have to make sure that the patient understood all instructions verbally and should be provided with a written version as well (Vila et al. 2020).

CONCLUSION

The findings of this rare and unusual case of a patient misuse of the OTC mouthwash presented with dryness, burning, and painful erythematous swelling intraorally. Also, chapped, dry, and painful swollen lips. Therefore, thorough instructions should be delivered clearly to patients before recommending or prescribing any type of medication. The patient felt more at ease from the initial visit to the oral medicine clinic. He strictly followed the prescribed therapies. More importantly, the treatment has an excellent impact on his daily routines including eating, drinking, and speaking.

Conflict of Interests: Authors declare no conflicts of interest to disclose.

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Medical Communication

Impact of Outpatient-Based Exercise Prescription on Stable Rheumatic Heart Disease and Post Coronavirus Infection Individual- A Case Report

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ABSTRACT

The outcome of covid-19 patients with co-morbidities has reported to be poor. Post covid exercise-based rehabilitation may have a major role in improving exercise tolerance and quality of life in post-covid-19 patients with co-morbidities. This case report discusses the clinical scenario of a 35-year-old person who is a known case of stable rheumatic heart disease since 29 years and recently diagnosed with covid-19 moderate pneumonia. He was referred to post covid outpatient department for post-covid physiotherapy rehabilitation. After one month of structured exercise-based rehabilitation, improvement recorded in his SF-12 and 6-minute walk distance. COVID-19 patients with co-morbidities seemed to have a poor prognosis, according to various studies. Post covid exercise-based rehabilitation may have a major role in improving exercise tolerance and quality of life in post-covid-19 patients with co-morbidities. There is no structured rehabilitative protocol designed for such unusual combination as of now hence our study focuses on this lacunae. This case report discusses the clinical scenario of a 35-year-old person who is a known case of stable Rheumatic heart disease in the last 29 years and recently diagnosed with COVID-19 moderate pneumonia. He was referred to post covid outpatient department for post-covid physiotherapy rehabilitation. The individual followed a structured exercise-based rehabilitation protocol for one month 6 times/week and improvement recorded in his SF-12 and 6-minute walk distance. There was improvement in six-minute walk distance by 150 meters and rate of perceived exertion was also improved from 9 score to 7 in 6-20 borg scale post one month. In comparison to pre-Rehabilitation, SF-12 improved the quality of life in both physical and mental score. This case report concluded that there was a definite improvement in the walking distance and quality of life of the patient after post COVID physiotherapy rehabilitation.

KEYWORDS: COVID-19 MODERATE PNEUMONIA, POST-COVID REHABILITATION, RHEUMATIC HEART DISEASE, SHORT FORM-12, SIX MINUTE WALK DISTANCE.

INTRODUCTION

Rheumatic heart disease (RHD) affects >39 million persons globally, with the highest prevalence is seen in low-income countries having limited health care facilities. It is an after effect of Rheumatic fever which occurs after a single or multiple episodes of sore throat due to group A beta-hemolytic streptococci. For these individuals, the COVID-19 outbreak poses a serious problem, as secondary prophylaxis and access to care is likely to be disrupted, due to possibly disabling effects of the disease. (Melo et al. 2018). Cardiovascular disease is the most common comorbidity found in COVID-19 patients (Enrique et al. 2020;

Müller-Wieland et al. 2020). The clinical manifestation of Rheumatic heart disease and COVID-19 is challenging, both diseases could develop fever, chills, dyspnea, fatigue, cough, and myalgia. This case is been recorded with the view of observing the effect of structured exercise program on post covid patient with stable RHD, attending post covid outpatient department (Sanyaolu et al. 2020).

COVID-19 patients display wide spectrum of clinical problems, ranging from respiratory failure, an overactive immune system, coagulation issues, renal failure, endocarditis to reduced exercise tolerance. The SARS-CoV-2 virus has the potential to invade human body cells and systems. COVID-19 predominantly affects the upper respiratory system (sinuses, nose, and throat) and the lower respiratory tract (bronchitis, pneumonia, and bronchitis). Since the virus enters host cells through the receptor for the enzyme

Article Information:*Corresponding Author: drismaarumani@gmail.com

Received 15/10/2021 Accepted after revision 21/12/2021

Published: 31st December 2021 Pp- 1417-1421

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.7>

angiotensin-converting enzyme 2 (ACE2), which is most prevalent on the surface of type II alveolar cells in the lungs, the lungs are the organs most impacted by COVID-19 (Harrison et al. 2020). The Novel Corona virus can impact the heart and circulatory system, the brain directly (encephalitis) and indirectly (e.g. secondary to hypoxia or vascular thrombosis), the kidney and renal function, blood coagulation, and the gastrointestinal tract, in addition to the respiratory system (Wade 2020; Verdecchia et al. 2020).

As per WHO following a severe COVID-19 sickness, exercise rehabilitation is a vital aspect of recovery. It can aid in improving fitness, reducing dyspnea, increasing muscle strength, improving balance and coordination, improving thinking, reducing stress and improving mood, increasing confidence, and increasing energy level (WHO 2020). There is no structured rehabilitative protocol designed for such unusual combination as of now hence our study focuses on this lacuna. The outcome measures used to assess the impact of exercise was 6-minute walk distance and for health-related quality of life was SF-12. The 6-minute walk test (6 MWT) is a submaximal exercise test that involves measuring walking distance in 6 minutes. 6-minute walk test routinely performed for patients with moderate to severe pulmonary disease to quantify their functional capacity. 6-minute walk test often captures the coexisting extra pulmonary manifestations of chronic respiratory disease, including cardiovascular disease, frailty, sarcopenia, and cancer (Agarwala and Salzman 2020).

In contrast with cardiopulmonary exercise stress testing, this test does not require complex equipment or technical expertise. The SF-12 is a health-related quality-of-life questionnaire that consists of twelve items that examine physical and mental health by measuring eight health areas. General Health (GH), Physical Functioning (PF), Role Physical (RP), and Body Pain (BP) are all physical health-related dimensions. Vitality (VT), Social Functioning (SF), Role Emotional (RE), and Mental Health Scales are all mental health-related dimensions (Huo et al. 2018; Agarwala and Salzman 2020). The aim of rehabilitation is to improve recovery and reduce disability in patients with COVID-19. As of now, no studies have been reported with such unusual combination of RHD and COVID-19 given a physiotherapy rehabilitation protocol, hence this case report will manifest a stepping-stone to form a standard COVID Rehabilitation protocol for rheumatic heart disease patients being affected with COVID-19.

METHODOLOGY

A 35-year-old male, a known case of stable rheumatic heart disease referred to post covid outpatient department, presented with complaints of grade 2 dyspnea on a modified medical research council scale and was gradual in onset. Aggravating factors for dyspnea were climbing stairs, walking more than 5-10 min, and relieving factors were rest or sitting on a chair. He also complained of sore throat and dry cough. After being diagnosed with COVID-19 moderate pneumonia, he was hospitalized for 2 days in a dedicated COVID-19 hospital setup, followed by 14 days of home-quarantine. He was on standard COVID-19 medical

management protocol as per the guidelines which included cholecalciferol capsules 1000 IU, vitamin C tablets 500 mg, and azithromycin 500 mg (Launois et al. 2012).

On general examination all his vitals were within the normal limits i.e., blood Pressure was 120/88 mmHg, heart rate was 67 beats per min, respiratory rate was 22 breaths/min, rate of perceived exertion was 11 using standard 6/20 rating of perceived exertion Borg scale. Oxygen saturation, was 98% at rest, measured using a standard pulse oximeter of Meditive Company. Systemic examination of the cardiovascular system revealed pan systolic murmur. He is on regular medication, which is a tablet, digoxin 0.25 mg for Rheumatic heart disease. Respiratory system examination shows reduced chest expansion and chest excursion. On auscultation, his breath sounds; air entry was reduced in bilateral middle and lower zones, bronchial type of breathing were heard which may be due to post covid pulmonary fibrosis, no foreign sounds (Williams 2017).

After the detailed assessment, a six-minute walk test was performed as per American thoracic society guidelines in which the patient completed a distance of 240 meters as compared to age predicted distance of 610 meters. He walked 61% less than the age predicted calculated, by formula. Pre- Six-minute walk test vitals were; blood pressure was 120/88 mm of Hg, a saturation of oxygen was 98% at room air, heart rate was 67 beats/min and the rate of perceived exertion was 9 on a 6-20 Borg scale. Post-test immediate his vital was; blood pressure 130/92 mm of Hg, oxygen saturation dropped to 93% immediate post 6-minute walk test, heart rate was 55 beats/min and his rate of perceived exertion was 11 on 6-20 Borg scale (Enright and Sherrill 1998; Issues et al. 2002; Williams 2017). Vital parameters settled to baseline in 5 minutes post-test. The exercise protocol was formulated base on the clinical assessment and six-minute walk distance as shown in table 1.

The individualized exercise prescription protocol was of 45mins-1hour duration consisting of warm-up, breathing exercises, circuit training included followed by a cool-down with a 1-month follow-up as shown in table 2. The entire rehabilitation session was uneventful. The outcome measures pre rehabilitation were SF-12 which scored SF-12 is physical score: 37.9548, mental score: 43.25045, and 6-minute walk distance in which the patient walked 8 laps in 6 minutes i.e. 240 meters, and rate of perceived exertion was 9 in 6-20 Borg scale. Written consent is taken from the patient to publish his case.

RESULTS AND DISCUSSION

- The patient 6-minute walk distance pre rehabilitation was 240 meters and post-rehabilitation, the total 6-minute walk distance increased to 390 meters as shown in Fig 1
- Rate of perceived exertion was 11 in 6-20 Borg scale pre rehabilitation and post-rehabilitation rate of perceived exertion was 7 as shown in Fig 2.
- The quality of life improved as compared to pre-rehabilitation.

- Pre rehabilitation scores of SF-12 were physical score: 37.9548, mental score: 43.25045 and post-rehabilitation scores of SF-12 were increased to, physical score: 44.61544, mental score: 56.83795 as shown in Fig 3.

Figure 1: Pre and Post 6-minute walk test

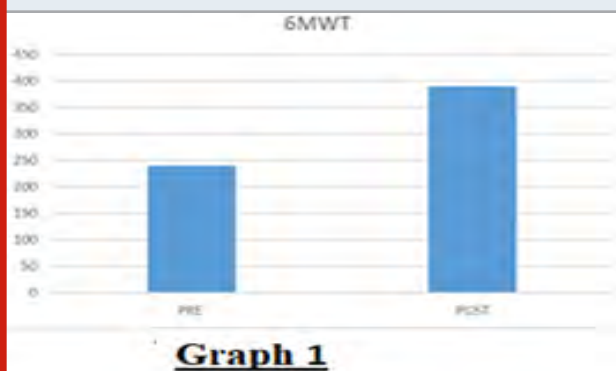


Figure 2: Pre and post rate of perceived exertion

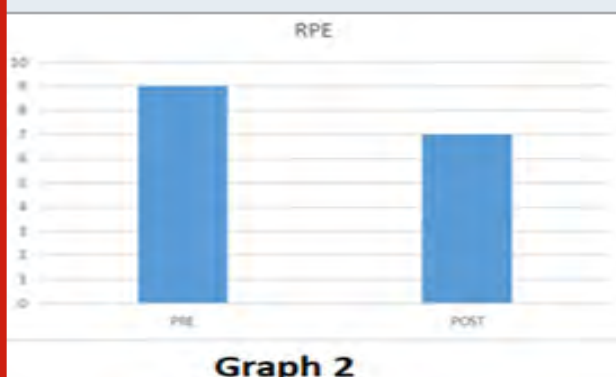
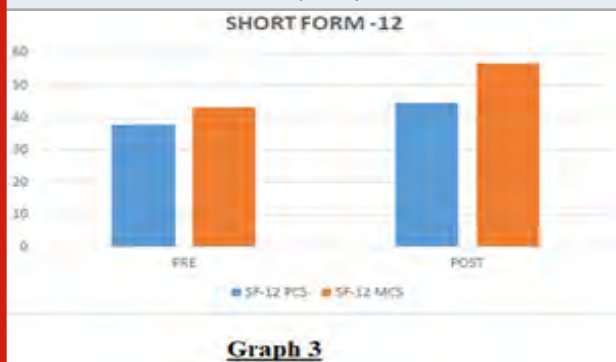


Figure 3: Short form-12 Pre and Post Physical Score (PCS), Pre and Post Mental Score (MCS)



Individuals with stable rheumatic heart disease have reported having reduced exercise tolerance and quality of life, which can be pronounced by post covid sequelae (Melo et al. 2018; Sanyaolu et al. 2020).

Detailed Exercise Program: In the current case report, we observed 61 % lower 6-minute walk distance as compared to the predicted 6-minute walk distance as per age, height

& weight. 6-minute walk distance significantly improved after the post covid exercise-based rehabilitation by 150 meters, which is more than the minimal clinical difference change (Wise and Brown 2005). Similar results have reported for improvement in 6-minute walk distance in post-covid-19 patients with co-morbidities. The rate of perceived exertion also significantly improved after the post covid exercise-based rehabilitation by 2 points, which is more than minimal clinical difference. Similarly, Claudio et al. also reported improvement in the rate of perceived exertion after inpatient rehabilitation in post-intensive care unit COVID-19 patients (Ries 2005; Medica et al. 2021). Quality of life has also significantly improved, which measured using the SF-12 scale. SF-12 improvement was 6.71 for the physical score and 13.63 for the mental score, which is more than its minimal clinical difference (Parker et al. 2013; Tozato et al. 2021).

There was not much improvement in physical score pre and post-rehabilitation, but adequate improvement in mental score by 13.63 points. Hence, there was an overall improvement in quality of life. Studies show improvement in quality of life after post-covid exercise-based rehabilitation. Wenguang Xia et al. also discussed improvement in SF-12 scored post rehabilitation (Li et al. 2021). Exercise training did not only increase the exercise capacity of the patient but also improved the quality of life as well (Luoto et al. 2012). The World Health Organization (WHO) has declared a global coronavirus pandemic for 2019. Since then, the disease has infected over 12 million people in countries worldwide. Several studies, including one meta-analysis, have reported cardiovascular involvement along with COVID-19 disease. The most common comorbidity in COVID-19 patients is cardiovascular disease. RHD and COVID-19 have similar clinical symptoms, including fever, chills, dyspnea, tiredness, cough, and myalgia in both cases (Murdoch 2009; Tozato et al. 2021).

However, in developing countries, COVID-19 was seen in conjunction with Rheumatic heart disease which is a sequela to infective endocarditis, and initial screening might well be ambiguous. Various studies have reported physiotherapy rehabilitation is beneficial in post COVID patients (Sheehy 2020; CURCI et al. 2020; Demeco et al. 2020). In our case report we have discussed a rehabilitation of an unusual combination of RHD and COVID-19 infection and to our knowledge rehabilitation of similar combination have not been published. Physiotherapy Rehabilitation could be a key strategy for reducing the impact of COVID-19 on an individual's health and function. It can also reduce Intensive Care Unit -admission related complications, such as Post Intensive Care Syndrome (PICS), Intensive care unit acquired weakness (ICUAW). The aim of rehabilitation is to improve recovery and reduce disability in patients with COVID-19. As of now, no studies have been reported with such unusual combination of RHD and COVID-19 given a physiotherapy rehabilitation protocol, hence this case report will manifest a stepping-stone to form a standard COVID Rehabilitation protocol for rheumatic heart disease patients being affected with COVID-19 (Barker-Davies et al. 2020).

Table 1

	Aerobic	Resistance
Frequency	4 Days Per Week	2 Days Per Week
Intensity	Mild To Moderate 40-59% Of Hrr/ 12-13 Of Rpe	60-70% Of 1rm
Time	More Than 30 Mins Per Day (Min 10 Min Of 1 Bout)	2-4 Set Of 8 -12 Reps Of
Type	Cycling And Treadmill	Each Of Major Muscle Group Multi Gym And Free Weights

Table 2

Warm up 10 minutes 10 reps each	Cool down 10 minutes 30 sec hold x 3 reps each	Breathing exercises 10 reps x 2 set	Upper-limb strengthening 10 reps x 2 set	Lower-limb strengthening 10 reps x 2 set
Neck movements: flexion- extension Side flexion (right-left) Rotations (clockwise and anticlockwise) <u>Shoulder</u> : elevation and depression Rotations (clockwise and anticlockwise) Flexion-extension Abduction-adduction <u>Elbow</u> : flexion-extension <u>Wrist</u> : rotations (clockwise and anticlockwise) Flexion-extension Trunk: rotations (right-left) Side bending (right-left) <u>Pelvis</u> : rotations (clockwise and anticlockwise) <u>Hip</u> : flexion and extension Abduction -adduction Knee: flexion-extension Ankle: rotations (clockwise and anticlockwise) Dorsiflexion Plantarflexion	Stretching of forearm flexors and extensors, biceps, triceps, trapezius, shoulder capsule, quadriceps, hamstrings, gastrosoleus.	Diaphragmatic breathing Thoracic mobility exercises Pursed-lip breathing in dyspnoea relieving position Segmental expansion exercises	Wrist Flexors Extensors <u>Elbow</u> Flexors (biceps) Extensors (triceps) <u>Shoulder</u> shrugs (upper trapezius) Flexors (anterior deltoid, coracobrachialis, pectoralis major) Abductors (middle deltoid, supraspinatus,) External rotators (posterior deltoid, infraspinatus, teres minor) Seated row and latpulldown (Latissimusdorsi) <u>Chest</u> protractors (pectoralis major) Retractors (rhomboids major and minor)	Squats (quadriceps and gluteus Maximus) Lunges (quadriceps and gluteus Maximus) Calf raises (gastrocnemius and soleus) Leg curls (hamstrings) Dynamic quads (quadriceps) Hip- flexors (ilio-psoas) Extensors (gluteus maximus) Abductors (gluteus medius and minimus) Adductors (adductor longus, brevis, magnus)

CONCLUSION

The findings of this case report concluded that there was a definite improvement in the walking distance and quality of life of the patient after physiotherapy rehabilitation. There was improvement in six-minute walk distance by 150 meters and rate of perceived exertion was also improved from 9 score to 7 in 6-20 borg scale post one month. In comparison to pre-Rehabilitation, SF-12 improved the quality of life in both physical and mental score.

Ethical clearance: Institutional ethics committee clearance was undertaken.

Ref. No. EC/NEW/INST/2019/377/46

Informed Consent: An informed consent was taken from the patient prior to collection of data. All necessary information was given to him and doubts were cleared.

Source of funding: self

Conflict of Interests: Authors declare no conflict of interests to disclose.

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Environmental Communication

A Review of Microplastics Risk Assessment in the Coastal Environment

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ABSTRACT

Pollution from microplastics has recently become a prevalent threat to the ecosystem. Microplastics with a dimension less than or equal to 5 mm are smaller. There are many ways that microplastics can reach the atmosphere. By various mechanisms, the breakdown of macro plastics will happen. Chemical degradation, tire abrasion, is the most common forms of degradation. Microplastics (MPs) pollution in the coastal and marine ecosystem is currently a global problem. Transferring MPs from land to sea and allowing them to enter the food chain has a direct negative impact on marine life and human health. The combined toxicity effects of MicroPlastics (MPs) and other contaminants in marine environments, as well as their toxicity effects and mechanisms based on a variety of environmentally important test organisms, were also covered in this study.

KEY WORDS: AQUATIC ANIMALS, MICRO PLASTIC, MARINE ENVIRONMENT, POLLUTION.

INTRODUCTION

Microplastics in the atmosphere of the ocean: Microplastics (MPs) are increasingly infiltrating the climate, according to accumulating evidence. Scientists, politicians, and the general public all over the world are debating the role of MPs. It's alarming to see how quickly Microplastics (MPs) are assimilated into the global culture. Marine environment ends up MPs methods (Stolte et al. 2014). MP particles have been found all over the country, from the coast to the interior. The origin and place of sources, as well as the local atmosphere, all contribute to the formation of MPs. MPs may come from a variety of places, such as unmaintained plastic waste on land or at ocean, goods transfer leakage, fishing accessories, waste water treatment plants, and so on. A variety of factors affect the distribution of Microplastics MPs in the atmosphere. Microplastics MPs are transported throughout the atmosphere through a variety of processes (Hidalgo-ruzet 2012; Karrman et al. 2016). One consideration is the buoyancy of plastic polymers; for example, since their density is lower than that of water, PE and PP float on the water surface. PVC and PET, on the other hand, have higher densities than water and are thus found

under the water's surface. Microplastics MPs' distribution in the atmosphere is determined by their particle size (Karrman et al. 2016).

Occurrence in beach sediments: MPs have been present in sediments since the late 1970s, according to studies. Spain, New Zealand, Canada, Bermuda, and Lebanon were among the first countries to make observations. This aids in comprehending the global distribution of MPs since the late 1970s (Lisbeth et al. 2015). MPs reach the marine environment primarily as a result of plastic litter from various sources. According to Karrman et al. (2016), the total amount of plastic waste produced by coastal countries worldwide in 2010 was 192 tons, with 2–5% of that being mismanaged and ending up in the ocean (Karrman et al. 2016).

MPs' effect on aquatic species: MPs will interact with their surroundings when they arrive in the aquatic environment through various carriers, and their biological fate and mobility will be determined by their size, shape, and other characteristics. MPs in water have a major impact on aquatic life. Marine animals absorb MPs, and this is the main pathway. MPs are contained in sediments, so animals that eat detritus are likely to be affected. The number of laboratories works recently investigated in MP ingestion by marine biota. An accede can be the primary cause of MPs

Article Information:*Corresponding Author: b.balasu84@gmail.com

Received 15/10/2021 Accepted after revision 15/12/2021

Published: 31st December 2021 Pp- 1422-1427

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.8>

particle ingestion in certain circumstances (Wright 2013; Karrman et al. 2016).

It may also cause inflammation and clog the digestive tract. MPs ingestion has been discovered in a number of marine species as a result of laboratory studies (Auta 2017). Through the use of a variety of applications, the presence of colorants in species indicated an anthropogenic origin. As a result, coastal areas are a hotspot for MP emissions, with filter-feeding bivalves being the most vulnerable. As a result, more research is needed to better understand MP accumulation rates and residence times across food webs. The overall distribution of approximately value (Fig 1) micro plastics studies have been conducted across the world (Silva et al. 2018).

Figure 1

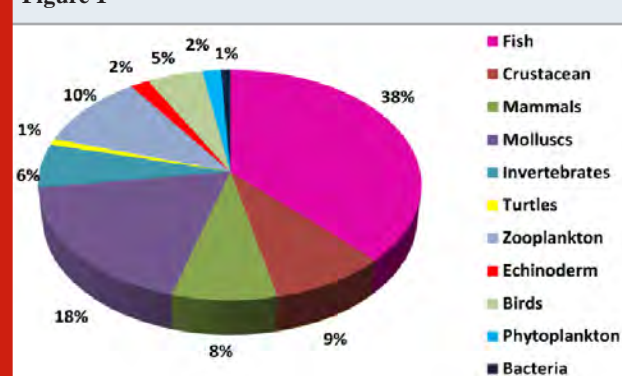


Table 1. Sampling equipment used to collect MPs Matrix.

Matrix	Equipment
Water	The trawl with rectangular opening and a net bag
Surface:	The bongo net using for collection
Mid water level	
Sediments	
Bottom sediments	Collection through box corer
Surface samples	The iron spoons using for collection
Seabed samples	core or bottom trawl
Biological tissue	Dissection & ejection

Sample Collecting Methods: This section describes the different sample collection methods used by many researchers during their study. The processing and handling of MP samples is a delicate procedure. As a result, determining the origins of contamination is important. Items such as synthetic fibbers, gears, clothing, and other unwanted items may contaminate Microplastics samples. The equipment to be fully carefully cleaned and to be sealed with polymer free clothing can be worn though for contamination prevention. The following methods of Microplastics sample collection are in the table (Silva et

al. 2018).

Water sample: A variety of nets are used to collect water samples. Neuston nets, manta trawls, and bongo nets are the most commonly used nets. The tail end of these nets has a sample collection bin. The microplastics sample collection nets ranges from 53m to 3mm were used (Silva et al. 2018).

Sampling of Sediments: This is a screening technique in which microplastics are obtained from beaches or the bottom of lakes. It is simpler and more convenient to obtain samples on beaches. During the sampling process, there is no one-size-fits-all approach. It is entirely dependent on the person and the sampling location. To collect samples from beaches, most researchers tend to use tidelines and sampling depth (Mai et al. 2018).

Biota sampling: The marine animals like fish, sea gulls, sea cow and other planktons are consumed microplastics, the biota sampling assessment use full for concentration of micro plastic level. Animals' digestive tracts are used to collect the samples. Laboratories dissect the digestive tracts of marine creatures to collect samples (Mai et al. 2018).

Sample separation and purification: Floatation is a term used to describe the process of Separation and purification of samples can be accomplished in a variety of ways. Density separation is the most common method for sampling microplastics. It is used to distinguish low-density particles such as sand, dirt, and sediments from higher-density particles. Microplastics like PP and PE have a lower density than seawater (1.10 g/cm³). To differentiate microplastics from higher density microplastics, such as PVC (1.40 g/cm³) or higher density microplastics, various density solutions are used. Saturated NaCl is widely used in this system due to its nonhazardous properties. It's also reasonably priced and easily available. The use of NaCl solution as a density separator has several drawbacks. During the extraction process, samples with a higher density (PVC) cannot be completely collected (Mai et al. 2018).

During the flotation method, another commonly used solution is ZnCl₂, which is effective in extracting almost all microplastics of various densities. The density of the most common products, such as PP and PE, is lower than that of ZnCl₂ and CaCl₂ solutions. The use of ZnCl₂ has the drawback of being harmful. As density separators, other flotation solutions are used, but the overall process remains the same (Stolte et al. 2014; Mai et al. 2018).

Sample purification: The flotation process is ZnCl₂ solution widely used, which is efficient in removing almost all microplastics of various densities. Most popular products, such as PE and PP, contain a lesser density than CaCl₂ and ZnCl₂ solutions (Stolte et al. 2014). The use of ZnCl₂ has the drawback of being harmful. As density separators, other flotation solutions are used, but the overall process remains the same. According to a recent analysis, vegetal material such as algae, seagrasses, and various small residues are abundant in microplastics samples collected from beaches (Herrera et al. 2018).

These materials can be sorted by sight or by sieving, but the remaining tiny residue cannot be sorted by sight. There has been a breakthrough in the removal of residues from microplastics samples, which takes less time and is more likely to extract all of the small vegetal residue from the microplastics sample. The method is based on a five-step digestion process that includes the use of chemicals such as HCL, NaOH, KOH, and H₂O₂, as well as a density separation process using 96 percent ethanol. When it comes to extracting vegetal pollutants from microplastic samples, the digestion method is extremely efficient (Herrera et al. 2018).

Digestion of samples: The most of samples are chemical or enzymatic digestion to break down the organic matter. Hence a chance of damaging plastics due to deterioration and mechanical friction and loss due to heating. The polymer detection system can be used later depending on the detection. The clean water samples only can be filtered directly, for example glass fiber or aluminum oxide filters (Herrera et al. 2018).

Acidic digestion: HNO₃ is one option for removing organic compounds. When compared to digestion in HCl, H₂O₂, and 324 NaOH, the degradation of biogenic compounds was between 94 and 98 percent. However, Avio et al. (2015) demonstrated that polymers and PE dissolving and agglutination occurred. HCl is not recommended because it does not fully kill organic matter and therefore is ineffective (Cole et al. 2011; Avio et al. 2015; Herrera et al. 2018).

Alkaline digestion: The NaOH or KOH solution can be used for sample digestion process (Cole et al. 2011). The 1M NaOH is high efficiency of 90% digestion. While increasing the temperature and morality to achieve the effective digestion process, but the use of 10 M NaOH degraded many polymers, including PC, cellulose acetate (CA), PET, and PVC (Dehaut et al. 2016; Herrera et al. 2018).

Oxidizing digestion: The efficient oxidizer H₂O₂ for removing organic material. The polymers are slightly getting more transparent, smaller or thinner while using 30% H₂O₂. However, after a seven-day digestion with 30% H₂O₂, only 70% of the 343 microplastics were removed. This was most likely due to the development of foam and, as a result, a material loss. PE and PS 345 were unaffected by a 15% H₂O₂ solution. After seven days of use of a 35 percent H₂O₂ 346 solution and a time span of seven days, only 25% of the organics were killed, according to another report. Frias et al. (2010) suggest using 10% H₂O₂ with an exposure period 348 of 18 hours due to the heavy reaction of plastics with 347 high concentrated H₂O₂ solutions (Nuelle et al. 2014; Herrera et al. 2018; Renzil 2019).

Enzymatic degradation: In certain trials, enzymes were used to remove organic matter and reduce a portion of the 352-biological tissue (Loderet et al. 2015). Cellulase 353 (> 30 U/ml), lipase (> 15,000 U/ml), chitinase (> 40 U/ml), and protease (1,100 U/ml) in technical grade were used to incubate microplastics and inorganic content (Cole et al. 2011; Loder et al. 2015; Renzil 2019).

Density separation: Many research (see compilation in review papers) use density separation. It's often used to separate microplastics from soil or other inorganic matter that didn't get digested by enzymes or chemicals. A high concentrated or even saturated salt 369 solution is mixed with the sample and shaken (Loder et al. 2015; Renzil 2019).

Water litter has an impact on oceans and marine life, according to Renzil (2019), posing a danger to natural populations of pelagic fish species at the base of the marine food chain. In the years (2013–2014), marine litter and microplastics were measured in the stomach contents of *Sardinia pilchardus* and *Engraulis encrasicolus* on a seasonal basis. In the Adriatic Sea, some planktivorous species are of great ecological and commercial value. Data was compared to potential factors that could influence ingested amounts, such as organisms, sampling season, biometry, and animal sex (Renzil 2019).

Almost all of the tested samples (80 organisms for each species) contained marine litter and microplastics (over 90% of samples from both species), but no meso- or macroplastics were found. The average number of registered items per person was 4.63 (*S. pilchardus*) and 1.25 (*E. encrasicolus*). Sardines had a higher percentage of microplastics of smaller sizes than anchovies. In sardines, sex, the Gastro Somatic Index, and the sampling season had no effect on the amount of ingested litter; however, anchovies showed differences related to both animal sex and the dominant color of ingested materials, with black and blue colors predominating (Mai et al. 2018).

Identification by chemical composition: The molecular structure of plastic polymers is used in this method to establish the polymer's origin (Bergmann et al. 2015). Furthermore, this process provides a simple way to identify samples using polymer recognition methods such as FTIR, Raman analyses, pyrolysis, GC, and MS (Mai et al. 2018).

Pyrolysis- GC/MS: This is a method for detecting microplastics in the atmosphere that is based on experience. The chemical composition of microplastic particles can be determined using thermal oxidation (Bergmann et al. 2015). The main advantage of this approach is that it can simultaneously analyze both the polymer shape and the organic additives present. Environmental samples are analyzed using gas chromatography and mass spectrometry (Mai et al. 2018).

FTIR spectroscopy: The polymer composition and origin of microplastic particles in samples can be determined using this method (Bergmann et al. 2015). Furthermore, it enables accurate particle detection using their unique IR spectra. This approach has the advantage of causing molecular vibrations when interacting with the sample. In the case of a plastic sample, this technique makes obtaining extremely complex IR spectra with distinct band patterns much simpler. Based on the oxidation level observed, FTIR spectroscopy also provides information on the weathering of sampled plastic particles (Bergmann et al. 2015).

Raman spectroscopy: One of the most dependable analytical techniques for determining the chemical composition of unknown plastic fragments is chromatography (Hidalgo-Ruz et al. 2012). Raman spectroscopy has the advantage of being able to analyze small samples (1 m) and providing a better response to non-polar functional groups than other analytical approaches (Mai et al. 2018).

Routes of exposure: Microplastics are a significant pollutant in the environment. Ingestion of microplastic-containing foods, inhalation of microplastics in the environment, and dermal contact with these particles contained in cosmetics, textiles, and dust are all ways for microplastics to reach the human body (Cox et al. 2019).

Ingestion: Ingestion is thought to be the most common way for humans to come into contact with microplastics (Galloway et al. 2015). Microplastics are expected to be consumed in amounts ranging from 39,000 to 52,000 particles per person per year based on food consumption (Cox et al. 2019). Particles may enter the gastrointestinal system through infected foods or mucociliary clearance after inhalation, causing inflammation, increased permeability, and changes in gut microbe composition and metabolism. Microplastics have been found in mussels, commercial fish, and other foods (Salim et al. 2013; Neves et al. 2015; Li et al. 2016; Cox et al. 2019).

Inhalation: Synthetic textiles, abrasion of materials (e.g., car tires, buildings), and resuspension of microplastics in surfaces are all sources of microplastics in the air. Outdoor concentrations of 0.3–1.5 particles m⁻³ and indoor concentrations of 0.4–56.5 particles m⁻³ (33 percent of polymers), including inhalable sizes, were one of the first measurements of microplastics in the air (Dris et al. 2017). Individual inhalation of 26 to 130 airborne microplastics per day have been estimated (Prata 2018). A male individual with light activity inhales 272 microplastics every day, according to air sampling using a mannequin (Vianello et al. 2019).

Dermal contact: While nanoplastics have been proposed, dermal contact with microplastics is thought to be a less significant route of contamination.

Microplastic toxicity pathways: Microplastics, which were once thought to be harmless particles with no toxicity, are now thought to be potentially harmful to species, depending on exposure and sensitivity. Microplastics' large surface area may trigger oxidative stress, cytotoxicity, and translocation to other tissues (Galloway 2015; Anbymani and Kakkar 2018).

Oxidative stress and Cytotoxicity: Oxidative stress may be caused by an overabundance of antioxidant responses. Because of their high surface area, release of oxidizing species adsorbed to their surface (e.g., metals), or reactive oxygen species released during the inflammatory response, microplastics can be at the root of this oxidative stress (Kelly and Fussel 2012; Valavanidis et al. 2013). For example, oxidative stress has been observed in zebrafish

and mice after exposure to microplastics (Lu et al. 2016; Deng et al. 2017).

After injection of a polypropylene (PP) prosthesis, an acute inflammatory reaction culminates in the release of oxidants (e.g., hydrogen peroxide, hypochlorous acid), causing the polymer to degrade, hydrolyze, crack, and additively leach, resulting in a positive feedback loop of free radical development and exposing possible pathways of plastic removal from the organism (Anbymani and Kakkar 2018).

Neurotoxicity: Contaminant use has been linked to neurotoxicity and neurodegenerative diseases. In vivo neurotoxicity has been observed in reaction to particulate matter exposure, most likely as a consequence of oxidative stress and activation of the brain's microglia (immune cells) caused by direct contact with translocated particles or the involvement of circulating pro-inflammatory cytokines (from other sources) (Vianello et al. 2019).

Microplastics as vectors of microorganisms and potentially toxic chemicals: Microplastics can pose a chemical and biological risk in addition to particle toxicity.

Within the body, monomer and additives from the microplastics matrix can leach, exposing tissues to chemicals such as phthalates and bisphenol A, which are known as endocrine disruptors – substances that interfere with endogenous hormones even at very low concentrations (Cole et al. 2011). Microplastics, in addition to their constituents, have a large surface area, making them susceptible to acting as vectors for microorganisms or chemicals they come into contact with. For instance, persistent organic pollutants (POPs) have been identified in microplastics recovered from the environment, including polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) (Vianello et al. 2019).

CONCLUSION

The findings of the present review are risk of microplastic ingestion was evaluated for the various species living in these habitats. Potential trophic transport routes for bioaccumulation of microplastics have been proposed to fill existing knowledge gaps, with a specific emphasis on routes involving some at-risk species. More research in the Indian scenario is required to fully assess the impact of bioaccumulation in specific species and on the coastal ecosystem as a whole. Given the prevalence of microplastics in aquatic habitats and their inability to be removed, we believe it is not too late to combine research on plastic alternatives. Furthermore, immediate steps should be taken to avoid the entry of plastics into the world.

ACKNOWLEDGEMENTS

This study was financially supported by the Government Arts College and the Centre for Advanced Study in Marine Biology, Annamalai University, Tamil Nadu, India.

Conflict of Interests: Authors declare no conflict of interests to disclose.

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Biomedical Communication

An Updated Review on Nanomaterials for Biomedical Advancements: Concepts and Applications

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ABSTRACT

The sphere of Nanotechnology encompasses most of our lives and houses biomedicine and biomedical advancements. Nanoparticles owing to their minuscule sizes and due to various physicochemical and electrical properties have been exploited in pharmaceutical industries, agriculture, packaging, cosmetic, food industries. Nanomedicine is a laboratory-designed molecular-level pharmaceutical material that has revolutionized diagnostic techniques and therapeutics. Nanoscience and nanotechnology and their wide applications have become spread field worldwide because nanomaterials have novel and unique properties. Nanotechnology involves understanding and manipulating materials normally in the size range of 1 to 100 nm, where they show completely novel physicochemical properties from their bulk counterpart. The capacity to study compounds at the molecular level has aided the hunt for materials with exceptional qualities for medical applications. Nanotechnology in recent days is applied in the designing of nano biosensors. Nanobiosensors are biological molecules immobilized onto the surface of a signal transducer. The application of nano biosensors in the field of disease detection has increased in recent years which has influenced in research of cancer and biosensing. Due to the high surface area of nanoparticles, they are important in the production of nano biosensors with high levels of sensitivity and diminish the response times. However, a comprehensive review regarding the type, mode of function, and their application in various diseases is missing. Nano Deterministic lateral displacement technology that provided exosome splitting based on size differences has resulted in providing the much-needed boost to cancer research. The time taken for cancer screening has been reduced drastically. This review aims to describe the utilization of nano deterministic lateral displacement technology, nano biosensors, and their applications in certain disease diagnoses.

KEY WORDS: BIOMEDICAL APPLICATIONS, CANCER, NANOBIOSENSORS, SARS-COV-2, SIGNAL TRANSDUCTION.

INTRODUCTION

There are several definitions and applications for nanotechnology. All definitions, however, emphasize the design and evolution of highly ordered bottom-up nanostructured materials that respond to specific stimuli. Surface chemistry and physics "tune" the uses of nanoscale materials (Fang 2018; Chaturvedi et al. 2019). The atom concentration on the surface of these systems can account

for up to 90% of their total mass, resulting in increased reactivity. In present days, visceral imaging technology and morphological diagnosis of tissues or cells are helpful in the primary detection of tumors or cancer. The frequently used diagnosis techniques, such as X-ray, magnetic resonance imaging (MRI), computed tomography (CT), endoscopy, and ultrasound, can only examine cancer only when there is a visible change to the tissue. For detection of cancer, nanoparticles are used to diagnose biomarkers of cancer for example cancer-associated proteins, tumor cells, and exosomes (Zhang et al. 2019).

An important advantage for the application of tiny particles for cancer diagnosis is based on the large surface

Article Information:*Corresponding Author: banerjeepradipto.123@gmail.com

Received 17/09/2021 Accepted after revision 18/12/2021

Published: 31st December 2021 Pp- 1428-1434

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.9>

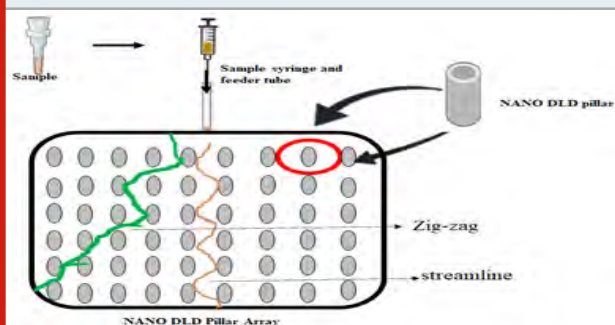
area to volume ratio compared to bulk materials and with this characteristic, surfaces of tiny particles can be compactly wrapped with antibodies, peptides, and other moieties (Zhang et al. 2019). The nanoparticles along with quantum-dots technology and gold nanoparticles and their combinatorial applications can be used in the diagnosis of cancer (Chandrasekaran et al. 2019).

Nanotechnology products have become increasingly useful in biomedicine and have led to the advent of a hybrid science named nanobiotechnology. Nanomaterials are used extensively in nanobiotechnology, including diagnostic, medication delivery systems, prosthetics, and implants. Because most biological processes are nanoscale, nanoscale materials fit nicely with biomedical equipment (He et al. 2018; Zhang et al. 2019). Inorganic and metal nanoparticles, carbon nanotubes, liposomes, and metallic surfaces are popular materials utilized to build nanotechnology goods. The above-mentioned keywords were searched on PubMed and a total of 45 papers were included in this mini-review. We focused on the use of nanoparticles in cancer detection, Nano-DLD technology, biosensors, and the assessment of toxicity levels in an *in vivo* model in this review study.

Nano-DLD (Deterministic Lateral Displacement)

Technology: One of the most essential factors in combating diseases like cancer is early detection. As was observed in Hawkes (2019), one-year survival for patients with colorectal cancer dropped from 97.7% when detected at Stage 1 to 43.9% when detected at stage 4. Classical techniques of early detection fall short due to poor tumor specificity in addition to high toxicity. Besides, most of the approaches target rectification of damage rather than proactive screening (Hawkes 2019).

Figure 1: Nano DLD pillar array separates particles based on the fluidic forces.



Lab on a chip technology has allowed easy separation of exosomes from body fluids (Chandrasekaran et al. 2019). The introduction of NanoDLD Pillar Array Technology allows splitting of exosomes based on their size. Upon entry of the fluid containing exosomes into the NanoDLD chip, smaller particles tend to move in a streamline manner whereas, bigger particles are deflected owing to their size (Smith et al. 2018). This technique allows for a gradient separation and the separated exosomes can be analyzed for biomarkers. Exosomes are nanoscopic particles that are released by both normal and tumor cells. These are powerful biomarkers, as they contain DNA, RNA, and proteins of the cell from which they are released. Due to their extremely

small size, the traditional methods of isolation of exosomes fail to produce a high yield (Zhang et al. 2017; Wu et al. 2020; Dash et al. 2021).

Nano DLD pillar array separates particles based on the fluidic forces and the strategic placement of the pillars within the chip. Particles that have a smaller diameter than the space between two adjacent pillars will follow a streamlined path whereas those with larger diameters will follow a zig-zag route. To optimize a critical diameter, essential for optimum separation of particles, separation of whole-blood components was attempted without dilution. It was seen that when the pillars were tilted at a certain angle, optimum separation was achieved. However, this model fails to address the displacement of particles caused due to anisotropic permeability effect (Vernekar et al. 2017; Wu et al. 2020; Dash et al. 2021).

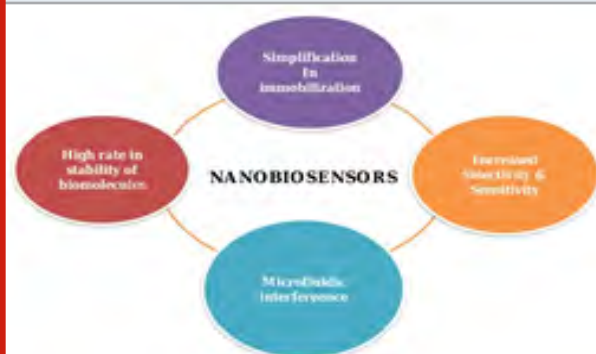
This issue was resolved by a comprehensive model that takes into account the displacement of small particles as well. This theory puts forward the notion that the trajectory of particles in the pillar array is largely dependent on the arrangement of the pillar arrays (Kim et al. 2017). To evaluate Nano DLD pillar technology as a point of care diagnostics, successful detection of albumin proteins at a concentration as low as 10ng/ml and nano-sized polymer vesicles at concentrations of 3.75ng/ml were successfully detected. This technology thus obviates the requirement of fluorescent labeling. The results obtained were in line with detection studies done with microcantilevers (Zeming et al. 2018).

The concentration of particles in the fluid plays a pivotal role in obtaining efficient separation. While working with blood, in the absence of anti-coagulating agents, clogging is a common issue in the microchannels, which ultimately renders the NANO DLD pillar array unusable. However, it was observed, that when the flow rate was controlled at 1ul/min and a voltage of 20V was passed as an anticoagulant, satisfactory purity levels were obtained up to 12 hours although the plasma purity dropped. In the absence of such voltage, the Nano DLD pillar array was unusable after 4 hours (Kang et al. 2018; Dash et al. 2021).

Nanobiosensors: Nanotechnology has highly influenced the field of biosensors, as their increased rate of sensitivity and selectivity. The emergence of nanotechnology design the materials to be prepared and fabricated in mini architecture at a tiny scale, producing tiny particles for successful assays with the integration of microfluidics devices (Chen et al. 2018). A biosensor is an analytical device that puts together a biologically active component with a signal transducer that can generate a computable signal tantamount to the concentration of chemical elements in any sample. Nano biosensors incorporate nanotechnology with biosensors (Jarockyte et al. 2020; Kveton et al. 2020; Sharifi et al. 2020). Apart from this, nano biosensors played a key role in the detection of breast cancer as a portable device and also in the field of clinical medicine. The interrelationship of biomarkers and nanomaterials is efficient for the advancement of the performance of nano biosensors for all categories of diseases and also for treatment (Sharifi

et al. 2020). Some of the major nano biosensors can be categorized based on whether nanoparticles, nanotubes, or nanowires have been used in the fabrication of the nano biosensors (Moon et al. 2020).

Figure 2: Application of nanotechnology in the advancement of nano biosensors



Nanoparticle-based biosensors include bulk acoustic wave devices (Liu et al. 2020). These devices use the acoustic properties of the analytes and amplify them to aid in detection. Nano biosensors fabricated with magnetic nanoparticles produce a magnetic flux that is detected under a microscope (SQUID microscope) (Reichert et al. 2018; Forsythe et al. 2018; Liu et al. 2020). In this technique antibodies against the epitope are attached to superparamagnetic particles and the magnetic flux is detected under the SQUID microscope. It was further reported that the unbound particles contributed very little to no noise in detection, thus a line can be drawn between bound and unbound magnetic labels. One of the most abundant utilization of nanotube-based biosensors is glucose biosensors that utilize carbon nanotubes and immobilizes glucose oxidase. Glucose oxidase breaks down glucose in the blood to gluconic acid, this is coupled with a redox reaction in the mediator that results in a release of electrons which are in turn detected by the electrode (Merinopoulos et al. 2020).

Nanoparticles made of metals are used to enhance detection along with sensor function. Further, they have been used to develop nano biosensors as their properties stand on principles of detection, therefore; they have been designed to perform as glucose biosensors. Nanoparticles mixed with solution suspension are then applied to sense glucose in various methods based on optical and electrochemical characteristics. Various researchers in the past have concentrated on the magnetic properties of nanoscopic materials employed in biomedical applications, such as immobilized enzymes for biosensor development. According to previous studies, graphene is a specialized sort of glucose biosensor that has generated encouraging results. They made a biosensor out of graphene and chitosan, which they drop-coated onto a glassy carbon electrode. Metal nanoparticles produced with graphene were subsequently employed as glucose biosensing with successful statistical findings (Lu et al. 2020).

Nanotechnology and SARS-CoV-2: The recent outbreak of the global pandemic that took the world by surprise

was found to be caused by the Severe Acute Respiratory Syndrome Corona Virus2 (SARS-CoV-2). Nanotechnology has great application in the development of COVID-19 drug delivery as there are high advantages like the small shape and size and morphology of the nanoparticles (NP) helps the drug delivery to invincible sites and diminishes the immune response by reticular endothelial cells. Nanoparticles have a large surface-to-volume ratio which helps drug docking and loading. Though the nanoparticles can pass through cell membranes mostly anionic and it is only happening due to surface charge modification. Nanotechnology in combination with large porous micro-particles (LPMP), solid lipid microparticles (SLMs) can be used to deliver drugs and can be adapted at antiviral drug therapy (Bhavana et al. 2020; McGrath et al. 2020; Zhu et al. 2020). The current technique for the detection of the virus is through reverse transcription polymerase chain reaction which is highly time-consuming given the rate of progression of the disease (Wong et al. 2020; Bhadra et al. 2020; Lu et al. 2020).

A novel technique using carboxyl polymer-coated magnetic nanoparticles allowed easy extraction and detection of SARS-CoV-2 RNA through reverse transcription-polymerase Chain Reaction. This technique combines both the lysis of viral particles along RNA binding and further reduces the time required for detection. The polymer-coated magnetic nanoparticles have high specificity for RNA binding, thereby producing a high throughput analysis (Zhao et al. 2020). However, magnetic nanoparticles require heavy instrumentation which poses problems in the portability area, but the higher output is available when used in a laboratory setup. Such a problem can be averted when using techniques such as colorimetric or electrochemical assays (Tymm et al. 2020). However, Nanobiotechnology, over the past few decades has played a pivoting role not only in the early detection of tumorigenic cells but also in several infectious diseases. These include techniques like biochips, Nanoproteomics, Nanobiosensors, Nanoparticle-based immunoassays, etc (Ellwanger et al. 2019; Wong et al. 2020; Bhadra et al. 2020; Lu et al. 2020).

Diagnosis of Infectious disease: As per the reports published by World Health Organization on foodborne diseases, 1 out of 10 people fall ill every year (420000 die each year) from consumption of contaminated food, with children under the age of 5 being at high risk (125000 die each year) (Cissé 2019). Oral diseases like tooth caries, endodontic infections, etc are most common across the world. Prevalence of infection in the oral cavity and its effect on human lifestyle decreases the quality of everyday life which denotes an urge for effective therapy. The emergence and application of antimicrobial photodynamic therapy (APDT) on infection of oral disorder has been increased tremendously in past years (Qi et al. 2019; Lu et al. 2020).

In the United States alone *Escherichia Coli* have been reported to cause over 73,000 illnesses. Classical methods for the detection of *E. coli* require amplification to bring the sample up to the detection limit. However, antibody-conjugated nanoparticles have proven to be a highly

sensitive and rapid immunological assay for the detection of bacterial cells without the requirement of amplification. This technique immobilizes several fluorescent dye molecules on each nanoparticle together on a silica matrix. Due to the presence of several surface antigens on the bacterium, numerous nanoparticles would bind to it resulting in amplified signal output. In a different approach, nanoparticles were found to have virucidal effects, owing to their size, surface area, and charge. Their small size allows effective membrane permeability within the foreign body and thus delivers virucidal drugs to the target (Singh et al. 2017; Russell et al. 2019; Lu et al. 2020).

In the modern science of treating infectious diseases, nanomedicine is a highly accepted approach for the advancement of nanotechnological systems in the field of disease diagnosis. For disease treatment, targeted therapy is the one of most enriched approaches to deliver the accurate quantity of therapeutic molecules for a long-term cure for the disease in the human body. For this advanced technology to apply and development of safer and more effective therapeutic nanoparticles is crucial and this is the main goal of nanomedicine. With proper design and decoration of the nanoparticle surface with polyethylene glycol (PEG), acetyl groups, or protein moieties (arginine-glycine-aspartate or RGD) peptide, albumin), retention time can be altered (Shreffler et al. 2019). Last few years, the application of nanoparticle dependent vaccines has gained high acceptance in the field of vaccine technology and immunological experiments. To get the proper results in immune responses nanotechnology has no comparison (Yetisgin et al. 2020).

The nanocarriers improve vaccination administration by preventing antigens from being degraded prematurely by proteolytic enzymes, enhancing antigen presentation by antigen-presenting cells (APCs), controlling release, and being safe for human usage. It was also shown that providing viral and bacterial antigens in combination with gold nanoparticles improved immune responses in the host against influenza, immunodeficiency virus, foot and mouth disease, and TB. *Mycobacterium tuberculosis* is successfully reduced in infected rats using plasmid DNA encapsulated in gold nanoparticles that encode mycobacterial hsp65 antigen. Some research has been done using hollow mesoporous silica, nanotube, and spherical forms of carbon nanoparticles as adjuvants to improve immunogenicity and deliver protein and antigenic peptides for viral infection. Some nanoparticles are based on silica which has utilization in the introduction on their surface with a proper functional group to access target cells for vaccine administration. One of the vast applications of inorganic nanoparticles is that they have low production cost, safer administration, and high reproducibility (Pati et al. 2018; Yetisgin et al. 2020).

Nanoparticles toxicity analysis toward its *in vivo* application: The toxicity of nanoparticles increases with their size. This is partly because tiny nanoparticles are more easily taken up into the cell or even near the nucleus (Serpooshan et al. 2019). Because their cellular absorption is restricted at the same dose, larger nanoparticles may be less harmful. A

few aspects should be thoroughly investigated to assess and forecast probable nanoparticle toxicity *in vivo* applications (Chen et al. 2018; Donahue et al. 2019; Shao et al. 2020). To begin with, *in vitro* cytotoxicity studies should be utilized with caution to extrapolate predicted outcomes *in vivo* research. Nanoparticles in an *in vivo* system would be subjected to far more complex perturbations due to a large number of proteins and tiny biomolecules present. Nanoparticles can be destroyed, absorbed by phagocytic cells, or transported away from the target location by the lymphatic system because of these nearby biomolecules. Assay responses acquired in a well-controlled environment, such as a culturing plate, may not necessarily match those obtained in an *in vivo* setting. As a result, drawing any inferences from the *in vitro* assay for nanoparticle responses in an *in vivo* system will be insufficient until at least animal model investigations are completed (Cao et al. 2021).

Second, the limits of present cytotoxicity or inflammatory reactions of cells to nanomaterials should be carefully noted, and more effort should be put into developing tools for better assaying nanoparticles. Traditional tests established for chemical poisons or microparticles have been used in studies of *in vitro* cytotoxicity and the inflammatory response to nanoparticles (Lih et al. 2018). These studies don't go into detail on how specific cells react to nanoparticles. Furthermore, because cells might behave differently depending on the assays used, the examination of these test data is prone to inaccuracy. The following are the limitations of existing cytotoxicity and immune response tests for nanoparticle evaluation. For starters, cells cannot be retrieved after a single test readout (Schaerli 2018), limiting the options for tracking changes in a cell's activity over time. Second, the results of the experiments are averaged over all of the cells present.

As a result, the reactions of a single cell to the nanoparticles cannot be recorded separately from the experiment. Third, nanoparticles within a cell may interfere with the fluorescence signal generated by the test dye. Nanoparticles can also interact with dyes and/or bind to them, changing their absorption and/or fluorescence. Nanoparticles can also adsorb to proteins and other biomolecules in the cell culture medium, interfering with the particles' usual interactions with cells (Faruqu et al. 2018). Furthermore, nanoparticles can attach to cytokines generated by cells, reducing the positive signal in an assay artificially. The technique of flow cytometry is widely employed in biological response experiments, although it necessitates the removal of cells from the cell culture plate, which may affect the cells' mortality. Finally, there have been no multiplexed investigations of nanoparticles in the same well as single cells. Because of these constraints, a solid assay that overcomes the aforementioned issues with traditional assays and can examine biological responses to nanoparticles in a multiplexed, high-throughput way is urgently needed (Jeon et al. 2019).

To measure cytotoxic and inflammatory reactions to nanoparticles in a multiplexed manner, cutting-edge single-cell test approaches have been created. The multiplexed analytical approach will be employed in numerous

nanoparticle safety investigations. Time-dependent investigation of a single cell's reactions to nanoparticles may reveal the mechanism of nanoparticle toxicity; these single-cell studies will be utilized in conjunction with standard bulk experiments (Yi et al. 2019). The methodologies

outlined will enhance nanotoxicological investigations and their uses mentioned in table 1. The larger nanotechnology community demonstrating the feasibility of a high-throughput, multiplexed analytical tool for investigating the safety of nanoparticles at the single-cell level (Jonghoon and Nam Sun 2011; Gibellini et al. 2020).

Table 1. Few important nanomaterials and their applications in the field of medical diagnosis and treatment involving use in drug delivery

Nanomaterial	Applications
Calcium Carbonate Nano Particles coated with Hyaluronic Acid activated by Glucose	In Oral delivery of Insulin (Liu et al. 2017)
Low molecular weight protamine nanoparticle with paclitaxel; enzyme activated	In Glioblastoma Therapy (Gu et al. 2013)
Nanocarrier containing Histidine-4 polyamidoamine dendrimer linked with disulfide bonds to Polyethelene glycol and Transferrin	As Anticancer drug delivery. (Shi et al. 2020)
Alginate and cystamine based nanogels	As anticancer drug delivery (Xu et al. 2018)
Melanin like nanoparticles	In imaging of tumors (Zhou et al. 2020)
Nanoparticles containing hyaluronic acid, chitosan, and lipoic acid are linked together. Polyaniline based nanoparticle-containing gold nanocomposite activated through electrical signals	In Breast Cancer therapy (Mutlu-Agardan et al. 2020) In the detection of Chronic Kidney Disease (Shaikh et al. 2019)

CONCLUSION

The study suggests that the exosome nanoscopic particles used as powerful biomarkers for malignant cells, as they contain DNA, RNA, and proteins of the cell from which they are released. Nano DLD pillar technology applied in diagnostics approach and successfully detects albumin proteins in living cells. Numerous nanoparticles can bind bacterium surface antigens, resulting in amplified signal output. By using different nanomaterials the recent outbreak of the global pandemic that took the world by surprise was found to be caused by the SARS-CoV-2 coronavirus. The current technique for detection of the virus is through a reverse transcription-polymerase chain reaction which is highly time-consuming given the rate of progression of the disease. It's tough to predict the future of any key technology. On the one hand, there is a common propensity to underestimate a technology's influence and rate of development. As other kinds of nanotechnology grow more common, the prominence of nanoparticles as the most potentially harmful variety may shift. Nanoparticles are becoming more widespread, and their properties are becoming more understood.

Conflict of Interest: Authors declare no conflict of interest to disclose.

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Biomedical Communication

On the Ten-Year Success in the Application of Partial Extraction Therapy: A Systematic Review

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ABSTRACT

Hürzeler presented the socket-shield technique (SST) more than 10 years ago. The partial extraction therapy (PET), a collective concept of utilizing the patient's own tooth root to preserve the periodontium and peri-implant tissue, has been remarkably developed. PET comprises a group of novel techniques for post-extraction implant placement. Several modifications of PET and simultaneous implant placement have been presented since its inception. Since its origin, several alterations have been employed in the methodology of partial extraction of the root and the simultaneous implant placement. A repeatable, predictable protocol is needed to provide tooth replacement in esthetic dentistry. Moreover, a standardized procedure provides a good framework for clinicians to report data relating to the technique with procedural consistency. This review aims to illustrate a reproducible and systematic protocol for the PET techniques with immediate implant placement at the aesthetic zone. The most used technique is the socket-shield technique, which is potentially offers promising results, minimizing the necessity for invasive bone grafts round implants in the aesthetic area, clinical data to support this is very inadequate. The limited research data existing is cooperated by a deficiency of well-designed prospective randomized controlled investigations. The present case studies and techniques are of actual incomplete technical value. Retrospective studies published in limited records but are of inconsistent plan. At this point, it is indistinct whether the socket-shield technique will offer a stable long-time outcome or not.

KEY WORDS: PARTIAL EXTRACTION THERAPY, PONTIC SHIELD, PROXIMAL-SOCKET SHIELD, ROOT SUBMERGENCE, SOCKET SHIELD TECHNIQUE.

INTRODUCTION

Qualitative and quantitative variations, which arise in the alveolar ridge next tooth removal, can complicate the implant-prosthetic restoration. Several socket and alveolar ridge preservation systems have been developed to minimize the alveolar ridge atrophy. The tooth root can be conserved to limit bone resorption under a fixed or removable denture (Pagni et al. 2012). PET, as a socket shield technique, was first introduced by Hürzeler in (2010) and this process was first carried out on dogs, followed by a single implant placement in a human as a proof of concept (Hürzeler et al. 2010). Finally, a fabricated screw retained abutment was

placed with an out of occlusion provisional crown. Many cases followed the concept and became published (Han et al. 2018; Gluckman et al. 2018; Schwimer et al. 2019).

The concept of PET is composed of four different techniques that aim to preserve slice of the tooth in the bone, thereby minimizing the loss of the bone vasculature and periodontal ligament attachment, thus eliminating the remodeling and resorption of both hard and soft tissues associated with tooth removal. Gluckman et al. (2016a), and Shaheen (2021) found that partial extraction therapy (PET) includes root submergence (RST), socket shield (SST), proximal socket shield (PSST), and pontic-shield (PST) (BUSER et al. 2000; Abadzhiev et al. 2014; Troiano et al. 2014; Al-Dary and Al Hadidi 2015; Durrani et al. 2017; Mitsias et al. 2017; Al-Dary and Alsayed 2017; (Durrani et al. 2017; Esteve-Pardo and Polis-Yanes et al. 2020; Abd-Elrahman et al. 2020).

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Received 19/10/2021 Accepted after revision 22/12/2021

Published: 31st December 2021 Pp- 1435-1443

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.10>

These systems have provided excellent mechanical, biological, and esthetic outcomes in the hands of experienced operators with meticulous treatment planning and case selection. In addition, a modified SST was presented by (Glocker et al. 2014). Han et al. 2018 used a 1.5-mm thick shield with the most coronal portion, while Guo et al. 2018 modified the SST by placing platelet-rich fibrin (PRF) in the gap between the root fragment and the implant and found that that peri-implant tissue was well preserved by the SST without significant peri-implant tissue resorption (Aslan 2018). The most commonly used indices for the evaluation of the aesthetic dimension of anterior single-tooth implants are the pink and white aesthetic score (PES/WES) indices, and they have been used in several studies (Belser et al. 2009; Buser et al. 2013; Mangano et al. 2014a; Zhao et al. 2016). Pink esthetic score evaluates the anterior esthetic of the implant-supported single crown on seven points, including mesial and distal papilla, soft-tissue color, contour, level, texture, and deficiency of alveolar (Fonseca 2018 and Mourya et al. 2019).

It comprises 10 variables such as mesial papilla, distal papilla, curvature of the facial mucosa, level of the facial mucosa, the root convexity, soft tissue color, and texture at the facial aspect of the implant site, tooth form, volume, color, surface texture, and translucency. A score of 2, 1 or 0 is assigned to all parameters. All parameters are assessed by direct comparison with the natural, contralateral reference tooth, estimating the degree of match or mismatch (Belser et al. 2009). Based on the Kaplan–Meier survival estimator, the cumulative implant survival rate (implant-based) was high. The complications were the infection of the root portion, with suppuration and fistula formation, which occurred in four cases at 83, 51, 59, and 12 months after implant insertion) and the infection of the root associated with peri implant mucositis in 1 case (at 113 months from the insertion of the fixture (Mangano et al. 2019).

Infection of the root membrane with fistula was determined in 50% of cases the occurrence of periimplantitis that caused the loss of two implants (at 12 and 59 months after insertion). In the remaining 50% of cases, however, the implant was not affected by the infection (Gluckman et al. 2016; Siormpas. et al. 2018). The prosthetic complications were divided into minor complications, such as no treatment needed or 60 min chair time and additional laboratory costs, repositioning of a loosened abutment, and removal of a fractured abutment or fabrication of new restorations. Static and dynamic occlusions were evaluated using standard occluding papers. All prosthetic complications were carefully registered and managed if possible, during the follow-up visits. Mangano et al. (2016) and Han et al. (2018) have shown a prosthetic complication such as abutment screw loosening, abutment fracture, and/or chipping/fracture of the ceramic restorations. Al-Dary and Alsayed (2017) replaced missing maxillary 2 central incisors with zircon cantilever bridge (Abd-Elrahman et al. 2020). This review aims to illustrate a reproducible and systematic protocol for the PET techniques with IIP at the aesthetic zone and summarize the clinical outcome of this technique during the last 10 years.

MATERIAL AND METHODS

An electronic exploration was achieved to identify related research. The search was restricted to May (2010) to October (2021), at the time of gathering of the information with the resulting databases from Medline/PubMed, Cochrane, Scopus, EBSCO host, Google website, Web of Science, and Wiley Library. The search terms included “Partial extraction therapy”, “socket shield technique,” “modified SST”, “root membrane technique”, “Pontic-shield technique”, “type of the final restoration”, and “immediate implant placement”, and case report, series, and clinical studies. The study was finalized manually by evaluating the particular reference tilts of similar articles. Studies published from (2010 to 2021) were included if they met the following measures: case report, case series, prospective and retrospective studies, clinical trial study, and involves the use of PET and procedures with IIP after tooth extraction.

The exclusion criteria were clinical studies on human and follow-up not less than 3 months after implant assignment. Two review authors (Al MM and A.M.A) evaluated the title, abstract, and available text of articles documented in the electronic search and the inclusion and exclusion criteria. All published papers related to PET reports were evaluated for relevance, eligibility, and data extraction. For all type of studies, the implant osseointegration, shield exposure, shield infection, shield migration, soft tissue contour, and type of prostheses were recorded. Radiologic result for buccal and/or crestal bone loss were assessed. The selected studies were analyzed for complications and adverse effects stated by corresponding author(s).

All data were extracted, and the contents were screened by the author. Full texts of the associated studies were reviewed for further assessment. This systematic review was designed in accordance with the Preferred Reporting Items for Systematic reviews (Moher et al. 2009) with some modifications specified by recent systematic reviews published in the previous studies (Siormpas et al. 2018; Blaschke and Schwass 2020; Ogawaa et al. 2021; Magadmi 2021). The extracted data from the nominated studies were as follows: author(s) name, publication year, type of technique used, arch, region, tooth type, causes of extractions, implant placement, loading protocol, final restoration type, complications, survival rate, and follow-up period (Table 1). The quality of each involved study was evaluated by the authors (Al MM and A.M). The included articles were evaluated using the Checklist for Systematic Review, Case Reports and/or Series. Data were organized and summarized in designed tables. The mentioned variables in all collected studies in any form were summarized and analyzed (Blaschke and Schwass 2020; Ogawaa et al. 2021; Magadmi 2021).

RESULTS AND DISCUSSION

The flowchart for the selection of articles based on their eligibility for the current systematic review is presented in Figure 1. The database search across literature resulted in 561 articles related to questions raised, and these articles were gathered and analyzed. The author further separated

the publications and removed similar studies and other papers articles not correlated to the question elevated. A total of 496 studies were removed, because they are duplicates or not related to the study. By screening 65 articles, 21 studies were omitted, because they were not related to the review, leaving 44 studies (Figure 1: Flowchart). Eight studies were included for each of clinical studies and case series, while the remaining articles were case reports (28).

Variables related to PER among clinical studies or both case series and reports were presented in Table 1. The extracted items were included the author(s) name, publication year, type of technique used, arch, region, tooth type, causes of extractions, implant placement, loading protocol, final prostheses type, complications, survival rate, and follow-up period. A total of 44 articles were included in the present review, as shown in Table 1. Eight clinical studies

and eight case series were conducted between 2014 and 2021. Majority of the case reports were about SST and immediate implant placement. All cases were followed up with minimum of 3 months and extended up to 10 years. All the parameters' data are represented and arranged. Graph 1 represents the outcome of screened studies in relation to PET with immediate implant.

The highest percentage of the type of technique used. The proportion of implant loading technique (immediate vs. delayed), arch involved maxillary or mandibular arch, the place of studies applied, and the ratio of each tooth type are shown. Parameters such as causes of extraction, follow-up period, and survival rate for each study are presented in Graph 2. The details of the materials used for final prosthesis and the number of screws retained or cemented prosthesis are shown in Graph 3.

Table 1. Qualitative analysis of studies included in this review and arranged ascending

Investigator(s) /Year	Arch/ Region/ Tooth	Extraction Cause /Loading Type	Technique Type/ Pink esthetic score/ Complications	Restoration Type	Survival Rate/ Follow-up
Hurzeler et al/ 2010/ SST	Case report/ Maxilla/ Right Central Incisor	Fracture (Trauma)	SST does not interfere with osseointegration, is beneficial in preserving bundle buccal bone plate, ↓resorption post extraction.	SRCC	100% 3 Months
Abadzhiev et al/ 2014/ SST	Prospective clinical and radiological trail study	NM SST cases (10) IIP/ CIIP & Graft (10)	CIIP [12% bone loss = 5 mm]/ SST [2% bone loss = 0.8 mm] Mean CBL=0.8 mm] Soft tissue volume or quantity of attached gingiva [CIIP = 18% SST implant = 2%] Aesthetic results [SST/ 98% Perfectly and 2% Very good] [CIIP/ 50%-Perfectly, 2% VG, 10/ Compromise, 20% Bad] SST for peri-implant tissue preservation in esthetics zone	CC & PFM Cs	80% 24 Months
Glocker et al/ 2014 Modified SST	Case Series / Maxilla/ Right Central Incisor; Left Lateral Incisor; Canine	Failure RCT (3) PFM bridge	MSST → prevents alveolar ridge resorption, cost-effective and minimally invasive. MSST avoids bundle bone resorption	All Ceramic Crowns	100% 6 Months
Troiano et al/ 2014/ Root-T-Belt Technique	Case Series/ Maxilla Incisors (4), Canines (3), Canines (3)	Failure of RCT (10)	No implant immobility, peri-implant radiolucency or infection, pain, and paresthesia in the treated area. Root MT conserves all dental structure, preserves peri-implant gingival, and results in more predictable bone structure. CBL, not more 1.5 mm; Mean crestal bone loss = 1.3, 6, 0.2 mm	SCRCs/	100% 1-72 Months
AlDary and Al Hadidi/ 2015/ SST	Case report Maxilla/ Left 1 st Premolar	NM Replace PFM 23-25	SST → ultimate aesthetic outcome, natural emergence profile, preserving soft and hard tissue.	ZCs /	100% 3 Months
BUSER et al/ 2017/ SST	Case report Maxilla/ Right Central Incisor	Failure RCT	Marginal tissues of mid-facial mucosa healthy, shallow probing pockets and no bleeding after gentle probing.	ZSRC/	100% 12 Months
Huang et al/ 2017/ RMT	Case report Maxilla/ Right Central Incisor	Post-Trauma Pain	PES score of 12 /more defined as perfect. case PES 13 RMT, human histologic good after 5 years of function	Ceramic Crown	100% 6 Months
Durrani et al/ 2017/ RMT	Case report Maxilla/ Left Central Incisor	Cariou Tooth	SST&PDL-mediated RMT may be future of aesthetics with hard and soft tissue with volume maintenance.	E-Max CC/	100% 24 months
Mitsias et al/ 2017/ RMT	Case report Maxilla/ Left Central Incisor	Trauma	Histological RMT prevents bone resorption of BBP of anterior maxilla, → maintains hard soft tissues; optimizes aesthetic result	CC /	100 % 60 Months
Roe et al/ 2017/ SST	Case report Maxilla/ Right Central Incisor	Failure RCT	Clinical, Stable preimplant architecture, no inflammation. Radiographic, Stable proximal bone levels, along with no pathology → FRF and implant surface. SST with IIPP maintains osseous and gingival architecture. Facial window approach improves access to residual root.	SR- PFM Crown/	100% 24 Months
Petsch et al/ 2017/ SST	Case report Maxilla/ Right Central Incisor	Failure RCT	In the presence of thin biotype, peri-implant tissues were well preserved, indicating successful operation. No-change in soft tissue, pocket depth plaque accumulation	All Ceramic Crown	100% 24 Months
Pour et al/ 2017/ SST	Case report Maxilla/ Left Canine	External resorption	SST favorable system dental practice → highly aesthetic, ↓time, expense, less psychological stress patient restorative team No added cost for patient, single surgical procedure, ↓morbidity.	SRCC	100% 3 Months

Continue Table 1

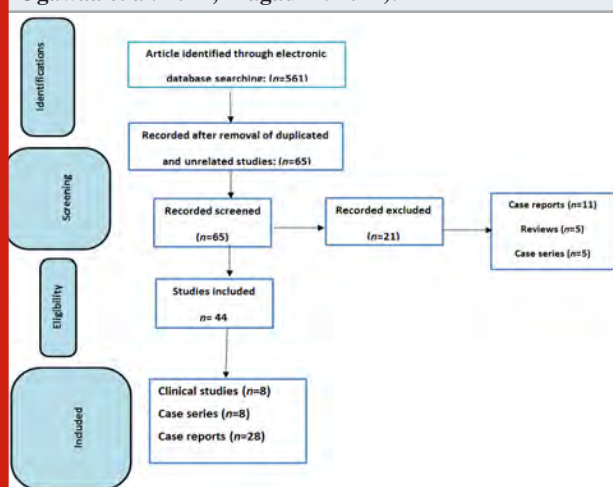
Investigator(s) /Year	Arch/Region/ Tooth	Extraction Cause /Loading Type	Technique Type/ Pink esthetic score/ Complications	Restoration Type	Survival Rate/ Follow-up
Baumer et al/ 2017/ SST	Randomize clinical trail 10 Patients Maxilla/ Right Central Incisor (2); Right Lateral Incisor (2); Maxilla/Right 1 st Premolar (1) Maxilla/ Left central Incisor (2); Left 1 st Premolar (2); Left 2 nd Premolar (1)	Failure RCT (4)& Cariou teeth (3)	SST Good implants in aesthetic zone ↔ 2 nd premolars SST [invasiveness at surgery time, high aesthetic with effective preservation of facial tissue contour. PES positive results in all cases (Mean 12) BBL -37.0mm (16.0– 66.0), Facial-mid average-33.0 mm, Loss Recession mesial 33.0, Bone marginal mm 17.0 distal. Volumetric analysis, low degree contour changes in extraction and IIP follow-ups. Mucosal recession at implant restoration was comparable to neighboring teeth.	All Ceramic Crowns	100% (51–63) 58 Months
Arora & Ivanovski/2017/ SST	Prospective clinical study / Maxilla / Centrals (46), Laterals (6), Canines (6)	NM 100 Pt (Type I-N33), (Type 2-N14); after 4-8 weeks; (Type 3-N19); after 8-16 weeks (Type 4-N44) ≥16 week	PES; Male; 10.52 ± 2.01/ Females; 9.40 ± 2.21 ^{SD} WES; Specialist; 8.47±1.54/ G. Practitioner; 7.90± 1.27 Gender ^{SD} , Specialist Vs General Practitioners ^{SD} Biological Complications (6) / Prosthetic 2 cases only No significant effects of time	SPFM & CPFM	88% 26.3 Months
Saeidi Pour et al/ 2017/ SST	Case Series/ Maxilla/ Right Canine	Failure RCT	SST is a minimally invasive implant approach offers less stress to patients and clinicians. SST → Results in soft-hard-tissue stability around implant and provides high esthetic outcome to patients	All Ceramic Crown	100% 3 Months
Hinze et al/ 2018 / SST	Prospective clinical study 15 patients 17 SST Maxilla / Anterior & Posterior ↔ 2 nd Premolar	NM	SST & CIIP with protypiozation → Preservation buccal root [buccal contour changes after tooth extraction SST; 8/15 Patients suffer recession SST; 0.31±0.64mm mesial&0.38±0.57mm distal-papilla↑ change GM Change buccal contours 0.37 – 0.32mm (0.17 ± 0.67)	PFM and CCs	100% 3 Months
Kumar & Kher/ 2018/ SST	Case report/ Maxilla/ Right Central Incisor	NM	Preservation of hard soft tissues both horizontally and vertically	E-Max CC	100%/ 3, 6, 12 Months
Han et al/ 2018/ Modified SST	Prospective clinical study/30 Patients Maxilla (34) Mandibula (6) Central (12), Lateral (10), Canines (5), Premolars (13), Parafunction/ Bruxism/Clenching (7)	Failure RCT (16) Cariou (14) L ₁ Plot Area 7.0 (8), 10.0 (3); TT ₂ (34), 13.5 (2) Diameter/mm 3.5(8), 4.0 (16), 4.5(11) 5.0(4), 5.5(1)	MSST with IIP, because root fragment does not affect osseointegration & benefits aesthetics; protects BBE resorption. Gender, Age Groups ^{§§} , Smoking ^{§§} / PFH ^{§§} Location Maxilla/Mandibula ^{§§} / Position ^{§§} / Length and Widths ^{§§} / Insertion torque ^{§§} / Implant stability at placement No biologic, 2.5%- Prosthetic or Mechanical complications	All Ceramic Crowns	100% 24 Months
Verma et al/ 2018/ SST	Case report Maxilla/ Right Central Incisor	NM	SST valuable, minimizes buccal contour after extraction. Results in healthy peri-implant soft tissue and preserves ridge.	Ceramic Crown	100% 12 Months
Guo et al/ 2018/ SST	Case report Maxilla/ Left Central Incisor	In-proper Post & Core	SST with PRF, IIP may be effective for the preservation and maintenance of stable peri-implant tissue.	All Ceramic Crown	100% 18 Months
Mattar AA/ 2018/ SST	Case report Maxilla/ Left Central Incisor	Grossly and PFM crown	SST & IIP prevent the collapse of thin buccal bone → excellent aesthetic. Inflammations in socket, changed of insertion	All Ceramic Crown	100% 18 Months
Investigator(s) /Year	Arch/Region/ Tooth	Extraction Cause /Loading Type	Technique Type/ Pink esthetic score/ Complications	Restoration Type	Survival Rate/ Follow-up
Esteve-Pardo, Esteve-Colomina / 2018 / SS	Case report Maxilla/ Left Central and Lateral Incisors	Roots subgingivally	SST & IIP → successful aesthetic restoration maintains tissue volume in aesthetic area. Two implants supported 6 Max anterior teeth with cantilever*	CS- Retained Cantilever bridge	100%/ 5-6 Months
Fonseca DL/ 2018/ SST	Case report Maxilla/ Right Central Incisor	Heavily restored failing	Case-selection, planning → Aesthetically challenging scenarios Positive aesthetic results-↑PES & WESs (≥12)	CSRC /	100% 24 Months
Schwimer et al/ 2018/ SST	Case report Maxilla/ Left 1 st Premolar	Failure of Implant (Peri- implantitis)	SST & IIP with provisionalization → Bone occupy space ↔ implant surface and SS as osseointegration outcome. ↑probing depth and crestal bone loss	Ceramic Crown	0.00% 24 Months
Aslan S/ 2018/ Modified SST	Case series Maxilla/ Right Central Incisor	Incomplete RCT	MSST maintains natural emergence profile and improved volume and contour stability can be obtained by retaining thin shield in IIP. Thin buccal bone (0.39mm) after 1 year.	E-Max CC	100% 12 Months
Gluckman et al/ 2018/ SST	Case report Maxilla/ Left Central Incisor	Post & Core/ Resorption	SST in conjunction with IIP and provisionalization positively supported facial ridge of implant.	Ceramic Crown	100% 12 Months
Dayakar et al/ 2018/ SST	Case report Maxilla/ Left Lateral Incisor	Cariou tooth	SST with IIP is a good alternative to preserve BCP in aesthetic area and healthy per-implant tissue	All Ceramic Crown	100% 3 Months
Patel et al/ 2019/ SST	Case Series Maxilla/ Left Central, Lateral Incisors, and Canine	RCT & Grossly carious	SST along with provision of smooth-surfaced DIs, stabilized cortical engagement, replacement missing maxillary anterior teeth. Patient reported no discomfort satisfied aesthetic	PFM Crown /	100% 12 Months
Habashneh et al/ 2019/ SST	Case Series Maxilla/ Right and Left Central Incisor; Right Lateral Incisor; Left 1 st and 2 nd Premolars	Failure RCT	SST and IIP Improved buccal contour stability/better esthetic SST with IIP is a minimally invasive approach that can preserve hard and soft tissue, contour of ridge can be implemented in areas of high aesthetic demands for better esthetic outcomes.	SR-PFMCs	100% 12 Months
Arabbi et al/ 2019/ SST	Case report Maxilla/ Left & Right Centr Incisors	Fractures of Crowns	SST with IIP technique of choice in aesthetic area → resulted in excellent aesthetic appearance	All Ceramic Crowns	100% 6 Months
Schwimer et al/ 2019 / SST	Case report Maxilla/ left 2 nd Molar	Fractures of Crowns	SST preserves tooth structure, maintain ridge implant sites. SST maintains alveolar ridge in posterior (MOLAR) at IIP site.	SR Restoration	100% 4 Months
Zuhr et al/ 2020/ SST	Case report Maxilla/ Right Central Incisor	Failure RCT	Shield around buccal aspect of implant was mobile 8-mm depth. Incisal edge of implant was lower to adjacent tooth, suggesting ongoing vertical growth of neighboring tooth.	All Ceramic Crown	0.00%
Sun et al/ 2020/ SST	Randomize clinical trail 30 Implants/ 30 Patients	NM	CIIP Plot Area pre: 11.33 ± 1.76 SST; PES score: 12.07 ± 1.62	SR-PFM	24 Months
Alshammari et al/ 2020/ SST	Case report Maxilla/ Left Central Incisor	Failure RCT	SST prevents soft and hard tissue changes during alveolar socket healing after extraction. PES score was 12.	SR Restoration	100% 36 Months
Hana et al/ 2020/ SST	Prospective clinical study 40 Patients Maxilla SST; (20/Patients) Central (8), Lateral (6), Canines (6) CIIP (20/Patients); Central (9), Lateral (6), Canine (5)	Failure RCT	SST; 12 Months (1) case internal (1) external shield exposure PES; Mean 12.3 CIIP; 12 Months (5) cases, need tissue graft manage recession & inadequate keratinized tissue affect aesthetic PES; Mean 9.6	PFM-CR	95% 3, 6, 12 Months
Polis-Yanes et al/ 2020/ /PSTs	Case report Maxilla/ Left and Right Cent Incisor	Failure RCT	Pontic Shield Techniques & SST procedures that should be considered in oral rehabilitation in selected cases	CC/ Bridge Cantilever	100%

Continue Table 1

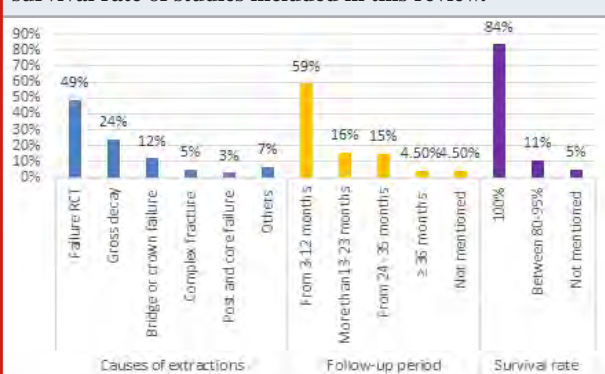
Investigator(s) /Year	Arch/Region/Tooth	Extraction Cause /Loading Type	Technique Type/Pink esthetic score/ Complications	Restoration Type	Survival Rate/ Follow-up
Abd-Elrahman et al/ 2020/ Pontic Shield Techniques	Randomize clinical trail	NM SST with IT (20- Exp G) IIP with IT (20 - Cot G)	PES 0.0-0.26 (0.15) 0.11-0.55 (0.31) ↑ 11-12mm ↓BBL & ↑ PES 0.03-0.44 (0.32) 0.25-0.51 (0.7) ↓ 13-9mm IIP ↓ PES	PFM	100% 6 Months
Dash et al/ 2020/ SST	Case report / Maxilla/ Left Central Incisor	Gross Caries/ Failure RCT	SST shows promising result in aesthetic dentistry, maintain diastema	PFM crown/	100% 6 Months
Mathew et al/ 2020/ SST	Case Series/ Maxilla / Centrals (5) Maxilla / Laterals (5) 5 cases; SST & 5 cases; CIIP	NM	SST prevents soft, natural, hard tissue changes, resorption, more aesthetically pleasing, and acceptable results PES Score 12.2* ^{sig} MBR 0.68mm PES Score 10.8 Marginal bone resorption = 0.88mm* ^{sig} # 5 ^{sig}	PFM Crowns	100% 3,6,12 Months
Nguyen et al/ 2020/ SST	Case Series Maxilla /anteriores/ Left Central & Lateral incisors Maxilla /anterior/ Left Central & Lateral incisors	2. Failure RCT 1. Previous trauma	SST preserve not only BB marginal but also inter-implant papilla. No changes in soft tissue dimensions. Marginal bone loss = 0.1 ± 0.2 mm Hard and soft tissues became very stable Well-preserved hard and soft tissue profiles are observed.	SRCC	100%/ 72, 60, 24 Months
Gluckman et al/ 2019/ MSST	Case report Maxilla /anterior/ Left Central incisor	Failure RCT (4)	PET is a collective concept of utilizing the patient's own tooth root to preserve the periodontium and peri-implant tissue.	SRCC	100% 12 Months
Alone & Niswade / 2021/ SST	Case report Maxilla/ Right Central Incisor	Cariou tooth	SST provides promising results with respect to soft and hard tissue preservation in cases of post extraction II	PFM Crown	100% 3 Months
Srivastava et al/ 2021/ SST	Case report Maxilla/ Left Cental & Lateral incisors	Grouse decayed teeth	SST with IIP in esthetic zone provides promising treatment in preserving both soft and hard tissue	PFM Crown	100% 3 Months
Oliveira et al/ 2021/ SST	Case report Maxilla /Posterior left #15	Cariou tooth	SST maintains alveolar bone preservation and contour tissue that facilitate 3D implant positioning, involves low cost good esthetic	SR- PFM	100% 3 Months

Abbreviations:
 Partial Extraction Therapy -PET; Root Submergence - RST; Socket Shield- SST; Proximal Socket Shield-PSST; Pontic-Shield- PST; Marginal Bone Loss- MBL; Crestal bone level -CBL; Crestal bone resorption- CBR; Buccal bone plate- BBP; BBL- buccal bone loss; Conventional immediate implant placement- CIIP; Modified socket shield technique- MSST; Immediate implant placement- IIP; Delayed - PRF; Buccal cortical plate - BCP; Root fragment - RF; Alveolar bone loss - ABL; Pocket Probing Depth— PPD; implant surfaces- IS; Randomized clinical trial- RCT; Pink esthetic score- PES; ↑-Increase; ↓-Decrease; ↔-Between; → Resulted in % - Percentage; ↔-Between; NM-Not mentioned; Zirconia Abutment-ZA; Ceramic Crown-CC; Zirconia Crown- ZC; Screw-retained Ceramic Crowns- SRCC; All Ceramic Crown-ACC; Screw Retained All Ceramic-SRAL; Screw Retained
 Retained Porcelain-Fused-Metal-SR-PFM; Porcelain-Fused-Metal-PFM

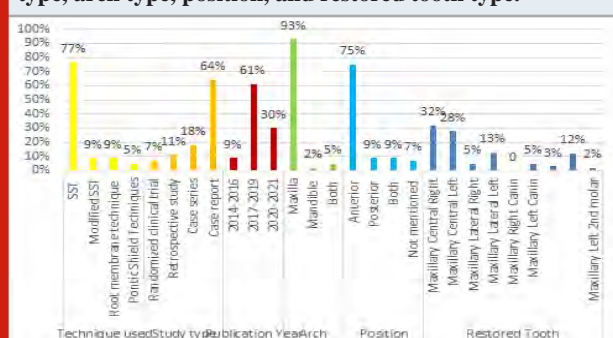
Figure 1: Flowchart of the study selection process (Moher et al. 2009; Siormpas et al. 2018; Blaschke and Schwass 2020; Ogawaa et al. 2021; Magadmi. 2021).



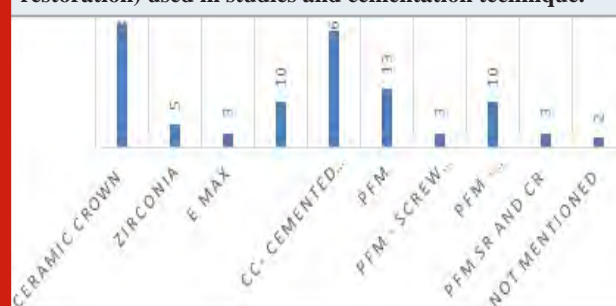
Graph 2: Causes of tooth extraction, follow-up period, and survival rate of studies included in this review.



Graph 1: Extracted data in relation to type of PET. Study type, arch type, position, and restored tooth type.



Graph 3: Numbers of different types of prostheses (final restoration) used in studies and cementation technique.



In addition to that the studies by Arora and Ivanovski (2018), Han et al. (2018); Hana et al. (2020); Mathew et al. (2020) recorded 102,33,25,13,7, and 3 maxillaries central, lateral, canine, 1st and 2nd premolar, and mandibular canines were recorded, respectively. Abadzhiev et al. (2014) (80%), Arora and Ivanovski (2017) (88%), Schwimer et al. (2018) (100)

%) ;Zuhr et al. (2020) (100.00%), Hana et al. (2020) (95%) found high percentage of success with different period of follow-up as recorded after each one. Canti-lever of 6 unites from maxillary canine in the left side into canine on other side with two abutments. Lateral's incisors were used by Polis-Yan et al. (2020).

Cemented retained cantilever all ceramic with abutment was lateral incisor and the pontic was the adjacent central incisors, while Abadzhiev et al. (2014) used mixed ceramic and PFM crowns for their final restoration after SST with or without IIP. Other authors used mixed PFM and CC as Arora and Ivanovski (2017) used Screw R PFM, cemented PFM. Pour et al. 2017 used SR CC, while Hinze et al. (2018) used PFM and ceramic crowns (Esteve-pardo and Colombia 2018). Various PET techniques have provided outstanding biological, mechanical, and aesthetic consequences in hands of knowledgeable clinicians with careful treatment arrangement and case collection. A uniform assessment of PET outcomes needs to be established to provide objective findings, in addition to a consistent protocol for root portions preparation and to place dental implants in the ideal place and achieve long term success of treatment. This review aims to determine the advantages of different PET techniques aesthetic outcome IIP in the aesthetic zone and the different types of final prostheses used (Esteve-pardo and Colombia 2018; Oliveira et al. 2021).

Among the PET techniques, SST is the most used technique because of its many advantages in cases of post extraction immediate implant with IIP, such as high stability and well-preserved hard and soft tissue; it preserves the buccal bone marginal and inter-implant papilla with minimum marginal bone loss, maintains alveolar bone level, and does not change soft tissue dimensions (Nguyen et al. 2020; Alone and Niswade 2021; Srivastava et al. 2021; Oliveira et al. 2021). This method is good alternative to preserve BCP in aesthetic area and healthy per-implant tissue, improved buccal contour stability and/or better esthetic outcomes can be achieved (Dayakar et al. 2018; Patel et al. 2019; Arabbi et al. 2019; Schwimer et al. 2019; Dash et al. 2020).

In a case series by Habashneh et al. (2019) and Alshammari et al. (2020) they show minimally invasive approach that can preserve hard and soft tissue and contour of ridge, and this method was implemented in areas of high aesthetic demands to achieve good esthetic outcomes. SST with IIRP preserved hard and soft tissue and kept it stable without any changes in dimension, resulting in optimum aesthetic results and improving and preserving the buccal contour of ridge areas of high aesthetic demands (maxillary anterior up to premolars) to achieve good esthetic outcomes (Glocker et al. 2014; Mitsias et al. 2017; Habashneh et al. 2019; Mathew et al. 2020; Nguyen et al. 2020; Germi et al. 2020). Tissue volumes remain unchanged, and good osteointegration was achieved (Troiano et al. 2014; Gluckman et al. 2016b; Baumer et al. 2017). In addition to the above characteristics, a group of clinical studies showed excellent scores for PES and was in clinical studies (Sun et al. 2020; Hana et al. 2020; Abd-Elrahman et al. 2020).

Ideally, a method for the prevention of alveolar ridge resorption should be cost-effective and minimally invasive. Various methods of guided bone regeneration (GBR) have been described to retain the original dimension of the bone after extraction. All these procedures are cost-intensive and technique-sensitive. The presented method is cost-effective but is a technique-sensitive SST that avoids the resorption of the bundle bone by leaving a buccal root segment (socket shield) in place (Mourya et al. 2019; Ogawa et al. 2021). The SST seems to be beneficial for ridge preservation despite its insufficient documentation. In this case report series, implants were placed immediately after extracting a hopeless tooth by using this technique, and the patient was followed up for 1 year to document functional and esthetic outcomes (Mourya et al. 2019; Ogawa et al. 2021).

PES was between 8–10 and 6–10 after 6 and 12 months, while previous studies recorded 12.2 PES with complete score for central incisors, recorded 13.5 mm, and recorded a mean PES of 12. Only a single article recorded PES and MBL for CIIP of 10.8 and 0.88 mm by, respectively. The MBL for SST was 0.1 ± 0.2 mm as determined in the previous studies and 0.17–0.22 mm as determined in the previous studies (Baumer et al. 2017; Zhu et al. 2018; Germi et al. 2020; Mathew et al. 2020; Sun et al. 2020; Mathew et al. 2020; Mathew et al. 2020). Other information in relation to case series are available in Table 1 and Graph 1.

The advantage of RST is inexpensive preservation of alveolar bone dimensions to provide a good retentive surface area for RDP or to preserve alveolar bone for a future dental implant, or to preserve the tissues' dimensions in the pontic's area under a tooth supported FDP, with a chance of developing bone and new cementum and connective tissue coronal to submerged segment. It also preserves the tissues next to a dental implant and improves the predictability of interdental papillae height in DIT (Roe et al. 2017; Petsch et al. 2017; Baumer et al. 2017; Pour et al. 2017; Kumar and Kher 2018; Verma et al. 2018; Guo et al. 2018; Mattar 2018; Patel et al. 2018; Schwimer et al. 2019).

In the aesthetic area, the preservation of the interdental papilla among two implants is one of the major challenges of implant rehabilitation, and the PSST was first proposed and described by involving the similar values of the SST, but the distal root piece was used instead of the buccal one. Consequently, studies about this technique are lacking (Chen et al. 2018). The complications observed during follow-up of case series include a shield failure caused by infection, a case of deficiency of alveolar ridge, a patient who had complications with the three other socket shields exposed caused by failure of soft tissue closure (Lagas et al. 2015; Gluckman et al. 2016b; Schwimer et al. 2019).

The pontic ST was recognized as the modified SST, and it was introduced to preserve both hard and soft tissues in the pontic extents following the same technique as the SST. However, instead of inserting an IIP in the socket, a bone grafting material was used to seal the socket, and the socket was closed by a repositioned flap, gingival graft, or membrane. Moreover, under the presence of an apical

pathology, the buccal pieces can be conserved, while all the other tooth structures and apical lesions are detached, which overcomes a matter that was identified with the use of RST (Nisar et al. 2020).

CONCLUSION

The findings of the present study suggests that although PET can be used for dental implant treatment, it remains difficult to predict long-term success of this technique until high-quality evidence becomes available. Studies published from 2010 to 202 were included. A total of 40 studies were included, as randomized controlled trial, cohort studies, clinical case reports, and case series. 123 patients were treated with PET, most of them underwent SST with IIP. The follow-up was conducted between 3–120 months after placement. Several complications were recorded, but it was manipulated. Most studies reported implant survival without complications (91%). Most of cases that were followed up for more than 12 months after implant placement achieved a good aesthetic appearance. The failure rate was low without the complications, although some failures occurred because of failed implant osseointegration, socket shield mobility and infection, socket shield exposure or migration, and apical root resorption.

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Biomedical Communication

***Bupleurum turcicum*: A Rich Source of Saikosaponin A and D with Potential Use as Adjunct for the Management of Acute Respiratory Diseases: A Meta Review**

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ABSTRACT

COVID-19 pandemic caused by SARS-CoV-2 has resulted in unprecedented havoc worldwide with significant morbidity and mortality. Till now, no effective antivirals are at disposal prompting researchers to explore potential lead molecules including from bioactive phytochemicals. An extensive literature search was carried out utilizing online resources; Google Scholar and PubMed to collect published reports on pharmacological potential of saikosaponin particularly in underexplored *Bupleurum* species. A number of molecular docking studies have reported promising antiviral effects of saikosaponins particularly of saikosaponin A, D, U and V with tremendous potential to be developed as anti-SARS-CoV-2 therapy. The search for potential sources of saikosaponin A and D led to the identification of *Bupleurum turcicum*; an unexplored, underutilized and endemic *Bupleurum* species. The observation that *B. turcicum* root extract contains highest amount of SSa and SSd among endemic *Bupleurum* species found in Turkey (*Bupleurum sulphureum*, *Bupleurum lycaonicum*, *Bupleurum turcicum*, *Bupleurum heldreichii*, *Bupleurum pauciradiatum*) and presence of significant amounts of antioxidant compounds led to the proposition of using *B. turcicum* extracts as adjunct therapy in the management of COVID-19. The proposal also relies on the evidence of SSa and SSd being effective against a number of viruses including SARS-CoV. This review discusses phytochemical composition of *B. turcicum* root, antiviral, immunomodulatory and anti-inflammatory potential of saikosaponins in view of its plausible usefulness in the management of COVID-19. *B. turcicum* is an underutilized species rich in saikosaponin A and D with potential antiviral properties which could be effective alternative therapy in COVID-19 management..

KEY WORDS: ANTIVIRAL, BUPLEURUM TURCICUM, COVID-19, SAIKOSAPONINS, SARS-COV.

INTRODUCTION

SARS-CoV-2 (COVID-19) infection started as an outbreak of pneumonia of unknown origin in Wuhan City, China during late December 2019 and soon declared as a global epidemic by February 2020 (Lu et al. 2020; WHO 2020).

Soon the sequencing of virus genome from patient samples and human to human transmission were confirmed by researchers (Zhu et al. 2020; Chan et al. 2020). Researchers began to explore novel therapeutic approaches to treat COVID-19 in view of its potential to rapidly develop into acute respiratory distress syndrome which can in some cases possibly cause multiple organ failure in the absence of effective drugs/vaccines (Patel and Jernigan 2020; Sahin et al. 2020; Gralinski and Menachery 2020).

Article Information:*Corresponding Author: f.masfoor@qu.edu.sa
Received 25/10/2021 Accepted after revision 18/12/2021
Published: 31st December 2021 Pp- 1444-1451
This is an open access article under Creative Commons License,
Published by Society for Science & Nature, Bhopal India.
Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.11>

It is reported that during the outbreak of SARS-CoV in 2003, traditional herbal medicines used in conjunction with conventional medicine were effective in alleviating the symptoms of SARS (Lin et al. 2003; Xiao et al. 2003; Zhao et al. 2003; Zhong et al. 2003). Though the mechanism of action of those herbal products was not fully understood, the general consensus was that they contain antiviral molecules which might be acting by inhibiting viral replication (Vlietinck et al. 1991; McCutcheon et al. 1995; Jassim and Naji 2003). Therefore, exploring natural products with demonstrated antiviral efficacy against other coronaviruses is one of the therapeutic approaches. Since SARS-CoV-2 is a betacoronavirus which invades the host cell through angiotensin converting enzyme 2 (ACE2) receptor similar to SARS-CoV, it is assumed that antiviral biomolecules that are active against SARS-CoV can be repurposed to be used in COVID-19 patients (Cheng et al. 2006; Huang et al. 2020; Shahrajabian et al. 2020).

In view of this, an extensive literature search was carried out utilizing online resources; Google Scholar and PubMed to collect published reports on herbal medicines and natural products with antiviral activity. The search was then tapered down to the natural compounds active against SARS-CoV wherein, saikosaponins widely distributed in *Bupleurum* spp., *Heteromorpha* spp. and *Scrophularia* spp were selected to explore further as saikosaponin A, B2, C and D have been reported to show significant antiviral activity against human coronavirus 229E *in vitro* (Cheng et al. 2006). The search was further narrowed down to *Bupleurum* spp. and *Bupleurum turcicum* was selected for this study since it contained highest levels of saikosaponin A and saikosaponin D amongst *Bupleurum sulphureum*, *Bupleurum lycaonicum*, *Bupleurum heldreichii*, and *Bupleurum pauciradiatum* (Kars et al. 2012; Huang et al. 2020).

Bupleurum L. genus belongs to Apiaceae family which consists of around 200 species distributed across Northern Hemisphere, Eurasia, and North Africa. The plants are often annual or perennial herbs/shrubs up to 2 m tall with simple, entire and alternate leaves and the flowers are small, radical and yellowish (Xie et al. 2009). Many plants particularly roots from *Bupleurum* genus are used in traditional systems of medicine across China, Japan, Korea and Taiwan in the treatment of fevers associated with common cold and malaria, inflammation, hepatitis, diabetes, cancer and for wound healing, while the essential oils are used as anti-inflammatory and antiseptic agents (Nose et al. 1989; Motoo et al. 1994; Benito et al. 1998; Van-Wyk and Wink 2004; Wu 2005). *Bupleurum* species are used as analgesics in amenorrhea and cholecystitis. They also find their use in nephrotic syndrome, autoimmune diseases deafness, dizziness, dry throat, vomiting, diarrhea and hemorrhoids (Ashour and Wink 2011). These plants reportedly contain diverse classes of bioactive components including essential oils, alkaloids, flavonoids, coumarins, polysaccharides, lignans, triterpene saponins, phytosterols and polyacetylenes. WHO monographs list *Bupleurum* species as commonly used medicinal plants of China and Korea and also officially listed in Japanese and Chinese Pharmacopoeias (Ashour and Wink 2011; Huang et al. 2020).

Bupleurum Bioactive Saikosaponins: Saikosaponins are triterpene oleanane saponin glycosides widely distributed bioactive compounds in *Bupleurum* spp amounting to up to 7% of the total dry weight in roots. Though saikosaponins are classified into seven types based on the type of aglycone (closely related oxygenated pentacyclic triterpenoidal structures), four different types namely saikosaponin-A (SSa), saikosaponin-B (SSb), saikosaponin-C (SSc) and saikosaponin-D (SSd) are considered as most commonly occurring and biologically active saikosaponins (Yuan et al. 2017). SSa, SSd and SSc are epoxy-ether saikosaponins designated as type I and SSb2 is a heterocyclic diene saikosaponin designated as type II (Lin et al. 2013; Huang et al. 2020).

Plant extracts/herbal medicines containing saikosaponins are commonly prescribed as anti-inflammatory and anti-infectious medicine in Asian countries including China, Taiwan and Japan. More than 120 types of saikosaponins have been isolated from *Bupleurum* species till date and some of them have significant bioactivity both *in vitro* and *in vivo* (Kim 2018). Previous studies have reported antioxidant, antidepressant, anti-inflammatory, antimicrobial, antiviral, anticancer, anti-tumor and immunomodulatory effects of saikosaponins (Wu et al. 2008; Wu et al. 2010; Jin et al. 2013; Sui et al. 2011; Wu et al. 2011; Ying et al. 2014; Zhang et al. 2014; Huang et al. 2020).

***Bupleurum turcicum*:** *B. turcicum* belongs to Apiaceae family which mostly has flower bearing shrubs (Davis 1972). The essential oils from flowers, fruits and roots contain 39 distinctive compounds (Table 1). Heptanal, pentadecane and undecane were major compounds of flower and fruit essential oils. The flowers contained 33.2% heptanal, 19.6% pentadecane, 6.6% undecane, while fruits contained 23.5% heptanal, 13.4% pentadecane and 8.9% undecane. On an interesting note, essential oil from root did not contain heptanal, pentadecane or undecane which were major compounds of flowers and fruits. The major compounds of root essential oil were pentacosane (9%), 1-undecanol (8.8%) and hexacosane (8.0%) (Huang et al. 2020).

The oils were evaluated for their antibacterial activity against *Staphylococcus aureus*, *Staphylococcus aureus*, *Escherichia coli*, *Escherichia coli*, *Bacillus cereus*, *Streptococcus salivarius*, *Pseudomonas aeruginosa*, *Pseudomonas aeruginosa* and *Proteus mirabilis*. It was found that the essential oils of flowers and roots did not show any activity against tested bacteria, while the oil obtained from roots exhibited good antibacterial activity which was comparable to that of chloramphenicol against *E. coli*, *B. cereus*, *S. salivarius* and *P. aeruginosa*. The observed phenomenon is attributable their chemical composition wherein, both fruits and flowers contained heptanal, pentadecane or undecane as major components which were not found in root samples. It was concluded that the essential oils from the roots of *B. turcicum* could be used as a potential source of novel antibacterial agents (Cheng et al. 2006; Kars et al. 2012; Saraçoğlu et al. 2012; Tykheev et al. 2020).

Table 1. Photochemical composition of *Bupleurum turcicum* essential oils

Compounds	Flowers (%)	Fruits (%)	Roots (%)	Compounds	Flowers (%)	Fruits (%)	Roots (%)
(E)-2-Decanal	0.4	0.5	0.9	Dodecanoic acid	0.4	0.8	
(E)-2-Nonenal	1.5	2.3	-	Farnesyl acetone	0.1	0.4	1.4
(E)-2-Octenal	0.5	0.2	-	Heneicosane	-	-	1.1
(E)-2-Undecanal	-	-	0.8	Heptacosane	0.3		3.6
(E)-Geranyl acetone	3.1	7.7	0.8	Heptadecane	0.8	0.8	0.6
(E)-Neralidol	-	0.5	-	Heptanal	33.2	23.5	-
(E)- β -Ionone	0.8	0.8		Heptanoic acid	1.0	0.7	-
(E,E)-2,4-Decadienal	0.8	0.7	0.8	Hexacosane	0.2	-	8.0
(Z)-Geranyl acetone	1.4	2.5	-	Hexadecane	0.2	0.2	0.4
1-Decanol	-	-	0.8	Hexadecanoic acid	1.9	2.8	0.2
1-Dodecanol	-	-	6.3	Hexahydrofarnesyl acetone	1.4	3.6	3.7
1-Heptadecene	0.5	0.5	-	Hexanal	0.9	0.5	-
1-Hexadecanol	0.3	0.7	-	Limonene	1.5	1.8	-
1-Octadecene	0.3	0.7	-	Nonacosane	0.2	-	2.4
1-Octen-3-ol	0.2	-	-	Nonadecane	-	-	0.5
1-Tetradecanol	-	-	2.2	Nonanal	0.3	0.4	0.2
1-Undecanol	-	-	8.8	Octacosane	0.1	-	3.9
2-Decyl acetate	-	-	4.5	Octadecane	0.2	-	0.5
2-Hexyl furan	0.3	0.3	-	Octanal	0.5	0.5	-
2-Octanone	0.3	-	-	Pentacosane	0.2	-	9.0
2-Pentyl furan	1.3	1.0	-	Pentadecane	19.6	13.4	3.0
2-Undecanone	0.3	0.7	0.5	Phytol	-	1.0	-
3,4-Dimethyl-5-Pentyl-5 HFuran-2-one	0.4	-	-	Spathulenol	3.5	5.9	6.3
4,8-Dimethyl-1,3,7-nonatriene	0.2	-	-	Tetracosane	0.2	-	5.4
6-Methyl-5-hepten-2-one	-	0.3	-	Tetradecane	0.3	-	-
Aristolene	-	0.2	-	Tetradecane	-	0.2	0.5
Benzyl salicylate	-	-	1.8	Tetradecanoic acid	0.6	2.6	-
Bornyl acetate	-	-	0.2	Tricosane	-	-	3.3
Caryophyllene oxide	3.5	2.4	-	Tridecane	1.8	1.9	1.8
Cuparene	-	-	0.5	Undecanal	-	-	0.5
Decanal	-	-	0.4	Undecane	0.6	8.9	5.5
Decane	-	-	0.1	α -Pinene	0.8	2.0	-
Dihydroedulan II	-	0.2	-	β -Caryophyllene	0.5	-	-
Docosane	-	-	1.4	β -Elemene	0.2	0.8	-
Dodecanal	-	-	0.4	γ -Muurolene	1.6	-	-

*Source: (Cheng et al. 2006; Kars et al. 2012; Saraçoğlu et al. 2012; Tykheev et al. 2020)

In an important study, the levels of phenolic compounds (catechin, quercetin, isoquercitrin), SSa, SSd and podohyllotoxin were determined in the root extracts of *B. sulphureum*, *B. lycaonicum*, *B. turcicum*, *B. heldreichii* and *B. pauciradiatum* using HPLC. *B. turcicum* was found to contain significant amounts of total phenolics (34.48 mg GAE/g extract), catechin (0.11 mg/g extract), quercetin (0.21 mg/g extract) and isoquercitrin (1.85 mg/g extract).

Furthermore, *B. turcicum* root extract contained highest amounts of SSa (12.99 mg/g extract) and SSd (17.96 mg/g extract) among all the other extracts investigated (Huang et al. 2020; Shahrajabian et al. 2020).

B. turcicum root extract exhibited significant free radical scavenging activity with lowest IC₅₀ value (57.3 μ g/mL) amongst other extracts in DPPH radical scavenging assay.

In antiproliferative assay, *B. turcicum* root extract exhibited potent activity against sensitive and drug resistant MCF-7 cells (Kars et al. 2012). The observation that *B. turcicum* root extract contains highest amount of SSa and SSd among endemic *Bupleurum* species also found in Turkey (*B. sulphureum*, *B. lycanicum*, *B. turcicum*, *B. heldreichii*, *B. pauciradiatum*) and presence of significant amounts of antioxidant compounds led to the proposition of using *B. turcicum* extracts as adjunct therapy in the management of COVID-19. The proposal also relies on the evidence of SSa and SSd being effective against a number of viruses including SARS-CoV (Huanga et al. 2020; Shahrajabian et al. 2020).

Antiviral properties of saikosaponins: Recent studies have evaluated the anti-SARS-CoV-2 effects of different saikosaponins found in *Bupleurum* Spp. using advanced molecular docking techniques and have found promising results with potential to be studied further with respect to saikosaponins A, D, U and V. Similarly, in the experimental setup saikosaponins isolated from *Bupleurum* spp. have been shown to possess potent antiviral activity against a number of viruses including herpes simplex virus, influenza virus (IAV), hepatitis B virus (HBV), hepatitis C virus (HCV), measles and varicella zoster viruses (Ashour and Wink 2011). Based on these findings, saikosaponins from *Bupleurum* Spp. have been proposed to be repurposed for the treatment of COVID-19 (Bahbah et al. 2020).

In a molecular docking study, SSa has been shown to possess significant affinity towards binding ACE 2 receptors through which SARS-CoV-2 infect lung epithelial cells (Yan et al. 2020). Another molecular docking simulation study showed that Saikosaponins exhibit high affinity towards RBD region of the spike glycoprotein of SARS-CoV (Goswami and Bagchi 2020). Another molecular docking study conducted to evaluate the affinity of Saikosaponins towards SARS-CoV-2 binding protein showed, Saikosaponins U and V to exhibit strong affinity towards SARS-CoV-2 binding protein. The study concluded that Saikosaponins U and V could be future research molecules for SARS-CoV-2 research (Sinha et al. 2020).

A study conducted to evaluate the antiviral and immunoregulatory activities of SSa and SSd against Porcine Coronavirus 2 (PCV2) showed that saikosaponins reduced the incidence and severity of PCV2-induced immunopathological damage in terms of pyrexia, weight loss, anemia, and internal organ oedema in mice. Immunoglobulin and protein absorption levels were also affected suggesting immunoregulatory effect of saponins (Yang et al. 2017). In another study, antiviral activity of saikosaponins against herpes simplex type I (HSV-1), vesicular stomatitis virus (VSV) and poliovirus type 1 was evaluated *in vitro* at non-cytotoxic concentrations. Buddlejasonin 4 was found to be potent against vesicular stomatitis virus (Bermejo et al. 2002; Shahrajabian et al. 2020).

Ushio and Abe evaluated the virus neutralizing effect of SSd against measles virus and herpes simplex virus *in vitro*

(Ushio and Abe 1992). SSd at above 5 μ M concentration rendered measles virus and herpes simplex virus (HSV) completely non-infective when incubated together for 10 min at room temperature. Lin et al (2015) evaluated the antiviral activity of SSa, SSb₂, SSc, and SSd against cultured hepatitis C virus (HCV) *in vitro*. The effect of saikosaponins on virus entry, RNA replication/translation and particle production were studied using different HCV genotypes, clinical isolates and infection of primary human hepatocytes. All saikosaponins were found to exhibit potent inhibition of HCV infections even at non-cytotoxic concentrations by targeting early steps of the viral life cycle. SSb₂ was found to significantly prevent virus entry by neutralizing virus particles, preventing their attachment and inhibiting viral entry/fusion.

SSb₂ also inhibited other genotypic strains and prevented HCV binding onto hepatoma cells thereby blocking HCV infection of primary human hepatocytes. The study proposed further research into SSb₂ to develop it as an HCV entry antagonist. These findings were reiterated by another study in 2019, wherein SSb₂ was found to inhibit viral entry, replication, and translation of hepatitis C virus (HCV) in a cell culture-derived HCV system *in vitro*. SSb₂ also inhibited daclatasvir-resistant mutant strains of HCV when used in combination with daclatasvir indicating potential antiviral effects of SSb₂. Antiviral activity of SSa, SSc and SSd against anti-hepatitis B virus (HBV) was evaluated *in vitro* using HBV-transfected human hepatoma cells (Lee et al. 2019; Shahrajabian et al. 2020).

SSc was found to inhibit DNA replication of HBV which was higher than that of lamivudine- a known antiviral drug in clinical use (Chiang et al. 2003; Chang et al. 2007). In another study SSd showed significant antiviral activity against HBV through inhibition of HBV-DNA replication (Yin et al. 2008). The anti-viral activity of SSa was evaluated against influenza A virus (IAV) infections *in vitro* and *in vivo*. SSa reduced replication of three different influenza A virus strains, including a H5N1 strain, in human alveolar epithelial A549 cells by downregulating NF- κ B signaling and caspase 3-dependent virus ribonucleoprotein nuclear export. SSa decreased viral replication, production of pro-inflammatory cytokines in H1N1 PR8 model of influenza A virus lethality in C57BL/6 mice. SSa also attenuated lung neutrophil and monocyte recruitment during the early stages of immune response to PR8 infection (Chen et al. 2015; Shahrajabian et al. 2020).

The antiviral activities of saikosaponins against human coronavirus (HCoV), which cause severe acute respiratory syndrome (SARS), were also studied using 2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-5-[(phenylamino) carbonyl-2H-tetrazolium hydroxide] (XTT) assay *in vitro*. Results indicated both SSa and SSb₂ showed significant antiviral activity against HCoV at concentrations of 0.25-25 mmol/L. Both saikosaponins exhibited no cytotoxic effects on target cells at tested concentrations. The antiviral activity of SSb₂ was more significant than that of SSa and mediated by inhibition of attachment and penetration of the virus to target cells (Cheng et al. 2006; Shahrajabian et al. 2020).

Immunomodulatory and anti-inflammatory activity of saikosaponins in view of COVID-19: SARS-CoV-2 much like Influenza A virus (IAV) can cause severe pneumonia resulting in morbidities and mortalities (Chen et al. 2015). Generally, infection starts in the upper respiratory tract epithelial cells and spreads aggressively into deeper regions of the lung parenchyma and might also enter into macrophages and dendritic cells (Spiegel et al. 2006; Manicassamy et al. 2010; Shahrajabian et al. 2020).

The infected cells release pro-inflammatory cytokines (IL2, IL7, IL10, IP10 and TNF α) and chemokines (MIP-1a and MCP1). Although these responses are important for controlling viral replication in the initial phase of infection through recruitment of immune cell into lungs, excessive pro-inflammatory components raise the levels of cytotoxic and pro-apoptotic products which damage lung tissue (Herold et al. 2008). Thus, natural products with anti-inflammatory and immunomodulatory properties could be useful in the management of COVID-19 (Huang et al. 2020). Kumazawa and coworkers investigated macrophage activation potential of SSa and SSd in mice. Intraperitoneal injection of saikosaponins induced a dose dependent activation of peritoneal macrophages leading to enhanced phagocytic activity, increased cellular lysosomal levels, induction of cytostatic activity and expression of Ia antigen on the cell surface. Authors opined that SSd could be a potent macrophage activator as it showed significantly higher activity compared to ginsenoside Rg1 and glycyrrhizin. Furthermore, SSd also modulates lymphocyte activity through suppression of T-cell and induction of B-cell response to different mitogens and up-regulates IL-2, IL-4 production in thymocytes through post-receptor signal transduction (Kumazawa et al. 1989; Ushio and Abe 1991; Kato et al. 1995; Lin et al. 2015; Huang et al. 2020).

Wong and coworkers reported *in vitro* suppression of OKT3/CD28-costimulated human T cell proliferation and inhibition of PMA, PMA/Ionomycin and Con A-induced mouse T cell activation by saikosaponin D *in vitro*. Examination of T cell activation signaling pathways suggested that saikosaponin inhibits T cell activation downregulating CD69, CD71 expression and IL-2 production through modulation of PKC pathway via PKCh, JNK, and NF- κ B transcription factors. It also downregulates CD25, IL-6, TNF α and IFN γ through NF- κ B, NF-AT and AP-1 (c-Fos) signaling pathways (Wong et al. 2009). These observations indicate that SSd could be a potential molecule with immunomodulatory functions (Leung et al. 2005; Huang et al. 2020).

SSa was found to inhibit inflammatory factors such as cyclooxygenase-2 (COX-2) and inducible nitric-oxide synthase (iNOS) and production of pro-inflammatory cytokines TNF α , IL1 β and IL6 in an experimental model of inflammation (lipopolysaccharide (LPS)-stimulated RAW 264.7 cells). The study concluded that SSa exhibits significant anti-inflammatory activity through regulation of inflammatory mediators and suppression of MAPK and NF- κ B signaling pathways (Zhu et al. 2013). These finding were reiterated in another study, wherein SSa significantly inhibited COX-2, iNOS, tumor necrosis factor- α , IL1 β and

IL6 in an obesity experimental model using mouse embryo fibroblast 3T3-L1 cells. The study also suggested SSa to be a potential therapeutic agent against obesity associated inflammation (Kim et al. 2015). SSa and SSd are reported to exhibit potent anti-inflammatory activity by inhibiting production of nitric oxide induced by lipopolysaccharide in BV-2 microglial cells. The inhibitory effect of both SSa and SSd were comparable to that of dexamethasone (Wang et al. 2017; Huang et al. 2020).

In vivo anti-inflammatory activity of SSa and SSd were evaluated in female albino rats by granuloma pouch method and antigranulomatous action by cotton pellet method. Oral administration of SSa and SSd showed significant anti-inflammatory effect without affecting hematocrit and plasma-11-OH-corticosteroid levels (Yamamoto et al. 1975). The anti-inflammatory effect of saikosaponins were evaluated in mice paw oedema model, wherein oedema was induced by injecting formalin into hind par and paw oedema index was used as a measure of anti-inflammatory effect.

Saikosaponins were found not only to decrease paw oedema but also decreased inflammatory metabolites including nicotinate, niacinamide, arachidonic acid (AA), and 20-carboxy-leukotriene B4 as evidenced by HPLC metabolomic study. The study concluded that saikosaponins exert their anti-inflammatory effect through regulation of nicotinate and nicotinamide and arachidonic acid metabolism (Ma et al. 2016). The saikosaponins have also been reported to exhibit anti-inflammatory effect in TPP-induced ear oedema model in rats (Recio et al. 1995). In experimental model of cigarette smoke induced lung inflammation in mice, SSa was found to inhibit inflammatory cell infiltration, nitric oxide production, TNF- α , and IL-1 β production, MPO and MDA levels in lung tissues (Chen et al. 2018; Huang et al. 2020).

CONCLUSION

The findings of the present study suggests that *B. turcicum* is an unexplored and underutilized species of genus *Bupleurum* L which is a rich source of saikosaponin A and D having potential antiviral properties against a number of virus including IAV, HBV, PCV-2, SARS-CoV and SARS-CoV-2. Further phytochemical characterization and standardization of *B. turcicum* root extract might be beneficial in finding safe and effective alternative therapy with respect to COVID-19.

Conflict of Interests: Authors declare no conflicts of interests to disclose.

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Biotechnological Communication

Production, Evaluation, Cultivation and *In vitro* Fertilization of Cattle Oocytes: Recent Trends in Reproductive Biotechnologies in Animal Breeding : A Review

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ABSTRACT

The article presents the results of assessing reproductive biotechnology for cattle breeding. The issues of obtaining genetic material from bulls-producers and oocytes from donor cows, for their further cultivation and obtaining embryos, in order to replicate highly productive offspring from valuable animals, are considered. Oocyte production was analyzed in three different ways: puncture, section, and aspiration. A total number of 156 oocyte-cumulus complexes (OCCs) were collected out of 40 ovaries by puncture – 50 pcs, section – 47 pcs, and aspiration – 59 pcs. The results showed that puncture and section gave significantly higher total OCCs per an ovary (4.16 and 4.0, respectively) than aspiration (3.68), but a higher number of normal (grade A and B) OCCs per an ovary was observed with aspiration (2.5) than with puncture (1.82) and section (2.00). During aspiration, oocyte-cumulus complexes were collected from the surface of follicles with a diameter of 3 to 8 mm using a needle. During puncture, all surfaces were pierced with a hypodermic needle; during section, incisions were made along the entire ovarian surface with a scalpel, that is, all sizes of superficial follicles were collected. OCCs were divided into 4 classes based on cumulus and nucleus cells: grade A - oocytes completely surrounded by cumulus cells; Grade B - oocytes partially surrounded by cumulus cells; Grade C - oocytes not surrounded by cumulus cells; and grade D - degeneration observed in both oocyte and cumulus cells. Grades A and B were considered normal, while grades C and D were considered to be damaged. The obtained oocytes were cultured and fertilized *in vitro*, which means that their fertilization with sperm occurred under artificially maintained optimal conditions outside the body. Normal fertilization results are zygote formation with male and female pronuclei (PN). As a result of the conducted fertilization of 251 mature oocytes, 142 fertilizations were successful, which amounted to 56.57%.

KEY WORDS: BIOTECHNOLOGY, CRYOPRESERVATION, EMBRYO TRANSPLANTATION TECHNOLOGY IN VITRO, OOCYTE AND SPERM.

INTRODUCTION

An essential factor contributing to the successful development of the livestock industry is the good reproductive performance of heifers, cows and bulls. For a breeding bull, an effectiveness indicator of reproductive capabilities is its sperm ability to fertilize an oocyte. Sperm products are usually purchased at livestock breeding enterprises specializing in breeding servicing bulls. At such enterprises, breeders conduct a comprehensive assessment and work to improve the economically useful qualities of animals. Sperm products are obtained from bulls that have passed the

assessment and received permission for use in accordance with the technologies provided by the enterprise (Luno et al. 2014; Morrell and Wallgren 2014; Iqbal et al. 2016; Alm-Kristiansen et al. 2018; Gorelik et al. 2021).

When obtaining semen, it is important to comply with all technological conditions, since the quality indicators are influenced by many factors, which include feeding and keeping bulls, hygiene of semen collection, composition of diluents, methods of freezing and thawing, and other factors. Sperm products obtained from breeding bulls are usually delivered to livestock enterprises frozen in liquid nitrogen (granules, pies). The use of artificial insemination and the development of cryopreservation technologies made it possible to obtain numerous offspring from a single sire (Luno et al. 2014; Morrell and Wallgren 2014; Iqbal et al. 2016). The biological characteristics of cows determine the

Article Information:*Corresponding Author: 6110699@mail.ru

Received 17/09/2021 Accepted after revision 20/12/2021

Published: 31st December 2021 Pp- 1452-1458

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.12>

bearing period of one calf during the year, which, in contrast to bulls-producers, is a limiting factor for replicating highly productive heifers from highly productive cows. Most farms raise and evaluate cows and heifers independently at all technological stages (Alm-Kristiansen et al. 2018; Gorelik et al. 2021).

In this connection, they have the opportunity to form groups of animals according to the desired phenotype and genotype, timely conduct a comprehensive assessment of the animal, including its productive potential and reproductive abilities, and, based on the data obtained, make a decision on its further use (Gritsenko 2016; Mostek et al. 2017; Chalova et al. 2020; Gorelik et al. 2021). The development of reproductive biotechnology in animal husbandry has made it possible to obtain many offspring from one highly productive cow within one year. This became possible thanks to the use of such methods as *in vivo* and *in vitro* embryo transplantation technology, as well as cryopreservation of genetic material (sperm, oocytes, embryos).

Freezing and storage of genetic material in liquid nitrogen is the most important method of animal reproductive biotechnology, however, the quality indicators of frozen and then thawed biomaterials suffer greatly, which significantly reduces the successful fertilization rates. For example, frozen-thawed spermatozoa, due to poor quality characteristics, often remain incapable of fertilizing an ovum. Some oocytes and embryos die during freezing and thawing (Kumar et al. 2016; Naresh 2016; Murphy et al. 2018; Nongbua et al. 2018; Tkachev et al. 2020b; Gorelik et al. 2021).

For these reasons, research to improve the quality of cryopreserved genetic material is being carried out by scientists around the world. Despite the fact that many positive points have already been achieved, this factor of reproduction requires a study and a scientifically grounded proposal to livestock breeders to increase fruitful inseminations using various technologies (Kumar et al. 2016; Naresh 2016; Murphy et al. 2018; Nongbua et al. 2018). There are two main types of embryo cryopreservation: slow freezing and vitrification. Slow freezing is an older method. In this case, the embryo is placed in an environment with a cryoprotectant, glycerin, or ethylene glycol, packed in a plastic straw and cooled at a rate of 0.50 per minute to -70 (Tkachev et al. 2020b).

Then the straw is touched with tweezers soaked in liquid nitrogen - seeding is carried out (freezing water in the embryo), slowly cooled to -350, and then transferred into liquid nitrogen, and freezing is completed to -1960. The disadvantage of this method is the formation of ice crystals both in the embryo itself and in the environment around the embryo. Ice crystals damage the membranes and membranes of the embryo, causing its death both during freezing and thawing (Youngs 2011; Konc et al. 2014). Cryoprotectants used in variable speed freezing are designed to prevent intracellular crystallization by osmotic dehydration of frozen water and replacing intracellular water with a cryoprotective solution. The degree of dehydration depends

on the duration of cooling or the temperature reached before the embryos are immersed in liquid nitrogen. The type of cryoprotectant and the cooling rate determine the way the embryos thaw (Niemann 1991; Asgari et al. 2012; Do et al. 2016; Tkachev et al. 2020a).

Vitrification is usually achieved by placing the embryos in a very high (5-6M) concentration of cryoprotectant solution, followed by rapid cooling ($> 2000^{\circ}\text{C}/\text{min}$) in liquid nitrogen. Vitrification is based on the ability of highly concentrated aqueous solutions of cryoprotectants to be cooled to very low temperatures. At low enough temperatures, the solution becomes very viscous so that it solidifies into an amorphous glass without the formation of ice crystals. Vitrified embryos should be stored at a temperature not exceeding -130°C . Above this temperature, the solution may become crystalline, which can damage the embryos. To avoid this damage, vitrified embryos must always be stored at temperatures below -130°C , and thawing must be done very quickly to avoid crystallization (Díez et al. 2012; Do and Taylor-Robinson 2020).

Currently, much attention is focused on the study of genetic material and preparations for compliance with their quality standards, including the study of oocytes obtained by different methods. For the successful development of animal husbandry with the introduction of modern biotechnological methods of reproduction, it is important, along with the assessment of bulls by the quality of the offspring and the qualitative characteristics of sperm, to study the qualitative characteristics of oocytes obtained by different methods (Andrabi and Maxwell 2007; Borunova et al. 2017; Tkachev and Tkacheva 2017; Do and Taylor-Robinson 2020).

It has been proven that the cytophysical state of the gametal cells of the donor cow directly affects the state and development of embryos. Therefore, it becomes important to assess the biological characteristics of oocytes obtained both during aspiration from the follicles of donor cows and during removal from the ovaries of slaughter animals. With the development of modern reproductive biotechnology, it has become possible to evaluate oocytes that have been fertilized and cultured at different stages of development. According to the available data, there are significant morphological variations among oocytes, which can affect the ability to develop and the potential ability of the embryo to implant (Sirard and Blondin 1996; Andrabi and Maxwell 2007; Spricigo et al. 2015; Iwasaki et al. 2018; Aguila et al. 2020).

Successful fertilization of oocytes and *in vitro* production of embryos depends on the quality and maturity degree of the oocytes upon receipt. The cumulus cells (CC) surrounding the oocyte play a key role in the maturation of oocytes and are known to supply nutrients, energy substrates, and / or messenger molecules for oocyte development (Vanderhyden et al. 1990; Prokofiev et al. 1992; Otoi et al. 1997). The CC concentration is highly dependent on the efficiency of oocyte collection. Several methods are used to collect oocytes from slaughterhouse ovaries of farm animals. A number of studies have been carried out to compare the effectiveness of methods for collecting oocytes from cattle

(Kątska 1984; Rizos et al. 2002; Bilodeau-Goeseels 2012; Do and Taylor-Robinson 2020).

MATERIAL AND METHODS

Under the research performed, the study used three methods for obtaining oocytes: puncture, section and aspiration for cultivation and *in vitro* fertilization of cow oocytes. The cow ovaries were collected from a local slaughter station and stored in a collection tube containing 0.9% saline in a thermos at 25-30°C. Each ovary was treated individually and the OCC was collected in one of the following three ways.

Puncture: the ovaries were placed in a Petri dish containing 5 ml of OCC's collection medium, held with forceps, and the entire surface of the ovary was punctured with an 18G hypodermic needle. **Section:** the ovaries were placed in a Petri dish containing 5 ml of OCC's collection medium, held with tweezers. The incisions were made over the entire surface of the ovary using a scalpel blade. **Aspiration:** visible follicles were aspirated using an 18G hypodermic needle attached to a sterile 5 ml disposable syringe containing 2 ml of collection medium. The medium, together with the collected OCCs, was then transferred to a 35 mm Petri dish. When collecting the OCC by puncture and cutting, the ovary was completely immersed in the medium. In all three methods, the Petri dishes were left at rest for 5 minutes, which allowed the OCC to settle. Excess medium was removed with a syringe without disturbing the oocytes at the bottom of the Petri dish.

Petri dishes were then examined under an inverted microscope and the total number of collected OCCs was counted. OCCs were divided into 4 classes based on cumulus and nucleus cells: grade A - oocytes completely surrounded

by cumulus cells; Grade B - oocytes partially surrounded by cumulus cells; Grade C - oocytes not surrounded by cumulus cells; and grade D - degeneration observed in both oocyte and cumulus cells. Grades A and B were considered as normal, while grades C and D were considered to be damaged (Khandoker et al. 2001; Khandoker et al. 2005; Khandoker et al. 2012). Follicular fluid was collected from all categories of morphologically healthy superficial follicles by aspiration using a 10 ml syringe with a 19G needle. The following criteria were applied to assess the follicle quality: good - uniform bright appearance, extensive and very fine vascularization and no free-floating particles in the follicular fluid, and atresic - loss of translucency, slightly or dull grayish and/or opaque appearance and free-floating particles in the follicular fluid. At each collection, the liquid from each follicle was combined, centrifuged 2 times at 3000 rpm for 30 minutes. The supernatant was collected and filtered through a 45 mm millipore filter and heated at 65°C for 1 hour.

RESULTS AND DISCUSSION

The results of obtaining OCC by three different methods are presented in Table 1. A total number of 156 oocyte-cumulus complexes were collected out of 40 ovaries by puncture – 50 pcs, section – 47pcs, and aspiration – 59pcs (Table 1). The results showed that puncture and section gave significantly higher total OCCs per an ovary (4.16 and 4.0, respectively) than aspiration (3.68); but a higher number of normal (grade A and B) OCCs per an ovary was observed with aspiration (2.5) than with puncture (1.82) and sections (2.00) (Table 1). During aspiration, OCC was collected from the surface of follicles with a diameter of 3 to 8 mm using a needle. During puncture, all surfaces were pierced with a hypodermic needle; during section, incisions were made along the entire ovarian surface with a scalpel, that is, all sizes of superficial follicles were collected.

Table 1. Obtaining oocyte-cumulus complexes (OCC) by different methods

Method		Puncture	Section	Aspiration
Number of ovaries		12	12	16
Total number of OCCs	n	50	47	59
Grade A	n per an ovary	4.16	4.00	3.68
	n	17	16	32
Grade B	n per an ovary	1.41	1.33	2.0
	n	5	8	8
Grade C	n per an ovary	0.41	0.67	0.5
	n	8	6	5
Grade D	n per an ovary	0.67	0.50	0.31
	n	20	18	14
	n per an ovary	1.67	1.50	0.87

Thus, the smaller number of OCCs collected by the aspiration method in this experiment may be due to the fact that aspiration did not receive OCCs from small follicles and some follicles that are deeply embedded in the ovarian cortex. Puncture and section techniques generate more

residuals, which can interfere with microscopic retrieval of oocytes and also require more washing than aspiration. As a result, the amount of OCC was removed from the cumulus cells due to repeated washing and, ultimately,

resulted in fewer normal OCCs compared to aspiration at the last observation (Chalova et al. 2020).

The result of the study is that aspiration of vesicular follicles with a diameter of 3 to 8 mm using an 18G hypodermic needle is the best method, a simple and effective way to extract morphologically normal oocyte-cumulus complexes from bovine ovaries. To obtain embryos, the method of oocyte aspiration from bovine ovaries with their subsequent cultivation, fertilization and cryopreservation was used. The ovaries were collected from local slaughterhouses and stored in a collection tube containing 0.9% saline in a thermos at 25-30°C. Each ovary was treated with three washes in Dulbecco's phosphate buffered saline (DPBS) and two washes in OCC collection medium (DPBS + 4 mg/ml of BSA + 50 IU/ml of penicillin). A total number of 134 ovaries from 67 cows were processed. The result of obtaining oocyte-cumulus complexes (OCC) is presented in Table. 2.

Table 2. The result of obtaining oocyte-cumulus complexes (OCC)

Method		Aspiration
Number of ovaries		134
Total number of OCCs	n	485
	n per an ovary	3.62
Grade A	n	268
	%	55.25
	n per an ovary	2.0
Grade B	n	76
	%	15.67
	n per an ovary	0.57
Grade C	n	51
	%	10.52
	n per an ovary	0.38
Grade D	n	90
	%	18.56
	n per an ovary	0.67

Table 3. Evaluation of oocyte maturation by expansion of cumulus cells

Indicator		Expansion degree of cumulus cells			
		Level 3	Level 2	Level 1	Total
Number of OCC	n	128	32	5	172
	%	74.42	18.61	6.97	100

As a result of the oocyte cultivation the stage of fertilization, 251 mature oocytes were obtained. Fertilization of prepared oocytes. *In vitro* fertilization means that the oocytes' fertilization with sperm has occurred under artificially maintained optimal conditions outside the uterus of the animal. For successful *in vitro* fertilization, bovine spermatozoa need to undergo a stage of capacitation in order to carry out an acrosome reaction to penetrate the oocyte

At present, there have not been developed the technologies for *in vitro* cultivation of bovine oocytes, which allow obtaining a sufficiently high yield of embryos. Oocyte maturation *in vitro* is subdivided into nuclear and cytoplasmic processes. Nuclear maturation includes resumption of meiosis and progression to the metaphase-II stage. Cytoplasmic maturation encompasses many cellular processes that must be completed in order for oocytes to be fertilized and develop into normal embryos and offspring. The rates of maturation and fertilization depend on the quality of oocytes, the sufficiency and efficiency of the environment, and the optimization of incubation conditions.

OCC of normal quality was cultured in TCM-199 medium (Sigma Chemicals, USA) supplemented with 2.5% BSA and 10% follicular fluid for 27 hours. After maturation, the degree of expansion of cumulus cells was determined under a microscope at 10-fold magnification as level-1: small expansion of the OCC; level-2: moderate expansion of the OCC, and level-3: significant expansion of the OCC. After that, half of the mature OCCs were taken from each drop, and oocytes were taken from the cumulus cells. Then the oocytes were placed on a glass slide covered with a cover slip (fixed with acetoethanol (acetic acid: ethanol, 1:3), stained with 1% acetoorcein) and examined under an inverted microscope at high magnification (100x) with immersion oil. The level of nuclear maturation was assessed. The assessment of oocyte maturation by the expansion of cumulus cells is presented in Table 3. As can be seen from the table, after 27 hours of cultivation, 74.42% of the oocytes matured to the stage of fertilization (Chalova et al. 2020).

The results of assessing the indicators of the oocyte nuclear maturation are presented in Table 4. After 27 hours of cultivation, 91.85% of oocytes-initiated meiosis, and 71.51% of oocytes reached the metaphase II stage. Thus, this method of OCC cultivation made it possible to achieve maturation of more than 70% of oocytes.

Table 4. Assessment of oocyte nuclear maturation

Indicator		Nuclear maturation of oocytes			Total
		Diplotene	Metaphase I	Metaphase II	
Number of OCC	n	14	35	123	172
	%	8.15	20.34	71.51	100

membrane and fusion with the oocyte plasma membrane. Normal fertilization results in the formation of zygotes with male and female pronuclei (PN). In order to fertilize the remaining matured OCCs, a fertilization medium was prepared and its pH was adjusted to 7.8 on the day of use. Sampels with frozen bullseed were thawed in a water bath at 37°C for 30-40 seconds. The concentration was approximately 250 µl thawed semen per 1 ml capacitation medium. Capacitation medium consisted of modified Tyrode's medium, without calcium ions. After incubation for

one hour, the upper layer of the medium with a volume of 0.5-0.8 ml, containing most of the motile spermatozoa, was removed from the tube and washed twice by centrifugation at 500 g for 7-10 minutes (Chalova et al. 2020).

After 15 min incubation with heparin (200 µg/ml), the suspension was diluted to a concentration of 50 million spermatozoa per ml. Then, droplets were prepared for insemination, covered with mineral oil and kept in a CO₂ incubator for 4-5 hours for pre-incubation. Then the remaining half of the matured OCC (the other half was used to assess the maturation of nuclei) was transferred into each of the sperm drops and incubated for 5 hours in an incubator at 38.5°C with 5% CO₂ in humidified air. After 5 h incubation, several OCCs from each drop were separated

from cumulus cells by repeated pipetting and fixed on a glass slide with acetoethanol (acetic acid: ethanol, 1: 3 v/v) and stained with 1% acetoorcein (Gorelik et al. 2021).

After drying, the slides were examined at high magnification (100x) using immersion oil to observe the formation of pronuclei: the presence of a male (PN) and female pronucleus (PN) - normal fertilization; an oocyte with one PN, asynchronous PN development / parthenogenetic activation, or one PN was hidden by lipid droplets and an oocyte with more than two PNs - polyspermia. For fertilization, 251 oocytes were selected from experiments on methods of oocyte elution. The results of *in vitro* fertilization are presented in Table 5. As a result, 56.57% of mature oocytes were fertilized. 142 zygotes were sent for cryopreservation for further study (Gorelik et al. 2021).

Table 5. Results of *In vitro* fertilization

Indicator	Normal fertilization	1 pronucleus, lack of fertilization or parthenogenesis	Polyspermia (more than 3 pronuclei)	Total
n	142	85	24	251
%	56,57	33,86	9,57	100

Thus, the use of modern reproductive biotechnological methods in animal husbandry makes it possible to increase the offspring number produced by highly productive cows and outstanding bulls-producers. Our research on the reproductive biotechnology application to replicate highly productive animals and increase the number of cattle showed good results. It is important to note that oocytes can be obtained both by cows' aspiration using OPU technology and at slaughter stations after slaughtering highly productive cows culled for various reasons. In this regard, it becomes necessary to study the development of oocytes and embryos at all stages of growth and development (Chalova et al. 2020).

CONCLUSION

The findings of the present study highlight an urgent issue for future researchers is the development and creation of nutrient media and optimal cultivation conditions, close to natural, both for oocytes and for embryos, which will increase the percentage of oocyte and high quality embryo yield. In addition, further development, improvement and implementation of *in vitro* embryo transplantation technology will allow maintaining and increasing the number of animals used not only in the industry of the agro-industrial complex, but it will also be useful when working with rare and endangered animal species.

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Biomedical Communication

Cissus quadrangularis Aqueous Extract Attenuates Angiotensin II-Induced Hypertension In Urethane-Anesthetized Rats

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ABSTRACT

Hypertension is a major cardiovascular problem resulting in significant mortality. *Cissus quadrangularis* having several pharmacological effects has not been evaluated for its ability to modulate blood pressure. Thus, the ability of *C. quadrangularis* aqueous extract (CQE) to modulate blood pressure was evaluated in normotensive and angiotensin II-induced hypertensive rats under urethane anesthesia. The animals were divided into four groups namely, control (saline injection), CQE (extract alone, 10 mg/kg), Ang II (Ang II alone, 0.5 µg/kg) and Ang II + CQE (Ang II + extract). All treatments were delivered by intravenous route and in Ang II + CQE group, Ang II was injected 30 min after injection of the extract. Hemodynamic parameters, systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial blood pressure (MABP), and heart rate (HR) were recorded by the BIOPAC system after the cannulation of the carotid artery and jugular vein. The results indicated that CQE lowered SBP, DBP, MABP and heart rate to varying degrees in normotensive rats compared to control groups. In case of angiotensin II-induced hypertension, CQE administration resulted in substantial decrease in SBP, DBP, and MABP which were raised by Ang II. CQE reduced SBP, DBP, and MABP by 12, 59, and 11%, respectively. It is worth noting that, while SBP was not brought down to baseline levels by CQE, DBP was, suggesting significant hypotensive/antihypertensive activity of CQE. Further research is required to determine the molecular mechanism of *C. quadrangularis* extract's hypotensive/antihypertensive action and to conduct clinical trials to establish its optimal use as an antihypertensive therapeutic.

KEY WORDS: ANTIHYPERTENSIVE, AT1, AT2, HADJOD, HYPOTENSIVE.

INTRODUCTION

Hypertension is a major cause of death from cardiovascular complications such stroke, chronic kidney disease, and congestive heart disease. Despite many pharmacotherapies and lifestyle changes, the treatment of hypertension is still being investigated and remains difficult, with blood pressure not remaining at a normal level in around half of hypertensive patients. Alternatively, researchers have

led concerted efforts to explore novel therapeutics with hypotensive/anti-hypertensive properties from medicinal plants/herbs. As an outcome, some of these medicinal plants with acclaimed hypotensive/antihypertensive properties have been validated, while others have been dismissed (Etuk 2006; Al-Anbaki et al. 2021; Azizah et al. 2021; Verma et al. 2021). *Cissus quadrangularis* Linn. (Vitaceae), often known as "bone setter" in Ayurvedic medicine owing to its bone fracture healing properties, is a tropical perennial plant found in India, Sri Lanka, Malaysia, Java, and West Africa. It is a perennial climber with four wings internodes and a slender, fleshy fibrous, smooth stem (Sundaran et al. 2020; Kaur et al. 2021). Aside from being used as a vegetable in India, it is also used as a folkloric medicine

Article Information:*Corresponding Author: f.masfoor@qu.edu.sa
Received 11/10/2021 Accepted after revision 21/12/2021
Published: 31st December 2021 Pp-1459-1462
This is an open access article under Creative Commons License,
Published by Society for Science & Nature, Bhopal India.
Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.13>

to treat menstruation irregularities, dyspepsia, flatulence, colic, convulsions, asthma, inflammation, infections, and obesity. A number of bioactive compounds, including flavonoids (quercetin, kaempferol, daidzein, and genistein), triterpenoids (friedelin, β -amyryn and 7-oxo-onocer-8-ene-3 β 21- α diol), stilbene derivatives (quadrangularin A, quadrangularin B, quadrangularin C, resveratrol piceatannol, pallidol, and parthenocissus), iridoids (6-O-[2,3-dimethoxy]-trans-cinnamoyl catalpol and 6-O-meta-methoxy-benzoyl catalpol, picroside and pallidol), phytosterols (β -sitosterol, β -sitosterol-O-b-D-glucoside and ketosterosterol), phenolic compounds and tannins have been isolated from the aerial parts of *C. quadrangularis* (Shah 2011; Sundaran et al. 2020; Zaki et al. 2020; Bafna et al. 2021; Kaur et al. 2021).

Various extracts and formulations of *C. quadrangularis* have been attributed with potential pharmacological effects including anti-osteoporotic, anti-obesity, antioxidant, antidiabetic, anti-nociceptive, anti-ulcer, analgesic and anti-inflammatory effects (Murthy et al. 2003; Shirwaikar et al. 2003; Jainu and Devi 2004; Jainu and Devi 2006a; Jainu and Devi 2006b; Oben et al. 2006; Rao et al. 2007; Mate et al. 2008; Lekshmi et al. 2015; Jain et al. 2020; Bafna et al. 2021; Kaur et al. 2021). Despite the fact that researchers have investigated into several pharmacological effects of *C. quadrangularis*, some of which have even entered clinical trials, its potential use against hypertension has yet to be revealed. Thus, the potential of *C. quadrangularis*

aqueous extract to modulate blood pressure and heart rate in urethane-anesthetized rats was investigated against angiotensin II-induced hypertension.

MATERIAL AND METHODS

C. quadrangularis aerial parts were collected from Mysore region in India, subsequently identified by Dr. Sharanappa and a reference specimen was retained in the laboratory. Normal saline, heparin and adrenaline were purchased from local pharmacy. Angiotensin II and urethane was procured from Sigma Aldrich, USA. All of the other chemicals and reagents used in the analysis were of the finest analytical purity. The sample was washed with running water to eliminate dirt before being spread out on large trays. The trays were placed in a shady area for 12 hours before being chopped into small pieces. The chopped pieces were then dried for 24 hours in a hot air oven set at 60°C. In a cyclonic laboratory blender, the dry material was pulverized until it passed through a 60-mesh screen. The powder was kept in an airtight container in the refrigerator for subsequent use. *C. quadrangularis* extract (CQE) was prepared by mixing the powder with hot distilled water at 70°C (1:8 w/v) and extracting it using a mechanical shaker for 24 hours. The extract was then filtered, and the residue was re-extracted with hot water for 12 hours before being filtered again. Both filtrates were pooled and evaporated in a flash evaporator set at 60°C to yield CQE, which was stored in an airtight container in the refrigerator for future use.

Table 1. Effect of *C. quadrangularis* extract on cardiac parameters (SBP, DBP, MABP and HR)

Groups	SBP	DBP	MABP	HR
Control	114.4 ^b \pm 1.8	85.4 ^b \pm 1.7	104.0 ^b \pm 3.3	271 ^b \pm 0.7
CQE	106.1 ^a \pm 3.8	70.6 ^a \pm 1.4	88.9 ^a \pm 2.9	258 ^a \pm 0.6
Ang II	190.4 ^d \pm 5.6	136.0 ^d \pm 1.7	159.9 ^d \pm 1.7	269 ^b \pm 1.2
Ang II + CQE	168.9 ^c \pm 3.2	85.4 ^c \pm 13.9	142.8 ^c \pm 2.6	270 ^b \pm 2.2

*Values were represented as mean \pm SD (n=6). Values with different superscript letters in columns differ significantly from each other at $p \leq 0.05$.

The research ethics committee approved the study (RU/REC-001/1.2/1441-42), and standard animal experimentation protocols were followed. Male albino rats weighing 350 \pm 30g were kept separately in polyacrylic cages in an air-conditioned animal house maintained at 25 \pm 2°C and 55 \pm 5% relative humidity. The animals were fed a standard laboratory pellet diet and were given free access to potable water. The rats were randomly categorized into four groups, each with six animals and received following treatment intravenously at the time of experiment: Group 1 – Control: received 0.2 mL normal saline. Group 2 – CQE: received CQE (10 mg/kg) dissolved in 0.2 mL normal saline. Group 3 – Ang II: received angiotensin II (0.5 μ g/kg) with 0.2 mL normal saline. Group 4 – Ang II+CQE: received angiotensin II (0.5 μ g/kg) + CQE (10 mg/kg) dissolved in 0.2 mL normal saline.

The hypotensive/antihypertensive activity of *C. quadrangularis* extract was evaluated using the procedure reported by Mohebbati et al. (2020) with some modifications. An intraperitoneal injection of urethane (1.25 g/kg) was used to anesthetize the animals. The rat's necks and inguinal regions were shaved using an electric shaver after the anaesthesia was confirmed. The jugular vein was surgically cannulated for medication delivery, while the left carotid artery got PE-50 tubing cannula to record cardiovascular data. The arterial cannula was connected to a blood pressure transducer (SS13L) and the venous cannula to a syringe using a three-way plastic stop cock and a stainless-steel needle at the end of the PE tubing. Before cannulation, both cannulae were prefilled with heparinized saline. Systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial blood pressure (MABP), and heart rate (HR) were all constantly monitored by the system (BIOPAC Systems

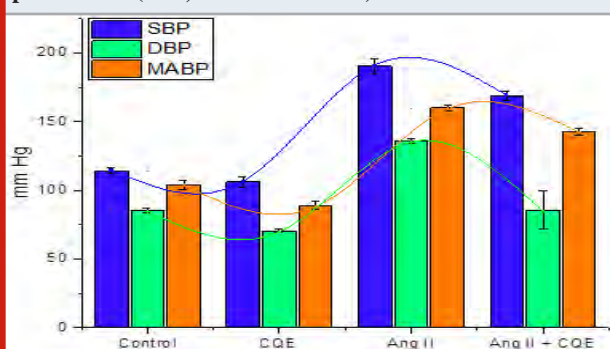
Inc, CA, USA). A decrease in SBP of 10-20 mm Hg was deemed significant hypotensive/antihypertensive activity. Data on different cardiac parameters (SBP, DBP, MABP and HR) were expressed as mean \pm SD in all groups. The data was subjected to one-way ANOVA and Tukey's post hoc tests using SPSS ver. 20.0 (IBM, USA) software. Values were considered significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

Hypertension is a major risk cardiovascular risk factor in reducing people's life expectancy and quality of life. Because the activation of the renin-angiotensin system (RAS) is thought to be a key precipitating factor in hypertension, medications that suppress the RAS may be therapeutically valuable (Chen et al. 2018). Because many medicinal plants have been shown to regulate the RAS system to varying levels (Etuk 2006), the current study explored into the hypotensive/antihypertensive potential of *C. quadrangularis* aqueous extract against angiotensin II-induced hypertension in urethane-anesthetized rats. Table: 1 summarizes the data on systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial blood pressure (MABP), and heart rate (HR) of different groups after injecting designated treatments intravenously after cardiac parameters were stabilized and responses were monitored for 15 minutes.

In control group, wherein saline was injected did not elicit any significant changes in any of the cardiac parameters, whereas the injection of *C. quadrangularis* extract at a dosage of 10 mg/kg significantly ($p < 0.05$) lowered SBP, DBP, MABP and heart rate to varying degrees in the CQE group. In group 3, conversely, angiotensin II (0.5 g/kg) injection resulted in a substantial rise in SBP, DBP, and MABP to the extent of 67, 59, and 54 percent, respectively, while heart rate remained unchanged compared to control group. CQE efficiently counteracted the blood pressure rising effect of angiotensin II in group 4 rats when CQE (10 mg/kg) was injected with angiotensin II (0.5 g/kg). CQE reduced SBP, DBP, and MABP by 12, 59, and 11%, respectively. It is worth noting that, while SBP was not brought down to baseline levels by CQE, DBP was, suggesting significant hypotensive/antihypertensive activity of CQE (Fig: 1).

Figure 1: Effect of *C. quadrangularis* extract on cardiac parameters (SBP, DBP and MABP)



Antihypertensive medicinal herbs usually modulate RAS by inhibiting angiotensin converting enzyme (ACE) activity or by modulating angiotensin II levels by inhibiting binding to AT1 and AT2 receptors (Mohebbati et al. 2020). Some investigations have found that antioxidant flavonoids like quercetin, triterpenes, anthocyanins and proanthocyanidins suppress RAS via reducing ACE and Ang II binding. As a result, natural products containing antioxidant compounds are thought to be beneficial against hypertension (Parichatikanond et al. 2012; Mohebbati et al. 2020). Various extracts of *C. quadrangularis* have been found to be efficient free radical quenchers, including nitric oxide (NO), superoxide anion, singlet oxygen, and hydroxyl radicals produced by the human body's cells as a result of oxygen utilization (Murthy et al. 2003; Dhanasekaran 2020; Jain et al. 2020; Bafna et al. 2021; Kaur et al. 2021).

These extracts have been shown to contain a wide range of chemical compounds, including flavonoids (quercetin, kaempferol, daidzein, and genistein), triterpenoids (friedelin, β -amyryn and 7-oxo-onocer-8-ene-3 β 21- α diol), stilbene derivatives (quadrangularin A, quadrangularin B, quadrangularin C, resveratrol piceatannol, pallidol, and parthenocissus), iridoids (6-O-[2,3-dimethoxy]-trans-cinnamoyl catalpol and 6-O-meta-methoxy-benzoyl catalpol, picroside and pallidol), phytosterols (β -sitosterol, β -sitosterol-O-b-D-glucoside and ketosterosterol), phenolic compounds and tannins, the majority of which have been associated with significant antioxidant and antihypertensive activities (Amarowicz 2007; Wang et al. 2007; Frombaum et al. 2012; Rodrigo et al. 2012; Kucharska et al. 2017). As a reason, the hypotensive/antihypertensive effect of CQE can be attributed to the antioxidant compounds' blocking of angiotensin II binding to angiotensin receptors.

CONCLUSION

The findings of the present study revealed that *C. quadrangularis* aqueous extract had strong hypotensive potential in normotensive rats and antihypertensive effect in angiotensin II-induced hypertension in urethane-anesthetized rats. Further research is required to determine the molecular mechanism of *C. quadrangularis*' hypotensive/hypertensive action and to conduct clinical trials to establish its optimal use as an antihypertensive therapeutic.

Conflict of Interests: Authors declare no conflicts of interests to disclose.

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Parasitological Communication

Prevalence of Swine Nematodes in Moscow, Russia

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ABSTRACT

To continue the fight against pig helminthiasis, a detailed analysis of the current prevalence of nematodes in these productive animals is required. The assessment of the material after the technological slaughter of pigs kept in the Moscow region revealed the presence of parasitization in their intestines of four nematodes (*Ascaris suum*, *Metastrongylus* spp., *Trichocephalus* spp., *Strongyloides* spp.) In the form of monoinvasion and any combination thereof. The total infestation of pigs with intestinal nematodes was 55.24%. *Trichocytic* invasion (*Trichocephalus* spp.) Occurred in pigs most often (23.77%), ascarous invasion (*Ascaris suum*) reached 22.38%, strongyloid invasion (*Strongyloides* spp.) Was noted in 9.09% of cases, metastrongylos (*Metastrongylus* spp.) was present in 1.75% of cases. In conditions of an increase in the severity of monoinvasion, the size of helminth eggs decreased in *Trichuris*: size in length by 5.9%, size in width by 7.4%, in *Ascaris*: length by 4.9%, width by 4.6%, in strongylate eggs a decrease in size is also noted. In the case of mixed *Ascaris-Metastrongylus* invasion, there was a clear antagonistic relationship, manifested in a decrease in the size of *Ascaris* eggs. The size of the eggs of *Trichuris* in the case of an invasion mixed with *Ascaris* decreased, which is apparently associated with the development of antagonism between them. Comparative analysis of information on the prevalence of nematodes in pigs in farms of the Moscow region and their size can help to increase the effectiveness of treatment and prophylactic measures against helminthiasis, and can be taken into account in the course of further examination of pigs in the Moscow.

KEY WORDS: EPIZOOTOLOGY, HELMINTHS, MONOINVASION, MIXED INVASION, PIGS.

INTRODUCTION

The processes of growth and development in productive animals are of great economic importance (Bespalov et al. 2018; Zavalishina 2018a). The implementation of these processes depends on the species characteristics of animals, on the state of many biological processes occurring in different cells and tissues of their body, the level of nutrition, quality of nutrition, the presence or absence of pathological processes (Vorobyeva et al. 2018; Zavalishina 2018e). Pigs are one of the most reliable sources of quality and affordable protein products (Maksimov et al. 2018; Zavalishina 2018c). Their development ensures the production of about 40% of all meat obtained in the world, the volume of which tends to grow. At the same time, the development of various diseases in animals during their entire ontogenesis, a significant proportion of which are various parasitic diseases, hinders the growth of the efficiency of pig breeding. Their presence contributes to the development of allergic processes in pigs,

morphological disorders in tissues, weakening of various life processes, including a decrease in the absorption of nutrients (Laha et al. 2014; Fava et al. 2020; Lobanova 2021).

The development of these processes inhibits the growth of animals, reduces their growth, reduces the number of obtained offspring and increases the likelihood of death of pigs (Kumsa and Kifle 2014). At the same time, the presence of parasites in the body of pigs aggravates the course of any concomitant diseases and reduces the effectiveness of any vaccination. All these circumstances reduce the quality of meat with frequent culling of organs (Nur-E-Azam et al. 2015). This dictates the need to control the level of prevalence of gastrointestinal helminthiasis in pigs at all stages of their maintenance (Allwin et al. 2015; Zavalishina 2018b). In pigs in the intestine, they are capable of parasitizing *Ascaris*, *Trichuris*, *Strongylate*, *Metastrongyla* individually and in various combinations (Yamov and Antropov 2008; Donnik and Sazhaev 2012; Ngowi et al. 2014; Allwin et al. 2015; Obebe et al. 2020).

A wide variation in the intensity of invasion by different types of nematodes is possible under conditions of monoinvasion and with mixed infection. The size of the

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Received 14/09/2021 Accepted after revision 15/12/2021

Published: 31st December 2021 Pp- 1463-1467

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Published by Society for Science & Nature, Bhopal India.

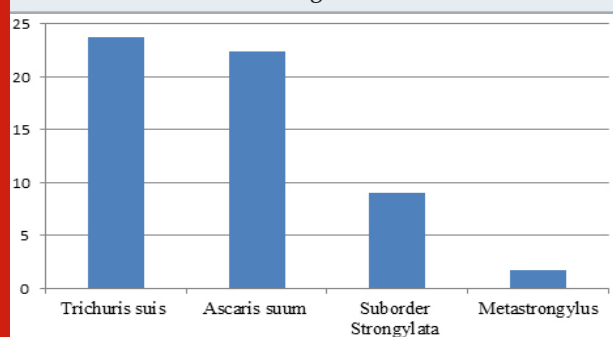
Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.14>

parasites themselves and their eggs may also vary. The dynamics of these indicators can be influenced by the level of activity of the immune system of animals, housing conditions, peculiarities of their feeding, and it is possible that the processes of interspecific relationships. The purpose of the study is to assess the prevalence of nematodes in pigs kept in the Moscow region of Russia.

MATERIAL AND METHODS

The assessment of the prevalence of pig helminthiasis was carried out in animals kept on the territory of the Moscow region. Pigs were taken to the slaughterhouse. 286 samples of pig feces were taken from the upper sections of their large intestine. The Kotelnikov-Khrenov method was used using a saturated solution of ammonium nitrate, which acts as a flotation liquid. The extent of invasion was revealed as the ratio of the number of invaded animals to the total population, expressing the value as a percentage. The intensity of nematode invasion (infestation with parasites of one animal) was determined using a Goryaev counting chamber. The species of the detected eggs was identified by the available morphological characteristics (Anne and Gary 2012). The process of morphometry of helminth eggs was carried out using the ImageJ software (Wayne Rasband of Research Services Branch of the National Institute of Mental Health). Statistical processing of the obtained data was carried out using the methodology for calculating the significance of differences in mean values according to the student's t-test.

Figure 1: Extensiveness of invasion in pigs by varieties of helminths in the Moscow region of Russia



RESULTS AND DISCUSSION

As a result of the study of collected fecal samples from pigs kept in the Moscow region, a general infestation of them with helminthiasis was revealed, the value of which was 55.24%. During the study, the following helminths were identified: *Trichuris suis*, *Ascaris suum*, *Metastrongylus* spp. and strongylata (*Strongylata suborder*), the severity of which is shown in Figure 1 and Table 1. This observation is consistent with the work of Russian and foreign researchers who confirm the wide prevalence of helminths found in the work carried out in pigs. The performed study found that the most common in pigs is trichoid invasion, the frequency of which was 23.77%, which is consistent with earlier studies

of domestic authors (Savelyev and Kulikova 2006; Laha et al. 2014; Petersen et al. 2020).

The incidence of ascariasis in pigs was 22.38%. This frequency is confirmed in the works of other researchers (Safiullin 2009). It can be assumed that the prevalence of ascarous infestation among the pig population in the regions of Russia ranges from 10.0% to 30.0%. The level of invasion by intestinal strongylates in the work carried out reached 9.09%, and the incidence of invasion by metastrongylae was the lowest 1.75%. It has been noticed that monoinvasions in pigs in the Moscow region of Russia occupy a leading place in the number of helminthiasis (in more than 50% of cases). Di-invasions occurred in about 20% of cases, and invasions by three types of parasites were quite rare, which is confirmed in earlier studies (Ngowi et al. 2014). Among the mixed invasions, the following variants were noted: *Ascaris* + *Metastrongyla*, *ascaris* + *Strongylata*, *Ascaris* + *Trichuris*, *ascaris* + *Trichuris* + *Strongylata*. *Ascaris* + *Trichuris*, which occurred in 6.99% of cases, were very frequent combinations in the study. The results of evaluating the size of the eggs of parasites are shown in tables 2 and 3. It was found that for certain types of helminths: *ascaris*, *strongylata* and *metastrongyla*, parasitism is characteristic when one animal is highly infested with parasites. It is rare in *Trichuris suis* nematodes, which is also confirmed by earlier studies (Allwin et al. 2015; Petersen et al. 2020).

It was found that the size of the eggs of *Ascaris* in the case of monoinvasion reaches - 77.2 μm x 63.1 μm with a low infection with parasites of one animal and 73.6 μm x 60.3 μm in conditions of high infection with parasites of one animal. In previous studies, it was proved that the length of the eggs of *Acan* be from 50 μm to 100 μm (Anne and Gary 2012). The value of the coefficient of variation in the size of *Ascaris* eggs with an increase in parasite infestation of one animal decreased for length from 9.88% to 6.11% (lengthwise), for width from 10.63 to 7.03%. In the case of an increase in the parasite infestation of one animal, the size of the *Ascaris* eggs decreased in length by 4.9%, in width by 4.6% (Petersen et al. 2020).

The size of *Trichuris* eggs was found to be 78.2 μm x 34.6 μm at a low invasion, and 73.8 μm x 32.2 μm in the case of an average invasion. The revealed sizes turned out to be somewhat larger than those observed in previous studies (Anne and Gary 2012). Apparently, this is due to the fact that in the work carried out, samples were taken from the upper sections of the colon and the eggs of *Trichuris* could be immature. The value of the coefficient of variation of the size of the trichuroz eggs increased little with the growth of the parasite infestation of one animal, which indicates a high reliability of the obtained numerical values of their sizes. In the case of an increase in the severity of invasion, the size of *Trichuris* eggs decreased: length by 5.9%, width by 7.4% (Petersen et al. 2020; Romashov et al. 2021).

The size of strongylate eggs varied significantly: with low invasion from 130.7 μm x 70.7 μm , with high invasion up to 86.3 μm x 51.3 μm . The value of the coefficient of variation for the length of the eggs was 27.67% and for the width it corresponded to 30.45%, indicating a significant

variability in their sizes. Apparently, this depends on the place of sampling of feces from the intestine (there was an immaturity of the eggs of these helminths), when several species from the Strongylata order were parasitized

together. At the same time, it is recognized that the eggs of helminths of this order belong to the genera *Hyostromylus*, *Trichostrongylus*, *Oesophagostomum* and *Globocephalus*, it is difficult to distinguish visually (Anne and Gary 2012; Petersen et al. 2020; Romashov et al. 2021).

Table 1. Distribution of nematodes among the surveyed pig population

Helminths	Number of positive samples, (n)	Extensiveness of invasion, %
<i>Trichuris suis</i> (monoinvasia)	42	14.68
<i>Ascaris suum</i> (monoinvasia)	25	8.74
Suborder Strongylata (monoinvasia)	21	7.34
<i>Ascaris suum</i> + <i>Trichuris suis</i>	20	6.99
<i>Ascaris suum</i> + <i>Metastrongylus</i>	7	2.45
<i>Ascaris suum</i> + suborder Strongylata	8	2.79
<i>Ascaris suum</i> + <i>Trichuris suis</i> + suborder Strongylata	2	0.69
<i>Trichuris suis</i> (total)	68	23.77
<i>Ascaris suum</i> (total)	64	22.38
Suborder Strongylata (total)	26	9.09
<i>Metastrongylus</i> (total)	5	1.75
Total:	158	55.24

Table 2. Sizes of helminth eggs in monoinvasion

Helminths	Number of samples (n)	The size of eggs under conditions of different invasion intensity (M±m), µm					
		low		average		high	
		length	width	length	width	length	width
<i>Trichuris suis</i> V	15	78.2±1.48 2.39	34.6±0.73 2.87	73.8±1.52 3.82	32.9±1.24 6.53	- -	- -
<i>Ascaris suum</i> V	15	77.2±3.75 9.88	63.1±3.07 10.63	- -	- -	73.6±1.12 6.12	60.3±1.25 7.03
suborder Strongylata V	12	130.7±10.65 27.67	70.7±6.52 30.45	- -	- -	86.3±2.25* 9.16	51.3±0.62* 4.08

*differences are significant in relation to parasite infestation of one animal (p <0.05) V - coefficient of variation, %

In the case of high parasite infestation of one animal with strongylates, the value of the variation coefficient decreased in length to 9.16% and in width up to 4.08%, which obviously needs to be regarded as a consequence of the presence of only one species of strongylate. Under conditions of increasing intensity of invasion by strongylates, the size of eggs decreased significantly: in length by 51.4%, in width by 37.8%. The size of the metastrongil eggs was 68.4 µm x 54.1 µm, which is higher than the values obtained by other researchers (Anne and Gary 2012). The value of the coefficient of variation for these eggs was not clarified, since metastrongylous monoinvasion was not widespread, and the presence of ascaris eggs does not make it possible to draw final conclusions about the severity of variation in the eggs of these helminths. that their size in length and width under

conditions of monoinvasion, as the severity of invasion in strongylates increases, statistically significantly decreases (p <0.05) (Petersen et al. 2020; Romashov et al. 2021).

From literary sources it is known that with increasing degree of invasion, helminths. Apparently, this regularity is also characteristic of the size of the eggs laid by helminths (Zavalishina 2018d; Nasurdinova and Zhigileva 2007). The conducted studies revealed that the sizes of eggs of ascaris under conditions of monoinvasion and mixed invasions, a combination of *Ascaris* and *Trichuris* differed little under conditions of the same severity of ascaronous parasitism. In the case of mixed ascaris-metastrongylous parasitism, there was a tendency to an increase in the length and an increase in the width of the ascaris eggs. It can be assumed that the

size of *A. suum* eggs is not affected by the concomitant trichurosis invasion, and metastrongylae apparently exhibit antagonistic effects on *Ascaris*, which is manifested in the size of their detected eggs. The sizes of eggs of *Trichuris*

under the conditions of mixed with *Ascaris* invasion decreased in length and width compared to the conditions of monoinvasion, which indicates the depressive effect of *A. suum* on *Trichuris suis* (Romashov et al. 2021).

Table 3. Sizes of helminth eggs with mixed invasion

Mixed invasion	Number of samples (n)	Size of eggs under conditions of different intensity of invasion (M ± m), µm					
		low		average		high	
		length	width	length	width	length	width
<i>Ascaris suum</i> (<i>Ascaris suum</i> +	8	77.2±2.63	60.3±2.26	-	-	-	-
<i>Trichuris suis</i>) <i>Ascaris suum</i>	5	-	-	-	-	76.2±1.4*	63.3*±0.82*
(<i>Ascaris suum</i> + metastrongylus) <i>Trichuris suis</i> (<i>Ascaris suum</i> + <i>Trichuris suis</i>)	8	73,8±0,36	33,8±0,27	-	-	-	-
<i>Metastrongylus</i> (<i>Ascaris suum</i> + etastrongylus)	5	-	-	-	-	68.4±2.15	54.1±0.32
*differences are statistically significant compared with monoinvasion (p <0.05)							

CONCLUSION

The finding of the present study suggests that the prevalence of infestation of pigs by intestinal nematodes in the Moscow region of Russia has reached 55.24%. Of the intestinal helminthiasis in pigs, the most common invasion is trichurosis - 23.77%, ascarous - 22.38%, strongylatous - 9.09% and metastrongylous - 1.75%. In the case of an increase in the intensity of the monoinvasion process, a decrease in the size of eggs was noted in all parasites. In the case of mixed invasion, a clear antagonistic relationship was noted, leading to a decrease in egg size. The information obtained on the interspecific relationships of helminths under conditions of mixed invasion can help in the development of anti parasitic measures in pigs.

Conflict of Interests: Authors declare no conflicts of interests to disclose.

ACKNOWLEDGEMENTS

The study was conducted at the expense of the authors. The study was approved by the local ethics committee of the Moscow State University of Food Production (Protocol №11 of January 17, 2018). Authors thank the administration of the Moscow State University of Food Production for the opportunity to research its basis.

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Biomedical Communication

Hepatoprotective Activity of *Helicteres isora* Ethanol Extract Against Paracetamol-Induced Liver Injury in Mice

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ABSTRACT

Helicteres isora L. is a medicinal plant which is used in several diseases, such as snake-bite, dog-bite, diarrhoea and constipation in a new born baby, gastrointestinal disorders, diabetes, cancer, and infections. This plant has also been used in the management of liver damage through traditional medicine. However, the hepatoprotective activity of *H. isora* L. ethanolic extract has not been reported so much. The present work was carried to investigate the hepatoprotective effect of *H. isora* L. against paracetamol-induced liver injury in Swiss mice. Paracetamol (PCM) is widely used as an analgesic and antipyretic drug which at high dose can lead to undesirable side effects, such as hepatotoxicity. Paracetamol induce hepatotoxicity was evaluated by an increase ($P<0.05$) in AST and ALT serum activity. Paracetamol hepatotoxicity was also manifested by an increase in ($P<0.05$) lipid peroxidation and depletion of reduced glutathione (GSH) in liver tissue. Ethanol extract of *H. isora* L. (250, 500 and 1000 mg/kg bw/day) significantly restored the PCM-induced alterations in the biochemical activities of blood and liver tissues. The hepatoprotective effect of *H. isora* L. was also confirmed by the histopathological examination of liver tissue. Histopathological examination of liver sections in mice administered with 1000 mg/kg bw/day doses of the extract were perfectly protected almost similar to those of untreated mice. The results indicated the hepatoprotective nature of studied plants extract against paracetamol induced toxicity. Our study scientifically validates the folkloric claim as well as traditional uses of *H. isora* L. as hepatoactive medicine. The results of this study suggests a new direction in the treatment of liver disease in future.

KEY WORDS: ETHANOL EXTRACT, HELICTERES ISORA L., HEPATOPROTECTIVE ACTIVITY, PARACETAMOL, SWISS MICE.

INTRODUCTION

Liver is the principal and metabolic organ involved in the metabolism of vital molecules. Liver diseases have become one of the major causes of morbidity and mortality all over world. From among, drug induced liver injury (DILI) is one of the most common causative factors that poses a major clinical and regulatory challenge (Rusmann et al. 2009; Sandhu and Navarro 2020). In spite of tremendous advances in modern medicine, there are hardly any reliable drugs that protect the liver from damage and/or help in regeneration of hepatic cell. Therefore, searching for effective and safe drugs for liver disorders are continues to be an area of interest. Plants and natural products have been used traditionally worldwide for the prevention and treatment of liver disease.

Scientific research has supported the claims of the medicinal efficacy of several of these herbal compounds, as evidenced from the voluminous work on their hepatoprotective potentials (Zhang et al. 2020; Shakya 2020).

More than 700 mono- and polyherbal formulations from over a hundred different plants are available for use (Lahon and Das 2011). *Helicteres* L. genus has pantropical distribution, comprising approximately 60 species in America and Asia. The hepatic protection exerted by the *H. isora* L., *H. hirsuta* and *H. angustifolia* species were reported in many researches (Maqboo et al. 2019; Bachar et al. 2020). *H. isora* L. (Sterculiaceae) is a shrub or small tree found in forests. Fruits, seeds, bark and roots of the plant are used (Kumar and Singh 2014; Fernandes et al. 2020; Nguyen 2020). *H. isora* L. has been used traditionally as an herbal drug for treatment of many ailments throughout south-east Asia since ancient times, in folk and tribal medicines. Roots of *H. isora* L. was reported to have hepatoprotective activity against carbon tetrachloride induced liver damage in rats (Chitra and Prema

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Received 14/10/2021 Accepted after revision 18/12/2021

Published: 31st December 2021 Pp- 1468-1472

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.15>

2009; Jain et al. 2014; Gaikwad and Dhasade 2019). *H. isora* L. bark ethanolic extract was demonstrated hepatoprotective activity against carbon tetrachloride induced liver damage in rats and mice (Fernandes et al. 2020).

Pharmacological studies of different plant parts of *H. isora* L. based on animal models have revealed its hepatoprotective property. There has been no scientific study on the hepatoprotective activity of arial parts of *H. isora* L. previously reported in literature. Our preliminary experimental results were highly encouraging as they revealed that AST, ALT levels were significantly lowered after oral administration of *H. isora* extract in paracetamol induced liver injury model. Thus, the present investigation confirms the hepatoprotective activity of *H. isora* and its effect on serum liver enzyme, MDA and GSH levels in paracetamol-induced liver injury in mice. The present study was with a view to justify the traditional use of the plant in the treatment of liver diseases.

MATERIAL AND METHODS

For drugs and chemicals, Silymarin (SILY) powder (procured from TRC Co., Ltd, Toronto, Canada) was used to make the suspension in doses of 75 mg/kg bw for the respective groups. Paracetamol (PCM) powder (procured from TRC Co., Ltd, Toronto, Canada) was used to make the suspension in a dose of 230 mg/kg bw for the respective groups. Malondialdehyde (MDA), 2-thiobarbituric acid were procured from Sigma – Aldrich (St. Louis, USA). Glutathione (GSH) was procured from TRC Co., Ltd (Toronto, Canada). 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) was purchased from Acros Organics (Leicestershire, UK). For the preparation of the extract, arial parts of *H. isora* L. were collected from Dak Mil district – Dak Nong province (Vietnam). Our Department of Botanical Museum has identified the plant. They were cut into small pieces and dried in Laboratory drying oven at 50 °C. They were then crushed into a coarse powder using a laboratory grinding mill with ring sieve, size 0.25 mm. Maceration extraction of *H. isora* L. in ethanol solvent (70%) followed by drying at 50 °C resulted in an average extract efficacy of 21%. The extract was stored at -20 °C.

For the experimental animals, outbred Swiss albino mice weighing 18-22 g of either sex, bred in Stem Cell Institute, University of Science, Vietnam National University Ho Chi Minh City were procured and used for the study. The animals were allowed food pellets (Pasteur Institute, Ho Chi Minh City) and water *ad libitum*. Animals were maintained in a control condition (12 h: 12 h dark and light cycle and room temperature). For the acute toxicity studies, acute oral toxicity study was performed as per OECD-423 guidelines. No toxicity was observed up to the dose of 5.0g/kg body weight. The extract was tested at three dose levels i.e., lower dose of 250 mg, medium dose of 500 mg and the highest dose of 1000 mg/kg body weight.

For treatment groups, animals were divided into 6 groups (n=10/group). Group 1 - Normal control: The animals received distilled water for 7 days. Group 2 - Induction of hepatotoxicity by using PCM: The animals received

distilled water for 7 days and given PCM single dose, 230 mg/kg bw orally on day 8. Group 3 - Positive control: Pretreatment with silymarin at 75 mg/kg bw/day for 7 days followed by a single dose of PCM on day 8. Groups 4 to 6 - Pretreatment with ethanol extract of *H. isora* L. at 250, 500 and 1000 mg/kg bw/day for 7 days followed by a single dose of PCM on day 8. Animals were sacrificed under light ether anesthesia 24-h after PCM administration. Blood was collected by cardiac puncture in plain tubes and liver was removed, rinsed in cold saline, blotted with filter paper and weighted (Girish et al. 2009). Activities of AST and ALT in the serum were Abbott™ Alinity™ Systems (Abbott, US).

For the estimation of hepatic MDA and GSH, a portion of each liver was homogenized (1:10, w/v) in 1.15% KCl solution at 0 – 5 °C. To a 2 ml of 10% homogenate pipetted into a 15 ml centrifuging tube were added 1ml of Tris-HCl buffer. The mixture was incubated at 37°C for 60 min. After incubation, 1 ml of 10% acid trichloroacetic solution was added and mixed vigorously. The mixture was centrifuged at 10,000 rpm for 30 min at 4°C. The resultant supernatant was used for various biochemical assays. Hepatic MDA and GDH were estimation according to the method described in the previous studies (Ohkawa et al. 1979; Moron et al. 1979). According to the method of Lowry et al. (1951), protein concentration was estimated using bovine serum albumin (BSA) as a standard. For the histopathological examination, a portion of each liver tissue was fixed in 10% buffered neutral formalin solution, embedded in paraffin, sectioned into 3 µm thick slices, and stained with hematoxylin and eosin (HE). The liver tissue injury was examined by a histopathological expert under optical microscope (Parmar et al. 2010; Chen et al. 2012). This work was carried out at Cho Ray Hospital, Ho Chi Minh, Vietnam. For the statistical analysis, all data were expressed as mean ± SD (n = 10). The data were analyzed by Student's t test and One way ANOVA using Sigma Plot version 11.0. Differences between groups were considered to be statistically significant at P < 0.05.

RESULTS AND DISCUSSION

PCM, a well-known compound for producing chemical hepatic injury in mice has been used as an experimental model to test the potential hepatoprotective activity by several investigators (Parmar et al. 2010; Nazir et al. 2020; Bouhlali et al. 2021). PCM treatment significantly also increased the serum enzyme levels, namely ALT and AST indicating chemical induced hepatocellular toxicity. Serum levels of these enzymes are very sensitive markers employed in the diagnosis of liver diseases. When the hepatocellular plasma membrane was damaged, the enzymes normally present in the cytosol were released into the blood stream. This was quantified to assess the type and extent of liver injury (Sallie et al. 1991; Shakya 2020; Alaraj et al. 2021). Our laboratory standardized the hepatotoxic dose of PCM in mice (unpublished observations). PCM at a dose of 240 mg/kg bw p.o resulted in death of 80 per cent of mice. The macroscopic appearance of the liver showed extensive areas of necrosis associated with haemorrhages.

PCM (230 mg/kg bw p.o) treatment significantly increased the serum liver enzyme levels (ALT and AST). The activity of ALT (1632.00 ± 448.09 U/L) and AST (766.00 ± 282.70 IU/L) was significantly higher ($P < 0.05$) in PCM treated group in comparison to normal control (ALT 25.22 ± 5.08 U/L; AST 78.22 ± 25.48 U/L) indicating a marked hepatocellular injury (Table 1). Besides, PCM treatment also increased ($P < 0.05$) in hepatic MDA level accompanied by significant decrease ($P < 0.05$) in GSH level (Table 2).

Fortunately, when reduced to a lesser dose of 230 mg/kg bw p.o, no mortality was observed and the hepatotoxicity that was produced with PCM resembled naturally occurring liver disease (Sherlock and Dooley 2002; Shakya 2020). PCM (230 mg/kg bw) given once orally showed hepatotoxicity as evident from biochemical and histopathological parameters of the study. The crude liver weight, which was increased after PCM administration may be due to haemorrhages, hydropic degeneration and fatty changes associated with necrosis.

Table 1. Effect of *H. isora* L. extract on serum marker enzymes (AST and ALT) on paracetamol induced hepatotoxicity in mice

Treatment	AST (U/L)		ALT (U/L)	
	0D	9D	0D	9D
Control	101.68 ± 13.11	78.22 ± 25.48	40.18 ± 11.59	25.22 ± 5.08
PCM	100.98 ± 8.98	$766.00 \pm 282.70^*$	38.80 ± 8.82	$1632.00 \pm 448.09^*$
PCM + silymarin 75	100.68 ± 48.22	107.27 ± 16.46	47.51 ± 28.76	33.19 ± 5.12
PCM + extract 250	95.29 ± 14.57	82.61 ± 14.47	52.68 ± 14.93	37.20 ± 5.05
PCM + extract 500	76.55 ± 11.86	82.98 ± 13.49	43.21 ± 10.71	35.27 ± 6.27
PCM + extract 1000	76.25 ± 10.84	78.74 ± 7.15	42.63 ± 6.46	32.83 ± 1.61

Value = mean±standard deviation; n=10. Analyzed using LSD's test. Significantly different from control: * $p < 0.05$. AST: aspartate aminotransferase, ALT: alanine aminotransferase.

The extract-treated mice (Group 4-6) resulted in comparable biochemical marker readings to those of normal control (Group 1) and silymarin-treated (Group 3) (Table 1). Previous studies have demonstrated that *H. isora* L. exhibited hepatoprotective effect (Kumar et al. 2006; Dhevi et al. 2008). The reduction of liver enzyme parameters (AST and ALT) were significant and showed as a specific marker of liver injury due to toxic drugs, alcohol and virus (Sherlock and Dooley, 2002; Shakya, 2020).

The protective effect may be the result of stabilization of plasma membrane thereby preserving the structural integrity of cell as well as the repair of hepatic tissue damage caused by PCM (Pari and Murugan 2004). So, these data demonstrated that the effects of toxicity induced by PCM on the liver function could be effectively counterbalanced by *H. isora* L. extract treatment. This fact was based on the tendency of liver enzymes to return towards their respective levels in the normal control group. Notably, the experimental mice treated with *H. isora* L. extract had significantly lower levels ($P < 0.05$) of liver MDA compared with the PCM-treated mice of Group 2. Moreover, there were no significant differences in the lipid peroxidation end product MDA between medium dose or high dose extract-treated animals (Group 5, 6) and control group (Group 1), and better than those recorded from silymarin-treated mice (Group 3) (Table 2). Therefore, the studied plant has great ability to reduced oxidative stress by preventing lipid peroxidation. In the current study, an increase in the hepatic MDA level suggested the enhancement of lipid peroxidation, consequently leading to hepatic damage as well as the inactivation of the antioxidant defense system

(Simeonova et al. 2014; Tsai et al. 2017; Moke et al. 2021).

Table 2. Effect of *H. isora* L. extract on hepatic MDA and GSH on paracetamol induced hepatotoxicity in mice

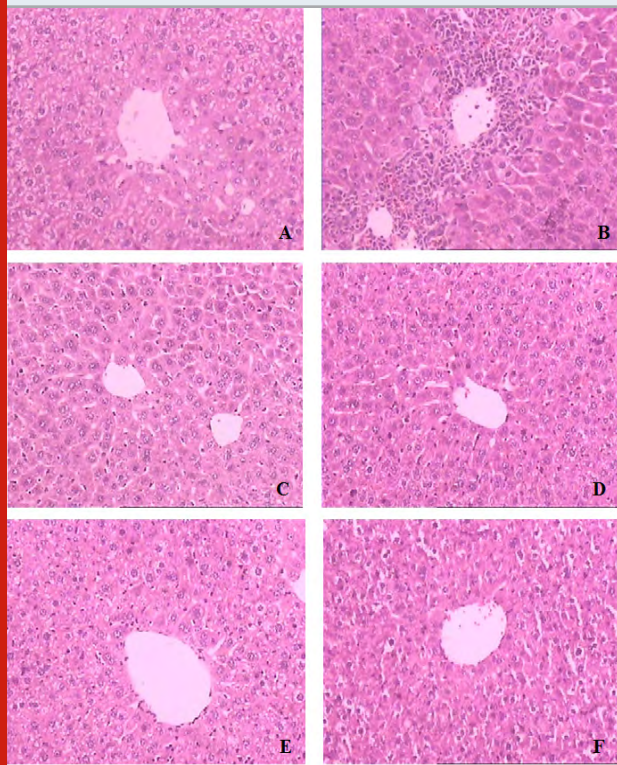
Treatment	MDA (nmol/g protein)	GSH (nmol/g protein)
Control	25.04 ± 1.93^c	327.87 ± 50.48^a
PCM	117.90 ± 9.29^a	33.65 ± 1.42^c
PCM + silymarin 75	54.51 ± 5.68^b	155.01 ± 19.30^b
PCM + extract 250	46.96 ± 11.35^b	61.63 ± 6.96^c
PCM + extract 500	34.09 ± 1.06^c	65.19 ± 6.46^c
PCM + extract 1000	30.98 ± 1.39^c	69.75 ± 19.21^c

Value = mean±standard deviation; n=10. Analyzed using LSD's test. abc: Indicate groups with statistical difference ($p < 0.05$). GSH: glutathione, MDA: malondialdehyde.

However, the increased hepatic MDA level decreased after treatment with the extract. The suppression of MDA production is likely to promote the activities of SOD, GPx, and GRd (Simeonova et al. 2014; Tsai et al. 2017). These results suggested that treatment with the extract may protect hepatic cells from further damage during cirrhosis. GSH in the PCM-treated mice were lower than in the normal mice ($P < 0.05$). When PCM is taken in at toxic doses, the compound is converted to a toxic form, NAPQI (N-acetyl-p-benzoquinoneimine). NAPQI can

rapidly react with glutathione (GSH) and lead to a 90% total hepatic GSH depletion. Treating the cirrhotic animals with silymarin significantly ($P < 0.05$) increased the GSH level and induced the survival of hepatocytes (Yen et al. 2007; Shakya 2020).

Figure 1: Hepatoprotective effect of *H. isora* L. extract on PCM-induced liver damage in mice (H&E stain 100 X). (A) Normal control group (B) PCM group (230 mg PCM/kg bw) (C) PCM + silymarin (75 mg/kg bw) (D) PCM + extract (250 mg/kg bw) (E) PCM + extract (500 mg/kg bw) (F) PCM + extract (1000 mg/kg bw)



However, the recorded result didn't show that *H. isora* L. extract had the capability of increasing GSH level in liver of experimental animals (Table 2). The histological examinations showed that PCM treatment induced inflammation in the liver tissue and blood congestion was observed (Figure 1B). Pretreatment with 250 or 500 mg extract/kg bw or 75 mg silymarin/kg bw markedly mitigated the inflammation in hepatic lobules (Figure 1C, 1D & 1E). Histopathological examination liver sections of mice administered with 1000 mg/kg bw doses of ethanol extract were perfectly protected almost similar to those of normal mice (Figure 1F).

Although the active compounds of the tested plant have not been well identified in the present study. Preliminary qualitative studies on various extracts suggested presence of phenolics, flavonoids, glycosides, tannins, carotenoids, ascorbic acid and saponins in different parts of *H. isora*; concentrations may vary according to season or part studied (Dayal et al. 2015). Especially, β -sitosterol was found to be the major active constituents in bark/stem of *H. isora* L. (Jain et al. 2009; Badgujar and Jain 2009; Chawla and

Bansal 2014). The reduced lipid peroxidation in mice liver homogenates were reported to be due to total phenolics or flavonoids contents of some medicinal plants. Many studies demonstrated that β -sitosterol protected liver damage and it may have beneficial properties in the prevention of liver toxicity (Kery et al. 2001; Sujila et al. 2014). The decrease in the status of the lipid peroxidation and upregulation of SOD and CAT could be attributed to the inherent antioxidant potential of β -sitosterol (Devaraj et al. 2020).

CONCLUSION

The results of this study suggests that the ethanol extract of *H. isora* L. arial parts possesses hepatoprotective activity in paracetamol-induced liver injury mice. Pretreatment with the extract has shown pronounced dose-dependent hepatoprotective effect. It could be attributed that the hepatoprotective activity of *H. isora* L. may be due to the presence of phenolics or β -sitosterol in the extract. Further studies can be carried out in order to explore the specific phytochemicals responsible for the hepatoprotective potential of *H. isora* L..

ACKNOWLEDGEMENTS

This study was financially supported by Dak Nong Department of Science and Technology, Gia Nghia, Daknong, Vietnam and Vietnam Academy of Science and Technology, Ha Noi, Vietnam.

Conflict of Interests: Authors declare no conflicts of interests to disclose.

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Biotechnological Communication

Enhanced Production of Citric Acid by Mutant PN12 of *Aspergillus fumigatus* Using Statistical Design

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ABSTRACT

Distillery spent wash is an unwanted residual liquid waste generated during alcohol production. It is a potential source for production of different industrially important products. Distillery spent wash is dark colored and has many organic compounds as a waste. In this experiment, removal of color and organic compounds was carried out by anaerobic treatment. The treated spent wash was utilized for citric acid production with the help of microorganisms. The current study was performed with the treated spent wash which was applied for high level of citric acid production by a mutant strain of *Aspergillus fumigatus* PN12. The parent strain *Aspergillus fumigatus* PN12 was mutagenized by UV exposure to enhance citric acid production. After UV exposure investigation, mutant strain was selected for optimization and statistical method. The best citric acid production obtained was, 26.45 g/L at 30 °C with pH 6.0, 0.1 g/L of KH_2PO_4 and $(\text{NH}_4)_2\text{SO}_4$ under OFAT. Under RSM optimization, maximum citric acid production was achieved as 30.89 g/L. Thus, the process optimization through the statistical approach resulted in a 1.16-fold enhancement in citric acid production as compared to that of the OFAT parametric conditions. Citric acid producing enzymes such as aconitase, NAD^+ isocitrate dehydrogenase and NADP^+ isocitrate dehydrogenase was studied. Maximum activity (U/mg) of aconitase (3.19 ± 0.023), NAD^+ isocitrate dehydrogenase (3.0 ± 0.15) and NADP^+ isocitrate dehydrogenase (2.91 ± 0.17) was observed at 96 h. The present study can conclude that spent wash is potential source for citric acid production. Utilization of mutant strain of *Aspergillus fumigatus* PN12 is beneficiary for large scale industrial fermentation and citric acid production.

KEY WORDS: ASPERGILLUS FUMIGATUS, CITRIC ACID, DISTILLERY SPENT WASH, FERMENTATION, UV EXPOSURE.

INTRODUCTION

Citric acid can be derived from natural sources (e.g., oranges, berries, limes, lemons, tangerines, and grapes) and synthetic sources (e.g., chemical reaction and microbial fermentation). Microorganisms can consume cheap raw materials and convert them into a value-added product such as organic acid. Generated wastewaters have many organic compounds which are utilized for production of various fermented products. Recently, spent wash generated from alcohol-producing industries is utilized for citric acid production. Citric acid has several extremely variable features and is purely a biological based product. So, it can be applied risk-free in the food and pharmaceutical industries. The benefit and versatility of citric acid increase its demand in daily life

consumption all over world (Sun et al. 2017; Aboyeji et al. 2020).

Increase in citric acid production is a major thing to overcome the demand in industries. Many techniques such as random mutations and selections are based on working hypotheses that are available for strain improvement. Selection of mutant strains is possible by the techniques of single-colony (single-spore) isolation and stable long-term maintenance of isolated strains. These are the principal ones that are generally employed on the vast scale. Various mutant strains of *Aspergillus* sp. are obtained and used for commercial production of citric acid. For this, mutagenesis is carried out by physical and chemical mutagens. Physical mutagens are UV and gamma radiation whereas chemical mutagens are ethyl methane sulfonate, N-Methyl-N-Nitrosoguanidine, ethidium bromide and acridine orange (Prasad et al. 2014; Alhadithy et al. 2020).

The Plackett-Burman design (PBD) is a statistical method used for screening the factors (media components) and finds

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Received 14/08/2021 Accepted after revision 18/11/2021

Published: 31st December 2021 Pp- 1473-1479

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.16>

the most significant influence on factors in a minimum number of experiments. After screening for the significant media components by the PBD method, the statistically based optimization using central composite design (CCD) and Box Behnken Design (BBD) was carried out under response surface methodology (RSM). The benefits of this method were the decreased number of experiments, time, and material resources. Furthermore, the analysis performed on the results can be easily realized with reduced experimental errors (Ayeeni et al. 2019). In the present investigation, efforts were made for raising potent of *Aspergillus fumigatus* PN12, a recently isolated fungal strain, for citric acid production by random mutagenesis using UV-irradiation. For further cost reduction, the ability of the selected mutagenized strain to utilize spent wash as carbon source was also examined. The aim of study was, production of citric acid using RSM with BBD by investigating the effects of parameters as well as citric acid fermentation in the optimized medium carried out in a 3 L bio fermenter.

MATERIAL AND METHODS

For the processing of raw material, chemical and medium component, distillery spent wash was obtained from sugar industry, Madhi, Surat, Gujarat, India. It was collected in an airtight sterile container and stored in refrigerator for the use of whole experiments. Spent wash contain high amount of sugars which can be utilized to convert valuable fermented products by fermentation process. Pretreatment of spent wash conducted in suspended growth reactor by physicochemical parameters. PDA medium, other mineral salt and nitrogen sources were purchased from Himedia laboratory, and S. D. fine (India). Citric acid enzymes reagents were supplied by Himedia Laboratory (India). All chemicals were of the highest purity and of an analytical grade.

For the isolation of fungal culture and gene sequencing, mutant strain *Aspergillus fumigatus* PN12 was isolated and identified as described in our previous published article (Aghera and Bhatt 2019). For mutagenesis, germicidal lamp (20 W) was used for UV mutation of *Aspergillus fumigatus* PN12 ($\sim 1 \times 10^6$ spores/mL). UV exposure was operated at a distance of 20 cm with short wavelength 254 nm. Treated spore were collected in sterile petri plate and it was exposed to UV light at the time interval of 3 min for 3-21 min. After UV irradiation to spores, 100-fold serial dilutions of potentially mutation-induced spores were plated to give 40 colonies or less per plate (Adeoye et al. 2015; Aghera and Bhatt 2019).

To begin the mutation process for *Aspergillus fumigatus* PN12, it was first subjected to UV radiation to develop its hyper-producing mutants for enhanced citric acid production. Then, in a dark room, the spore dilutions (0.1 mL) from UV treated mutants were spread onto PDA media containing 2% triton X-100 as colony restrictor. Untreated spores; also plated as control. All processes were carried out in strict aseptic conditions in laminar airflow. The selected mutants were cultured in submerged fermentation (SMF). Citric acid production process using a mutant was then

optimized to enhance citric acid production by the mutant in SMF (Javed et al. 2010).

$$\text{C.F.U / mL} = \frac{\text{No. of colonies on agar plate} \times 1}{\text{Amounted plated (0.1 mL)} \quad \text{Dilution factor}}$$

For the assessment of various process parameters such as nutritional and environmental values were optimized by one at a time. The experiments were conducted in a 250 mL flask containing 100 mL of wastewater (treated spent wash). After inoculation (fungi) maintain under following operational conditions; these were pH (4, 4.5, 5, 5.5, 6, and 6.5), temperature (25, 28, 30, 35, and 37 °C), $(\text{NH}_4)_2\text{SO}_4$ concentration (0.05 to 0.3 g/L) and KH_2PO_4 concentration (0.05 to 0.3 g/L).

For the optimization of citric acid production, a statistical approach was considered. An optimization statistical method called as Response Surface Methodology (RSM) with Box Behnken design (BBD). This technique was employed to investigate the optimum conditions for microbial production of citric acid yield from post methanated wastewater using potent fungal culture *Aspergillus fumigatus* PN12. Four independent parameters namely pH, temperature, $(\text{NH}_4)_2\text{SO}_4$ concentration (g/L), and KH_2PO_4 concentration (g/L) were employed for the study. Each of the independent variables was studied at three different levels as per BBD in four variables with a total of 28 experiments (Table 2). Citric acid production corresponding to the combined effects of four components were studied in their specified ranges (Table 1). The parameters were chosen and their levels were based on preliminary optimization experiments carried out in our laboratory and as discussed above. The design was planned for performing an experiment in a laboratory using Design-Expert version 11.0 (Stat-Ease, Inc.) software (Aghera and Bhatt 2019).

To study the enzymatic activity, acotinase, NAD⁺ Isocitrate dehydrogenase and NADP⁺ Isocitrate dehydrogenase enzymes were studied as described our previous published article (Aghera and Bhatt 2019). For the determination of citric acid, sugar consumption and dry biomass, the citric acid concentration was estimated by pyridine-acetic anhydride method at 427 nm using a UV visible spectrophotometer and HPLC method. Sugar consumption and residual sugar was measured by using DNSA method. Dry cell mass was estimated by gravimetric analysis (Miller et al. 1959; Aghera and Bhatt 2019).

RESULTS AND DISCUSSION

UV Mutagenesis: After UV mutagenesis of PN12 with different time intervals, the kill/survival curve was prepared and the time of exposure giving 0.04×10^3 CFU/mL was selected (Figure 1). Mutant strain PN12 survived of growth still 21 min with exposed of UV light (Figure 2). Colonies that changed their color to blackish brown were picked from this plate and grown on slants for making a further selection. On the other hand, it was not showed results in chemical mutagenesis. Mutant colonies were changed their color

greenish to brown color and user for further investigation (Abirami et al. 2018).

Figure 1: Survival / kill curve for UV treated PN12

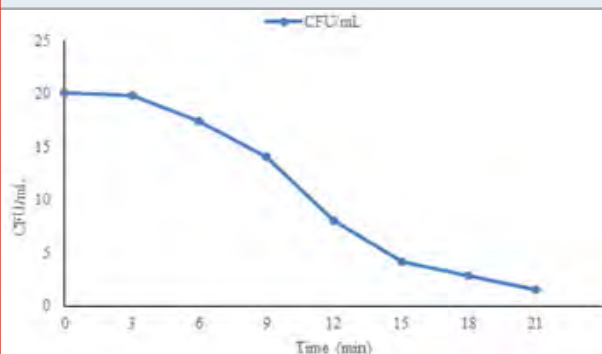
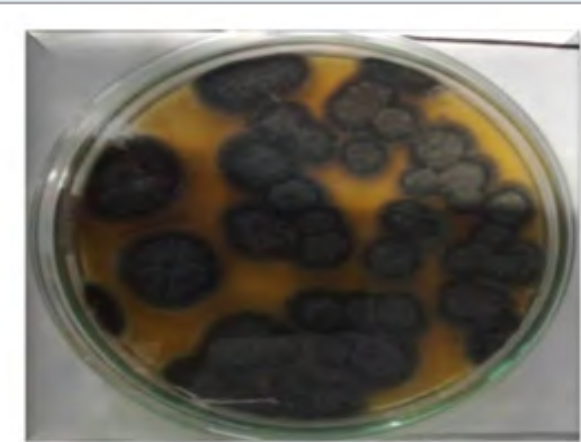


Figure 2: UV mutant strain PN12



Screening of mutation based on citric acid production:

The selected mutants from UV exposure of PN12 were used for the high yield of citric acid in submerged fermentation. Mutant PN12 treated with UV (treated for 21 min) gave remarkable production of citric acid after 96 h. Citric acid production by the selected mutant could be further enhanced by the optimization of the submerged fermentation parameters. Javed et al. (2010) stated that all mutants showed better citric acid production when compared to the parent strain. Our observations were in accordance with Abirami et al. (2018) said that the fermentation process was carried out by using three types of inoculum i.e., *Aspergillus niger* spores and mycelium and also mutated *Aspergillus niger* mycelium for citric acid production. The highest yield of citric acid (10g/100mL) was observed in the mutated mycelium with compared to the spores and normal mycelium (Javed et al. 2010; Abirami et al. 2018).

Optimization of environmental and nutritional parameters for citric acid production:

Effect of pH on citric acid production: The hydrogen ion concentration influences citric acid production, therefore in this optimization study, the initial pH of the PMWW was varied from 2 to 7 (Figure 3). The production of citric acid was found to be maximum (17.52 ± 0.22 g/L), when the pH

of the medium was maintained at 6.0. However, it was also noted that the medium amended with pH 6 consumed reducing sugar, (56 ± 0.8 %), produced dry biomass (10.74 ± 0.9 g/L/h), and maximum productivity (111.87 ± 0.3 mg/L/h) within 96 h. Mutant *Aspergillus fumigatus* PN12 exhibited good production in a narrow pH range from 5.0 to 6.0 with marked less productivity at pH up to 7.0. The obtained results suggested that the change in pH affected the citric acid production. This may be related to the transport of the molecules across the membrane, which was considered a rate-limiting step for the citric acid production (Figure 3). The noted results were in accordance with the work of Emeka et al. (2012) who reported higher citric acid production by *Aspergillus* EGN003 at pH 5.5. However, the substrate used as a banana peel by *Aspergillus niger* MTCC 282 has been investigated to increase drastically under 3 to 5 ranges (Emeka et al. 2012; Abirami et al. 2018).

Figure 3: Effect of pH on citric acid production, consumed sugar and productivity

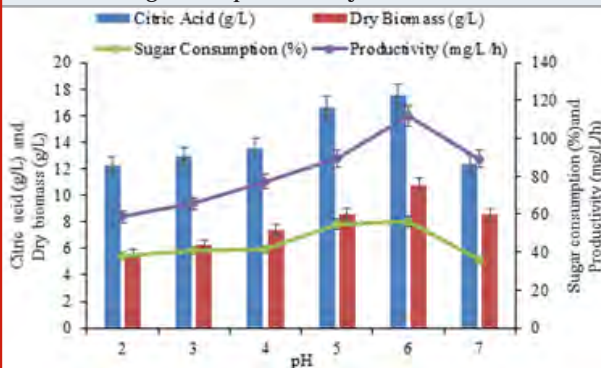
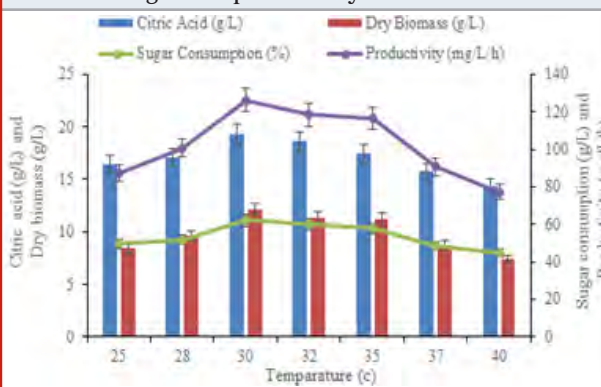


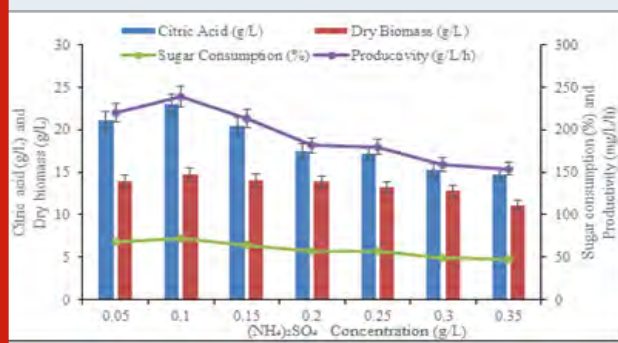
Figure 4: Effect of temperature on citric acid production, consumed sugar and productivity



Effect of temperature on citric acid production: The selection of incubation temperature is guided by the optimum growth temperature of the culture. In the present investigation, the biosynthesis of citric acid by *Aspergillus fumigatus* PN12 determined at a different temperature range from 25 to 40 °C. The obtained results suggested that maximum citric acid production (19.36 ± 0.7 g/L), consumed reducing sugar (62.12 ± 0.55 %), produced dry biomass (12.12 ± 0.39 g/L/h) and maximum productivity (126.25 ± 0.21 mg/L/h) were observed at 30°C. Thus, the optimal temperature for citric acid biosynthesis and

growth of the organisms were found to be the same as the organism has growth optima at 30 °C. As the temperature was increased from 25 to 30 °C there was a gradual increase in the enzyme activity. However, with further up to 30 °C temperature there was a decrease in citric acid production (Figure 4). This study was supported by Ali et al. (2015) used arginine as a nutritional ingredient for citric acid production by *A. ornatius* at 30°C. In contrast, Xu et al. (2015) investigate the best production of citric acid at 37.5 °C from cassava and corn powder by *A. niger* (Emeka et al. 2012; Xu et al. 2015).

Figure 5: Effect of $(\text{NH}_4)_2\text{SO}_4$ concentration on citric acid production, consumed sugar, and productivity



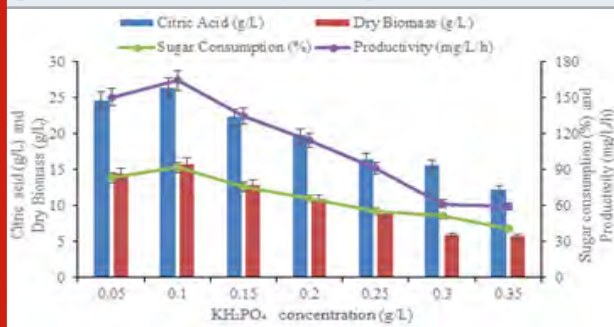
Effect of $(\text{NH}_4)_2\text{SO}_4$ concentration on citric acid production: Fungal isolate *Aspergillus fumigatus* PN12 exhibited maximum production of citric acid when $(\text{NH}_4)_2\text{SO}_4$ supplemented in the medium. In order to optimize the concentration of $(\text{NH}_4)_2\text{SO}_4$ in the medium for maximum production, the extent of production was monitored at $(\text{NH}_4)_2\text{SO}_4$ concentration varying from 0.05 to 0.35 g/L (Figure 5). However, increasing $(\text{NH}_4)_2\text{SO}_4$ supplement in PMWW, the production efficiency of the culture increased proportionally and maximum citric acid production (22.98 ± 1.2 g/L), consumed reducing sugar, (72.43 ± 0.5 %), produced dry biomass (15.8 ± 0.17 g/L/h) and maximum productivity (164.58 ± 0.32 mg/L/h) was achieved with 0.1 g/L $(\text{NH}_4)_2\text{SO}_4$ in 96 h.

Table 1. Coded and actual level of variables used in BBD for citric acid production.

Variables	Symbol	Coded level of variables		
		-1	0	1
Temperature (°C)	A	28	30	32
pH	B	5	6	7
$(\text{NH}_4)_2\text{SO}_4$ concentration (g/L)	C	0.05	0.1	0.15
KH_2PO_4 concentration (g/L)	D	0.05	0.1	0.15

Further increase in $(\text{NH}_4)_2\text{SO}_4$ concentration in PMWW up to 0.1 g/L had no major effect on the production of citric acid. Based on the result observed, the citric acid production was significantly affected through fungal culture connected to the above or lower at 0.1 g/L of ammonium sulphate

Figure 6: Effect of concentration of KH_2PO_4 on citric acid production, consumed sugar and productivity



(Figure 5). The obtained results and findings are also similar to Max et al. (2010) they detected that lab-scale examination was supplemented by ammonia salt which was responsible for the decline in pH which gave a favorable fermentation process. Shankar et al. (2016) also reported NH_4Cl as the best nitrogen source for enhancing the citric acid production using *A. niger* (Max et al. 2010; Shankar et al. 2016).

Table 2. Box-Behnken design and results for optimization of citric acid production

Run	A	B	C	D	Experimental value
1	0	-1	-1	1	20.13
2	0	0	-1	1	19.15
3	0	0	1	1	20.87
4	0	0	0	0	28.81
5	0	0	-1	-1	12.11
6	0	0	0	-1	27.95
7	1	0	0	1	15.55
8	0	1	0	1	14.1
9	-1	0	0	1	18.23
10	1	-1	0	0	14.72
11	-1	-1	0	0	11.44
12	0	-1	0	-1	10.59
13	0	1	-1	0	16.16
14	-1	0	-1	0	14.7
15	1	0	0	-1	12.73
16	-1	0	1	0	25.75
17	-1	0	0	-1	11.35
18	0	-1	-1	0	10.55
19	0	1	0	-1	12.11
20	0	-1	0	1	7.06
21	1	0	1	0	20.11
22	0	1	1	0	24.88
23	0	0	-1	0	27.99
24	0	0	-1	-1	30.89
25	0	-1	1	0	17.7
26	-1	1	0	0	23.5
27	1	0	-1	0	15.1
28	0	0	1	-1	12.91

Table 3. Analysis of variance (ANOVA) for citric acid production: Individual and interactive effects of all four variables.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Mean vs Total	8723.34	1	8723.34			
Linear vs Mean	258.60	4	64.65	1.71	0.1811	
2FI vs Linear	44.58	6	7.43	0.1535	0.9857	
Quadratic vs 2FI	734.75	4	183.69	27.15	< 0.0001	Suggested
Cubic vs Quadratic	72.49	8	9.06	2.93	0.1258	Aliased
Residual	15.46	5	3.09			
Total	9849.23	28	351.76			

Figure 7: 3D surface figure showing the interaction between temperature and pH

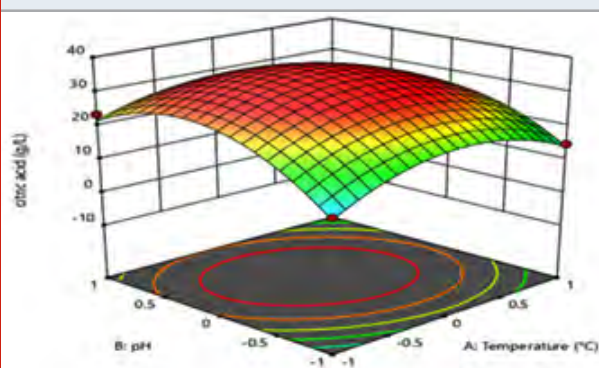
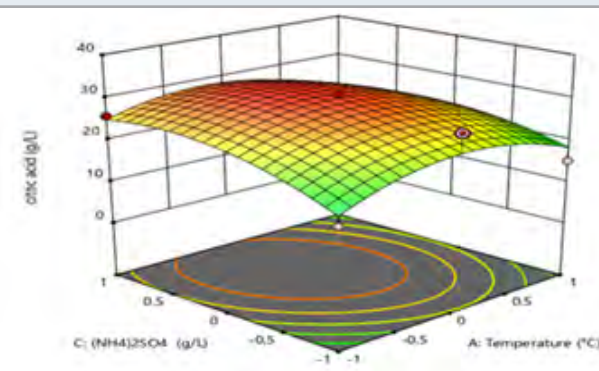


Figure 8: 3D surface figure showing interaction between temperature and $(\text{NH}_4)_2\text{SO}_4$

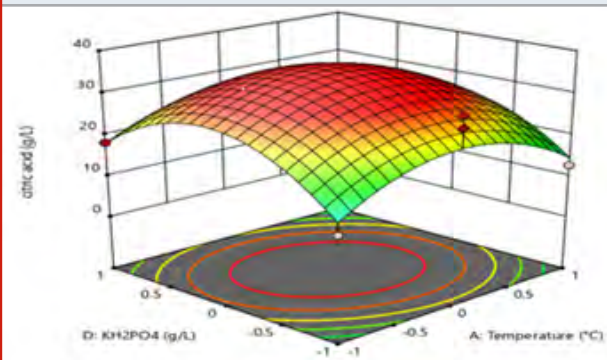


Effect of KH_2PO_4 concentration on citric acid production: Among different concentrations of KH_2PO_4 from 0.05 to 0.35 g/L tested in raw material as post methanated wastewater for citric acid production. It was noticed that maximum production of citric acid (26.45 ± 0.2 g/L) by mutant strain PN12, it was noted that the medium amended with 0.1 g/L KH_2PO_4 with consumed reducing sugar (91.60 ± 1.0 %), produced dry biomass (15.8 g/L/h) and maximum productivity (164.58 mg/L/h) of citric acid within 96 h. However, we reached to the conclusion that the citric acid production was drastically changed by increased or decreased 0.1 g/L concentration of KH_2PO_4 (Figure 6). The observed results were similar to those previously carried out by Qurban et al. (2017) their work

recommended that the restriction of phosphate might be absolutely affected on production of citric acid, while the high presence of phosphate could reduction in fixation of CO_2 and responsible for the production of sugar acids (Qurban et al. 2017; Abirami et al. 2018).

Citric acid production by response surface methodology involving Box-Behnken design: The citric acid production was carried out by optimizing environmental and nutritional parameters viz. pH, temperature, $(\text{NH}_4)_2\text{SO}_4$, and KH_2PO_4 at specified ranges as shown in (Table 1). The results of the 28 run BBD for citric acid production is shown in (Table 2).

Figure 9: 3D surface figure showing interaction between temperature and KH_2PO_4



Where, Y was the predicted response, and A, B, C, and D were uncoded values of pH, temperature, $(\text{NH}_4)_2\text{SO}_4$ concentration, and KH_2PO_4 concentration, respectively. For each run, the experimental responses along with the predicted response were calculated from the regression Equation (1).

The statistical analysis of experimental results suggested that the model was highly significant ($p = < 0.0001$) as shown in (Table 3). Fisher's F' and Student's t-test were used for the testing of the analysis of the model. For the determination of the significance of the regression coefficient of variables, Student's t-test was applied. p-values less than 0.0500 indicate model terms are significant. In this case pH, $(\text{NH}_4)_2\text{SO}_4$, KH_2PO_4 , temperature and temperature, pH and pH, $(\text{NH}_4)_2\text{SO}_4$, and $(\text{NH}_4)_2\text{SO}_4$, KH_2PO_4 , and KH_2PO_4 were significant model terms. A p-value of less than 0.05 indicated the test parameter was significant. The interaction

effect among four variables was highly significant with $p = < 0.0001$. The model was significant, it was implied by Model F-value of 10.96. According to interaction effects were highly significant with p values less than 0.05. The expected R^2 estimation of 0.358 was practically close with the adjusted R^2 estimation of 0.484, which determined that the BBD configuration was in great concurrence with the observed and expected reaction (Abirami et al. 2018).

Interpretation of the model: The Figure representation provides a method to visualize the relationship between the experimental and response levels of each four variables and the type of interactions between test variables. (Figure 7) illustrates the variation in the citric acid production as a function of simultaneous change in the temperature (A, in Figure 7 indicated as A: temperature)

and (B: pH in Figure 7 indicated as B: pH) whereas, temperature and pH were kept at the middle level of the range (at '0' value).

Figure 8 and 9 demonstrates the interactive effects of temperature (A in Figure 8 indicated as A: temperature) and (C, in Figure 8 indicated as C: $(\text{NH}_4)_2\text{SO}_4$, and (A in Figure 9 indicated as A: temperature) and (D, in Figure 9 indicated as D: KH_2PO_4), respectively. Biological citric acid production highly varies in presence of nutrient components. Contour plot suggested that the level of temperature and pH should be kept at 0 levels (i.e., 30 °C and 6.0, respectively) while, $(\text{NH}_4)_2\text{SO}_4$ and KH_2PO_4 concentration should be maintained at low level (0.05 g/L and 0.05 g/L, respectively) in process. The middle-level ranges of all four variables have shown optimum interactive effects on citric acid production as it can be illustrated from the contour plots.

Table 4. The Effect on specific activities (U/mg protein) in *A. fumigatus*

Enzymes	12h	24h	48h	72h	96h	120h
Acotinase	1.01±0.09	1.64±0.12	2.21±0.19	2.80±0.08	3.19±0.023	2.54±0.01
NAD ⁺ Isocitrate dehydrogenase	0.86±0.02	1.56±0.06	1.89±0.15	2.50±0.23	3.0±0.15	2.34±0.10
NADP ⁺ Isocitrate dehydrogenase	0.95±0.11	1.35±0.03	1.75±0.01	2.27±0.05	2.91±0.17	2.48±0.19

$(\text{NH}_4)_2\text{SO}_4$ and KH_2PO_4 is an important nutrient for the production of citric acid. In this study, high nutrient concentration did not allow the high citric acid production indicating the inhibitory effect of high nutrient content in the fermentation medium. Crolla et al. (2001) also studied the effect of nutrient concentration on citric acid production. They reported maximum citric acid production after 96 h of fermentation. The significant effect of KH_2PO_4 on citric acid production (Crolla et al. 2001; Hyder et al. 2011; Aghera et al. 2019).

Enzymatic study: To determine the biological perceptive for guideline of citric acid synthesis, Specific exercises of aconitase, NAD⁺ isocitrate dehydrogenase and NADP⁺ isocitrate dehydrogenase were concentrated from UV treated *Aspergillus fumigatus* PN12 regarding citric acid synthesis. The acceptance of different compounds during the citrus extract beneficial system likewise strengthens the active job of microorganisms in the produced cycle. As introduced in (Table 3.5) specific action of aconitase in high citric acid producing strains of *A. fumigatus* during the evolution time of 96 h. It is seen that the action level of aconitase in PN12 is reliably higher all through the whole time of aging. Aghera et al. (2019) investigated that aconitase activity high during citric acid fermentation utilizing *A. fumigatus* PN12. NAD⁺ isocitrate dehydrogenase and NADP⁺ isocitrate dehydrogenase displayed nearly less specific activity. That likewise during citric acid fermentation high and brings down just as, the amount of aconitase, NAD⁺ isocitrate dehydrogenase, and NADP⁺-isocitrate dehydrogenase was essentially expanded and decreased (Aghera et al. 2019).

CONCLUSION

The findings of the present study suggests that the citric acid is an industrially important molecule which play an important role in preprocessing, bioremediation, and brewing industries. It is a natural preservative and is also used to add an acidic (sour) taste to food and soft drinks. This study investigated on an environmentally friendly and economic approach to produce citric acid from wastewater. Mutant strain was the survive and of growth was observed till 21 min with exposer of UV. The process optimization through the statistical approach resulted in a 1.16-fold enhancement in citric acid production as compared to that of the OFAT conditions. Thus, produced citric acid by the collective action of citrate formation enzymes where *A. Fumigatus* PN12 playing their significant role in citric acid fermentation.

ACKNOWLEDGEMENTS

This study was financially supported by the Gujarat Biotechnology Research Center (GBRC). Authors thank the institute for the culture identification and Dr Jyoti Vivecha for the statistical study.

Conflict of Interests: Authors declare no conflicts of interests to disclose.

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Medical Communication

Early Diagnosis of Oral Cancer: Knowledge and Perception of Oral Health Care Providers in Qassim, Saudi Arabia

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More than 570,000 new oral cancer cases are diagnosed, and around 300,000 deaths of this dreadful disease take place annually. The most widely recognized reason for morbidity and mortality globally is cancer. Early detection and regular follow up of high-risk patients can curb the mortality and morbidity rate. Dentists play a vital role in the early detection of oral cancer; therefore, assessing their knowledge and practice for early detection of oral cancer is crucial. A cross-sectional study was designed to assess the dentists' knowledge and practice for the early detection of oral cancer. The study was conducted in the Qassim region among dental interns, general dental practitioners (GPs), specialists, and consultants. The questionnaire consisted of 14 closed-ended questions that contain socio-demographic characters of participants, information regarding the participants' knowledge, and practice related questions. Data collected and analysed in SPSS software v. 21 and descriptive analysis was done. A total of 159 participants in the study. Regarding the potential risks for oral cancer, 48.4% and 34.6% believed viruses and hereditary. Regarding the high-risk sites for oral cancer, 38.4% of the participants choose soft palate complex, lateral border of the tongue, and floor of the mouth as high-risk sites. The majority of the participants, i.e., 70.4% did not use any adjunctive screening tools. 57.2% feel they do not have sufficient knowledge concerning the early detection of oral cancer. The vast majority, i.e., 87.4% agreed that they need more information and continuing education for the same. Dentists' comprehension must be reinforced and constantly updated by continuing education programs.

KEY WORDS: CONTINUING EDUCATION, DIAGNOSTIC AIDS, EARLY DETECTION, ORAL CANCER, QASSIM.**INTRODUCTION**

The most widely recognized reason for morbidity and mortality globally is cancer. More than 570,000 new oral cancer cases are diagnosed, and around 300,000 deaths of the disease annually (Hung et al. 2020). Majority of oral cancer diagnosed at late stages that will lead to disfiguration and results in severe loss of oral function and chronic discomfort, including difficulty in chewing, swallowing and speaking (Shanmugavel and Shive, 2010). Also, in these patients with advanced disease along with surgical intervention results in distortion, social/psychological problems, increased morbidity level, and uncommonly death (Decuseara et al. 2011). The comparatively low survival rate is attributed to late diagnosis, which occurs in more than half of all cases (Clovis et al. 2002). Varela et al. (2017) investigating the relationship between late diagnosis, stage, and death rate would 80% increase if the disease was detected and treated earlier. Early detection is the single most critical intervention

influencing survival (Awan et al. 2014; Varela et al. 2017). Several dynamic factors were involved in the early detection of oral cancer from a primary care viewpoint, including general dental practitioners (GPs) and dentists' professional knowledge of oral cancer, saving services of the primary care, and the impact of the doctor-patient relationship (Gomez et al. 2010; Rawal et al. 2018; Alqutaibi et al. 2020; Morikawa et al. 2021).

The access of oral cavity is easy and can be examined with little discomfort. Dentists, as primary care providers, can easily incorporate the screening protocol into their routine examinations (Clovis et al. 2002). Dentists have precise knowledge about oral cancer to diagnose individuals at risk, which is vital. Examining the whole oral cavity and running for appropriate treatments can contribute to reducing the oral cancer occurrence, morbidity, and mortality (Rawal et al. 2018; Alqutaibi et al. 2020; Morikawa et al. 2021). National Institute for Health and Care Excellence (NICE, 2017) guidance recommends that GPs refer to an oncologist if the patient has an unexplained persistent neck lump or an unexplained persistent mouth ulcer for three weeks. Oral cancer is influenced by numerous factors, such as alcohol,

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Received 19/09/2021 Accepted after revision 15/12/2021

Published: 31st December 2021 Pp- 1480-1485

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.17>

tobacco, familial and genetic predisposition, shisha, viruses, and more. Viruses have been positively associated with the development of oral malignancy. HPV, especially HPV 16 type, are the common viruses implicated in oral cancer (Rawal et al. 2018; Alqutaibi et al. 2020; Morikawa et al. 2021).

Genetic susceptibility has been known to be an important risk factor in the occurrence of oral squamous cell carcinoma. Sharp teeth that occurred by a fracture due to caries or trauma and long-standing irritation has been proposed to promote neoplasm in the presence of other risk factors. Shisha smoking has been associated with oral cancer in many studies (Kumar et al. 2016; Patil et al. 2019). Statistically, the smokeless tobacco habit is considered a significant risk factor associated with oral cancer among the Saudi population. Numerous studies show an absence of dentists' knowledge and a negative approach toward oral cancer investigation and routine oral examination (Kumar et al. 2016; Gupta et al. 2016; Alqhtani et al. 2019; Jafer et al. 2020). Greenwood and Lowry (2001) showed that the majority of the dentists focus their examination on teeth and the procedure-related instead of high-risk sites like the floor of the mouth. Our study aims to assess the knowledge and practice in early detection of oral cancer among the practising dentists in Qassim region of Saudi Arabia.

Table 1. Socio-Demographic Characteristics of the Participants

Variables	n	(%)
Gender		
Male	77	48.4%
Female	82	51.6%
Designation		
Intern	51	32.1%
GP Dentists	71	44.7%
Specialist	34	21.4%
Consultant	3	1.9%
Region		
Qassim	159	100%

MATERIAL AND METHODS

The Ethical Committee in Buraydah Colleges approved the current study. The questionnaire consisted of 14 closed-ended questions that contain socio-demographic characters of participants such as gender, designation, and region. Furthermore, information regarding the participants' knowledge, such as the causes/risks for oral cancer, signs, and symptoms of oral cancer, high-risk sites of the disease, and knowledge regarding the obstacles of the early detection of oral cancer. Practice related questions were framed in the last part of the questionnaire such as the use of any adjunctive screening tools in their clinics with specifying the type of tool if used, how to examine a patient for signs and symptoms of oral cancer and if we should do a periodic oral cancer examination for the high-risk patients.

Also, we asked about the referral point if suspected oral malignancy. Two self-reported questions about if the participants feel they have sufficient knowledge concerning the early detection of oral cancer and if they need more information or training on oral cancer. This cross-sectional survey was conducted in the Qassim region among dental interns, general dental practitioners (GPs), specialists, and consultants in both private and public sectors. The goal of the questionnaire was explained to participants, and all necessary information was provided to them. Data collected and analysed in SPSS software version 21 and descriptive analysis was done.

Table 2. Dentist's Knowledge about Oral Cancer

Variables	n	%
Knowledge regarding causes of oral cancer		
Smoking	153	96.2%
Alcohol	143	89.9%
Viruses (HPV)	77	48.4%
Hereditary	55	34.6%
Smokeless tobacco (Chewing)	147	92.5%
Shisha	120	75.5%
Sharp cusp/Long standing irritations	131	82.4%
Knowledge regarding signs and symptoms		
A lump / non-healing ulcer that develops in the mouth or in the neck	50	31.4%
TMJ fusion	0	0.0%
Dysphagia	4	2.5%
Options 1 & 3	104	65.4%
None of these	1	0.6%
Knowledge regarding high risk sites for oral cancer		
The soft palate complex	24	15.1%
The lateral tongue	9	5.7%
Floor of the mouth	10	6.3%
Options 1 & 2	55	34.6%
All of the above	61	38.4%
Knowledge regarding obstacles early detection of oral cancer		
Warning signs are actually manifestations of more advanced disease	7	4.4%
Early oral cancer is generally asymptomatic and may be difficult to see	51	32.1%
The oral cancer exam may not be done by enough clinicians on their patients at risk	16	10.1%
All of the above	85	53.5%

RESULTS AND DISCUSSION

The demographic characteristics of the participants, 51.6% were females and 48.4% males. The study comprised 44.7%

GP dentists, 32.1% dental interns, 21.4% specialist dentists and 1.9% consultants (Table 1).

The potential risks/causes for oral cancer were smoking and smokeless tobacco, respectively 96.2% & 92.5%, which viruses and hereditary are considered to be the least risk factors, i.e., 48.4% & 34.6% respectively. Regarding the high-risk sites for oral cancer, 38.4% of the participants choose soft palate complex, lateral border of tongue, floor of

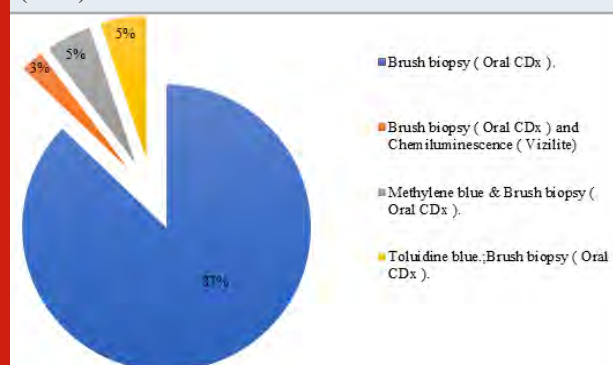
mouth as high-risk sites. 32.1% of the participants believed that early oral cancer is generally asymptomatic and may be difficult to see (Table 2).

Regarding the usage of screening tools and their relations with designation among participants, consultants and specialists used adjunctive screening tools more compared to GPs and dental interns ($P < 0.05$) (Table 3). Regarding types of screening tools, brush biopsy (Oral CDx) was the most commonly used (Figure 1).

Table 3. Prevalence of usage of screening tool and its relations with designation among participants

			Designation				Total	X ² value	P value
			Consultant	GP Dentist	Intern	Specialist			
Do you use any adjunctive screening tools at your clinic?	No	N	2	55	42	13	112	27.130	<0.001
		%	66.7%	77.5%	82.4%	38.2%	70.4%		
	YES	N	1	14	5	19	39		
		%	33.3%	19.7%	9.8%	55.9%	24.5%		
	I don't know what are the screening tools	N	0	2	4	2	8		
		%	0.0%	2.8%	7.8%	5.9%	5.0%		
Total		N	71	51	34	159			
		%	100.0%	100.0%	100.0%	100.0%			

Figure 1: Type of screening tools used by participants (n=39)



About examining the patient for signs and symptoms of oral cancer, 56.6% opined unusual lumps, sensitivity and non-healing ulcers in and around the head & neck were considered vital. But 40.9% choose to palpate the lumps in and around the head & neck region while examining. Regarding the referral point, if suspected oral malignancy, 58.5% considered oral medicine/oral pathology, 30.8% considered oral & maxillofacial surgery, and 10.7% considered tertiary care hospital, respectively (Table 4).

Self-reported questions regarding the knowledge for the early detection of oral cancer, 57.2% of the participants lack the same. The majority of the dentists, 87.4%, agreed that they need more information and continuing education programs for the early detection of oral cancer (Table 5).

In the present study distribution of participants are as follows, 44.7% general dental practitioner, 32.1% dental interns, 21.4% specialist, and 1.9% consultants. Tobacco, both smoking and smokeless form, were considered as a potential causative agent for oral cancer, 96.2% and 92.5%, which is similar with other studies showed that tobacco in both forms is the main risk factor of oral cancer (Clovis et al. 2002; Kumar et al. 2016; Rawal et al. 2018). Shisha, which is prevalent in this geographic area, 75.5% of the participants considered as a risk factor. Patil et al. (2019) showed an association between shisha smoking and oral cancer. Viruses (HPV) as a causative agent, 48.4% of the participants considered HPV as a causative factor of oral cancer. Previous studies have showed the HPV role should be considered more seriously, and HPV infections should be focused on screening (Poelman et al. 2018; Keser et al. 2021).

Regarding the signs and symptoms of oral cancer, a study done by Rethman et al. (2010) showed that the non-healing

ulcer is a clinical presentation of the early stage of disease. 96.8% of our participants agreed with the same. The knowledge findings regarding the clinical presentation of oral cancer in our study population are similar to a study done in the past in United Arab Emirates (Hashim et al. 2018). The familiarity of anatomy and health of different structures of the oral cavity will help the dentists in easily identifying the signs and symptoms of oral cancer than any other health professionals (Lingen et al. 2008; Galvão-Moreira and da Cruz 2017; Nagao et al. 2020).

Table 4. Practice Related Questions

Variables	n	(%)
Do you use any adjunctive screening tools at your clinic?		
Yes	39	24.5%
No	112	70.4%
I don't know what are the screening tools	8	5.0%
How you will examine a patient for signs and symptoms of oral cancer?		
By palpating areas of the patient face, neck and mouth for unusual lumps or sensitivity	65	40.9%
By talking with the patient about any unusual sores or non-healing ulcers	2	1.3%
Options 1 & 2	90	56.6%
None of these	2	1.3%
Practice regarding providing periodic oral cancer screening to the high-risk patients		
Yes	151	95.0%
No	8	5.0%
Practice regarding the referral point if suspected oral malignancy		
Oral & Maxillofacial Surgery	49	30.8%
Oral Medicine / Oral Pathology	93	58.5%
Tertiary Care Hospital	17	10.7%

Table 5. Self-Reported Questions

Variables	n	(%)
Do you feel that you have sufficient knowledge concerning early detection of oral cancer?		
Yes	68	42.8%
No	91	57.2%
Do you need more information or training on oral cancer?		
Yes	139	87.4%
No	20	12.6%

38.4% of the participants choose soft palate complex, lateral border of the tongue, and mouth floor as high-risk sites. 6.3% of the participants opined floor of the mouth as a high-risk site. Whatever, the lateral side of the tongue and floor of the mouth are the high predilection sites for cancer

in most parts of the world except for South and Southeast Asia, where buccal mucosa is commonly involved (Lingen et al. 2008; Galvão-Moreira and da Cruz 2017; Nagao et al. 2020). The overall usage of an adjunctive screening tool for oral cancer among the participants was low, and specialist dentists reported more usage than others. In our study, among those who used screening tools, 87% of the dentists used brush biopsy alone. Many screening tools have been used to help in the early detection of oral cancer (Masthan et al. 2012; Walsh et al. 2013). According to Cochrane Systematic Review done by Macey et al. (2015) and a study done by Morikawa et al. (2020), scalpel biopsy is considered as the gold standard in oral cancer screening and diagnosis. Dentists also can utilize other less invasive techniques such as vital stains like Toluidine blue, exfoliative cytology, fluorescent imaging, etc. for this purpose (Wan and Savage 2010; Murgod et al. 2011; Awan et al. 2014; Vinuth et al. 2015; Morikawa et al. 2021; Jafer et al. 2021).

The lesser usage of a screening tool for early detection of oral cancer could be attributed to inadequate exposure or importance given to the practical teaching of these techniques during their undergraduate training (Wan and Savage 2010; Murgod et al. 2011; Jafer et al. 2021). The study findings showed that almost all participants believed that periodic oral screening should be done for individuals above 40 years of age and also for high-risk patients like tobacco, alcohol users, etc. According to Sankaranarayanan et al. (2015), oral visual screening can reduce oral cancer-related mortality in high-risk individuals. In Oral visual screening, a visual and physical examination of intraoral mucosa is done using a mouth mirror and gauze under bright lights.

They are used for signs of oral potentially malignant disorders (OPMDs), which is then followed by extra-oral examination using gloved hands and good lighting to examine the skin and palpate muscles, TMJ and lymph nodes for any abnormality (Sankaranarayanan et al. 2015). Despite good knowledge of the oral anatomy and risk factors of oral cancer, the lack of confidence in our study population is obvious. Studies conducted by Diamanti et al. (2002) and Anandani et al. (2015) also reported that dentists were reluctant to perform oral screening and biopsy techniques for detecting oral cancer (Diamanti et al. 2002; Anandani et al. 2015; Morikawa et al. 2021; Jafer et al. 2021).

Some of the limitations should be considered before generalizing the findings for our study. As this is a questionnaire study, the reported responses may vary from the actual clinical practices and knowledge regarding oral cancer. Thus, there could be a presence of social desirability bias in reported responses, resulting in underestimation. It is apparent in our study that there are inconsistencies between dentists, which necessitates further study to understand the barriers and concerns in oral screening and associated procedures for oral cancer.

CONCLUSION

The findings of the present study indicates that the current dental education curriculum still lacks the training of

graduates in early detection and in conducting biopsy procedures. The majority of the study participants wanted to have more information and training through workshops and continuing education programs on oral cancer detection and related procedures. Training on use of chairside invasive and non-invasive diagnostic procedures especially for interns and GPs, screening of high-risk population at community level can reduce the mortality rate.

conflict of Interests: Authors declare no conflicts of interests to disclose.

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Pharmacological Communication

Pharmacological Effect of Verapamil on Brain Oxidative Parameters and Mania-Like Behaviour in Paradoxical Sleep Deprived Mice

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ABSTRACT

Although the implication of calcium signalling in the aetiology of anxiety remains elusive, drugs modulating calcium (like calcium channel blockers) have been discovered to be somewhat beneficial as treatment option for anxiety related disorders. This study was therefore undertaken to assess probable ameliorative potential of verapamil against manic-like (stereotype behaviour) and anxiety-like symptoms in mice exposed to sleep deprivation. Mice were allotted into five treatment groups (n=5): group 1 and 2 received 10 mL/kg distilled water, groups 3 and 4 verapamil (25 and 50 mg/kg) while group 5 received astaxanthin (50 mg/kg) which served as the reference drug. Treatment was for 7 days and animals were sleep-deprived on the final 72 hours. Various behavioural tests to determine degree of stereotypical behaviour and locomotor activity were carried out. Anxiety test was done via the aid of a light/dark box and plus maze while stereotype behaviour was assessed utilizing an open field box. Oxidative stress parameters; malondialdehyde and glutathione were assessed. Histopathological perturbations in the caudate putamen were also recorded. Data were subjected to ANOVA at $\alpha 0.05$. The results obtained suggest that verapamil significantly suppressed stereotyped behaviour and reduced the incidence of manic-like behaviour which was induced by paradoxical sleep deprivation. Verapamil also significantly restored antioxidant levels and protected against loss of caudate neurons. In conclusion, verapamil ameliorates manic-like symptoms and anxiety in mice deprived of sleep, while protecting brain neurons against oxidative stress damage induced by sleep deprivation.

KEY WORDS: ANXIETY, CALCIUM CHANNEL BLOCKERS, NEUROPROTECTION, STEREOTYPE BEHAVIOUR, VERAPAMIL.

INTRODUCTION

Sleep is considered the body's own way of replenishment and restoration. Optimally, adults require at least a minimum nine (9) hours of uninterrupted sleep each night. Rapid eye movement (REM) sleep, is an essential part of the sleep-wake process characterized by rapid side-to-side movements of the closed eyes, atonia, arousal and low amplitude mixed-frequency brain waves, relatively similar to those experienced during the waking state (Colavito et al. 2013). The REM sleep is an indispensable physiological

occurrence of which its chronic deprivation can result in distinct behavioural and cognitive alterations (Hirshkowitz et al. 2015; Gonzalez-Castañeda et al. 2016; Ben-Azu et al. 2020). Studies have posited that anxiety is usually associated with substantive loss of REM sleep, aggressive behaviour, memory deficit, including oxidative stress.

Therefore, paradoxical sleep deprivation (PSD) is considered a good animal model for inducing mania-like behaviour, as it stimulates some aspects of a manic episode, such as hyperactivity, hypersexuality and aggressive behaviour (Martins et al. 2008; Benedetti et al. 2008; Mathangi et al. 2012). Apart from PSD, there is also extensive subjective evidence that stressful experience and other psychosocial factors contribute to the initiation and frequency of manic phases (Koyuncu et al. 2019; Baker et al. 2019; Ben-Azu et

Article Information:*Corresponding Author: tonyeduviere@yahoo.com

Received 28/10/2021 Accepted after revision 23/12/2021

Published: 31st December 2021 Pp- 1486-1492

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.18>

al. 2020). Mania which is a distinctive period of aberrantly and sustained elevated, energetic, irritable or elated mood, lasting for a week or any period until hospitalization aberrant is necessary (Paul and Allan 2012; Dailey and Saadabadi 2021).

Mania is the most distinguished stage of bipolar condition which is a foremost cause of disability, stigma, and cognitive impairment (Martinez-Aran et al. 2004; De Miranda et al. 2020). The possible causes of mania include; high levels of stress, alterations in sleep pattern or insomnia, use of recreational drugs or alcohol, seasonal changes, child birth or could be even due to a major significant change in the life of an individual such as going through a divorce. The indicators of mania may consist of increased talkativeness or activity, loss of appetite, disinhibition, decreased need for sleep, reckless behaviour or grandiosity, which causes severe mood distortion which affects normal functioning of the individual in daily living, psychomotor agitation, hyper-sexuality, and increased reward-seeking behaviours (American Psychiatric Association 2000). The most generally used medications for mania/mania-like behaviour include; lithium, some anticonvulsants (valproate, carbamazepine), atypical antipsychotics (quetiapine, olanzapine, risperidone, ziprasidone, aripiprazole, clozapine), standard antipsychotics (e.g., haloperidol, chlorpromazine), and benzodiazepines (e.g., lorazepam, clonazepam) (Eduard and Sanchez-Moreno 2008; Dailey and Saadabadi 2021).

These medications might be hindered by side effects (antidepressants), habituation and tolerance (benzodiazepines), or inefficacy in some anxiety disorders (buspirone) (Goldstein 1985). Therefore, medications with probable theoretical efficacy and low side effect profile are sought after. Calcium channel blockers (CCBs) seem to meet these criteria. The CCBs, are more generally used as treatment options for cardiovascular disorders (cardiac arrhythmias, angina pectoralis, and hypertension) (Das and Plow 2010). However, the discovery that CCBs crosses the blood-brain barrier and act directly on neuronal cells cross by binding to numerous brain regions suggesting that they might offer some beneficial effects as treatment options for neuropsychopathological disorders and suggests even reduce anxiety (Urien, et al. 1987; Umukoro et al. 2010). Verapamil is among the most studied CCBs, and has shown positive activity in neurological related disorders (Umukoro et al. 2010; Dailey and Saadabadi 2021).

Dubovsky (1994) emphasized that intracellular calcium (Ca^{2+}) plays a fundamental role in various physiologic processes, namely; action potential, release of neurotransmitters, and functioning of receptors implicated in neuronal periodicity and mood disorders. He pointed out that different antimanic drugs have calcium antagonist properties. Although the precise mechanism of CCBs in these conditions remains unknown, the interference of these drug class with calcium metabolism is a key mechanism these of their action in mental disorders (McCarty et al. 2021). Since a recent longitudinal study has posited that (i) insomnia and other associated sleep glitches worsen before a manic episode, (ii) lack of sleep can trigger mania, and

(iii) that sleep problems also adversely affect mood and contribute to relapse, we decided to induce mania in mice by the process of PSD employing the platform over water model in this research while investigating the possible ameliorative potential of verapamil against such manic-like symptoms. Note that PSD is not able to cause mice to exhibit bipolar disorder, but does induce a mania-like behaviour (McCarty et al. 2021).

MATERIAL AND METHODS

Male mice (Albino Swiss) weighing 22.0 ± 2.0 g, used for this study were procured from the Animal House, College of Health Sciences of Delta State University, Abraka and were held in rectangular plastic cages at ambient temperature. They were fed with balanced rodent diet and had unrestricted access to water ad libitum. Animal handling was done according to internationally established guidelines on animal care and handling with necessary approval from the Ethical Committee of the University. Mice were individually suspended on grids (2.5 cm apart) over water in a rectangular thermoplastic cage (1.2 cm beneath the grid). Although this design predominantly targets REM sleep, a significant loss of non-REM sleep, accompanied by a significant amount of stress is unavoidable. At the expiration of the 3-day sleep deprivation period, the outcome of sleep restrictions on motor function and manic-like symptoms was assessed between 9:00 am and 12:00 noon.

The rodent sleep deprivation model was chosen to mimic circadian rhythm changes associated with mania. This model offers significant relevance as regards face validity since sleep loss is a notable feature of mania, and can therefore trigger this disorder in patients (Wehr 1992; Mansell and Pedley 2008; McClung 2011). Moreover, sleep deprivation induces several manic like behaviours, such as insomnia, hyperactivity, aggressive actions toward other animals, hyper sexuality (an increase in mounting behaviour), and stereotypy (sniffing and rearing) (Hicks et al. 1979; Fratta et al. 1987; Gessa et al. 1995; Abrial et al. 2015). These behaviours were measured in the animals and all data were presented as mean \pm standard error of mean of the grouped mice. The result was analysed using ANOVA and post hoc tests (Student's Newman-Keuls) were utilized to determine the source of main significance for every test, which was set at $\alpha 0.05$ (De Miranda et al. 2020).

RESULTS AND DISCUSSION

Verapamil on mania-like behaviour, motor activity and anxiety in sleep-deprived mice: The behavioural test involved the utilization of open field chamber test which revealed that sleep deprived mouse exhibited behavioural patterns similar to manic symptoms similar of bipolar disorder. There was an observed increase in hyperactivity (increased number of lines crossed) and stereotype behaviour (rearing and grooming). These results are in tandem with former studies, which indicated that REM sleep restrictions can induce manic-like behaviour in mice (Malkoff-Schwartz et al. 1998; McClung, 2007; Salvatore et al. 2010). Previous studies also demonstrated that PSD induces hyperactivity (increased locomotor activity),

which treatment with lithium attenuated (Benedetti et al. 2008; Armani et al. 2012; De Miranda et al. 2020). As obtainable in Table 1 and Figure 1 below, sleep deprivation significantly increased manic-like behaviour in PSD mice.

However, the results obtained revealed that verapamil, at both doses, caused a significant reversal in hyperactivity and the various stereotype behaviours. Thus, indicating the probable use of verapamil in the reduction or attenuation of manic like symptoms.

Table 1. Effect of verapamil on manic-like behaviour induced by paradoxical sleep deprivation in mice

Treatment	Grooming Frequency	Rearing Frequency	Number of lines crossed
VEH 10 mL/kg	10.67±0.95	19.83±2.09	143.70±8.19
VEH 10 mL/kg + SD	18.50±0.85#	37.67±2.08#	213.50±10.22#
VPL 25 mg/kg + SD	13.00±0.77*	27.50±1.61*	161.70±8.39*
VPL 50 mg/kg + SD	10.83±0.79*	20.67±1.05*	146.00±12.88*
AXT 50 mg/kg+ SD	14.33±0.84*	27.67±2.93*	185.50±4.87*

Figure 1: Effect of verapamil on motor coordination in mice subjected to paradoxical sleep deprivation.

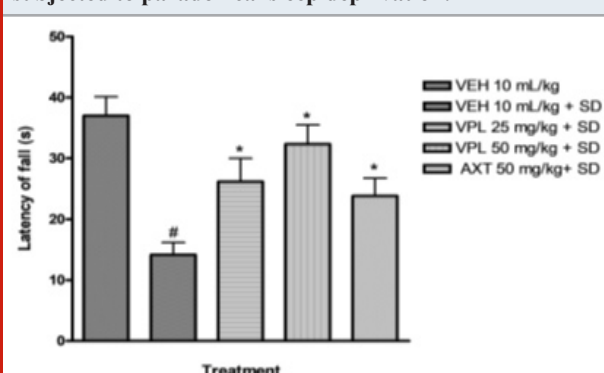


Table 2. Effect of verapamil on anxiety in sleep deprived mice using the light/dark box

Treatment	Duration of mice exploration (per 5 min)	
	Light Compartment (s)	Dark Compartment (s)
VEH 10 mL/kg	155.50±7.06	141.20±6.65
VEH 10 mL/kg + SD	100.20±5.87#	199.80±5.87#
VPL 25 mg/kg + SD	139.80±4.83*	158.80±5.03*
VPL 50 mg/kg + SD	148.20±6.25*	147.20±6.44*
AXT 50 mg/kg+ SD	122.00±6.86*	178.00±6.86*

Table 3. Effect of verapamil on anxiety in sleep deprived mice using the plus maze

Treatment	Open arm frequency	Exploration of mice (per 5 min)		
		Closed arm frequency	Open arm duration (s)	Closed arm duration (s)
VEH 10 mL/kg	4.33±0.33	6.17±0.87	105.50±8.61	194.50±8.61
VEH 10 mL/kg + SD	1.83±0.31#	12.33±0.80#	43.67±6.74#	256.30±6.74#
VPL 25 mg/kg + SD	3.12±0.31*	9.10±0.54*	83.58±7.64*	215.20±7.83*
VPL 50 mg/kg + SD	3.38±0.45*	8.42±0.65*	94.33±7.21*	207.20±6.87*
AXT 50 mg/kg+ SD	3.00±0.37*	9.16±0.70*	73.00±8.76*	227.00±8.76*

The tests for anxiety were conducted using a light/dark box and plus maze. Sleep deprived animals spent longer period in the dark box compared to animals that were not sleep deprived. In the plus maze, the sleep deprived mice expended much time in the closed arm than open arm; signifying classic indication of anxiety. However significant variations were observed when verapamil was administered – the mice spent lesser period in the dark box and more in the light box compared to the vehicle plus sleep deprived

group, and for the plus maze, the mice spent significant time in open arm than closed arm, thus signifying that verapamil could possibly be used to attenuate the symptoms of anxiety associated with sleep deprivation (Table 2 and Table 3).

Each bar represents mean ± S.E.M of grouped mice (n=6). # specifies significant ($p < 0.05$) difference compared to vehicle. * specifies significant ($p < 0.05$) difference compared to the vehicle + SD. (One-way ANOVA and

post-hoc test; Student-Newman-Keuls). VEH: Vehicle, AXT: Astaxanthin, VPL: Verapamil, SD: Sleep deprivation. under/beneath All the Tables and figures, except for figure 6.

Verapamil on brain oxidative stress in sleep-deprived

Figure 2: Effect of verapamil on brain malondialdehyde levels in mice subjected to sleep deprivation

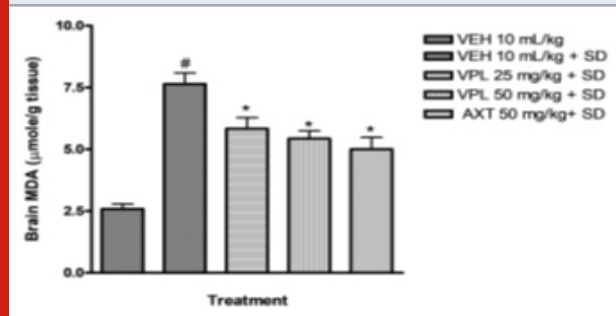
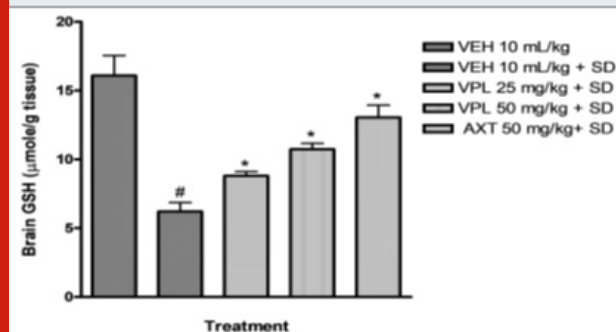


Figure 3: Effect of verapamil on brain glutathione levels in mice subjected to sleep deprivation.



mice: In the present study, alteration of pro-oxidants and antioxidant enzymes were seen alongside the behavioural alteration. Marked reduction in glutathione levels was observed (Figure 3) alongside increase in malondialdehyde levels (Figure 2).

This agrees with previous findings which reported increased products of inflammation, lipid peroxidation, including alterations of the major antioxidant enzymes in bipolar disorder (BD) subjects particularly within the mania spectrum (Kuloglu et al. 2002; Ranjekar et al. 2003; Ozcan et al. 2004). In addition, animal studies also have earlier shown that PSD increases brain lipid peroxidation (Kumar and Garg 2008; Garg and Kumar 2008). In the group that received verapamil, a reversal in the decrease of glutathione levels was seen, and a decreased malondialdehyde levels was observed further indicating the probable use of verapamil in the reversal of manic-like symptoms induced by sleep deprivation (De Miranda et al. 2020).

It is known that the brain is highly prone to oxidative damage due to high rate of oxygen utilization and reduced antioxidant defense systems and the depletion of antioxidants action in discrete parts of the brain following PSD is an indication of free radical generation which could cause neuroinflammation (Tabet et al. 2000; De Miranda

et al. 2020). Furthermore, PSD alters membrane fluidity, calcium ion (Ca^{2+}), concentration, gene expression, and enhances metabolic rate (Ramanathan et al. 2002; Singh et al. 2008; Mathangi et al. 2012; Ben-Azu et al. 2020).

Verapamil on pain and nociception in sleep-deprived

mice: Results obtained indicate that verapamil produced an anti-nociceptive effect by reducing the pain threshold and increasing reaction time of PSD mice as recorded by the two tests; hot plate (Figure 4) and tail flick (Figure 5). This gives an indication that verapamil may be useful in the mitigation of pain in patients who experience sleep difficulties. This also supports previous studies which showed that PSD contributes to hyperalgesia. It is, thus, logical to infer that PSD aggravates chronic pain symptoms in patients (Smith et al. 2000). Also, patients with pain disorders normally suffer anxiety due to the strain associated with living with pain. Note that the effect of verapamil on pain differs depending on dose, route of administration and pain test utilised (Tamaddonfard et al. 2014).

Figure 4: Effect of verapamil on reaction to nociception in mice subjected to sleep deprivation using the hot plate

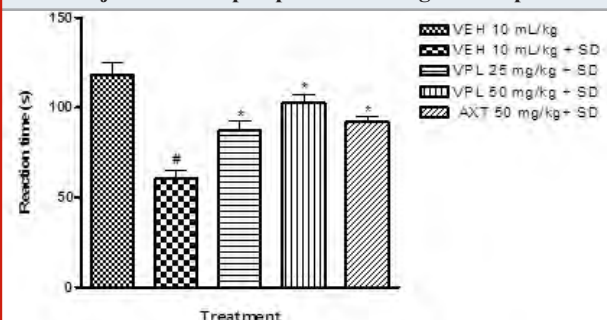
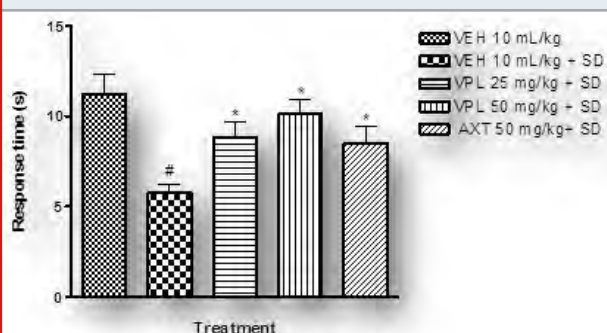


Figure 5: Effect of verapamil on response to nociception in mice subjected to sleep deprivation utilizing the tail flick test



Supporting, sleep loss increases the experience of pain (Lautenbacher et al. 2006). Casual manipulations in experimental animals have revealed that sleep restrictions heighten nociception. Clinical trials have established that complete, partial, or selective sleep restriction (deprivation) increases pain, thus lowering pain thresholds (Lentz et al. 1999; Roehrs et al. 2006; Schuh-Hofer 2013; Schrimpf et al. 2015; Faraut et al. 2015). Despite this literary evidence,

the principal brain mechanism at the core of the impact of sleep deficits on nociception remains unknown (Stroemel-Scheder et al. 2020).

Figure 6: Photomicrograph of the caudate putamen of mice after paradoxical sleep deprivation

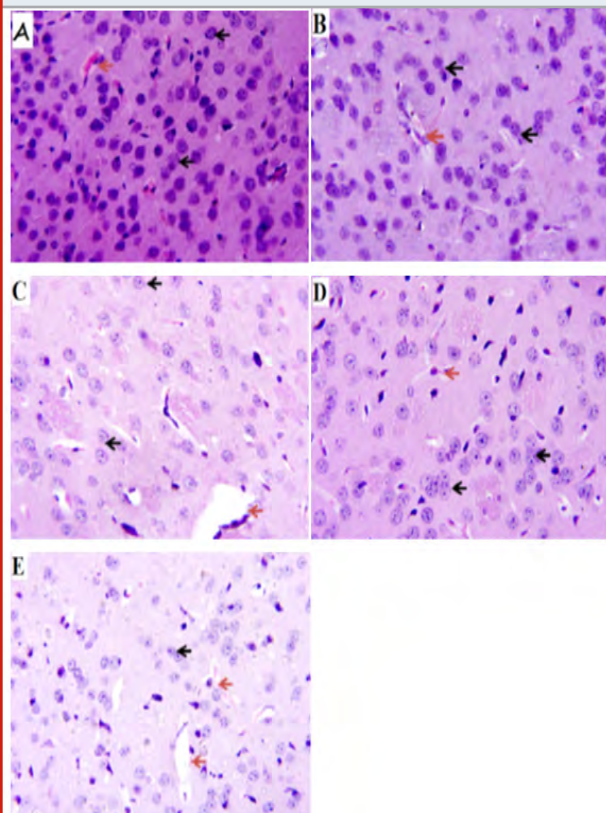
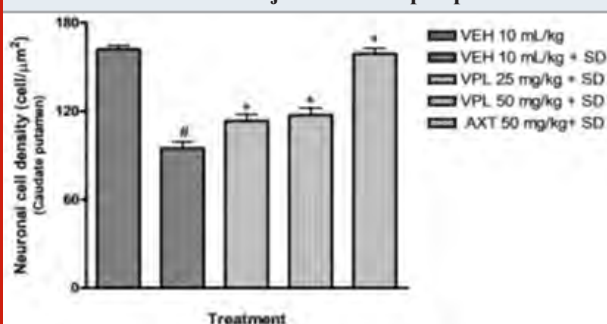


Figure 7: Effect of verapamil on viable caudate putamen neurons in mice after subjection to sleep deprivation



Verapamil on caudate putamen neurons of sleep-deprived mice: In order to further evaluate the effect of verapamil on manic-like symptoms, histology of the caudate putamen was done after sacrificing on the 7th day. The Hematoxylin and Eosin (H&E) staining of the caudate putamen (as seen on slides in Figure 6) revealed that PSD increased the magnitude of caudate putamen neuronal damage and subsequently decreased viable neuronal population in mice brains (Figure 7). In contrast, verapamil treatment revealed that there was a reduction in the extent

of neuronal damage and also an increase of viable neuronal cells in PSD mice.

Counts were based on neuronal nuclei present in three (3) squares per slide, using the pre-calibrated Image J software.

CONCLUSION

The finding of the present experiment suggests that verapamil attenuated anxiety/manic-like behaviour induced via deprivation of REM phase sleep, through a mechanism believed to be associated with reduced oxidative stress, and neuronal damage. Verapamil also significantly restored antioxidant levels and protected against loss of caudate neurons. In conclusion, verapamil ameliorates manic-like symptoms and anxiety in mice derived of sleep, while protecting brain neurons against oxidative stress damage induced by sleep deprivation.

ACKNOWLEDGEMENTS

This work was technically supported by the laboratory technicians of Pharmacology laboratory, DELSU.

Conflict of interests: Authors declare no conflicts of interests to disclose.

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Biomedical Communication

Impact of Increased Screen Time on Physical and Psychological Health of Indian Students During COVID-19

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ABSTRACT

Now in the 21st century, both physical and psychological health are associated with technology. By the late March 2020, the Indian government announced multiple lockdowns to fight with COVID-19 pandemic which included the shutdown of all educational institutes due to which all schools, college, and universities had shifted all their classes online by using video conferencing apps which also accounted for a large proportion of tasks that resulted in increased screen time after lockdown compared to pre-lockdown during COVID-19. So, the present study was conducted to determine the impact of increased usage of the screen on Psychological and physical health during the COVID-19 among Indian Students. A cross sectional survey was conducted on Indian students by using self- made Questionnaire with snowball sampling method. A total of 210 responses were received online from various schools and colleges students between 15th August to 30th August 2020. The results showed a statistically significant difference between the screen time spent before and during COVID ($t= 19.96$; $p<0.01$). Similarly, a statistically significant positive correlation at 0.01 level was found between screen usage time during COVID-19 pandemic with backache (0.62), neck pain (0.71), headache (0.50), and weight gain (0.52) and with mental health (0.40). Hence the study concluded positive association between screen time with physical and psychological health during COVID-19 among Indian students. It further implies that adequate preventive health measures need to be incorporated among students. There is a scope of further study to identify the impact of over usage of screen on health of individuals of specific population i.e., either adolescents or adults' group and also on the larger sample size to get more reliable and accurate results.

KEY WORDS: CORPOREAL HEALTH, COVID-19, ERGONOMICS, PHYSICAL AND PSYCHOLOGICAL HEALTH.

INTRODUCTION

The musculoskeletal ailments have been ranked tenth by the World Health Organization for number of years with more common physical complaints like back ache, neck pain, and headache amid adolescents. It has been found in various studies that excessive usage of screen are linked with physical complaints. Subsequently, as a period of screen usage increases which leads to continuous stress on muscles and lack of convalescence from such muscle tension and ultimately leads to change in muscle activity (Sjolie 2004; Hakala et al. 2006; Torsheim et al. 2010; Vos et al. 2015).

Students have been found more prone to neck pain with the prevalence of neck pain among adults ranging from 12 to 34% because of the attainment of sitting postures for longer duration (Green 2008; Gheysvandi et al. 2019).

Student's mental health is gaining more attention day by day internationally and it has been considered as a public health challenge nowadays (Storrie et al. 2010). Both anxiety and depression are deleterious to academic and social engagement in everyday student's life (Byrd and McKinney 2012; Salzer 2012). Consequences of depressive disorders can be manifested as depressed mood, decrease cognitive function, lack of a sense of subsisting, attentiveness, as well as lack of energy (Grotan et al. 2019). In turn, depression and anxiety frequently influence memory and attentiveness, which makes it tougher to obtain new knowledge and subsist with the examination environment. This will also reinforce

Article Information:*Corresponding Author: shaillygupta9@gmail.com

Received 25/10/2021 Accepted after revision 15/12/2021

Published: 31st December 2021 Pp- 1493-1500

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.19>

feeling of fear and insufficiency, and in numerous people, it will undergo the warmth of anxiety and depressed mood in a vicious circle (Rice et al. 2006; Grotan et al. 2019).

Most of the Physical health ailments are associated with new age technology, usually resulting in neck pain and back ache. Children are having sedentary lifestyle due to increased usage of screen making them prone to cardiovascular diseases, cancer, diabetes, etc. The direct impact of increased screen time has led to attainment of same posture for longer duration and monotonous movements (Straker et al. 2008). Excessive neck flexion leads to the increased risk of neck pain and its indirect effects incorporate decrease in energy expenditure that may be accountable for increasing weight (Straker and Howie 2016; Stiglic and Viner 2018). Normal people recognize increased screen time influences their lifestyle negatively which is associated with behaviour that incorporates lesser exercise, over eating which causes deleterious effects on sleep, social well-being, and diet. It also has been found a direct relationship with increased screen time on a variety of health issues that strongly include adiposity, unhealthy diet, depressive symptoms, and quality of life (Marsh et al. 2013; Stiglic and Viner 2018; Ashton and Beattie 2019).

Increased screen time adversely affects the activities like social contact, physical activity, or may foster a sedentary lifestyle and good sleep hygiene (Costigan et al. 2013; Lissak 2018). It has been found a positive relation between screen time duration and severity of anxiety and depressive symptoms (Hoare et al. 2016; Stiglic and Viner 2018). There is also evidence that high screen time is associated with adverse effects on peevishness, depressed mood, cognition and socio-emotional evolution, leading to low grades in exams (Ashton and Beattie 2019; Smith et al. 2020). By the late March 2020, Indian government announced series of lockdowns to combat with COVID-19 pandemic declared by the World Health Organization on 11 March, 2020 after spreading to various countries, which included closure of all public activities that required public gatherings like malls, schools, colleges, offices etc (Kumar and Dwivedi 2020).

This resulted in most people working from their home through digital media or the internet to continue their job and work (De et al. 2020). Even though, the cases of pandemic has been reduced in many countries, but in case of India, the number of cases of COVID 19 is still surging continuously but still some activities throughout the country has been allowed by taking appropriate measures using WHO guidelines (Kumar and Dwivedi 2020). In terms of education, there is increase in the usage of online services during and after lockdown as compared to pre-lockdown as schools, colleges, universities have shifted all their classes online by using mode of video conferencing apps which also constitutes a large proportion of work i.e. assignments, teaching, examinations etc. (De et al. 2020; Khan and Smith 2020; Pandya and Lodha 2021).

It has taken a dramatic shift in digital usage which impact on all aspects of work and life during COVID-19 pandemic. During this COVID-19 pandemic, there is growing concern about the impact of increasing exposure of screen on health

of children and young adults as physical activity outside home was not permitted due to increased chances of getting infected (Chen et al. 2020; Guo et al. 2021). Moreover, the association of screen time with limitation of Physical activity, adiposity, increase in energy intake or reduction in metabolic rate has been found (Iannotti et al. 2009; Guo et al. 2021). Thus, the objective of present study intent to evaluate the effect of increased usage of the screen on Psychological and physical health midst of COVID-19 pandemic among Indian Students.

MATERIAL AND METHODS

An observational cross-sectional study was conducted to evaluate the effect of increased screen time on the physical and mental aspects of health of Indian students during COVID 19 pandemic. This study was approved by the Ethical Committee of Xcell Physiocare and followed the Declaration of Helsinki ethical principles for Medical Research Involving Human Subjects. Snowball sampling method was adopted to assemble the samples. The self-made questionnaire was framed as Google form having consent, demographic details, and 2 sections. The questionnaire was carried forward through various social networking platforms to college and school students of India and requested to share further among their peer groups respectively. A total of 210 responses of students were received online from various schools and colleges between 15th August to 30th August 2020. The physical and mental health of students having increased usage of the screen was assessed on 5-point Likert scale questionnaire comprising of two sections. In the first section, students were asked about the mode and duration of screen time for study purposes daily. The second section included questions on student's physical and mental health while using the screen for study purposes during COVID-19 pandemic.

Table 1. Demographic Variables of Participants

Variables	Participants, N (%)
Gender	
Males	69 (32.8)
Females	141 (67.14)
AGE (In Years)	
≤ 12 Years	25 (11.90)
12 – 24 Years	97 (46.19)
25 – 38 Years	88 (41.90)
>38 Years	0
Qualifications	
Primary	21 (10)
High School	19 (9.04)
Diploma	4 (1.90)
Graduate	75 (35.71)
Post Graduate	88 (41.90)
Doctorate	3 (1.42)

For the data analysis: The data of the study was compiled in the Microsoft Excel sheet and then it was analyzed on descriptive statistics of SPSS version 26.0. The reliability

of the questionnaire was tested using Cronbach alpha (α). The demographics of the respondents were analyzed using descriptive statistics. Spearman correlation was applied to determine the correlation between screen usage time during COVID and physical health problems (neck pain, weight gain, backache, and headaches) and mental health. A paired t-test was applied to find the difference in screen time usage before and during COVID. The level of acceptable significance was set at $p < 0.01$.

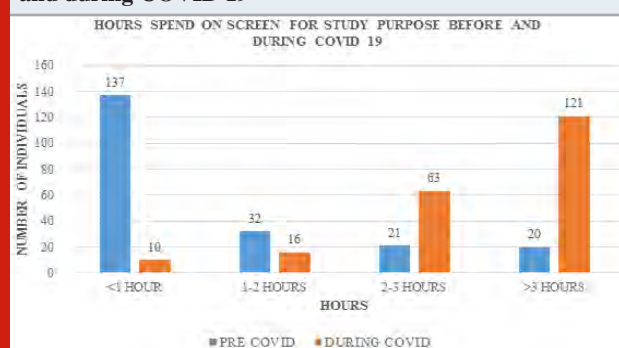
RESULTS AND DISCUSSION

The questionnaire was found to be reliable as the value of Cronbach alpha (α) came out to be 0.7 respectively.

Demographic Data: With the help of various social networking sites, 210 individual's responses were collected. The number of female's respondents (67.14 %) were more than the number of male respondents (32.8%). A good chunk of respondents belongs to the age group of 12-24 years. Majority of the respondents belong to Delhi. Table 1 depicts the Demographic details of the respondents respectively.

Section A: Screen usage time: This section of the questionnaire concerned about the time used on the screen for study purposes before and during COVID. The bulk of them were spending more than 3 hours on screen for study purpose during COVID. The comparison of time spent on screen before and during COVID is illustrated in figure 1. A statistically significant difference in time duration spent on screen before and during COVID was observed ($t = 19.96$; $p < 0.01$) (Table 2). Another question in this section includes the mode of the screen mostly used for study during COVID. Most of the respondents used mobile phones (64.2%), whereas 33.3% were using laptops, 1.9% were using computers and 0.4 % responded tab as their mode of the screen widely used for educational purposes during this pandemic.

Figure 1: Hours spend on screen for study purpose before and during COVID 19



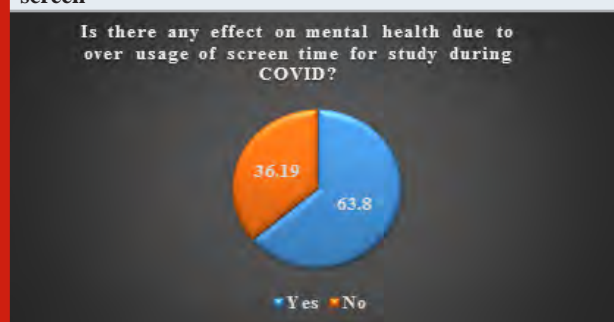
Section B: Physical and mental health: The section includes questions that determined the impact of screen usage on physical as well as mental health. On asking about the physical health issue which was faced most due to increased screen usage during COVID, the majority of people reported neck pain (63.3%) as the major issue faced due to screen usage. This was followed by eye problems (59.04%), headaches (56.19 %), and backache (29.52 %).

Another question asked about was "How often do you feel a problem of neck pain while studying on-screen during COVID?" 54.76% of respondents felt neck pain every time they used screens during this pandemic.

Table 2. t-test for time spend on screen pre and during COVID 19

PARAMETER	T	p-value
Screen usage time pre and during COVID	19.968	0.00*
*= value is significant at 0.01 level		

Figure 2: Effect on mental health due to over usage of screen



Another question stated, "How often do you feel a problem of backache while Studying on-screen during COVID?" 44.7 % responded that they felt backache every time. In other questions asking about eye problems and headache, 53.33 % of individuals responded they felt eye problems most of the times whereas 39.04 % responded they had headache every time while using the screen for study purposes during COVID. Another question asked whether there was any change in their weight during COVID as compared to pre-COVID. The majority of them responded that their weight was increased as compared to pre-COVID (61.42%). On another question asking about physical activity, 88.5 % responded that their physical activity was affected due to increased screen time during COVID.

On another question regarding the sleep quality, 46.6 % answered that their quality of sleep was hampered most of the times during COVID due to increased usage of screen. In the question concerned about mental health, 63.8 % responded that their mental health was affected due to over usage of screen during COVID. (Figure 2). Other question in this regard stated "if they felt that their mental health is affected due to over screen usage, then which problem you are facing due to over usage of screen time for study during COVID?" 69.9% responded they felt anxious due to over screen usage whereas 48.8 % responded lack of confidence as a major issue faced by them followed by panic disorder (24.8 %), depression (23.3 %), while 5.2 % responded to other problems which included lack of concentration, feeling agitated, and irritated due to over screen usage. These responses to the questions concerning physical and mental aspects of the health can be seen in Table 3.

A statistically significant positive correlation was found between the screen time usage with back ache ($r=0.62$), neck pain ($r=0.71$), headache ($r=0.50$), weight gain ($r=0.52$) and mental health ($r=0.40$) respectively (Table 4). This study was conducted to determine the impact of increased usage of the screen on psychological and physical health during the COVID-19 pandemic among Indian students as it has been observed that there was a dramatic shift in digital usage in the education system during the outbreak of the COVID-19 pandemic due to the closure of schools, colleges, and other

educational institutions (De et al. 2020; Khan and Smith 2020). So in order to preserve the regularity of education, all education institutions have switched classes and all assignments work to the online mode which was responsible for increasing screen time during COVID 19 as compared to pre-COVID times which further led to various physical and psychological problems among students. This study has also found a significant increase in the time duration spent on-screen during COVID 19 as compared to pre-COVID which was in line with findings of some studies (Schmidt et al. 2020; Guo et al. 2021).

Table 3. Impact of over screen usage on Physical and Mental health

Variables	Every time	Most of the time	Quite often	Rarely	Not at all
How often do you feel a problem in your eyes while studying on-screen during COVID?					
N (%)	23 (10.95)	112 (53.33)	50 (23.80)	18 (8.57)	07 (3.33)
How often do you feel back pain while studying on-screen during COVID?					
N (%)	94 (44.76)	36 (17.14)	31 (14.76)	17 (8.09)	32 (15.23)
How often do you feel neck pain while studying on-screen during COVID?					
N (%)	115 (54.76)	46 (21.90)	25 (11.90)	16 (7.61)	08 (3.80)
How often do you feel headache while studying on-screen during COVID?					
N (%)	82 (39.04)	59 (28.09)	20 (9.52)	25 (11.90)	24 (11.42)
How often do you felt that your sleep quality has been affected due to increased screen time because of studying during COVID?					
N (%)	25 (11.90)	98 (46.66)	62 (29.52)	17 (8.09)	08 (3.80)
Variables	Increased	Decreased	Same as before		
Do you feel any change in your weight during COVID as compared to pre-COVID?					
N (%)	129 (61.42)	47 (22.38)	34 (16.19)		
Variables	Yes	No			
Do you think increased screen time for study affects your physical activity during COVID?					
N (%)	186 (88.57)	24 (11.42)			
Variables	Backache	Neck pain	Eye problems	Headaches	Others
Which problem do you face most while studying on-screen during COVID (You can respond to more than one option)?					
N (%)	62 (29.52)	133 (63.33)	124 (59.04)	118 (56.19)	04 (1.90)
Variables	Anxiety	Depression	Loss of confidence	Panic disorder	Others
If you think that your mental health is getting affected, then which problem you are facing due to over usage of screen for studying during COVID (You can respond to more than one option)?					
N (%)	93 (69.92)	31 (23.30)	65 (48.87)	33 (24.81)	07 (5.26)

The analysis of this study revealed a strong association between physical complaints which included backache and neck pain with overuse of screen for study purpose which was similar to the results found in other studies indicating that the use of computer/ laptops for more than 2 hours a day is a threshold for neck pain and more than 5 hours per day for lower back pain (Hakala et al. 2006; Torsheim et al. 2010). Another study has been done in this regard in which the reported time spent on screen i.e. computer/ laptops for educational purpose (17.9 ± 12 hours per week) were associated with both chronic neck and back pain as most of the time digital academic studies required sitting position for longer duration which is responsible for causing muscles to stress in a particular position which further led

to musculoskeletal related problems (Casas et al. 2016). It is a specific risk factor but not a sufficient or mandatory cause of physical health problems among students.

Further, more use of electronic devices has been considered a risk factor associated with the presence of migraine or other types of headaches. Xavier MK found the usage of the screen for more than 4 hours were directly associated with headache (Xavier et al. 2015). Singh and Balhara (2020) also suggested that it is the time to revisit the recommendations on Screen time for Children & Adolescents by WHO (Singh and Balhara 2020). The present study also found a significant association of headache with increased time spent on screen for studies due to which students tend to get

a very little time for recreational activities. This encourages them to maintain faulty body postures for a long time for study purposes that leads to overload on a visual system that triggers headache among students especially during the time spent on the screen (Oksanen et al. 2007; Smith et al. 2009).

The study showed a significant positive correlation between the increase in weight with increased time spent on the screen during COVID. Various studies support our findings in which they reported an association of overweight and obesity with screen time as it requires prolonged sitting for studying during the pandemic (Duncan et al. 2012; Stiglic and Viner 2018). One of the underlying reason could be the adoption of a sedentary lifestyle by the people in order to combat with this pandemic by maintaining social distancing due to which opportunities for physical activities were reduced with the closure of gyms, and parks especially in urban areas where they lived in a small apartment or likely they don't have access to safe outdoor space where they can

maintain social distancing (Marsh et al. 2013; Rundle et al. 2020). However, on the contrary, in Germany, due to their increase in habitual Physical Activity, their overall Physical activity duration was found out to be more than they used to do earlier (Schmidt et al. 2020). Lesser Physical activity has affected the health-related Quality of life during COVID times as compared to before (Wunsch et al. 2021).

Recently, Kovacs et al. (2021) also found similar patterns where online Physical Education increased the odds of healthy levels of Physical activity and screen time in moderately affected European countries (Kovacs et al. 2021). Apart from this finding, our study also showed the impact of screen time on physical activity and sleep quality. Similar findings were reported by Guo et al. (2021) Most of the respondents reported that their sleep quality was affected. This could be due to the lack of physical activity in which they have stated that sleep quality among the Indian population was affected due to a decrease in physical activity during this pandemic (Sharma et al. 2020; Guo et al. 2021).

Table 4. Correlation between screen usage time and various parameters

Parameters	Correlation (R)	P-Value
Screen usage time during COVID and backache	0.622	0.00*
Screen usage time during COVID and neck pain	0.719	0.00*
Screen usage time during COVID and headache	0.503	0.00*
Screen usage time during COVID and Weight gain	0.524	0.00*
Screen usage time during COVID and mental health	0.406	0.00*

*= Correlation is significant at 0.01 level

COVID-19 is a crucial time for everyone but for students, it is difficult to cope up with this time as they are worried about their future, exams, classes, etc. To maintain the continuity of their education they need to spend more time on screen as compared to pre-COVID-19 for classes, assignments, etc. which leads to lower psychological well-being. More usage of the screen is likely to develop poor emotion regulation, lower self-control, less socially active in making friends, and low curiosity in other things (Twenge and Campbell 2018). Also, Parents do play a major role during this juncture of time. It has been seen that Mother, who usually is the closest to child, have higher screen time then child will also have the higher screen time. As technology is not confined to the educated or technology geeks, preschool children usage was found to be higher than school going children. Various studies depicted the trend of Increased screen time affecting paediatric patients and different age groups simultaneously. (Ishtiaq et al. 2021; Sultana et al. 2021; Lim 2021).

The present study has found a significant positive correlation of screen time during COVID-19 with mental health. Various studies have also found the diagnosis of depression, anxiety, behavioural problem, poor mental health and various other mental health illness among children and

adolescents with more screen time in comparison with the low users of the screen. However, the ones having known mental ailments were affected worse than normal ones. (Allen and Vella 2015; Babic et al. 2017; Pandya and Lodha 2021; Neophytou et al. 2021; Henson et al. 2021). As continuously growing concerned on online education during pandemic, students are not able to adapt themselves in the environment of online education and therefore the basic expectation of the students from the online education curriculum was not fulfilled, ultimately leading to mental health ailments and negativity about themselves. By virtue of the observation, the present study depicts general health care issues among the students who were taking classes online during this pandemic.

Limitations and future scope of the study: Some limitations of this study include: Self-reported data from selected participants and relying on other networks for sharing the Questionnaire. The age group is not specified as it involves both adolescents and adults group. There is a scope of further study to identify the impact of over usage of screen on health of individuals of specific population i.e., either adolescents or adults' group and also on the larger sample size to get more reliable and accurate results.

CONCLUSION

The findings of the present study concluded that the increased time spends on the screen has a great impact on physical and mental health. Among the physical health problems, major issues faced by students are mainly backache, neck pain, and headache. Whereas, anxiety is the major mental health issue reported by students. With this study, we can imply that proper preventive measures should be incorporated into the daily schedules to prevent students from physical and mental ailments. Also, the college authorities should plan the schedule of teaching and assignments in such a way that it should be compatible with the students

Conflict of Interest: Authors declare no conflict of interests to disclose.

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Biotechnological Communication

Screening and Evaluation of a Potential Bacteriocin Producing *Lactobacillus* in Milk and Dairy Products Collected from Puducherry

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ABSTRACT

In an array of identifying safe antimicrobial compounds, bacteriocin producing *Lactobacillus* strain have been investigated in this study from the daily consuming food resources of humans. Till now, the best studied bacteriocins are nisin A produced by *Lactobacillus lactis* and pedocin PA-1 synthesized by *Pediococcus acidilactici* which have been accredited as a preservative in the food industries by the World Health Organization (WHO). For this study, four different milk and dairy products viz., curd, cheese, yoghurt and butter were collected from the local markets of Karaikal region, Puducherry, India and were used for the isolation of *Lactobacillus* species using MRS agar. Totally, five morphologically distinct strains were collected and were initially named as MPD 1 to MPD 5. During the screening process of bacteriocin production, the strain MPD 5 showed maximum antimicrobial activity against *Vibrio cholerae* MTCC 3906 with 900AU/ml. This strain was molecular identified as *Lactiplantibacillus plantarum* MDP 5 based on 16S rRNA partial sequence method. This 16S rRNA partial sequence was submitted to the NCBI nucleotide GenBank and provided with the accession number, MW301154.1. Further, this strain revealed an enhanced production of bacteriocin using the standardized physicochemical factors such as pH 7, 35°C, 2% fructose and 1% peptone. Furthermore, these optimal conditions revealed more than 2-fold increase in the bacteriocin production. All the above information suggesting the possibilities of bacteriocin for the bioindustrial production using the *L. plantarum* MDP 5 of this study and its future prospects for the investigation of biocidal activities against many highly infectious pathogens of human and veterinary.

KEYWORDS: ANTIMICROBIAL, BACTERIOCIN, GROWTH KINETICS, LACTIPLANTIBACILLUS PLANTARUM, MILK AND DAIRY PRODUCTS, OPTIMIZED PRODUCTION.

INTRODUCTION

Lactobacillus species are common for the application of fermentation as well as preservation of broad class of milk, vegetable, meat products, etc. There are antimicrobial compounds derived from the *Lactobacillus* strains viz., diacetyl, hydrogen peroxide, organic acids as well as bacteriocins which reveal a key role in assuring the safety and lengthening the shelf-life of many food products. Since

the last 20 years, antimicrobial significance of *Lactobacillus* strains has been globally studied (Daeschel 1989; Jack et al. 1995). Increasing number of consumers demanding the organic based bioactive products in the recent years enhance the interest of applications using naturally derived inhibitory substances which could alternate the chemical counterpart. Among such natural bioactive compounds, bacteriocin could be in the leading interest which attract many researchers globally (Heredia-Castro et al. 2015; Balan et al. 2019a; Balan et al. 2019b).

Synthesis of bacteriocin is a common phenomenon among *Lactobacillus* species. Bacteriocins are bioactive proteins which usually act antagonistic towards Gram-positive

Article Information:*Corresponding Author: drsumathimicro@gmail.com

Received 09/10/2021 Accepted after revision 12/11/2021

Published: 31st December 2021 Pp- 1501-1507

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.20>

bacteria or closely related to the synthesizer microbe and the appreciable bioactivities were recorded against *Staphylococcus aureus*, *Clostridium tyrobutyricum*, *Listeria innocua*, *Listeria monocytogenes* and *Bacillus cereus* (Klaenhammer 1988). Further, bacteriocin rarely evidenced its bioactivities against gram negative bacteria such as *Helicobacter pylori*, *Salmonella typhimurium*, *Campylobacter jejuni* and *Escherichia coli* (Rammelsberg and Radler 1990; Reis et al. 2012). Bacteriocin production have also been reported from *Leuconostoc*, *Carnobacterium*, *Pediococcus*, and *Lactococcus* species (Kohoutova et al. 2020; Nebbia et al. 2021).

The best studied bacteriocins are nisin A produced by a *L. lactis* and pedocin PA-1 synthesized by a *Pediococcus acidilactici* which have been accredited as a preservative in the food industries by the World Health Organization (WHO) (Biscola et al. 2013). Bacteriocins have many positive features which compel it special for numerous different applications. Bacteriocin from *Lactobacillus* are often having extreme thermal and pH tolerance. Further, these peptides are also known for their colourless, odourless, and tasteless, which further enhance their added advantage for many potential usefulness (Negash and Tsehai 2020). In the present investigation, fermented food from the local markets of Karaikal, Puducherry have been aimed for the isolation and screening of bacteriocin producing *Lactobacillus* strains which explore the significance of indigenous microbiota. Indigenous microbes of various natural or geographical habitats have unique biochemical, functional as well as metabolic properties (Balan et al. 2013a; Balan et al. 2013b; Ahmad et al. 2020; Chen et al. 2021). Hence, this study concentrated on native species and the potential isolate have further been characterized for its enhanced bacteriocin production.

MATERIAL AND METHODS

Sampling and isolation of *Lactobacillus* strains was performed using four different milk and dairy products viz., curd, cheese, yoghurt and butter were collected from the local markets of Karaikal region, Puducherry, India. All the samples were individually processed for the isolation of *Lactobacillus* strains. One gram of sample was serially diluted with pre-sterilised bacteriological saline water and spread plated on MRS agar medium (HiMedia, Cat. No. M641) plates. After two days incubation at 37°C, morphologically distinct colonies were axenic cultured in newly prepared MRS agar plates. The purity of the bacterium was microscopically confirmed after Gram's staining method (Harley 2013). All the axenic strains were stored under lyophilized conditions for further use.

Screening of bacteriocin producing bacteria was done using an indicator pathogenic bacterium, *Vibrio cholerae* MTCC 3906 purchased from the Microbial Type Culture Collection, CSIR-Institute of Microbial Technology, Chandigarh, India. The strain was overnight cultured at 37°C on tryptone soya broth (HiMedia, Cat. No. LQ508) and the OD of the culture was tuned to an inoculum concentration of 10⁵ CFU/ml for all the bioactivity studies. The quantitative bacteriocin production was determined by microtiter well-plate assay

as earlier described in the previous studies (Kang and Lee 2005). The bioassay was performed in 96-well flat bottom polystyrene microtitre plates with lids (Tarsons, India). In this assay, well plates were filled with 100µL of serially diluted bacteriocin containing cell free supernatant followed by the addition of 20µL indicator strain, *V. cholerae* MTCC 3906 at a concentration of 10⁵ CFU/ml assessed using optical density at 620nm and 80µL tryptone soya broth. The growth control well plate has 100µL of phosphate buffer (pH 7), 20µL of indicator strain and 80µL tryptone soya broth.

The susceptibility control well plate has 100µL of 4mg/ml streptomycin containing phosphate buffer (pH 7), 20µL of indicator strain and 80µL of tryptone soya broth. The well plates were incubated for 6hrs at 37°C, further, the absorbance was estimated at 620nm with a microplate reader (Biotek Elx808, WI, USA). The growth inhibition percentage were estimated as given below: Percentage growth inhibition = $[(1 - (As/Ac)) \times 100]$, where As denotes the absorbance of wells having the test samples and Ac denotes the absorbance of wells without any added bioactive sample (control). Bacteriocin activity was represented here with the arbitrary unit (AU) in which the AU is defined as reciprocal of the maximum dilution of sample forming 50% growth inhibition of indicator strain.

The study of extracellular or intracellular bacteriocin production was performed with the individual isolates. The strains were cultured in 30ml screw cap tubes containing aliquot MRS broth (HiMedia, Cat. No. M369). After two days incubation at 37°C, centrifugation was performed on the cultured broths for the separation of cell pellet and cell free supernatant at 3000rpm 15 min. The separated cell pellet dissolved in 50ml phosphate buffer and sonicated at 20KHz for 45 sec. using Ultra-sonicator (Hielscher, USA). The same centrifugation condition was applied to remove the cell debris formed during the sonication process and the supernatant was used for the estimation of bacteriocin production whereas the cell free supernatant collected from cultured broth was instantly applied for the bacteriocin bioassay. This experiment revealed whether the isolate produced extracellular or intracellular bacteriocin or observed from both locations.

Molecular identification of potential bacteriocin producer was done using 16S rRNA partial sequence using the Eubac set of primers, 27F (5'-AGAG TTTG ATCM TGGC TCAG-3') and 1492R (5'-GGTT ACCT TGTT ACGA CTT-3'). The phylogenetic tree was drawn with neighbour joining procedure (Saitou and Nei 1987) against the maximum similar sequences available in the NCBI nucleotide GenBank database using MEGA 7 software (Kumar et al. 2016). Optimization of enhanced bacteriocin production was performed in this study in which the potential strain was standardized for the possible enhanced production by adopting search technique varying one condition at a time and the standardized conditions were fixed for subsequent estimations. The optimization of bacteriocin production was studied in 250ml conical flask with 100ml working volume of yeast glucose broth [10] has the following composition of yeast extract (10g/L) and glucose (20g/L) with the final pH of 7.5 ± 0.2 along with

the subsequent conditions of 37°C temperature, agitation at 150rpm and one ml inoculum. The inoculum preparation and bacteriocin quantification was performed using the same applied procedure as mentioned in the screening of bacteriocin producing bacteria.

Growth kinetics profile as a function of time on bacteriocin production was carried out with the potential strain. It was studied for the required time of maximum bacteriocin production with reference to the cell biomass concentration at 12hrs regular time intervals from 0hrs to 144hrs. The estimations were performed with a portion of cultured broth next by the separation of cell biomass and cell free supernatant with the help of centrifugation at 3000rpm for 15 min. Bacterial cell growth was estimated from the dry weight of cell biomass resulted from the hot air oven dried centrifuged cell pellets at 50°C for 30min. and bacteriocin quantitative estimation was directly determined from the cell free supernatant.

Recapitulation of different biotic and abiotic growth parameters was performed in this study. Optimizing parameters like pH, temperature, carbon and nitrogen take part a significant role in the enhanced bacteriocin production. Influence of pH conditions from pH 5 to 9 were studied for the effective medium optimization, similarly, various temperature conditions from 20 to 50°C were investigated in the production medium for the enhanced production. Similarly, various carbon sources like fructose, galactose, sucrose, maltose, cellulose and starch were utilized at 2% concentration in the production medium as well as various nitrogen substrates like ammonium nitrate, malt extract, tryptone, beef extract and peptone were used at 1% concentration.

RESULTS AND DISCUSSION

Lactobacillus were generally regarded as safe (GRAS) microorganisms which playing a key role in the fermentation of foods. *Lactobacillus* strains were known to produce various antimicrobial compounds such as bacteriocins, D-isomers of amino acids, reuterin, hydrogen peroxide, acetaldehyde, CO₂, and diacetyl (Cintas et al. 2001). Bacteriocins were ribosomally produced antimicrobial compounds which were active against bacterium of same species or other genera. Bacteriocins have been reported from both gram positive and negative bacteria. Nowadays, bacteriocin producing *Lactobacillus* have gained attention because of their non-toxic status and its uses as potential food preservative (Diop et al. 2007; Nebbia et al. 2021).

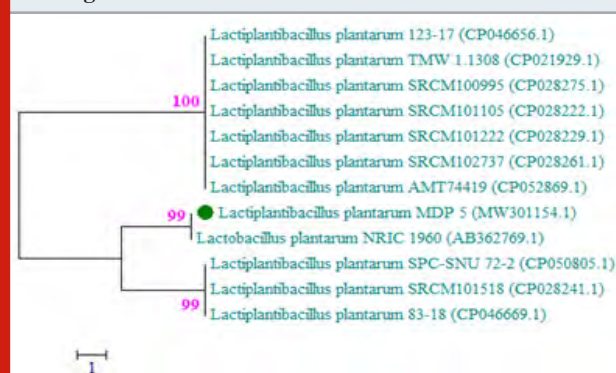
In this study four different fermentation products of milk and dairy such as curd, cheese, yoghurt and butter were taken for the isolation of *Lactobacillus* strains using MRS medium. Initially, the samples were serially diluted, spread plated on MRS agar medium and incubation for two days. There were five morphologically distinct strains obtained as axenic culture and these strains were initially referred using the voucher names viz., MPD 1 to MPD 5 in which the MDP represents the abbreviation of Milk and Dairy Products followed by serial number of the bacterial strains. All the strains were individually MRS broth cultured using screw

cap tubes and screened for the intracellular and extracellular production of bacteriocin (Fig. 1).

Figure 1: Axenic strains cultured in 30ml screw cap tube containing MRS broth for the screening of bacteriocin production



Figure 2: The phylogenetic tree of *L. plantarum* MDP 5 based on evolutionary analysis was studied using Neighbor-Joining method.

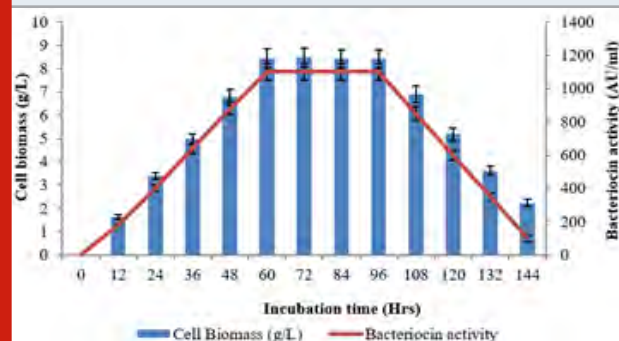


Among the isolates, only two strains revealed bacteriocin production viz., MDP 2 and MDP 5 through its antimicrobial activity against *V. cholerae* MTCC 3906 in which MDP 2 showed intracellular production and MDP 5 showed extracellular production and the rest of the strains revealed negligible bacteriocin activities. Further, the strain MDP 5 evidenced maximum bacteriocin activity of 900AU/ml followed by the strain MDP 2 with 150AU/ml. Based on the appreciable production of bacteriocin, the strain MDP 5 was chosen for molecular identification and enhanced bacteriocin production studies. Similar to this study, an appreciable bacteriocin producing *L. plantarum* ST664BZ procured from a traditional cereal beverage of Bulgaria (Todorov and Dicks 2006). Likewise, bacteriocin purified from *Enterococcus faecalis* and *E. hirae* of homemade milk and dairy products revealed strong growth inhibitory activity against many foods borne pathogens (Sonbol et al. 2020).

The molecular identification of the isolated potential bacteriocin producing *Lactobacillus* strain, MDP 5 of this investigation was carried out with the 16S rRNA partial gene sequence method. The 16S rRNA partial gene

sequence region was amplified using a PCR and the total sequence length of the amplified product was 1501bp. Based on BLASTn homology search, the amplified region revealed 100% sequence similarity with an available nucleotide database of the NCBI GenBank sequence and it was a type strain, *L. plantarum* NRIC 1960 with the accession number AB362769.1. Based on this highest gene sequence homology, the isolate MDP 5 was identified as *Lactiplantibacillus plantarum* as well as the sequence was deposited in NCBI nucleotide GenBank and provided with the accession number MW301154.1. The genus *Lactobacillus* included in the family *Lactobacillaceae* and the phylum Firmicutes. The phylogenetic tree of *L. plantarum* MDP 5 was illustrated using the neighbour joining method with the twelve sequences of highest homology obtained from the NCBI GenBank nucleotide collection (Fig. 2). The exact molecular identification procedure was carried out for the identification of *L. plantarum* collected from the native fruits of Ecuadorian Amazon and a fermented drinks of China (Garzon et al. 2017; Pei et al. 2020).

Figure 3: Growth kinetics profile as a function of time on bacteriocin production using the *L. plantarum* MDP 5



The growth kinetics profile of *L. plantarum* MDP 5 with regarding to the bacteriocin synthesis as a function of incubation time was examined (Fig. 3). The bacteriocin production was examined from the initial incubation period till the decline growth phase. Further, the peak production of bacteriocin was recorded during the end of exponential or beginning of stationary growth phase of the potential bacterium (60hrs) and it was maintained till the end of stationary or initiation of decline growth phase (96hrs) (Balan et al. 2019a; Balan et al. 2019b). Furthermore, the highest production of bacteriocin, 1100±50AU/ml was achieved with the 8.43±0.42 cell biomass concentration. This growth dependant pattern of metabolite production revealed it is a primary metabolite (Balan 2014). The main difference between antibiotics and bacteriocins is that antibiotics were produced in the stationary growth phase and hold as secondary metabolites whereas bacteriocin synthesized during primary growth phase and act as primary metabolites (Beasley and Saris 2004; Balan et al. 2019a; Balan et al. 2019b).

Optimization of physicochemical parameters plays an important factor in the production process because it decides the commercialization value of any products

(Sankar et al. 2013). Because each microbial strain has its own functional, metabolic and biochemical uniqueness, hence worth standardization of various physicochemical conditions requires to be done for the enhanced production of all the microbial based products (Mani et al. 2016; Balan et al. 2012; Balan et al. 2019a). Hence, this recapitulation study of pH, temperature, carbon and nitrogen parameters was carried out for the potential bacterium, *L. plantarum* MDP 5 of this study.

Figure 4: Effect of various pH conditions on the Bacteriocin production using the *L. plantarum* MDP 5

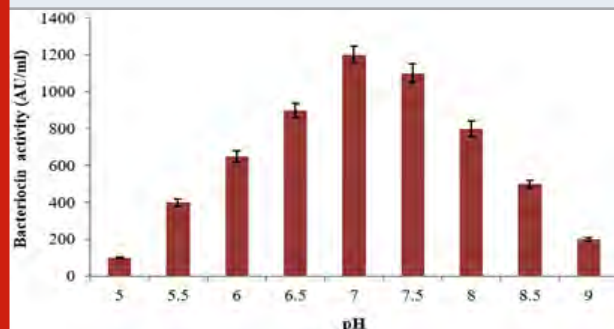
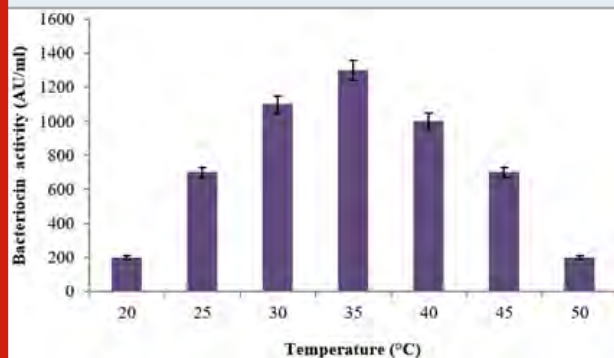
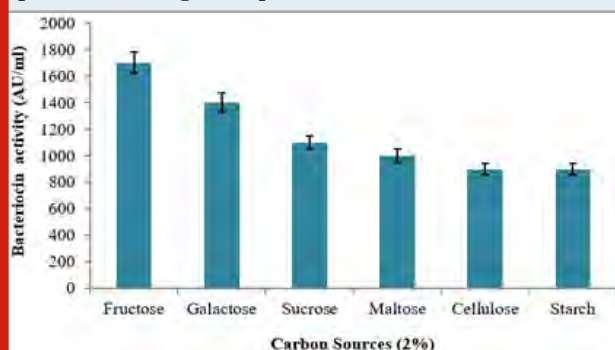
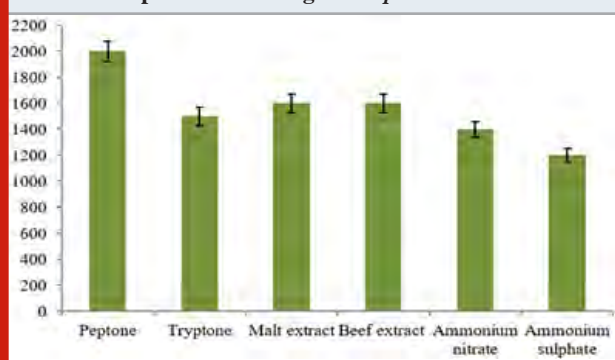


Figure 5: Influence of different temperature on the Bacteriocin production using the *L. plantarum* MDP 5



Among the tested pH conditions, the potential strain revealed maximum bacteriocin production at the pH 7 with 1200±50AU/ml activity followed by pH 7.5 and 6.5 with 1100±50AU/ml and 900±40AU/ml activities (Fig. 4). Further, the least bacteriocin activity of 100±5AU/ml was recorded at pH 5. This study proves the strong dependence of the *L. plantarum* MDP 5 on the hydrogen ion concentration of the surviving medium. A similar study was performed on temperature at varied conditions, the maximum bacteriocin production was observed at 35°C with 1300±60AU/ml activity followed by 30°C and 40°C with 1100±50AU/ml and 1000±50AU/ml activities and least production was recorded at both 20°C and 50°C with 200±10AU/ml activity (Fig. 5). From the above study, both the increasing and decreasing temperatures from 35°C revealed negative effects on bacteriocin synthesis. Close to this study, a probiotic *L. plantarum* BC-25 cultured in MRS broth revealed maximum production of bacteriocin using 35°C and pH 6.8 (Zhou et al. 2015; Zhang et al. 2020).

Figure 6: Study of various carbon sources on the Bacteriocin production using the *L. plantarum* MDP 5**Figure 7: Study of different nitrogen sources on the Bacteriocin production using the *L. plantarum* MDP 5**

The influence on various carbon sources at 2% concentration was used for the production of bacteriocin using the potential bacterium, *L. plantarum* MDP 5 (Fig. 6). Among the tested carbon sources, monosaccharide substrate of fructose revealed the highest production of bacteriocin with 1700 ± 80 AU/ml activity followed by another monosaccharide substrate of galactose with 1400 ± 70 AU/ml activity. Further, a moderate bacteriocin production was observed with 1100 ± 50 AU/ml and 1000 ± 50 AU/ml while using the disaccharide sources of sucrose and maltose and a least production was evidenced using polysaccharide substrate viz., cellulose and starch in which both has 900 ± 40 AU/ml activities. However, *L. acidophilus* AA11 showed maximum production of bacteriocin using 0.5% lactose as its sole carbon source (Abo-Amer, 2011). In support to the present study, *L. plantarum* Q7 revealed appreciable production of bacteriocin using fructose in the MRS medium (Zhang et al. 2020).

Likewise, the effect of different nitrogen sources at 1% concentration was investigated for the bacteriocin production in this study. Interestingly, organic nitrogen substrates revealed good sources for the bacteriocin production when compared to inorganic substrates used in this investigation (Fig. 7). Among them, use of peptone in the production medium evidenced maximum bacteriocin activity of 2000 ± 80 AU/ml followed by malt extract, beef extract and tryptone with 1600 ± 80 AU/ml, 1600 ± 80 AU/ml and 1500 ± 80 AU/ml activities, respectively. Further, low bacteriocin production was recorded in ammonium

nitrate and ammonium sulphate with 1400 ± 60 AU/ml and 1200 ± 50 AU/ml activities, respectively. However, *L. plantarum* ST13BR evidenced maximum production of bacteriocin in the combination of the meat extract and yeast extract with 1:1 ratio as its sole nitrogen source (Todorov 2004). Similarly, *L. brevis* C23 showed maximum bacteriocin production using meat extract as its sole nitrogen source (Sreedharan et al. 2021). From the above findings, all the four physicochemical parameters utilized in this study revealed the enhanced production of bacteriocin.

CONCLUSION

The findings of the present study explored a new bacteriocin producing *L. plantarum* MDP 5 isolated from the milk and dairy products collected from Puducherry. The bacterium showed a growth dependent synthesis which revealed its peak production at earliest time of stationary growth phase. Further, this study evidenced enhanced bacteriocin production through the easily lab consumable physicochemical conditions which suggesting its feasibilities for commercialization opportunities. Moreover, this bacterium revealed a strong antimicrobial activity against *V. cholerae* MTCC 3906 which holds as baseline data for the possible future investigation regarding its antimicrobial applications against many deadly human and animal pathogens.

ACKNOWLEDGEMENTS

The study was supported by the Department of Microbiology, Faculty of Science, Annamalai University, Chidambaram, Tamil Nadu, India. Authors thank the department for providing lab facilities and supporting the research work.

Conflict of Interests: Authors declare no conflict of interest to disclose.

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Dental Communication

Effect of Different Finishing and Polishing Methods for Color Stability of the E-Max Dental Ceramics: *In vitro* Study

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ABSTRACT

In order to assess the influence of finishing and polishing on the surface brightness and color stability of the ceramic veneer, fifty specimens were fabricated with 10 mm diameter and 2 mm thickness using IPS E-Max Ceramic. After glazing, 10 specimens were untouched as control group, and the other 40 specimens were abraded using 125 μ m diamond bur to create surface roughness. Forty specimens were divided into four groups (n=10), in group 1: specimens were finished using diamond point, in group 2 specimens' surface was polished with a polishing kit, Group 3: Each specimen surface was polished with the polishing kit as in protocol 2 and was polished a polishing past and group 4 Each specimen was glazed by heating at 621°C for 3 minutes followed by a temperature increase of 83°C/min up to 918°C for 30 seconds. Color measurement was performed using spectrophotometer. Color stability data were analyzed using two-way ANOVA and Tukey's HSD test ($\alpha=0.05$). For Ra values, paired-samples t-tests were used to analyze the data and compare groups. The change in L and E showed a significant difference among the study groups; (group 1, group 2, group 3 and group 4) with respect to three variables L, a and b. A significant difference was noted when compared each group with the control; however, only group 2 showed a significant difference from group 4; the remaining groups demonstrated similar findings for all three variables. The study displayed a significant impact of the finishing and polishing technique on the surface brightness and color stability of ceramic restoration. However, it was evident that combination of two or three polishing techniques which includes polish kit and glaze enhances the surface finish and adds color stability by alternating the yellow – blue axis (increase in b) and red-green axis (decrease in a).

KEY WORDS: COLOR STABILITY, COMMISSION INTERNATIONALE DE L'ECLAIRAGE, FINISHING, GLAZING, POLISHING, VENEERS.

INTRODUCTION

Aesthetics is one of the pioneer aspects of a smile which adds up to the confidence and personality of the person. The appearance of the dental teeth is not only important for the patient but also for the dentist. With advanced technology, manufacturers produce wide range of range of materials to match the shade of the teeth varying across the globe (Vieira et al. 2013; Vanlıoğlu and Kulak-Özkan 2014). With advancement and greater awareness, patient demands for good functional and highly esthetic restoration. Hence, it is utmost important to select appropriate veneer materials with excellent esthetic quality. The recent developed dental

ceramics presents desirable characteristics that highlights them as best choice as indirect restoration (Kilinc and Turgut 2018; Sarıkaya et al. 2018).

Dental ceramic is a dental material used extensively by the dentist for veneers, braces, inlays, onlays and crown restoration. The material is biocompatible, esthetics, insoluble and hard enough for a perfect and long-term restorations (Sarıkaya et al. 2018; AlMawash et al. 2020). Several different types of ceramics are available which includes pressed ceramics that mimics the natural tooth properties such as light transmission, color reproduction and texture. Studies have shown that the surface smoothness and color stability are interrelated. Hence after finishing and polishing procedures the optical properties of dental ceramics which enhances the surface finish to allow for

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Received 12/10/2021 Accepted after revision 25/12/201

Published: 31st December 2021 Pp- 1508-1513

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.21>

proper light reflection (Sarikaya. and Güler 2011; Volpato et al. 2016; AlMawash et al. 2020).

Restorations in the oral environment deteriorate with time under constant exposure to external factors and saliva. Along with surface properties, discoloration of the restoration is equally affected in long term. Authors demonstrated long term exposure to external factors such as forceful bite, diet and habits the restoration surface which affects the color stability and brightness of the restoration (Pires-de et al. 2009; Hamza et al. 2017). These craters or surface defects produced leads to staining, plaque retention, gingival irritation, and recurrent caries causing the adherence of agents responsible for changing the color. Hence, dentist imposes great emphasis on polishing and finishing of the surface for better quality finish and restoration (Cengiz et al. 2014; Shetty et al. 2020). Nonetheless, limited literature supports the importance of types of polishing in creating a smooth finish for color stability and brightness. Thus, the study aims to evaluate the effect of the types of finishing and polishing technique on surface brightness and color stability in ceramic veneers.

MATERIAL AND METHODS

A total of 50 disc-shape specimens were fabricated with 10 mm diameter and 2 mm thickness using IPS E-Max Ceramic Material (Ivoclar Vivadent, Schaan, Liechtenstein) (Fig. 1). Each sample was fabricated initially as a wax cylindrical pattern using a split brass matrix. The wax patterns were then invested with an investment material (IPS Empress Esthetic Speed Investment; Ivoclar Vivadent AG). Wax elimination was completed in temperature of 750°C for 6 min using oven (Meditherm 200 MP, Bego, Canada). Upon burnout, IPS e-max Press ingots (Ivoclar Vivadent, Schaan, Liechtenstein) were heat pressed using Programat® EP 3000 Oven (Ivoclar Vivadent, Schaan, Liechtenstein) at temperature of 920°C. After bench cooling, the specimens were treated with 2% hydrofluoric acid for 10 min and with 50 µm Al₂O₃ at 0.2 MPa pressure for 5 sec. The specimens were then inserted in a digital ultrasonic cleaner (JP-4820, Skymen, Shenzhen, China) for 10 min, followed by stratification of the ceramic using color A₂ of the Vita shade scale (Vita Zahnfabrik, Bad-Säckingen, Germany). After cleansing, 10 specimens were untouched as control group, and the other 40 specimens were abraded using 125µm diamond bur in a unidirectional motion to create surface roughness simulate. The 40 specimens were divided into four groups (n=10), and a different finishing and polishing method were completed for each group as following:

Group 1: Each specimen was finished using diamond point 862C 012 (Drendel+Zweiling, Germany) (Fig. 2), intermittently in one direction, with a cooling and high speed (Kavo do Brasil Industria, SC, Brazil), for 30 seconds. The specimen was then washed with air/water spray to remove the residues. **Group 2:** Each specimen surface was polished with a polishing kit (Ceramaster Finishing and Polishing Kit; Shofu Inc) (Fig. 3) using a slow-speed handpiece, as recommended by manufacturer. **Group 3:** Each specimen surface was polished with the polishing kit as in protocol

2 and was polished a polishing past (OptraFine polishing Paste; Ivoclar vivadent, Germany) (Fig. 4).

Group 4: Each specimen was finished as in protocol 1, and then polished with polishing kit as in protocol 2. Each specimen was then auto-glazed by heating at 621°C for 3 minutes followed by a temperature increase of 83°C/min up to 918°C for 30 seconds. Color measurement of all specimens was completed using a laboratory spectrophotometer (HunterLab LabScan XE spectrophotometer; the sotto group, United Kingdom). (Fig. 5). This instrument takes measurements at 400nm-700nm and gives readings at intervals of 10nm. It measures the reflected colour with 0°/45°, and use an inclusive EasyMatch QC software for analyzing colour data. Calibration was performed before take the measurement of each group, and and it is built in calibration. The average color of each group will be compared with the color of the control group. The Commission Internationale de l'Eclairage (CIE L*a*b*) color space is employed to determine color differences. ΔL*, Δa*, and Δb* are differences between two colors in the CIE-based color space. In present study, the colorimetric values of ΔL*, Δa*, and Δb* are measured from differences in the respective L*, a*, and b* values. The total color difference ΔE*ab between two colors, each given in terms of L*, a*, and b*, was calculated from the following formula: $\Delta E^*_{ab} = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$. Differences in color (ΔE) were calculated according to the following equation:

$$\Delta E = [(L_2 - L_1)^2 + (a_2 - a_1)^2 + (b_2 - b_1)^2]^{1/2}$$

Because the most important factor affecting the result of shade appearance of a new restoration is the Value "brightness", the differences in Value (ΔL) was calculated as well for each group comparing to the control group using the following equations:

$$\Delta L = [(L_2 - L_1)^2]^{1/2}$$

The formulae comprise of three variables L, a and b that represents the chromatic changes in the veneers. Each specimen was tested thrice followed by an average value for accurate measurements. This process was repeated for each specimen. In additions, after the color data was collected for each group (n=10) the spectrophotometer was recalibrated. Data collected were analyzed using a statistical program software, SPSS 13 for Windows (SPSS Inc., Chicago, IL, USA). The Normality distribution of the data was assessed using Levene's Test. Means and standard deviations of the 4 study groups based on the finishing and polishing techniques. The outcomes of these 4 groups were compared with the control group and among the study groups to evaluate impact of the finishing and polishing on the color stability and brightness of the surface using ANOVA and Tukey's HSD test (α=0.05).

RESULTS AND DISCUSSION

The present study demonstrated a positive effect of the type of polishing method employed on the color stability of the

restoration. The ΔL and ΔE showed a significant difference among the study groups; (group 1, group 2, group 3 and group 4) with respect to three variables L, a and b.

Table 1 presents mean and standard deviation of the CIE coordinates for color stability. The control group mean calculated for the three variables, L, a and b were as follows, 56.13, 0.887 and 6.67. Comparatively, the highest mean value for CIE coordinates obtained among the 4-study group was for group 1 [59.66 (1.670), [0.892 (0.11)] and [7.27

(0.498)] whereas the least value obtained was for group 4, L [58.36 (2.20)], a [0.727 (0.127)] and group 2, a [5.77 (0.96)] (Fig 1). A significant decrease in the L and a was noted; however, b increased after polishing with a paste and glazing. It was observed that using a polishing paste and glazing enhances the color components for the veneer natural appearance and esthetics. Furthermore, comparing the 4 groups, highest maximum value measured was in group 2 for L (68.25) and b (1.71) and group1 (0.69). Whereas the minimum value obtained group 2 (52.01), group 3 a (0.49) and group 4 b (4.07) (table 2).

Table 1. Mean and standard deviations (SD) for the CIE coordinates.

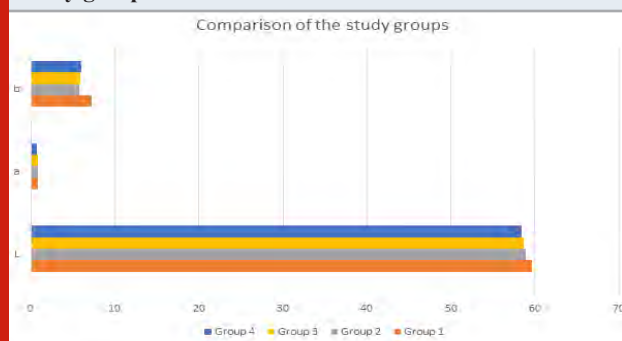
	Mean L	Mean a	Mean b	ΔL	ΔE	ANOVA p value
Control	59.66	0.887	6.67	-	-	0.01
Group 1	59.66 (1.67)	0.892 (.11)	7.27 (.498)	4.93 (1.07) ^a	4.487 (.897) ^a	
Group 2	58.88 (5.90)	0.832 (.37)	5.77 (0.96)	2.65 (0.77) ^b	2.70 (0.03) ^b	
Group 3	58.68 (3.45)	0.79 (.347)	5.85 (1.185)	1.93 (0.824) ^{bc}	2.33 (0.517) ^{bc}	
Group 4	58.36 (2.20)	0.727 (0.127)	6.01(1.34)	1.409 ^c	1.688 ^c	

Dissimilar superscript alphabets show significant difference among the study groups (ΔE & L)

Table 2. The maximum and minimum for the CIE coordinates

	L min	L max	a min	a max	b min	b max
Control	56.13	62.05	0.69	1.05	0.81	8.1
Group 1	56.1	62.05	0.69	1.05	6.72	8.1
Group 2	52.01	68.25	0.51	1.71	4.91	8.31
Group 3	53.81	64.1	0.49	1.59	4.63	8.82
Group 4	53.9	61.09	0.51	0.92	4.07	9.02

Figure 1: Comparison of the CIE coordinates among the study groups



The ANOVA for change in color, ΔL and ΔE displayed a significant difference $p < 0.01$. Comparatively, ΔL and ΔE mean value is suggestive of a significant difference in the effect of the polishing and finishing technique. Following the statistical calculation, the ΔL and ΔE showed that

highest degree of change was observed in group 4 with minimum value of 1.688 and 1 (1.409) whereas group 1 demonstrated least value of ΔL (4.93) and ΔE (4.487). Moreover, a significant difference was noted when compared each group with the control; however, only group 2 showed a significant difference from group 4; the remaining groups demonstrated similar findings for all three variables. Hence, displaying bur finishing without glazing and polishing kit significantly increase the surface roughness and color instability (table 1).

The present study assessed the effects of the 4 types of finishing and polishing methods on the color stability of the veneers. The difference of the composition and polishing methods play an important role in the final appearance and color stability of the e.max CAD ceramics. Each type of the polishing method applied has its own importance in producing a finishing look that reflects upon a natural appearance. This was evidently displayed in the present study when a significant difference was observed when compared to the control and among the study groups except few. Hence, the null hypothesis can be partially rejected. Surface roughness is one of the main contributors for the material strength as well as the color stability of the restorations (Gönülol and Yilmazl 2012; Maciel et al. 2019). Finishing the restoration with diamond bur leaves behind great amount of surface roughness in form of surface defects and craters that alters the light perception leading to color change (Köroğlu et al. 2016; Aldahian et al. 2021). In the present study, the ANOVA showed a significant difference among the study groups; however, no significant effect was observed if the surface was only polished with polishing paste after diskling.

In recent clinical practice, Sof-lex discs and shofu are popular polishing tools to produce smooth finish over the ceramic surface; however, they did not demonstrate any effect of glazing (Özarslan et al. 2016). On the contrary, few others emphasized greatly on the importance of glazing to alter the color gradient axis (Rashid et al. 2014; Sarikaya and Güleç 2019; Kim et al. 2021). Similarly, in the present study, it was observed that finishing the surface with polishing paste after using shofu did not positively influence color change; however, adding glaze to the surface protects the surface from extrinsic stains and plaque. Hence, a significant impact was observed between group 2 and group 4 indicating combine effects of paste improves surface smoothness and glazing enhances the color stability by altering the color axis; yellow – blue axis (increase in b) and red- green axis (decrease in a). Hence, this showed that the low surface roughness reduces the chance for adhesion of colorants and plaque leading to better color stability. Nevertheless, to ensure evident color stability along with surface brightness, glazing is important (Kim et al. 2021). To understand the importance to replicate the natural color of teeth two valuable variables are required to explore greatly, the translucency and optical properties. Optical properties play a vital role in light transmission and reflection at the surface of the restoration (Bagis and Turgat 2013; Yılmaz et al. 2019; Kim et al. 2021).

According to Shahmiri et al. (2018), the appearance of the restoration greatly influences its optical property. Studies have shown that these properties are greatly influenced by the surface roughness of the restoration (Shahmiri et al. 2018). The change in the surface polish and finish causes a change in light beam reflection at different angles producing variation in color perception (Bagis and turgat 2013; Shahmiri et al. 2018). In the present study ΔL and ΔE were comparable, which showed a positive correlation. As the study moved from burs to disc followed by glazing along with polishing paste, the finer the surface became and produce a translucent and brighter surface of the restoration. Nevertheless, optical properties do not only depend upon the color but is greatly influence by the thickness and type of porcelain used as well (Alkhudhairy et al. 2020).

Previously, dentist employed using superfine diamond burs and polishing brushes to smoothen out the restorative surfaces. These polishing discs are impregnated with aluminum oxide that contributes to smoother surface preparation. However, the geometry of the restoration is a limiting factor (Jefferies et al. 1998; Saker and Özcan 2020). Moreover, studies have shown that polishing the anatomical contours is often difficult, which leaves rough areas that causes difference in color at different spots especially in the posterior region of the mouth (Ehrmann et al. 2019; Saker and Özcan 2020). On the other, some studies support the use of polishing wheels consist of a flexible rubber-like material, a polymerized resin impregnated with an abrasive that polish effectively in contoured areas (Jung 1997). To demonstrate the impact of different finishing tools, the present study, 4 groups were prepared, the specimen in group 1 and 2 were polished using diamond burs and shofu. Among these tools dentist commonly uses rubber cups, which are compressible, and easily polishes deep

areas; however, adding up polishing paste in group3 further aided the finish of surface but not at a significant rate (Ishii et al. 2020).

Some dentist believes glazing the surface of the restoration completely finishes the product and provides a good shine to the surface (Al-Wahadni et al. 1998; Saker and Özcan 2020). These creates the surface stain free and reduces the color shift of the restoration. Many dentists believe quick polishing and finishing at chairside is reasonable to save time; hence, avoids glazing (Al-Wahadni et al. 1998; Saker and Özcan 2020). Nonetheless, the studies have shown glazing the surface although adds up an extra step but reduces the number of polishing products needed in the dental operator; hence, demonstrated a significant change that is decrease in L^* and a^* , and increase in b^* . Hence, authors concluded change significantly after glazing process, with respect to the nature and type of composition of the ceramic (Vichi et al. 2018; Alencar et al. 2021).

Other factors have also been identified as variables influencing the color stability and brightness of the restoration. One of the main factors is type of ceramic and its composition (Turgut and Bagis 2011; Boaventura et al. 2013). Literature supports ceramic laminate veneers are often preferred for their color stability and esthetics (Vanlioğlu and Kulak-Özkan 2014; Perroni et al. 2018). Moreover, it has been observed that the color stability of veneer restoration is greatly affected by firing temperature, number of firings, surface smoothness, ceramic thickness, manufacturer, and type of supporting structure (Vanlioğlu and Kulak-Özkan 2014; Alencar et al. 2021). However, the present study only evaluated direct impact of the color stability and surface brightness using the effect of the varying finishing and polishing techniques.

Certain limitations were identified with regards to the clinical perspectives. Methodological limitations are also inherent in in-vitro studies. Studies have demonstrated that aging of the restoration is an important factor that influences color stability over the period. Oral environment consists of variety of fluids whose prolong exposure negatively influences the surface of the restoration and color stability. Temperature changes and pH level in the oral cavity also greatly effects the ceramics restoration surfaces. In addition, in the present study flat specimen surfaces were used whereas clinically the surfaces are contoured such as an irregular geometric structure of convex and concave surfaces. Moreover, the present study only used one single type of ceramic to achieve a standard outcome to analyze the impact of polishing technique. Hence, in future studies comparison of the different types of material should be made to evaluate the impact of composition of ceramic influencing the surface finish (Alencar et al. 2021).

CONCLUSION

The findings of the present study displayed a significant impact of the finishing and polishing techniques on the surface brightness and color stability of ceramic restoration. However, it was evident that combination of two or three polishing techniques which includes polish kit and glaze

enhances the surface finish and adds color stability by alternating the yellow – blue axis (increase in b) and red-green axis (decrease in a). Hence presenting greater finish of the surface reduces the porosity of the surface and enhances the esthetics.

ACKNOWLEDGEMENTS

This study was supported by the Dentistry Research Center at King Saud University. Authors thank the collefore for supporting in specimen testing.

Conflict of interest: Authors declare no conflicts of interests to disclose.

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Biotechnological Communication

Development of An Efficient Protocol for Rapid Propagation of *Curculigo orchoides* Using Leaf Explant Culture

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ABSTRACT

Curculigo orchoides is one of the most common medicinal plants used by diverse cultures and tribal groups. The roots of the plant are used medicinally in Asian countries. *Curculigo orchoides* have the ability to regenerate through seeds and tubers, but the regeneration rate is low. Plant tissue culture method was believed to have potential for rapid multiplication of this medicinal plant. An efficient protocol for rapid propagation of *Curculigo orchoides*, of the family Amaryllidaceae, was developed using leaf explants culture. The leaf explants (1 cm x 1 cm squares) cultured on Murashige and Skoog (MS) basal medium were supplemented with various concentrations and combinations of auxins and cytokinins with temperature $25 \pm 2^\circ\text{C}$, relative humidity 85-90% and photoperiod of 12 hours light (2000-3000 lux). Callus induction was obtained within 4 weeks, 2,4-D at 3 mg/l formed profuse callus and the degree was found to be the highest (+++) among all the treatments. The best response to shoot induction, with maximum shoot number 5.33 (mean number of shoots per explant) was obtained using 1.0 mg/l 6-benzyl aminopurine (BAP) in combination with 1.0 mg/l Kinetin. *In vitro* shoots were induced for rooting on 0.5 mg/l of NAA supplemented medium. In order for seedlings propagated *in vitro* to adapt to natural conditions, plants were grown on a substrate coir: husk ash: sand (with the ratio of 0.5: 0.5: 1) in a greenhouse (humidity: 70%, temperature: 28-30 °C) gave 88.33% survival rate after 8 weeks of culture. With the results received, this is an effective approach to propagating *Curculigo orchoides*.

KEY WORDS: CALLUS, CURCULIGO ORCHOIDES, MEDICINAL PLANT, GREENHOUSE, SUBSTRATE.

INTRODUCTION

Curculigo orchoides Gaertn. (Amaryllidaceae), which has been used as a traditional herbal medicine in subtropical regions of Asia. It is believed to be a tonic for the treatment of declined physical strength. The plant possesses uterine stimulant, hypoglycaemic, spasmolytic and anticancer, phagocytic, immuno-adjuvant, antineoplastic, immuno-stimulant and hepatoprotective activities (Aruna and Sivaramakrishnan 1990; Dhar et al. 1968; Kubo et al. 1983; Oru and Kogyo 1983; Latha et al. 1999). The active compounds in this plant have been reported to include flavones, glycosides, steroids, saponins, triterpenoids (Misra

et al. 1984; Misra et al. 1990; Xu et al. 1992; Rajesh et al. 2000; Singla and Singh 2021).

Flavonoids are an important class of plant secondary metabolites, they have various biological activities, such as antiallergic, antiinflammatory, antioxidant, antimicrobial, anticancer, antistress and antidiabetic properties (Yamamoto et al. 2001; Cazarolli et al. 2008; Cushnie et al. 2011; Chauhan et al. 2021; Singla and Singh 2021). The major active component of rhizomes of *C. orchoides* was reported to be flavones. In which curculigoside was considered as a marker to evaluate the quality of medicinal herbs, curculigoside has been shown to have regulatory effects on bone metabolism via anti-oxidative activities in rats and osteoblasts and prevent osteoporosis (Asif 2012; Liu et al. 2021; You and Xu 2021).

Conventionally, the plant propagates through seeds

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Received 13/09/2021 Accepted after revision 14/12/2021

Published: 31st December 2021 Pp- 1514-1519

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.22>

and grows only during rainy season. Poor seed setting and germination restrict its abundance in nature, and overexploitation has led to the plant's current endangered status. Low-frequency regeneration of shoot buds has been reported from shoot tip culture in *C. orchoides* (Augustine

and Souza 1997; Shrivastava et al. 1998; Liu et al. 2021; You and Xu 2021). Tissue culture is considered as an effective method to propagate and source this medicinal plant material. In this study, we recorded the rapid process of *C. orchoides* through callus of explant leaves.

Table 1. Effects of auxins on callus induction from leaf explant of *Curculigo orchoides* after 4 weeks of culture in MS medium

Auxin	Concentrations (mg/L)	Explant response (%)	Morphology and colour of callus	Degree
2,4-D	0	20	Soft, white, wet-looking non morphogenic callus	+
	1	50	Soft, White, light greenish compact callus	++
	3	70	White, light embryogenic callus	+++
	5	100	White, light greenish hyperhydric callus	++++
NAA	1	40	Soft, white, wet light greenish compact callus	+
	3	65	Soft, white, wet-looking non morphogenic callus	++
	5	85	White, light greenish compact callus	+++
+: poor callus, formation, ++: minor callus, formation, +++ : average callus formation, ++++: moderate callus formation, +++++: profuse callus formation.				

MATERIAL AND METHODS

Leaves of *C. orchoides* Gaertn. were collected from natural places of Dakmil, Daknong, Vietnam (altitude 545 m above sea level, coordinates: 12°31'665" N, 107°38'240" E). The plant is distributed in evergreen forest, the average annual rainfall is 2.500 mm, the temperature varies from 14 to 35 °C. The leaves were washed for 5-7 minutes under continuous stream of running tap water to remove soil particles. Explants were surface sterilized with 0.1 % (w/v) of HgCl₂ for 3-5 minutes and then instantly rinsed three times using autoclaved double distilled water under aseptic condition in Laminar chamber. The sterilized explants were then implanted onto the Murashige and Skoog's (1962) agar medium. The media were adjusted to pH 5.7 before addition of 0.8% agar (w/v) and autoclaved at 121 °C for 15 minutes. For the culture medium, the sterilized young leaves were cut into 1 cm x 1 cm squares (Fig 1A) and these explants were inoculated on Murashige and Skoog's (1962) basal medium (MS medium) supplemented with various concentrations of auxins 2,4 Dichlorophenoxyacetic acid (2,4-D), and NAA (Table 1). Later different concentrations and combinations of BA and Kinetin were added on MS basal medium for

differentiation of the callus (Table 2). The excised shoots were transferred to medium for rooting.

The *in vitro* raised shoot lets were sub-cultured on MS medium supplemented with various concentrations of NAA (Table 3). *In vitro* rooted plantlets were transplanted into trays containing a mixture of substrates (sand, rice husk ash and coir) with different ratios (Table 4) and covered with transparent polyethylene bags to maintain high humidity. They were kept in the greenhouse for further acclimatization. The medium was solidified with 0.8 % agar. The pH of the medium was adjusted to 5.8 before autoclaved at 121°C for 20 minutes. Each experiment was repeated thrice. For the culture conditions, the culture bottles were maintained in the culture room with temperature 25 ± 2°C, relative humidity 85-90% and photoperiod of 12 hours light (2000-3000 lux). At 30 days interval, subcultures were done. The regenerated shoots were cultured in separate bottle for optimum growth.

RESULTS AND DISCUSSION

Induction of Callus, shoot bud proliferation and Induction of Root: The manipulation of plant growth

regulators was essential to optimize the induction of callus. After 4 weeks of observation, all the plant growth regulators tested on leaf explants showed stimulate of callus formation (Table 1). However, the effect of callus formation produced from the explants varied from each plant growth regulator. The callus formed was compact, light green in colour with some white callus distributed on the top of the light green callus (Fig 1B). This result was also consistent with that of Patel et al. (2011), on the MS media with 8 μ M BA, about 69 % cultures responded in terms of embryogenic calli, while about 40 % cultures developed embryogenic calli on MS media with 15 μ M BA. Earlier, similar (89 %) response was obtained with 8 μ M BA for direct embryogenesis (Thomas and Jacob 2004; Patel et al. 2011). Rapid shoot multiplication is an important stage in plant tissue culture. This stage has an important role to produce many shoots to create seedlings.

Table 2. Effects of growth regulators on MS medium for shoot regeneration of *Curculigo orchioides* after 4 weeks of culture

Growth Regulators (mg/L)		No. of shoots/ callus	Shoot height (cm)
BA	Kinetin		
1.0	0.5	2.00 ^d	3.47 ^a
2.0	0.5	2.33 ^{cd}	3.13 ^{ab}
3.0	0.5	3.00 ^{bcd}	2.67 ^{abc}
1.0	1.0	5.33 ^a	3.00 ^{ab}
2.0	1.0	4.67 ^{ab}	2.77 ^{abc}
3.0	1.0	4.00 ^{abc}	2.17 ^{cd}
1.0	1.5	4.33 ^{ab}	2.77 ^{bc}
2.0	1.5	4.67 ^{ab}	2.17 ^{cd}
3.0	1.5	3.67 ^{abcd}	1.60 ^d
CV		17.65	10.68
MSD _{0.01}		1.57	0.66

The average score with different letters are significantly different at $p = 0.01$ level. z: Letters a, b, c, d, in the same column represented the differences among treatments by t Tests (MSD).

To achieve this goal, the culture medium needed to be supplemented with a plant growth regulator. One of the commonly used substances is BA. BA is used in variable amounts or binds with other substances such as NAA or IBA, IAA, kinetin to stimulate multiple shoots in tissue culture. The obtained results showed the effectiveness of the application of plant growth regulators in the rapid multiplication of shoots in *Curculigo orchioides*. Among the various treatments to study shoot induction in *Curculigo orchioides*, the best response with maximum shoot (5.33 mean number of shoots per explant) was obtained after four weeks of subculture, using 1.0 mg/l BA in combination with 1 mg/l kinetin (Fig 1C).

Figure 1: The formation and development of plants from leaf tissue of *Curculigo orchioides*. A. leaf explants; B. Callus; C. the formation shoots from callus; D. developing plant.

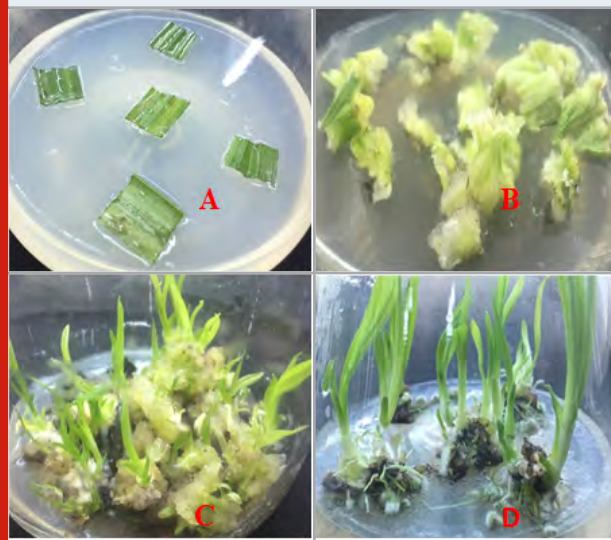


Table 3. Effect of NAA on the growing response of *Curculigo orchioides* after 4 weeks of culture.

Growth regulators NAA (mg/L)	Plant height (cm)	No. of roots	Root length (cm)
0.0	3.33 ^a	1.67 ^c	2.33 ^{bc}
0.1	3.90 ^a	2.67 ^c	2.83 ^{abc}
0.3	4.17 ^a	4.17 ^b	3.17 ^{ab}
0.5	4.67 ^a	5.00 ^{ab}	3.47 ^a
0.7	4.73 ^a	5.17 ^{ab}	2.33 ^{bc}
1.0	4.60 ^a	5.50 ^a	2.17 ^c
1.5	4.17 ^a	6.17 ^a	2.00 ^c
CV	12.32	11.26	13.87
MSD _{0.01}	1.27	1.19	0.88

The average score with different letters are significantly different at $p = 0.01$ level.

z: Letters a, b, c, d in the same column represented the differences among treatments by t Tests (MSD).

Response of BA and kinetin was found to be the best combination for bud break response as well as shoot bud regeneration in *Curculigo orchioides* from callus. Similar findings have been reported by various authors, such as on MS medium supplemented various concentrations of BAP (2-10 M) or Kin (2-10 M), cultured leaf segments produced swelling on its adaxial surface near the cut end which later transformed into somatic embryos (Thomas and Jacob 2004). *Embryogenic callus* was induced from rhizome explants, and the maximum induction frequency (62%) was obtained in medium containing 0.5-3 mg/L of 2,4-D and 0.5 mg/L BAP (Nagesh et al. 2010). The optimal conditions for callus proliferation of *Curculigo orchioides* were Half-

strength MS medium (½ MS) given 0.5 mg/L KIN and MS medium supplemented with 0.5 mg/L KIN (Nguyen et al. 2021). The stage of rooting from shoots is a step to create a complete plant (full of roots, stem, and leaves) to help plants adapt at the nursery stage. Using the shoots of the *Bumphythus* plant obtained from the shoot multiplication treatment with a height of 2 cm and transplanted into the medium containing plant growth regulators to determine the suitable environment for the ability to form and develop roots of *Curculigo orchioides* (Nguyen et al. 2021).

High cytokinin concentrations (0.5 - 10 mg/L) often inhibit or delay root formation, while also inhibiting root growth and inhibiting the effect of auxin rooting. For this reason, cytokinins are generally not used in shoot rooting-stimulating media for seedling production. Most plants require auxin to induce rooting. The most effective auxins in rooting stimulation were IBA and NAA. The shoots of *Curculigo orchioides* *in vitro* were cultured on plant growth medium (MS + 1mg/L of indole-3-butyric acid (IBA), after 4 weeks of culture, the following results were obtained: 100% rooting shoots; The average number of roots is 5 roots/plant; Average height of the tree: 4.5 cm. The shoot regeneration from the proximal rhizome plate was greater than that from the shoot tip explants on the medium supplemented with 6-benzylaminopurine (BAP) and kinetin (KN) (each at 1 mg/L). Optimum root induction

was achieved on half-strength MS medium supplemented with 1 mg/L of indole-3-butyric acid (IBA). When these embryos were transferred to MS medium with ½ strength nitrogen salts and supplemented with lower concentration of BA (0.44µM), developed shoots and roots (Augustine et al. 2008; Nagesh 2008; Nguyen et al. 2021).

Hardening and Acclimatization of plantlets: The greenhouse and field have substantially lower relative humidity, higher light septic environment that are stressful to micropropagated plants compared to *in vitro* conditions. The benefit of any micropropagation system can, however, only be fully realized by the successful transfer of plantlets from tissue-culture vessels to the ambient conditions found *ex vitro* (Hazarika 2003). The substrate to bring seedlings to the nursery has a great influence on the adaptability and growth of plants. Because at this stage, the growing substrate has the function of keeping the seedlings in a fixed position, a source of water and nutrients for the plants. A growing substrate is considered ideal if it is porous enough, breathable, holds and drains well, and is free of pests and weeds. When studying the differences of roots in different growing substrate, Long (1933) found that the main cause of the above phenomenon was due to the difference in the moisture holding capacity and aeration of the growing medium (Long 1933; Hazarika 2003; Nguyen et al. 2021).

Table 4. Effect of substrate to adapt and grown of *Curculigo orchioides* after 8 weeks of culture.

No of test	Experiment			Survival (%)	Number of roots (root/plant)	Root length (cm)	Number of leaves (leaf/plant)	Plant height (cm)
	Coir (%)	husk ash (%)	Sand (%)					
1	0	0	100	66.67 ^{bc}	14.00 ^a	3.50 ^c	6.33 ^{bcd}	10.50 ^{def}
2	0	100	0	73.33 ^b	4.33 ^{cd}	5.33 ^{ab}	6.67 ^{abcd}	11.33 ^{cde}
3	100	0	0	46.67 ^f	6.33 ^{bc}	3.17 ^c	5.33 ^d	9.50 ^f
4	0	50	50	65.00 ^{bcd}	8.33 ^b	6.50 ^a	6.00 ^{cd}	10.17 ^{ef}
5	50	0	50	55.00 ^{def}	5.33 ^{cd}	6.00 ^{ab}	7.67 ^a	12.50 ^{abc}
6	50	50	0	48.33 ^{ef}	3.00 ^d	6.17 ^{ab}	6.67 ^{abcd}	12.67 ^{ab}
7	25	25	50	88.33 ^a	5.67 ^{cd}	6.67 ^a	7.00 ^{abc}	13.50 ^a
8	25	50	25	70.00 ^b	4.67 ^{cd}	5.00 ^b	5.33 ^d	12.17 ^{bc}
9	50	25	25	58.33 ^{cde}	3.33 ^d	4.83 ^b	5.67 ^d	11.50 ^{bcd}
10	33	33	33	63.33 ^{bcd}	3.67 ^d	5.83 ^{ab}	7.33 ^{ab}	12.33 ^{abc}
	CV			7.19	17.60	10.33	8.07	4.23
	MSD _{0.01}			10.60	2.40	1.27	1.20	1.14

The average score with different letters are significantly different at p = 0.01 level.

z: Letters a, b, c, d, e, f in the same column represented the differences among treatments by t Tests (MSD).

Research results in this experiment showed that different growing substrate affect the adaptability and growth of plants at the nursery stage (Fig 2, Fig 3 and Table 4). The suitable substrate for the adaptability of plants when brought to the greenhouse is No.7 (coir: husk ash: sand in the ratio of 0.5:0.5:1) with 88.33% of live plants; root length: 6.67 cm; plant height: 13.50 cm), healthy plants and enlarged roots. The substrate was suitable for the ability to grow the number of roots No.1 (coir: husk ash: sand in the ratio

0:0:1) and treatment 5 (No.5: coir: husk ash: sand in the ratio 1: 0:1) had the highest number of leaves received at 7.67cm. Many recorded on the influence of the growing substrate on the adaptability *Curculigo orchioides* when introduced into the greenhouse, such as: Nagesh et al. (2010), recorded in vitro cultured plants brought to the nursery with a mixture of organic compounds. garden soil: farm soil: sand= 2:1:1, (autoclaved before seedling; maintained at room temperature 25 °C) resulted in 100%

of seedlings surviving 6 weeks of cultivation; Thomas and Jacob (2004), Peat: sand=1:1 for 90% survival rate (Nagesh et al. 2010; Nguyen et al. 2021).

Figure 2: Effect of substrate to adapt and grown of *Curculigo orchioides*.



Figure 3: *Curculigo orchioides* in vitro plants grown in greenhouse at 8 weeks.



Organic fertilizers: garden soil=1:1 (moisture: 70-80%) for a survival rate of 70% soil: sand: compost=1:1:1 for 95% survival rate is the result recorded by Patel et al. (2011). In addition to the growing substrate factor, a number of other factors such as the quality of seedlings to the greenhouse, humidity, temperature, and greenhouse conditions also affect the adaptability of plants. In this period, the target of interest is the percentage of plants that are adapted to natural conditions. Therefore, the treatment of coir: husk ash: sand in the ratio of 0.5: 0.5: 1 was selected as the substrate to bring the seedlings to the greenhouse (Nagesh et al. 2010; Sahay and Braganza 2017; Nguyen et al. 2021).

CONCLUSION

The study suggests that 2,4-D at 3 mg/l concentration was found to forming callus the highest among all the treatments. The highest shoot length, number of shoots was obtained at MS medium supplemented with 1 mg/l BA and 1 mg/l kinetin. The highest number of roots and root length was obtained on medium supplemented with 0.5 mg/l NAA at rooting stage. The highest percentage of plant survival was achieved by transplanting of the plantlets to pots containing coir: husk ash: sand in the ratio of 0.5: 0.5: 1(v/v/v).

ACKNOWLEDGEMENTS

This study was financially supported by National Key

Laboratory of Plant cell technology - Institute of Tropical Biology - Vietnam Academy of Science and Technology. The department also helped with experimental equipment. Moreover, Authors thank Daknong Department of Science and Technology, Gia Nghia, Daknong, Vietnam for the assistance.

Conflict of Interests: Authors declare no conflict of interests to disclose.

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Biotechnological Communication

Investigations on Bacterial Load in the Rural and Urban Indoor and Outdoor Environment of Gwalior, Madhya Pradesh, India

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ABSTRACT

Comparative analysis of airborne bacterial load in the rural and urban indoor and outdoor environment is of utmost importance to evaluate the wellbeing hazards linked with contamination of airborne bacteria in the indoor environment. The present study was conducted during December, 2020 to March, 2021 among 50 randomly selected rural and urban (Adupurajagir and Gwalior, respectively) dwellings to determine the indoor and outdoor bacterial load. The airborne bacterial load 562.35 CFU/m³ and 2593.75 CFU/m³ were recorded in the indoor environment of a modular kitchen in Gwalior city and traditional kitchen in Adupurajagir village, respectively. In addition, bacterial load of respectively 1215.13 CFU/m³ and 783.03 CFU/m³ was calculated in the open space at both study sites. Based on morphological characteristics five bacterial species (spp.) were identified *Staphylococcus aureus* spp, *Bacillus* spp, Coagulase-negative *Staphylococcus* spp, *E-coli* spp, and *Micrococcus* spp. By gram staining method the most common bacteria were gram-positive (+ve) [n=85, 54.48% (37.17% cocci, 17.94% bacilli)] followed by gram-negative (-ve) [n=71, 45.51% (23.07% cocci, 21.79% bacilli)] identified. Pearson's correlation coefficient was employed between bacterial load and physical factors of the indoor environment in the rural traditional kitchen. Bacterial load (CFU/m³) showed a significant correlation with temperature (p < 0.001). However, a non-significant correlation was recorded with relative humidity (p > 0.01). High bacterial load was found in the rural traditional kitchen's indoor environment compared to urban modular kitchen. Outcomes from this study revealed that bioaerosol sampling could deliver fruitful knowledge about the variation of air quality and prevent possible hospital admissions.

KEY WORDS: AIR QUALITY, BIOAEROSOL, GRAM STAINING, PASSIVE AIR SAMPLING, PEARSON'S CORRELATION.

INTRODUCTION

People spend 90% of their lives indoors, where the contaminated air in dwellings may be making people sick. In these areas, they are exposed to various airborne microorganisms like bacteria. According to previous studies, approx. 10-35% of indoor air contamination is caused by aerial microbial flora (Mirhoseini et al. 2016; Fujiyoshi et al. 2017; Andualet et al. 2019; Hui et al. 2019). Therefore, poor indoor air quality (IAQ) can lead to disorders known as sick building syndrome (SBS), building-related illness (BRI), chronic inflammatory response syndrome

(CIRS) and numerous negative exposures (Mirhoseini et al. 2016; Hui et al. 2019). The most common bacterial species identified in indoor environment were *Bacillus*, *Staphylococcus*, *Arthrobacter*, *Micrococcus*, *Streptococcus*, *Diphtheroid*, *Pseudomonas*, *Exiguobacterium*, *Enterobacter*, *Escherichia coli* (*E-coli*) and *Sphingomonas* (Mirhoseini et al. 2016; Bolookat et al. 2018; Andualet et al. 2019; Sivagnanasundaram et al. 2019).

It has been investigated that pathogenic bacterium like *Staphylococcus aureus* (methicillin resistant) and *Pseudomonas* species were nosocomial infections in nature and developed multi-antibiotic drug resistance which may be accountable ineffective cure (Kunwar et al. 2019). Therefore, indoor air can be more polluted and harmful in terms of health issues including upper respiratory infections

Article Information:*Corresponding Author: shivomsingh@itmuniiversity.ac.in

Received 15/09/2021 Accepted after revision 14/12/2021

Published: 31st December 2021 Pp- 1520-1525

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.23>

(URI) and lower respiratory infections (LRI), etc., over outdoor air (Baldacci et al. 2015; Kim et al. 2018; Smith et al. 2000). Though indoor environments are believed to be safer, but they can pollute with micro-pollutants when their load raised from recommended parameters than associated with outdoor exposure. A sampling of bioaerosols is a well-known technique to find out the bacterial load, as it permits a significant evaluation, hence the results were evaluated in the form of colony forming unit per cubic meter (CFU/m³). According to the National Institute of Occupational Safety and Health (NIOSH) and the American Conference of Governmental Industrial Hygienists (ACGIH) the recommended parameter of the total count of bioaerosols is 1000 CFU/m³ and the culturable total number of bacteria is 500 CFU/m³ (Cox and Wathes 2020).

Recent researches have exposed the load of bacteria in different indoor environments (Lazaridis et al. 2015; Mirhoseini et al. 2016; Bolookat et al. 2018; Kunwar et al. 2019; Sivagnanasundaram et al. 2019). However, the indoor airborne bacterial concentration is likely enhanced by the physical parameters of the indoor environment (thermal condition, humidity, and ventilation framework) as well as natural activities of human (sneezing, coughing and talking) by spreading micro droplets (Fujiyoshi et al. 2017; Andualem et al. 2019; Hui et al. 2019; Kunwar et al. 2019; Roslund et al. 2019). Therefore, there is need to investigate bacterial load in the indoors, physical parameters, and micro-climatic variations of the indoor environment. Indoor airborne bacteria can affect human health as various skin and respiratory infections (Fujiyoshi et al. 2017; Roslund et al. 2019; Sharma et al. 2020).

Nonetheless, contamination of bacteria in the kitchen may also be contributable to poor indoor air quality. Hence, kitchens are supposed to be another contributing factor in the burden of airborne bacterial infections. It is important to determine the bacterial load in the indoor environment and its comparison with outdoor environment to find out the risk from indoor environment generated bacterial diseases/disorders. The present work focused on the airborne bacterial load in the indoor environment of rural traditional kitchen and urban modular kitchen and their open space environment. Furthermore, it also analyzes the relationship between high bacterial loads with physical factors of indoor environment of rural traditional kitchen.

MATERIAL AND METHODS

The present study was accomplished from December, 2020 to March, 2021 among 50 randomly selected dwellings from rural (Adupurajagir village) and urban (Gwalior city) to compare indoor and outdoor airborne bacterial load to analyze the relationship between high bacterial load with the physical environment and cooking pattern of rural and urban people. Therefore, traditional and modular kitchens were selected from rural and urban dwellings respectively as indoor and their open space as outdoor environments. Gwalior is a major city in Central India in the state of Madhya Pradesh. The city is located at 26.22° N latitude and 78.18° E longitudes, 300 km from Delhi. Adupurajagir village is located in the Gwalior districts of Madhya

Pradesh. The village is located at 26.113491°N latitude and 78.226425°E longitudes (Fig. 1).

Figure 1: Areas in (a) Adupurajagir village and (b) Gwalior city where airborne bacterial samples were collected. (Source: <https://www.google.co.in/maps>)



Bacterial samples were collected from the kitchen and open space in morning cooking hours at 9.00-11.30 am. The passive air sampling technique was used, the Petri plate was placed at the center of the sampling area for 1 hour, 1 meter away from the wall, and 1 meter above the height of the human breathing zone (1/1/1 standardized method) (Bolookat et al. 2018; Andualem et al. 2019). Physical parameters such as thermal condition, humidity, and ventilation framework were recorded. To minimize the mixing of outdoor bacterial samples, all ventilations were remained closed and movement of inhabitants prohibited during the indoor sampling. At the time of sampling, other microbial safety measures were followed (Napoli et al. 2012; Sharpe et al. 2020). After sampling all samples were transported on the same day without any delay to the CTR (Centre for Translational Research) laboratory, Jiwaji University, Gwalior, and incubated at 37 °C for 24-48 hours. Bacterial colonies growing on culture media were expressed and calculated in the form of colony forming unit per cubic meter (CFU/m³) by applying the equation Andualem et al. (2019).

$$N = \frac{a * 10000}{bt * 0.2}$$

Where N = Indoor airborne bacterial CFU/m³, a = Colony counts per Petri plate,
b= Surface area of Petri plate in
cm²t= Time of air exposure in minutes l

Based on microscopic examination, purification by sub-culturing for another 24-48 hours at 37 °C on the same culture media was used to attain pure culture isolates. The identification of the obtained colonies was based on gram staining and their colony formation characteristics such as shape, size, opacity, and color (Becerra et al. 2016; Mirhoseini et al. 2016). A light microscope was used to assess the morphology of bacteria at 100X magnification under oil immersion. The bacterial generic identity was achieved based on the taxonomic classification (Goodfellow et al. 2012). Descriptive statistics were used to depict the airborne bacterial load. To evaluate comparison, between averages bacterial load in the rural and urban indoor

and outdoor environment one-way analysis of variance (ANOVA) was employed. In addition, to analyze the correlation of airborne bacterial load with physical factors of indoor environment Pearson's correlation coefficients were employed.

RESULTS AND DISCUSSION

Bacterial load: The present study was employed at a preliminary stage to compare the airborne bacterial load in the outdoor and indoor environment of rural traditional and urban modular kitchen among Adupurajagir village and Gwalior city respectively. The minimum and maximum bacterial load were estimated in the urban modular kitchen (511.12 CFU/m³) and a rural traditional kitchen (2621.16 CFU/m³) respectively. The mean bacterial load was 2593.75 CFU/m³ in the rural traditional kitchen, 783.03 CFU/m³ in rural open space, 562.35 CFU/m³ in urban modular kitchen, and 1215.13 CFU/m³ in the urban open space environment. The bacterial load of the indoor environment of rural traditional kitchen among Adupurajagir was found highest with the mean value of 2593.75 CFU/m³. Furthermore,

bacterial load of the indoor environment of urban modular kitchen among Gwalior was observed lowest with the mean value of 562.35 CFU/m³ (Table 1).

On the other hand, the sum of outdoor bacterial load of Adupurajagir and Gwalior was 77520.32 CFU/m³ and 120297.87 CFU/m³ with the mean bacterial load of 783.03 CFU/m³ and 1215.13 CFU/m³ respectively (Table 1). Although there is no general threshold estimation concerning airborne bacterial load in the indoor environment, the WHO suggested that a total load of microbes in the indoor environment should not surpass 1000 CFU/m³ (Hänninen 2011). The Sanitary Standards of the European Commission for non-industrial premises reported that > 50 CFU/m³, < 100 CFU/m³, < 500 CFU/m³, and < 2000 CFU/m³ is considered very low, low, high, and very high microbial load (Colbeck and Whitby 2019; Kotgire et al. 2020). Taking these standardized data into consideration, the mean bacterial load of the indoor environment in the rural traditional kitchen much higher than that outdoor. Moreover, the mean bacterial load of the indoor environment in the urban modular kitchen shows lower values.

Table 1. Descriptive statistics analysis of airborne bacterial load of indoor air environment in kitchen and open space environment among Adupurajagir village and Gwalior city (n = 50).

Bacterial CFU/m ³							
RK		RO		UK		UO	
Mean	2593.75	Mean	783.03	Mean	562.35	Mean	1215.13
Median	2594.95	Median	773.24	Median	550.44	Median	1192.62
Mode	2621.16	Mode	773.24	Mode	537.33	Mode	1153.31
Standard Deviation	23.614	Standard Deviation	44.161	Standard Deviation	43.992	Standard Deviation	68.971
Minimum	2555.63	Minimum	707.71	Minimum	511.12	Minimum	1048.46
Maximum	2621.16	Maximum	891.19	Maximum	655.29	Maximum	1310.58

RK=Rural Kitchen; RO = Rural Open space; UK = Urban Kitchen; UO = Urban Open space.

Table 2. One way ANOVA test results on rural and urban airborne bacterial load in indoor environment of kitchen and open space environment at study sites.

Source of Variation	Analysis of Variance					
	SS	df	MS	F	P-value	F crit
Between Groups	285805.8	99	2886.927	0.025533	< 0.001	1.684883
Within Groups	22612981	200	113064.9			
Total	22898787	299	115951.8			

SS = Sum of the Square; df = Degree of Freedom; MS = Mean Square.

One way ANOVA test results was demonstrated to differentiate the mean airborne bacterial load among study sites. Whereas the sum of square between groups was 285805.8 and within groups was 22612981 and total number of mean square was 115951.8 estimated. The ANOVA test

reflected that there was a significant mean airborne bacterial load difference among study sites at $p < 0.001$ with total degree of freedom was 299 (Table 2).

Morphology analysis: In this study, rural and urban airborne bacterial load in the indoor environment of the

kitchen and open space environment contains a diversity of airborne bacteria. Five bacterial species (spp.) were identified as *Staphylococcus aureus* spp, *Bacillus* spp, Coagulase-negative *Staphylococcus* spp, *E-coli* spp, and *Micrococcus* spp. Gram +ve bacteria were found maximum than gram -ve (Fig. 2).

Figure 2: Mean of isolated gram +ve and gram -ve bacteria from rural and urban kitchen and open space environments (n = 156).

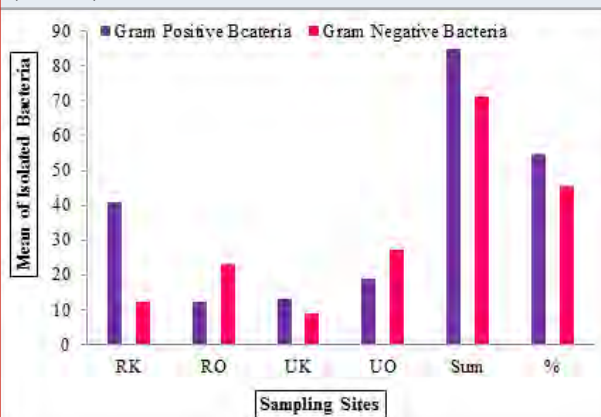
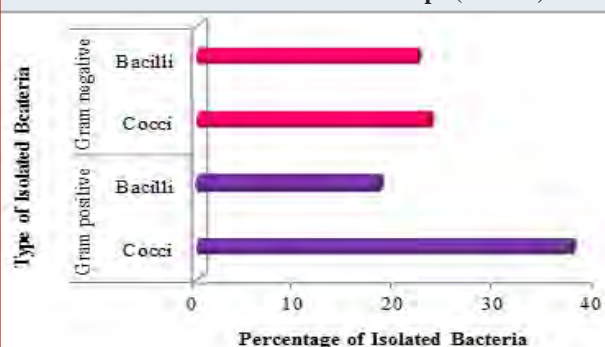


Table 3. Summary of bacterial isolates from rural and urban kitchen and open space environments (n = 156).

Sampling site	No. of isolates	Gram +ve		Gram -ve	
		Cocci	Bacilli	Cocci	Bacilli
RK	53	29	11	4	8
RO	35	7	5	13	9
UK	22	9	4	2	7
UO	46	13	6	17	10
Sum	156	58	26	36	34

Figure 3: Percentage of isolated gram +ve and gram -ve bacteria on the basis of their cellular shape (n = 156).



A total of 156 bacterial colonies were isolated from the rural traditional kitchen (53), rural open space (35), urban modular kitchen (22), and urban open space (46). All bacterial isolates were identified on their colony morphology, shape, size, opacity, and color. Among them, 54.48% and 45.51% belong to gram +ve and gram -ve

bacteria respectively. Therefore total 84 gram +ve (cocci = 58, bacilli = 26) and total 70 gram -ve (cocci = 36, bacilli = 34) bacteria were isolated from study sites (Fig. 3). In addition, the highest percentage of gram +ve cocci (23.07%) and lowest percentage of gram +ve bacilli were (17.94%) reported in Adupurajagir village. This was followed by the percentage of gram -ve bacteria in which the highest 23.07% cocci and lowest 21.79% bacilli were reported in Gwalior city. However, the highest percentage of gram +ve cocci were 37.17% and the lowest percentage of gram +ve bacilli was 17.94% estimated in the rural traditional kitchen and outdoor environment among Adupurajagir. Whereas, the mean of 56 gram +ve cocci and 26 bacilli and 36 gram -ve cocci and 34 bacilli were recorded (Table 3). Nonetheless, the high load of gram +ve cocci was observed then gram -ve airborne bacteria. This research also reflects the same outcomes as gram +ve bacteria were isolated more than gram -ve bacteria (Kotgire et al. 2020).

Characterization of identified bacterial species: Five bacterial species were identified *Staphylococcus aureus* spp, *Bacillus* spp, Coagulase-negative *Staphylococcus* spp, *E-coli* spp, and *Micrococcus* spp. *Staphylococcus aureus* spp and *Micrococcus* spp be owned by the flora of the human dermis and are usually members of the microbiota of the body, it is aptly that this microbiota may be originated from the dermis flora of the inhabitants of their dwellings. *Micrococcus* spp and *Staphylococcus aureus* were isolated from all the sampling sites (Fig. 2), it is considered to be an emerging nosocomial pathogen.

Micrococcus spp can cause pneumonia, septic shock, meningitis, and endocarditis. Conversely, *Staphylococcus aureus* is gram +ve bacteria that may cause disease symptoms through the production of toxins for example food poisoning, human dermis infections, pneumonia, bone infections, urinary tract infections, and diarrhea. *Bacillus* spp can stay alive in the harsh environmental conditions in the air due to its ability to form spores and show resistance against common disinfectants which are used in daily disinfection practice in dwellings. This gram +ve bacterium may be found on dirt particles and paper. Its presence on dirt particles in the air may consequence in settling on food or food contact surfaces, hence ensuring its survival in the kitchen and posing a possible threat to women and their children (< 5 years old) because of determined sex disparities across many proportions, women's household works such as cooking may be more exposed than men's (Kotgire et al. 2020).

In addition, improper cleaning and poor infrastructure (ceiling, mud walls, roof made by wood or leaves, short height of roof, and gap between wall and roof) were observed during sampling in the traditional kitchen among dwellings of Adupurajagir village, which may lead to serious bioaerosol infectivity of food such as queasiness, vomiting, diarrhea, wound, and central nervous system (CNS) infections and people with the weakened immune system are prone to *Bacillus* spp. *E-coli* is the most abundant species in the hospital environment however, in this study it was isolated from the kitchen environment among Adupurajagir village and Gwalior city also. This gram

-ve coliform bacterium of the family Enterobacteriaceae commonly lives in the human intestine. People inside the dwellings among both study sites may be exposed to *E-coli* from contaminated food and water due to poor hygienic practices were observed. Most strains of this bacterium are not harmful but some strains are contagious by produce toxins that cause illnesses such as septicemia, neonatal meningitis, bloody diarrhea, urinary tract infection, and gastroenteritis. Coagulase-negative *Staphylococcus* spp is referred from gram +ve (Kotgire et al. 2020).

This species is commonly found as a food-associated saprophyte and also present on the human dermis and mucous membrane. Overcrowded dwellings with poor ventilation framework found in Adupurajagir village. It was observed that villagers believe in living in joint families rather than nuclear families, which is considered to be the main basis of Coagulase-negative *Staphylococcus* spp occurrence within sampling sites. Coagulase-negative *Staphylococcus* spp is identified as is one of the main pathogens of nosocomial infection also shows methicillin resistance in nature and developed multi-antibiotic drug resistance which may be responsible for the ineffective treatment. Findings say that most of the bacterial species are airborne in the residential environment associated with the human dermis. All identified bacterial species have colonized all the sampling sites within the dwellings (Table 3). It should be considered that all the isolated bacterial species are identified as highly infectious and disease-causing or opportunistic pathogenic. Future research would help to find out the possible source of the subsistence of pathogenic bioaerosols within the kitchen and outdoor environment of the dwellings (Magd et al. 2020; Kotgire et al. 2020).

Pearson's correlation coefficients between bacterial load and physical factors of indoor environment in rural traditional kitchen: During measurement of physical factors of the indoor environment in the rural traditional kitchen, it was observed that all measured rural traditional kitchens did not have a ventilation framework. They use unclean fuel (wood, dung cake, and crop residues) over LPG for cooking. The relative humidity (RH %) and indoor temperature (T °C) ranged from 61% - 90% and 10 °C - 21 °C respectively. Bacterial load (CFU/m³) showed significant correlation with temperature ($p < 0.001$). However, a non-significant correlation was found with relative humidity ($p > 0.01$). The thermal condition of the indoor environment exhibited a significant correlation with airborne bacterial load in the rural traditional kitchen ($r = 0.9090$) while there was a non-significant correlation with the relative humidity ($r = 0.0006$) (Bragoszewska et al. 2017; Magd et al. 2020).

Hence the airborne bacterial load will increase as the indoor temperature increases and the bacterial load decreased with reduced relative humidity. This is attributed because of decreased metabolism and physiological activities of bioaerosols under dry environmental conditions (Bragoszewska et al. 2017). The difference of bacterial load in the indoor environment of rural and urban kitchen caused by outdoor climate and physical factors of the indoor

environment like ventilation framework of kitchens, thermal condition, and relative humidity. The difference amongst the bacterial load of indoor and outdoor environment at the study sites due to the microclimatic variations, construction material, vehicular pollution, outdoor levels, and daily household activities. The bacterial load of the outdoor environment in these settings reflects the variation of biological sources and the geochemical processes affecting indoor and outdoor relationships of airborne bioaerosols (Nasir et al. 2012; Magd et al. 2020).

Taking the account into consideration, the impact of physical factors of the indoor environment of rural kitchens may significantly affect the spread of diseases, as the lungs of exposed persons are more susceptible to infections due to heavy microbial load. In addition, relative humidity and poorly ventilated indoors also affect their health. There is no prominent evidence but the above-mentioned conditions which are analyzed in rural kitchens might influence the spread of dangerous coronavirus due to the poor health conditions and the increased load of aerosols. Recent research also confirmed that the number of positive cases varied between indoor and outdoor environments among rural and urban areas. Therefore, the indoor environment without a ventilation framework with increased temperature may be more vulnerable to the spread of coronavirus infection among residents (Magd et al. 2020).

CONCLUSION

The study suggests that the microbial air quality analysis of the indoor environment is necessary to provide variation of air quality and prevent possible wellbeing vulnerability allied with it. High bacterial load was found in the indoor environment of the rural traditional kitchen as compared to the urban modular kitchen due to poor ventilation framework and usage of unclean fuel over LPG for cooking. It is important to determine the airborne bacterial load to find out the risk from the indoor environment generated bacterial diseases/disorders. Significance of this study is that bioaerosol sampling could deliver fruitful knowledge about the variation of air quality and in future prevent possible hospital admissions. The study was planned to make a comparison of bacterial load in rural and urban indoor and outdoor environments, to specify the bacterial load in traditional kitchens of rural dwellings.

ACKNOWLEDGEMENTS

The study was financially supported by Madhya Pradesh Council of Science and Technology (MPCST) Bhopal, Madhya Pradesh, India (Grant Ref. No. R&D (Vet.)/17-18/06&28/03/32018). Authors would like to acknowledge Centre for Translational Research laboratory (CTR lab), Jiwaji University, Gwalior, India for their infrastructural support.

Conflict of Interests: Authors declare that they have no conflict of interests.

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Psychological Communication

Prediction of Resilience Based on Personality Traits of Female Adolescent Students

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ABSTRACT

In high school students' anxiety is very common problem for all. Nevertheless, the students predict to be stressful increasing with several sources of stress especially with Academic stress. How to respond to stress and challenges affect the entire life of them. The current study tries to investigate the relationship between resilience and personality traits. Moreover, the other important objective is to examine whether big five personality traits predict resilience of high school female students or not. Till now, very limited studies have been conducted in India in context to high school female students. Enrolled in academic session 2020-21, 85 students studying Humanities in 12th standard, in GGSSS, Delhi, India were selected to participate in the study. The standardized questionnaires of the Ego Resilience Scale and Big Five Personality Test were used as the tool of the study. The data was collected using both the questionnaires and interpreted using Pearson correlation and multiple linear regression at a significance level $p < 0.05$. As a result of analysis, Pearson Correlation shows that all the personality traits E, A, C, O have a significant positive correlation with Resilience ($r = .539$, $r = .734$, $r = .792$ and $r = .751$ respectively) but N has a significant negative correlation with resilience ($r = -.684$). Furthermore, the results reveal a statistically significant relationship between the variables of Conscientiousness, Neuroticism, and Agreeableness, and the participants' resilience scores ($R = .859$, $R^2 = .738$, $p < .05$). These three personality traits together account for 73% of the total variance in resilience ($F = 75.94$, $p < .05$). It is concluded that Understanding of the levels and relationship among Personalities and Resilience of adolescents can assist in predicting the performance level of the students and in future intervention programs can be developed accordingly for the students for a successful and satisfactory life.

KEY WORDS: ADOLESCENTS, BIG FIVE PERSONALITY TRAITS, PERSONALITY, RESILIENCE, STUDENTS.

INTRODUCTION

After a stressful and traumatic life event, some people adjust themselves by showing a stable, well-functioning path, while other people may face stress after that event. Resilience is a powerful process that combines good adaptation within the context of great difficulty (Oshio et al. 2018). On a daily basis, a remarkable amount of physical and mental growth is experienced by students. Among school, work, social life, and co-curricular activities, adolescents face lots of new challenges and experiences (Anderssen 2021). The competition in itself is quite stressful, and the uncertainty over the exam dates adds to the students' miseries (Kar et al. 2021). The inability to handle performance pressure, meet parental demands and accomplish aspirations may lead to

psychological distress and may lead to suicidal behavior. (Edlina et al. 2020; Karet et al. 2021).

Resilience gives them the capability to handle these head-on, recover from any obstacle and support them to approach new circumstances, people or experiences with a positive mindset and confidence which helps them succeed (Anderssen 2021). Accordingly, difficulties faced by students adjusting to their new situations and studies adversely influence their life experience and academic achievements. If they are unable to overcome the difficulties, they may not be able to reach their preset goals. Some may adjust easily while the rest find it extremely difficult as they face many changes in many aspects of their lives, such as geographical location, climate, food, language and educational systems (Singh 2021). Previous researches indicated that the concept of resilience was fundamental to people dealing successfully with change (Wang 2009; Raghavan and Sandanapitchai 2019; Fuente et al. 2021).

Article Information:*Corresponding Author: sonysharma6644@gmail.com

Received 25/10/2021 Accepted after revision 25/12/2021

Published: 31st December 2021 Pp- 1526-1530

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.24>

Women often have the added responsibility of demonstrating their abilities during daily challenges. They need to strengthen the ability that enables them to face challenges in different situations (Webb 2018). In rural areas, the condition of the female is challenging with early marriage, illiteracy, poverty, unemployment, etc. As very few studies are conducted on female students, therefore, this study has purposively selected this population to fill the gap in literature about personality traits and resilience levels of female adolescent students. Personality comprises of attitudes, moods, and opinions displayed in dealing with other people. The current study also reports the personality trait variable, which is supposed to be associated with resilience, consistent with the big five perspectives (Hulya 2017; Holzman 2020). Academic achievement is also influenced by personality traits. eg., conscientiousness has reliably appeared as a constant predictor of exam performance (Chamorro 2003; Fuente et al. 2021).

Groupings of Big Five traits have also been found to expect various educational results. Specifically, openness to experience and conscientiousness predict course outcome, and openness to experience, conscientiousness, and agreeableness predict overall academic performance. In contrast, academic achievement is negatively related to neuroticism (Chamorro et al. 2003, Samsilah et al. 2021). Several factors may help in developing resilience, but an individual's personality traits are important. There is ample evidence that Big Five Personality traits can influence Resilience among adolescents as found in previous studies. For instance, it is reported that resilience was negatively associated with neuroticism, and was positively related to conscientiousness and extraversion (Campbell et al. 2006; Fuente et al. 2021).

Previous findings suggest that resilient children have higher thinking and problem-solving skills. Further, researchers confirm that personality traits such as flair, motivation, and understanding contribute to resilience, and resilience enhances personality traits (Annalakshmi 2008; Edlina et al. 2020). Recently, many studies have been conducted to observe the relationship between the big five personality traits and resilience like 'resilience as a buffering variable between the big five component, Building Emotional Resilience with Big Five Personality Model, still there is a severe shortage of the studies that works with ego resiliency and big five personality traits of Indian high school female students (Sahni et al. 2021; Fuente et al. 2021). Moreover, it is required to enhance the literature of resilience and personality traits in Indian high female context as well as to investigate the personality traits that predict resilience in high school female students so that they would train accordingly to successfully deal with the upcoming adversities in life (Sonam 2021). This study aims to find if there is there a relationship between resilience and Big five personality traits and if the big five personality traits can predict resilience.

MATERIAL AND METHODS

This is a descriptive study with quantitative approach. A random selection of 85 female students, mean age ± 17.43

studying Humanities in class XII, session 2020-2021 for the sample the study was made. From GGSSS No. 1 JJC Bawana, Delhi, India from Sec- A, B, and C every odd roll no. students were selected. By meeting them in their classrooms the data was collected by the researcher. Aim of the study as well as the method to filling up the tools was clearly explaining to them. Then both of the tools were circulated and students were requested to fill up the tools to the best of their knowledge. All the difficult statements were explained by the researcher. Once the questionnaires were filled up researcher collected both questionnaires and ensured the confidentiality and anonymity of responses.

For Tool I, The Ego Resilience Scale, Ego resilience scale consists 14 items to determine the ego-resilience of the individual. To recognize the emotion, behavior, and motivation, the answers were evaluated on a four-point Likert scale where (1) representing "Does not apply at all" to (4) demonstrating "Applies very strongly". Each statement was positively stated and scored. Total scores for ER-89 can vary from 14- 56 where; 14 reflected Low resiliency Traits to 56 signified as Very High Resilience Traits. Internal consistency of this scale was 0.82 (Edlina et al. 2020).

For Tool II, Big Five Personality Test, Big five personality scale included 50 statements to assess the intensity of the different personality traits. Scores achieved in this scale were evaluated on a Five Point likert Scale where (1) signifying "disagree" to (5) representing "agree". The overall point of each personality trait was measured by summarizing all the points obtained in that aspect. Internal consistency of Conscientiousness is 0.81, Extroversion was 0.86, Agreeableness was 0.77, Neuroticism was 0.83 and Openness to experience 0.82 (Sonam 2021).

SPSS software had been used to analyze the data. To identify the relationship between ego resilience and big five personality traits descriptive analysis and Pearson correlation analysis were implemented. In addition, to measure whether personality traits can predict the level of resilience, a multiple linear regression analysis was conducted (Hulya 2017). Consent from the school has been attached below, and after permission from the school authority, data was collected only from those students who had agreed to take part in the study voluntarily.

Consent Form: Consent from the school for the present study was duly taken in writing, and after permission from the school authority, data were collected only from those students who had agreed to take part in the study voluntarily.

RESULTS AND DISCUSSION

Data from given Tables have reflected that the neuroticism personality trait had a statistically negative correlation of moderate level ($r = -.684$) with resilience which showed higher the resilience lower the neuroticism trait, whereas Conscientiousness had a statistically positive and strong correlation with the greatest score ($r = .792$) reflected 'higher the resilience higher the conscientiousness' trait. Likewise, Openness to experience also had a statistically

positive and strong correlation ($r=.751$) with the second greatest score and very close to conscientiousness which also presented 'higher the resilience higher the openness to experience' trait. Moreover, agreeableness and Extroversion were in third and fourth position to statistically positively and intermediately correlate ($r= .734$ & $r= .539$) with resilience. As per the findings, if the scores of resilience increased then the scores of conscientiousness, openness to experience, extroversions and agreeableness also increased proportionally whereas neuroticism score had been decreased at 0.05 level of significance. Additionally, the next question of the present study was to investigated whether the level of resilience had been predicted by the personality traits of the participants. For that, being predictor variables the big five personality traits had been entered to perform a multiple linear regression analysis. The results of the analysis presented in Table 3:

Table 1. Description of scores of Ego Resilience scale and Big Five Personality traits

	Mean	Standard Deviation	Min	Max
ERS	32.19	11.18	11	49
E	30.14	9.31	10	50
A	30.36	10.05	12	49
C	29.81	9.85	10	48
N	27.41	10.16	7	49
O	29.04	8.84	9	49
Age	17.43	.6	16	18

Note*: E, Extroversion; A, Agreeableness; C, Conscientiousness; N, Neuroticism; O, Openness to Experience; ERS; Ego Resilience Scale

Table 1 evidently showed the results of descriptive analysis of all the variables illustrating that the Mean (SD) of Ego Resilience scale, Extroversion, Agreeableness, conscientiousness, Neuroticism, and Openness to experience was 32.19 ± 11.18 , 30.14 ± 9.31 , 30.36 ± 10.05 , 29.81 ± 9.85 , 27.41 ± 10.16 , and 29.04 ± 8.84 respectively. It also appears that in personality traits, Extroversion had a maximum score i.e. 50 and minimum score was in Neuroticism i.e 7. The Ego Resilience scale had a maximum score of 49 and a minimum of 11. First question of the present study investigated the relationship among the level of resilience and personality traits of participants.

Table 2, demonstrated the correlation among the scores of ego resilience scale and big five personality traits obtained by the participants which exposed statistically significant relationships.

The results of the analysis presented that there is a statistically significant relationship found among the scores of participants of ego resilience and the variables of conscientiousness, neuroticism, and Agreeableness i.e ($R=.859$, $R^2= .738$, $p<.05$). Above mentioned variables of personality explained 73% of the entire variance in resilience ($F=75.94$, $p<.05$). As per the standardized

coefficient of regression (β), on resilience the order of predictor variables of relative importance is as follows: conscientiousness ($\beta= .413$, $p<.01$), neuroticism ($\beta= -.304$, $p<.01$), and agreeableness ($\beta=.276$, $p<.05$).

Table 2. Correlation among the scores of ego resilience and big five personality traits

	ERS	E	A	C	N	O
ERS	1.000	.539	.734	.792	-.684	.751
E	.539	1.000	.812	.663	-.337	.681
A	.734	.812	1.000	.734	-.511	.712
C	.792	.663	.734	1.000	-.578	.831
N	-.684	-.337	-.511	-.578	1.000	-.700

The present study investigated the relationship between ego resilience and the big five personality traits and whether the levels of resilience are predicted by the traits of personality or not. As a result of analysis, a significant but negative relationship was recognized between resilience and neuroticism. Resilience helps students in being constant and keep moving on whatever the conditions but neuroticism trait of personality consists of self-doubt, nervousness, negative feelings and weak coping skills (Campbell et al. 2006; Fuente et al. 2020). For abovementioned reasons it is expected that neuroticism trait and resilience correlate negatively with each other that find reliable with the previous studies (Campbell et al. 2006; Fuente et al. 2020; Sonam et al. 2021). In addition, extrovert students possess the qualities to assess more flexibly and possessively in wide range of opportunities that helps them handle the challenges and hardship with the availability of additional personal resources. Moreover, they also possess the qualities to find social support at the time of adversities. Therefore, it is not unexpected that extroversion and resilience correlate positively with each other which is consistent with earlier studies (Costa and McCrae 1992; Fuente et al. 2020; Sonam et al. 2021).

Students with open to experience personality trait possess qualities like enthusiasm, courage, they know how to stand against the strict rules with critical and innovative thinking and possess a tendency to be exceptional, intellectual, and independent for their rights. For these reasons, open to experience students are able to deal with life's hardships and difficulties which elucidate the statistically significant and positive relationship between resilience and openness to experience trait of personality. It is also found similar to previous studies (Fayombo 2010; Sonam et al. 2021; Kocjan 2021).

Students who possess Agreeableness trait of personality have qualities like well behavior, helpfulness, co-operation and compassion. Because of these qualities they receive additional mental and emotional support at the time of challenges and less struggles in relations. Hence, students with more mental and emotional support ultimately become more resilient which provide the reason of statistically positive and significant relationship between resilience and

agreeableness. Results of the study were similar to earlier studies (Hulya 2017; Fuente et al. 2020; Kocjan 2021; Sonam et al. 2021). Further, students with conscientiousness personality were explained with qualities like being persistent, plan-oriented, organized and patient. Such qualities allow them to focus on certain points and take

action. Hence, it helps them achieve goals and contributes to their level of resilience. Therefore, results of present study showed statistically positive correlation between resilience and Conscientiousness. Findings of the present study were similar to earlier studies (Hulya 2017; Fuente et al. 2020; Kocjan 2021; Sonam et al. 2021).

Table 3. Multiple Regression Analysis on Prediction of level of Resilience through Big five Personality Traits of participants

Model	B	SE	Beta	T	P	Zero-order r	Partial R
Constant	18.051	4.226		4.271	.000		
Conscientiousness	.469	.101	.413	4.623	.000	.792	.457
Neuroticism	-.335	.078	-.304	-4.305	.000	-.684	-.431
Agreeableness	.307	.094	.276	3.258	.002	.734	.340
R= .859		R= .738					
F= 75.94		p<0.05					

So, it is clearly elucidating from the results of the study that if a student has a stronger personality, then he/ she would have stronger resilience too which helps them face competitive examinations, support students in adjustment and learning and provide enough strength to the female to handle all the life-changing and simultaneously enhance their performance level. Lastly, a multiple linear regression analysis was run to examine whether the big five personality traits predicted level of resilience. Results found that the dimensions of conscientiousness, neuroticism, and agreeableness accounted for 73% of the total variance of resilience. This finding appears to be reliable with the findings of previous studies (Campbell et al. 2006; Cetin et al. 2015; Hulya 2017; Kocjan 2021).

CONCLUSION

The findings of the present study conclude that big five traits of personality can be used to address resilience. And Resilience has a significant positive relation with Conscientiousness, agreeableness, extroversion, and openness to experience personality traits and a negative relationship to neuroticism. Moreover, by recognizing risk and protective factors of students, intervention programs to enhance resilience can be designed in schools. In addition, if a student has weak resilience, then training should be given to him/her so that he/she could meet the life challenges after passing school, meet the challenges of excessive competition, monetary needs, and survival in society.

Conflict of Interests: Authors declare no conflict of interests to disclose.

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Nutritional Communication

Formulation and Quality Evaluation of Foxtail Millet and Semolina Incorporated Ready-To-Cook Upma Mix

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ABSTRACT

Traditional cuisine has risen to the top of the consumer's list of preferences. Many traditional cereal-based dishes have been processed, and instant mixes such as instant upma, idli, and dosa have been made. Comfort foods are foods that require little or no processing or cooking before consumption, making them more convenient for the user. Ready to Cook (RTC) and instant foods have grown highly popular as a result of increased urbanization and industrialization, owing to today's lifestyle and the need for quick-to-serve cuisine. The study's goal is to develop an RTC upma mix and evaluate its physicochemical, texture, sensory, and *in vitro* glycemic index. The millet was collected and pre-processing was done to develop the ready-to-cook mixes. The levels of millet incorporation ratio were 60% (V1), 70% (V2), and 80% (V3) levels. Standard procedures were used to determine the physicochemical, textural, sensory, and *in vitro* glycemic index. The nutritional composition of V3 was found to be high, with moisture, carbohydrate, protein, fat, fibre, soluble fibre, and insoluble fibre content of 6.4 percent, 73.6g, 8.5g, 6.2g, 6.6g, 2.4g, and 0.56g, respectively. The textural profile showed a significant difference ($p < 0.05$) between variations in comparison with the standard. Results on sensory evaluation showed that 70% foxtail millet upma mix shows high acceptable than other. The mean *in vitro* glycemic index of the control upma mix was 68.7 ± 0.12 , while the mean estimated glycemic index value of V1, V2, and V3 was 50.5 ± 0.5 , 49.8 ± 0.4 , and 49.2 ± 0.6 respectively. Thus, the result evident that all the developed RTC upma mixes exhibited a low glycemic index and were useful for Diabetic subjects and easy to carry as a journey food.

KEY WORDS: FOXTAIL MILLET, GLYCEMIC INDEX, ORGANOLEPTIC, READY-MIX, UPMA.

INTRODUCTION

The Indian Ready to Cook (RTC) food market has progressed from its humble beginnings as a flimsy substitute for a home-cooked meal or dining out. A rapidly urban lifestyle, rising disposable income, and a growing percentage of continent Indians with a sophisticated palate are all favorable demographic variables driving the adoption of RTE and RTC foods in India. Diversification of food production must be encouraged at the national and household level with improved yields and household techniques (Singh and Raghuvanshi 2012). Some of the highly nutritious agricultural foods are not being used as human foods because of the unawareness of people. Millets are one of them. Millets are being used as bird and animal feed. Millet has many nutritional and medical properties reported (Yang et al. 2012). Millets are rich sources of phytochemicals,

micronutrients, and antioxidants, such as phenolic acids and glycosylated flavonoids (Stanly et al. 2013).

Millets also contain water-soluble gum-glucans, which help with glucose metabolism. As a result, millets can be included in a diabetic diet to help with glucose control. Foxtail millet (*Setaria italica* L.) is important underutilized minor millet that sustains well in adverse conditions. Nutritionally it's superior to rice and wheat, and therefore provides proteins, minerals, and vitamins to the poor hooked into such grains (Tylor and Emmanbux 2008). In hyperlipidemic rats, foxtail consumption lowers plasma triglycerides, perhaps preventing cardiovascular disease (Lee et al. 2010; Stanly et al. 2013).

In humans with type 2 diabetes, high consumption of millet-based dietary fibre improves glycemic control, lowers hyperinsulinemia, and lowers plasma lipid contents (Jali et al. 2012; Kamatar 2013). Upma is a popular South Indian breakfast or snack cuisine that may be made in a short amount of time. Upma is a thick porridge made from dry roasted semolina and is typical Indian breakfast food.

Article Information: *Corresponding Author: karunafsnthara@gmail.com

Received 18/09/2021 Accepted after revision 20/12/2021

Published: 31st December 2021 Pp- 1531-1537

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.25>

In both Asian and Western cuisines, semolina is a primary ingredient. Semolina made from foxtail millet could be a novel product made from small millets that can provide consumers with the natural health benefits of foxtail millet while also allowing millet products to be used in new ways (Dharmaraj et al. 2016).

The millet's widespread use was still limited, owing to the lack of a variety of food products available on the market. Because millet semolina does not contain gluten protein, it might be used in place of wheat semolina, which could be beneficial to diabetes people. So yet, no one has used foxtail millet to make semolina or use it in the formulation of instant upma mix. With all these considerations in mind, the goal of this study is to develop a ready-to-cook (RTC) upma mix and assess its physicochemical, textural, sensory, and *in vitro* glycemic index.

MATERIAL AND METHODS

The physical properties of foxtail millet such as thousand-grain weight, thousand-grain volume, hydration capacity and index, swelling capacity and index and cooking quantity/characteristics millets and the functional properties such as bulk density, water absorption capacity, oil absorption capacity, swelling power, solubility, the solid loss were analyzed in foxtail millet. The analysis procedure of the AOAC method (2007) was used to examine millet nutritional qualities such as pH, total titratable acidity, moisture, carbohydrate, and energy value, crude protein, crude fibre, Ash, total starch, Amylose content, total sugar, dietary fibre, and mineral composition.

The anti-nutritional parameters such as tannin, total phenolics, and trypsin inhibitor were determined using standard procedures. For the creation of the upma mix, basic materials such as foxtail millet, semolina, Bengal gram dhal, mustard seeds, salt, green chili, carrot, beans, peas, and curry leaves were purchased from the local market. Millet was cleaned and washed under running water before being soaked for 30 minutes. After draining the grains and parboiling them for 10-15 minutes, the boiled grains were dried in the dryer to 14% moisture. Foxtail millet was converted into suji using a pulverizer and sieved (700mesh) as Millet suji. Dried vegetables were made by placing them in boiling water for 3 minutes and then cooling them immediately. At 60°C, carrots, chiles, and curry leaves were dried. Three variations of upma mixes were prepared by incorporating foxtail millet and semolina at different levels. Semolina in the ratios 0:100 (standard), other three variations of Upma mixes at 60%, 70%, 80% levels of millet suji. Below table-1 shows the composition of different ingredients for Upma mix preparation. The foxtail millet suji was roasted in a non-stick pan on slow flame with constant stirring till (5-7 min). The samples were cooled, packed in a container, and stored in normal condition.

According to the method of Basantpure et al. (2003), the physical properties of the upma mix were analysed. Moreover, the rehydration ratio (RR), which is a measure of the dehydrated product's water absorption was determined. Dry foxtail millet suji-based upma mixes (100 g) were

reconstituted with a measured amount of hot water (150-200 ml) and whisked on a low flame until the desired consistency was achieved. By using the AOAC technique (2000), all varieties of RTC upma were analysed for nutritional content, such as availability of carbohydrate, protein, fat, total fibre, soluble and insoluble fibre content, and the changes in nutritional quality on drying were documented. All the variations of millet upma mix with the respective control product were subjected to texture analysis using a Texture Analyser (TVT-300XP, Perten Instruments, Sweden) after the preparation.

The following Texture Profile Analysis (TPA) was used to determine the hardness, adhesiveness, springiness, and stickiness. All texture analysis measurements were repeated at least three times for each sample, and the mean values were calculated. The acceptable level of variations studied for sensory quality based on appearance, colour, flavour, taste, texture, and overall acceptability using a 9-point Hedonic scale by a panel of 10 judges scorecard with scores ranging from 9 to 1, in which 1 = dislike extremely, 5 = neither like nor dislike, and 9 = like extremely was used. Granfeldt et al. (1992) described a method for determining the *in vitro* starch digestibility of the formulated RTC upma mix. After that, the estimated glycemic index was determined using Goñi et al. (1997). The data was compiled and analysed using statistical methods such as mean, SD, ANOVA. All these were performed and the results separated, using the Multiple Range Duncan Test ($p < 0.05$) and using the statistical software of SPSS 16.

Table 2. Physical and functional properties of foxtail millet

Parameters	Foxtail millet
Thousand-grain weight (g)	2.64±0.005 ^{ab}
Thousand grain Volume (ml)	2.96±0.05 ^b
Hydration Capacity (g/1000 seeds)	2.01±0.02 ^{ac}
Hydration Index (%)	76.9±0.05 ^c
Swelling Capacity (ml/1000 seeds)	0.21±0.02 ^b
Swelling Index (%)	6.72±0.02 ^{ab}
Cooking Quantity	193.3±11.9 ^a
Cooking time (minutes)	15±0.24 ^{ab}
Bulk Density (g/ml)	0.52±0.005 ^a
Water Absorption Capacity (g/g)	1.18±0.05 ^b
Oil Absorption Capacity (g/g)	1.06±0.02 ^a
Swelling Power (g/g)	5.23±0.4 ^c
Solubility Per gram (%)	6.2±0.55 ^b
Solid Loss Per gram (%)	29.3±0.86 ^a

Values are the means ± standard errors of means (SEM) of 3 determinants. Means with the same superscript are not significantly different using Duncan's Multiple Range Test ($p < 0.05$).

RESULTS AND DISCUSSION

The results and discussion of this study were discussed in the following heading like physical and functional, nutritional and anti-nutritional, rehydration ratio, textural, organoleptic, and *in vitro* glycemic responses of the RTC upma mixes.

The table-2 depicts the physical and functional properties of foxtail millet. In here the physical properties like thousand-grain weight, thousand-grain volume, hydration capacity, hydration index, swelling capacity, swelling index, cooking quantity, and cooking time showed the values of 2.64g, 2.96ml, 2.01g/1000seeds, 76.9%, 0.21ml/1000seeds, 6.72%, 193.3g, and 15min respectively. The functional properties of foxtail millet were bulk density, water absorption capacity, solubility per gram and solid loss per gram was 0.52g/ml, 1.18g/g, 1.06g/g, 5.23g/g, 6.2%, and 29.3%. Thilagavathi et al. (2015) found that pearl millet has the highest thousand-grain weight (11.39g), followed by Kodo millet (2.45g), and small millet (2.45g) (2.23g). In this study, the selected foxtail millet grain weight was found to be more or less linked to Kodo millet grain based on the above literature. Thilagavathi et al. (2015) compared WAC of various types of millet with wheat and soybeans flour and found out that it ranged from 74.08 to 76.83 ml/100 g, 74.08 to 78.83 ml/100g, and (58.17- 60.02 ml/100g) for millet, wheat, and soybean flour respectively (Thilagavathi et al. 2015).

Butt (2010) also observed some variation in different millet flours and this may be due to different protein concentrations, their degree of interaction with water, and conformational characteristics. The oil absorption capacity according to Amir et al. (2016) on finger millet flour was found to be 1.93g/g and that of pearl millet flour is 1.60g/g. There is an advantage for the best organoleptic characteristics of a meal that the high water and oil absorption capacity of the flour can positively influence the flavor, moisture, and fat content in food. The bulk density (798.10 kg.m-3) of foxtail millet were comparable with Subramanian and Viswanathan (2007). According to Ojediran et al. (2010) the bulk density, true density and porosity of pearl millet varied from 646.40-811.40 kg.m-3, 953.26-960.59 kg.m-3 and 15.17-32.64 per cent, respectively. The 1000 kernel weights of foxtail millet were observed as 2.45g in the past (Sunil et al. 2016).

The table-3 shows that the nutrient content such as protein (6.8g), high fibre content (4.9g), and calcium (43mg). The iron content was 9.2g and the amylose content was 28.4g. A considerable amount of energy source was noticed in foxtail millet (333.5kcal). Protein synthesis was attributed heavily to the germination process due to the involvement of microbial organisms in the flour. Sambavi et al. (2015) and Punia et al. (2003) revealed similar results in the production of cookies using a mix of foxtail millet and wheat flour and the nutritional evaluation of kangni (*Setaria italic*). The anti-nutrient compositions of foxtail millet were 0.36g of tannin, 65.6g of phenolics, and 0.24g of trypsin inhibitors.

As a result changes in tastes and lifestyles backed by urban living will significantly impact food demand and consumption patterns (Goyal and Singh 2007; Sambavi et al. 2015). Balasubramanian et al. (2014) used pearl millet

semolina to make upma dry mix. Before the preparation of semolina, pearl millet grains were hydrothermally treated to reduce anti-nutritional factors and inactivate lipase activity. Pasrija and Punia (2000) reported that both pressure-cooking and solar-cooking significantly reduced the phytic acid and polyphenol contents of cowpea cultivars. The reduction was greater when the seeds were soaked or dehulled prior to cooking (Pasrija and Punia 2000; Balasubramanian et al. 2014).

Table 3. Nutritional and Anti-Nutritional properties of foxtail millet

Parameters	Foxtail millet
Nutritional parameters	
pH	7.0±0.10 ^a
Ash(g)	2.1±0.015 ^{ab}
Total titrable Acidity	0.54±0.01 ^a
Moisture(g)	15±0.15 ^c
Crude Protein(g)	6.8±0.01 ^{bc}
Crude Fibre(g)	4.9±0.04 ^c
Carbohydrates(g)	70±1.10 ^a
Fat(g)	2.9±0.60 ^{ab}
Energy (Kcals)	333.3±0.77 ^{abc}
Total Starch(g)	18.5±0.01 ^c
Amylose content(g)	28.4±0.1 ^b
Sodium(mg)	16±1.0 ^{ac}
Potassium(mg)	347±1.0 ^{ab}
Iron (mg)	9.2±0.1 ^a
Calcium(mg)	43±1.00 ^b
Phosphorus(mg)	265±1.00 ^{ac}
Anti-Nutritional parameters	
Tannin (mg)	0.36±0.010 ^a
Total Phenolics (mg)	65.6±0.20 ^b
Trypsin (mg)	0.19±0.010 ^{ac}

Values are the means ± standard errors of means (SEM) of 3 determinants. Means with the same superscript are not significantly different using Duncan's Multiple Range Test (p < 0.05).

Table 4. Rehydration ratio of upma mix

Variation	Rehydration ratio	
	Initial weight	Final weight
Standard	30g	40g
V1	30g	50g
V2	30g	60g
V3	30g	80g

In comparison to the standard sample in the table, the V3 sample shows a high rehydration ratio with a higher ability to reabsorb the water content. Among the three variations,

low rehydration was noticed in V1 (50g). Amount of water adsorption was also affected by bulk and particle's surface composition, particle size, porosity, and internal molecular structure of powder (Bhandari et al. 2013). Yadav and Sharma (2008) did their study in soy fortified upma. The

incorporation of vanaspati and spices in pre-cooked dried Kabuli channa increased the re-constitution time due to the production of a hydrophobic oil coating, which hindered water penetration in the grain. Balasubramanian et al. (2014) showed similar rehydration ratios (2.4 to 3.3) in pearl millet semolina (Balasubramanian et al. 2014).

Table 5. Nutrient analysis of RTC upma mix

Variations	Moisture	Carbohydrate (g)	Protein (g)	Fat (g)	Fibre (g)	Soluble fibre(g)	Insoluble fibre(g)
Standard	3.74±0.05 ^a	79.79±0.05 ^{ab}	5.38±0.01 ^{ac}	6.8±0.01 ^c	5.0±0.01 ^{ab}	2.4±0.01 ^b	0.28±0.01 ^c
V1	5.2±0.01 ^{ab}	74.3±0.01 ^a	8.6±0.01 ^b	6.1±0.01 ^c	6.2±0.01 ^a	2.0±0.01 ^{ab}	0.32±0.01 ^b
V2	6.2±0.01 ^{ac}	73.1±0.01 ^{ab}	8.2±0.01 ^b	6.4±0.01 ^c	6.4±0.01 ^{ac}	2.3±0.01 ^{ab}	0.42±0.01 ^b
V3	6.4±0.01 ^b	73.6±0.01 ^c	8.5±0.01 ^a	6.2±0.01 ^{ac}	6.6±0.01 ^a	2.4±0.01 ^{ac}	0.56±0.01 ^c

Values are the means ± standard errors of means (SEM) of 3 determinants. Means with the same superscript are not significantly different using Duncan's Multiple Range Test ($p < 0.05$).

Table 6. Textural characteristics of RTC upma mix

Sample	Cohesiveness (N)	Stickiness (N)
Standard	0.52±0.01 ^a	-2.5±0.01 ^{ab}
V1 (60%)	0.43±0.01 ^c	-1.6±0.10 ^b
V2 (70%)	0.05±0.01 ^{ac}	-1.6±0.10 ^a
V3 (80%)	0.00±0.00 ^b	-3.1±0.15 ^c

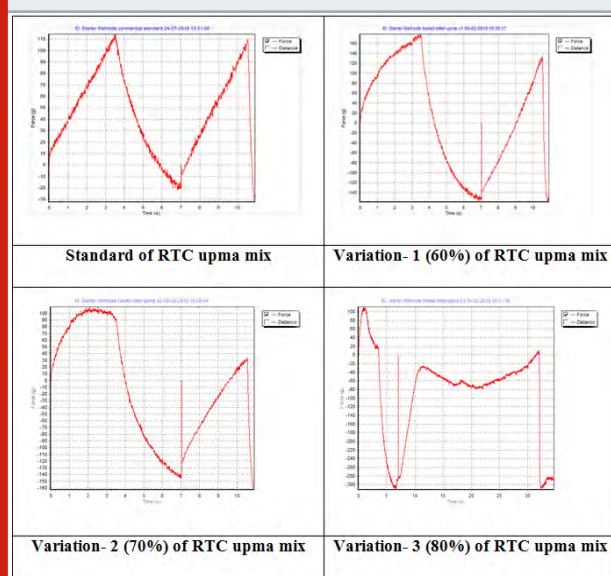
Values are the means ± standard errors of means (SEM) of 3 determinants. Means with the same superscript are not significantly different using Duncan's Multiple Range Test ($p < 0.05$).

From this table -5, it was predicted the nutrient analysis of RTC upma mix. In comparison to the standard, V3 had a high nutrient composition upma mix, with moisture, carbohydrate, protein, fat, fibre, soluble fibre, and insoluble fibre content of 6.4 percent, 73.6g, 8.5g, 6.2g, 6.6g, 2.4g, and 0.56g. The nutritional content of the selected fibre rich food items was determined to be good, and the fibre rich product was discovered to be a good source of minerals (Bora and Kulshrestha 2014). There was a substantial difference between the standard and the three variations upma mix in moisture, carbohydrate, and fibre at the 5% level, according to Duncan's multiple range test. During decortication, milling, soaking, heating, roasting, germination, and fermentation are just a few of the traditional/household processing methods employed in food formulations (Aisoni et al. 2018).

Compare with the standard high cohesiveness was found in V1 and maximum stickiness was noticed in the V3 upma mix. Fig-1 illustrates the textural profile curves of RTC upma mix variation and its standard. Duncan's multiple range test showed that there was a significant difference ($p < 0.05$) between the standard and the variations. Shivli

et al. (2013) found that adding durum wheat semolina (SSCSBS) to cooked Multigrain Semolina Blend reduced hardness and springiness while increasing cohesiveness, gumminess, and chewiness (MSB). Cohesiveness is defined as the ratio of the positive force area during the second compression for that during the first compression. The lowest cohesiveness was observed in V3 (0.00N) and the lowest stickiness was noticed in V1 and V2 (-1.6N). Stickiness was the important factor that affects the texture of the upma due to the presence of the soluble fibre content (Shivli et al. 2013; Aisoni et al. 2018).

Figure 1



When compared to other adjusted combinations, the V2 upma formulation made with 70% foxtail millet suji received the highest sensory ratings for colour (7.60), appearance (7.60), taste (6.60), flavour (7.00), texture (6.50), and overall acceptability (8.90). This could be

owing to the use of fine foxtail millet semolina in the right proportions, resulting in an upma with pleasing colour, flavour, and texture. Similarly, Itagi et al. (2012) and Adegunwa et al. (2014) found that the acceptable levels of foxtail millet flour in readymade foxtail millet mix

for diabetics, multigrain semolina blend for upma, and composite millet-wheat were 80 percent, 50 percent, and 100 percent, respectively, in readymade foxtail millet mix for diabetics, multigrain semolina blend for upma (Itagi et al. 2012; Adegunwa et al. 2014).

Table 7. Mean sensory analysis of RTC upma mix

Variations	Appearance	Colour	Flavour	Texture	Taste	Overall acceptability
Standard	8.10±0.55 ^b	8.20±.52 ^b	8.90±.30 ^d	7.75±.55 ^b	8.05±.39 ^b	8.20±.52 ^b
V1	8.20±0.78 ^{efg}	8.00±0.66 ^{cde}	8.10±0.73 ^{bc}	7.30±0.94 ^{abc}	7.80±1.03 ^{ab}	8.30±0.31 ^{fg}
V2	7.60±1.42 ^{cdefg}	7.20±1.31 ^{abcd}	7.00±1.24 ^{ab}	6.50±1.08 ^a	6.60±1.07 ^a	8.90±0.67 ^a
V3	7.20±0.78 ^{abc}	7.30±1.05 ^{bcd}	6.90±0.99 ^{ab}	7.10±0.87 ^{abc}	6.90±0.87 ^{ab}	8.00±0.66 ^{de}

Values are the means ± standard errors of means (SEM) of 3 determinants. Means with the same superscript are not significantly different using Duncan's Multiple Range Test ($p < 0.05$).

Table 8. *In vitro* glycemic response of RTC upma mix

Variations	Glycemic Index	Glycemic load
Standard	68.7±0.12 ^a	13.7±0.06 ^{ac}
V1(60%)	50.5±0.5 ^{ab}	10.1±0.1 ^{ab}
V2(70%)	49.8±0.4 ^c	9.9±0.1 ^b
V3(80%)	49.2±0.6 ^b	9.8±0.1 ^c

Values are the means ± standard errors of means (SEM) of 3 determinants. Means with the same superscript are not significantly different using Duncan's Multiple Range Test ($p < 0.05$).

Table -8 shows the glycemic responses of the formulated RTC upma mixes. Compare with standard, V1 (50.5), V2 (49.8), and V3 (49.2) had low GI values which indicate the addition of foxtail increases will decrease the GI value. As a result, all of the low GI values reported for various upma mixes were around 50, showing that all of these products have a low GI and are suitable for diabetic diets. According to Grandfeldt et al. (2000), limited grain processing and the manufacture of thicker flakes may aid in the development of flakes with reduced glycemic and insulinemic properties. Studies in this direction may be helpful for the preparation of finger millet flakes with a lower glycemic response. Livesey et al. (2008) stated in their meta-analysis study that foods with low GI caused significantly lower postprandial glycaemic response, they are considered better for people with diabetes. Monro (2014) suggests that the integrity of the dietary fibre is important to impart the desirable functional benefits like lower digestibility (Monro 2014). The effect of foxtail millet intervention on blood glucose might be explained by the slow digestion of carbohydrate and moderate glycemic index (Ren et al. 2016). In addition, the bioactivated fibers, flavonoids, polyphenols and other phytochemicals in foxtail millet might be other contributors to its glucose lowering effect (Sharma et al. 2018).

CONCLUSION

The findings of the present study determined that incorporating varying degrees of foxtail millet and semolina improved the quality of the RTC upma mix in terms of nutritional, textural, sensory attributes, and glycemic response. Variation 3 (80:20) RTC upma mix showed high nutritional composition than other variations with a 5% significant difference level. Based on textural characteristics, variation 1 showed a significant difference ($p < 0.05$) than variations. Through the glycemic response of the RTC upma mix showed a low glycemic response (GI less than 55) for all the three variations (V1, V2, and V3) when compared with standard. As a result of the current study, it was concluded that foxtail millet has a higher nutritional value grain than rice and wheat, and will almost certainly improve glycemic control in diabetic patients.

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Biotechnological Communication

Development and Optimization of Floating Microspheres in Amethopterin

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ABSTRACT

Due to the complexity of gastric emptying, as well as its considerable variability, the *in vivo* efficacy of drug delivery devices cannot be predicted. When it pertains to drugs with an absorption window in the upper small intestine, a controlled drug delivery system with a longer residence period in the stomach may be of considerable practical significance. Recent developments have shown that floating microspheres are particularly well suited for mixing sustained and delayed releases to achieve a variety of release models with a minimal risk of dumping. The aim of present investigation is to develop and analyze the floating microspheres of amethopterin, which after oral administration could increase the gastric residence time and enhance the bioavailability of the drug by sustained release and minimize the dose dependent side effects as well as improves patient compliance. Floating microspheres of ethyl cellulose, Polyvinyl alcohol and polyvinyl pyrrolidone-K90 were formulated by emulsification solvent evaporation technique. The various parameters of prepared microspheres were studied for SEM, flow properties, buoyancy, yield, percent drug loading, *in vitro* dissolution studies, stability in different pH and FTIR studies. Microspheres prepared with different concentrations of polymers were spherical in shape with smooth surface. The size of microspheres was in range of 256.02 μm and 362.84 μm . Good drug entrapment and buoyancy were observed for formulation F2. The *in vitro* drug release after 6h was found to be in range from 58.15% to 96.28%. It was established that the newly created floating microspheres of Amethopterin provide an appropriate and practical solution for the sustained release of medication over a longer period of time, resulting in increased oral bioavailability, effectiveness, as well as better patient compliance.

KEY WORDS: AMETHOPTERIN, ANTINEOPLASTIC, BUOYANCY, FLOATING MICROSPHERES.**INTRODUCTION**

Oral route of administration is the most convenient and widely used method of drug administration, and the development of stomach specific oral controlled- release delivery systems is a challenging job due to the variation of pH in different segments of gastrointestinal tract, the fluctuation in gastric residence time and the difficulty in localizing an oral delivery system in a selected region of the gastrointestinal tract. Rapid gastrointestinal transit can prevent the absorption of complete drug in the absorption zone and reduce the efficacy of administered dose since the majority of drugs are absorbed in stomach or upper part of small intestine. Polymers are generally employed in the development of floating microspheres. A number of different substances have been investigated for the preparation of floating microspheres; these materials include polymers

of natural origin or synthetic origin and also semisynthetic substances. Floating microspheres can be prepared by using both hydrophilic and hydrophobic polymers. The concept of floating or porous microspheres can also be utilized to minimize the irritant effect of weakly acidic drugs on stomach by avoiding direct contact with the mucosa and providing a mean of getting low dosage for prolonged periods (Bulgarelli et al. 2000; Davoudi et al. 2013; Prakash et al. 2015; Srikar et al. 2018; Mishra et al. 2020; Birajdar et al. 2021; Kumar et al. 2021).

Amethopterin synonym Methotrexate (MTX) (Figure 1) is an antineoplastic agent whose mechanism is similar to alkylating agents. It is a highly toxic drug with a very low therapeutic index. It causes toxicities like stomatitis, gingivitis, glossitis, ulceration, and bleeding of the mucous membrane when given orally and hematological effects like leucopenia, thrombocytopenia, anemia, hemorrhage from various sites in single-dose intravenous administrations, and also some hepatic toxicities by administering as conventional dosage forms. Sustained and targeted delivery of amethopterin will reduce these toxicities considerably

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Received 25/09/2021 Accepted after revision 21/12/2021

Published: 31st December 2021 Pp- 1538-1543

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.26>

by maintaining a low and constant level of drug in the blood. Therefore, floating microspheres have emerged as an efficient means of prolonging gastric residence time, targeting stomach mucosa, and enhancing the bioavailability. Floating microspheres remain buoyant due to lower density than the gastric and intestinal fluids. They are not subjected to 'all or nothing' gastric emptying nature of single unit system and releases the drug in a controlled fashion. In the current research, emulsification solvent evaporation including EC, PVP-K90, and PVA polymers were used to form hollow microspheres containing Amethopterin. (Chen et al. 2000; Singh et al. 2000; Shishu et al. 2007; Garg et al. 2008; Brunton et al. 2011; Rathor et al. 2011; Abbas et al. 2020; Kumal et al. 2020; Tomar et al. 2021; Thakur et al. 2021; Kharb et al. 2021).

MATERIAL AND METHODS

Amethopterin was obtained as a gift sample (Naprod Life Science Mumbai) whereas ethyl cellulose (EC), polyvinyl pyrrolidone K-90 (PVP K-90) and polyvinyl alcohol (PVA) were obtained from Merck India, Mumbai. Other chemicals used were of analytical grade. Floating microspheres containing amethopterin were prepared by emulsification solvent evaporation technique using EC, PVP-K90 and PVA polymers for microspheres (Soppimath et al. 2001) (Table 1). Amethopterin (100 mg) was weighed accurately and dissolved in 8 mL ethyl alcohol, followed by the addition of 2 mL isopropyl alcohol and 5 mL methylene dichloride. The polymer solution was slowly introduced into 100 mL of 1% polyvinyl alcohol aqueous solution while stirring at 250 rpm using a mechanical stirrer equipped with a 3-blade propeller. The solution was stirred for 10 minutes and microspheres were collected by filtration. The floating microspheres were collected by decantation, while the non-floating microspheres were discarded along with any polymer precipitates. The microspheres were dried in an oven at 50°C for 2 hours, weighed and then stored in a desiccator at room temperature for further use.

The developed floating microspheres were evaluation considering following parameters:

- **Surface Morphology:** The prepared microspheres were fixed on a brass stub using double sided adhesive tape and then made electrically conductive by coating, in vacuum, with a thin layer of gold for 30 minutes and then examined by scanning electron microscope (SEM) operated at 10kV (JOEL-JXA840A electron probe micro-analyser, Japan) (Jain et al. 2005) (Figure 2(A) and 2(B))
- **Flow Properties:** Flow properties were determined in terms of Carr's index (CI) using the following formulae:

$$CI = \frac{\rho_t - \rho_b}{\rho_t}$$

where,

ρ_t = Tapped density and ρ_b = Bulk density

The angle of repose $[\Theta]$ of the microsphere, which measures the resistance to flow, was determined by fixed funnel method, using the following equation:

$$\tan \Theta = h/r$$

where,

h = height of the pile and r = radius of the cone formed after making the microspheres flow through glass funnel from a fixed height (Kale et al. 2007).

The particle size of floating microspheres was performed with the help of optical microscope for randomly selected sample for all formulations (Table 2).

- **Buoyancy test for microspheres:** In triplicates, microspheres (100mg) were dispersed in solution composed of HCl (300 mL, pH 1.2 at 37°C) containing Tween 20 (0.02% w/v) to simulate gastric conditions (Patel et al. 2011, Raveendra et al. 2012).

The use of 0.02% Tween 20 was to account for the wetting effect of the natural surface-active agents, such as phospholipids in the gastrointestinal tract (GIT) (Jain et al. 2009).

The mixture was stirred on magnetic stirrer at 100 rpm and 37±0.5°C. After 12 hours, the floating particles were separated by filtration. The sinking particles were separated by filtration. Both particle types were weighed after drying at 40°C overnight. The buoyancy was determined by the weight ratio of the floating particles to the sum of floating and sinking particles. Percent buoyancy of formulations is shown in Table 3.

$$\text{Buoyancy(\%)} = \frac{Q_f}{Q_f + Q_s} \times 100$$

where,

Q_f = Weight of floating microspheres and Q_s = Weight of settled microspheres

- **Yield of Microspheres:** The prepared microspheres were collected and weighed. The actual weight of obtained microspheres divided by the total amount of all drug and polymers solid material that was used for the preparation of all the microspheres (Patel et al. 2006). Yield of microspheres is shown in Table 3.

$$\text{Yield (\%)} = \frac{\text{Actual weight of the product}}{\text{Total weight of the excipients and drug}} \times 100$$

- **Percent Drug Loading:** Amethopterin content in the floating microspheres was estimated by a UV-Visible Spectrophotometer (Lambda 25, Perkin Elmer, US) method based on the measurement of absorbance at 303 nm in distilled water (Semalty et al. 2007). Microspheres equivalent to 100 mg were weighed and added in 100 ml of distilled water. The volumetric flask was stirred continuously for 24 hours on a magnetic stirrer. At the end of 24 h sample was withdrawn, diluted suitably and measured spectrophotometrically at 303 nm for drug content. Quantitative estimation of

amethopterin was calculated by using equation obtained by Linear regression analysis of calibration curve in distilled water. The drug loading in microspheres was estimated using the formula:

$$\text{Drug Loading (L)} = (Q_m / W_m) \times 100$$

where,

W_m is the weight of microspheres and Q_m is the quantity of drug present in W_m of microspheres (Semalty et al. 2008). Percent drug loading of various formulations is shown in the Table 3.

- In vitro dissolution studies:** *In vitro* dissolution studies were performed using USP XXIII, Type-II (paddle) dissolution apparatus. The accurately weighed sample (50 mg) of formulations from F1 to F6 were dropped individually into 500 mL of phosphate buffer (pH 7.2) maintained at a temperature of $37 \pm 0.5^\circ\text{C}$ and stirred at a speed of 50 rpm. At different time intervals, 1mL aliquot of the sample was withdrawn and the volume was replaced with an equivalent amount of buffered dissolution medium kept at 37°C . The collected samples were filtered and diluted with 9mL of phosphate buffer and analyzed at λ_{max} 303nm using a UV- Visible spectrophotometer (Lambda 25, Perkin Elmer, US) against the buffer taken as blank. (Figure 3) Percent cumulative drug release from floating microspheres was calculated using Beer- Lambert's Equation. The drug release was calculated using various models and shown in Table 3 (Patel et al. 2005).
- Stability of microsphere at different gastric pH:** Floating microspheres are low density systems that have sufficient buoyancy to float over gastric contents and remain in stomach for prolonged period where they are exposed to different pH and different enzymatic conditions which can influence their physiochemical properties and drug release behavior and can alter their physiochemical properties and drug release behavior and can alter their stability characteristics. To test this hypothesis, drug loaded microspheres were subjected to different pH media where they encountered different ionic strengths and enzymatic conditions and the change in their properties was elucidated by counter checking their particle size. The pH dependent stability studies were carried out in following media:

- a) pH 1.1: 12 mL HCl (32%) with 1188 mL H₂O.
- b) pH 3.5: 150 mL solution (10.5g citric acid + 100mL NaOH of 1M + 395.5 mL H₂O) with 100mL HCl.
- c) Simulated Gastric Fluid (SGF): 0.2% NaCl, Pepsin 0.7% HCl with pH 1.2.

10 mL of simulated fluid were added to 10mg of microspheres. The samples were analyzed after a period of 12hours in each of the above media. The above time intervals were selected for the study based on expected

formulation residence time in stomach. Particle size was determined on the preset time periods (Kalaria et al. 2009). The results are recorded in Table 4.

- IR Spectroscopic Studies:** Drug-Polymer interaction was studied by FT-IR spectroscopy (Shimadzu Affinity I, FT-IR spectrophotometer). Samples were prepared by triturating 10 % of the drug or microspheres with 95% of KBr in glass pestle-mortar. The IR spectra of the drug and the microspheres were recorded, the identical peaks of the drug and drug with polymers concluded that neither the polymer nor the method of preparation has any significant effect on the drug stability (Jain et al. 2006; Kaushik et al. 2010).

RESULTS AND DISCUSSION

The floating microspheres of amethopterin were prepared by emulsion solvent evaporation method using ethyl cellulose, polyvinyl pyrrolidone and polyvinyl alcohol (Saravanan et al. 2011; Ahmadi et al. 2020; Ramadan et al. 2020; Maddibovina et al. 2020).

Figure 1: Amethopterin

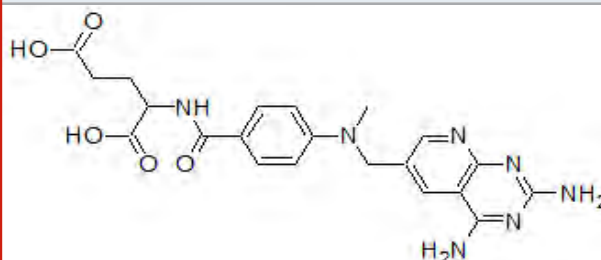


Table 1. Composition of floating microspheres

Ingredient	F1	F2	F3	F4	F5	F6
Drug	0.1	0.1	0.1	0.1	0.1	0.1
EC	0.5	1.0	1.5	0.5	1.0	1.0
PVP-K90	-	-	-	1.0	0.5	1.5
PVA	1.0	1.0	1.0	1.0	1.0	1.0

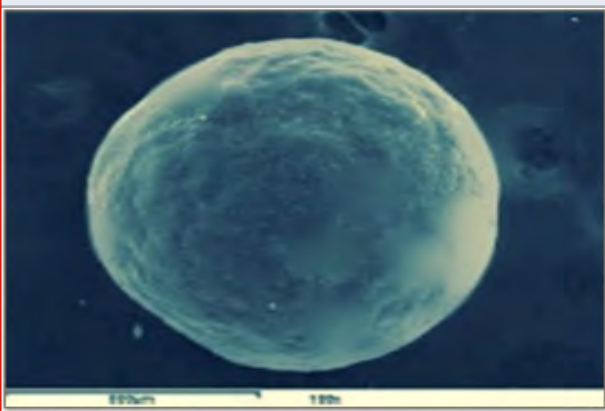
EC: Ethylcellulose; PVP K-90: Polyvinyl pyrrolidone K-90; PVA: Polyvinyl alcohol.

The prepared floating microspheres were evaluated for different physicochemical tests such as particle size, true density, flow properties, drug content, *in vitro* float ability and *in vitro* drug release studies (Mahor et al. 2020). Scanning electron microscopy showed that F1, F2, F4, F5, F6 formulation produced spherical microspheres compared to F3. The scanning electron microscopy confirmed the hollow nature of microspheres with pores on the surface of floating microspheres, which imparted floating properties to the prepared floating microspheres.

Table 2. Flow Properties of floating microspheres

Formulation code	Angle of Repose [°]	Carr's Index [%]	Average Particle size [μm]
F1	28.35±0.670	18.32±0.364	342.32±3.240
F2	24.64±0.502	15.25±0.342	362.84±2.452
F3	23.54±0.341	12.54±0.322	356.42±0.2465
F4	21.65±0.422	17.53±0.232	260.56±1.8602
F5	24.52±0.438	13.66±0.522	256.02±2.5446
F6	22.61±0.246	12.42±0.426	276.24±2.6773

Results are shown as Mean ± Standard Deviation, (n=3)

Figure 2(A): The cluster of microspheres**Figure 2(B): A single microsphere**

To estimate flow properties of prepared floating microspheres, micromeritic properties like particle size and true density were determined. The densities of floating microspheres were found to be less than density of gastric fluid, therefore tended to float over gastric fluid. So, the prepared microspheres combine the advantages of multiple unit systems and good floating properties. However, like all floating systems their efficacy is dependent on the presence of enough liquid in the stomach, requiring frequent drinking of water. Particle size analysis of different formulation was done by optical microscopy. The average particle size for microspheres was in the range between 256.02 μm and 362.84 μm. The average particle size of microspheres was found to be increasing with the increase in concentration of polymer (Ramadan et al. 2020).

Table 3. Release parameters of floating microspheres

Formulation code	Drug release after 6 h	% Drug loading	% Buoyancy	% Yield
F1	71.81% ±1.28	84.36±0.36	96.28±1.48	78.96±2.64
F2	92.71% ±2.36	58.84±0.82	94.36±1.86	95.38±0.38
F3	80.32% ±1.10	53.92±1.46	90.62±1.54	82.56±1.64
F4	68.22% ±1.46	95.36±2.36	60.26±2.62	70.48±1.74
F5	67.57% ±1.48	42.62±1.68	54.56±2.84	76.32±1.38
F6	58.15% ±1.02	76.38±1.72	68.38±1.36	82.93±1.76

Results are shown as Mean ± Standard Deviation, (n=3).

Drug content in F1, F2, F3, F4, F5 and F6 formulation were estimated by UV spectrophotometric method. Percent loading efficiency were found in the range of 42.62% to 95.36%. Formulation F4 containing ethyl cellulose (0.5%) and polyvinyl pyrrolidone (1%) showed maximum loading of drug up to 95.36%. The rank order of percent loading was found to be as followed F4>F1>F6>F2>F3>F5. *In vitro* drug release studies of all the formulations were performed in phosphate buffer of pH 7.2 at 303 nm.

Significant difference was observed in the release pattern of amethopterin floating microspheres EC, PVA and PVP. It was found that the drug release from the formulations were distinguishably different for the different polymers used in the formulations. The rank order of drug release after 6 hours was found to be 92.71, 80.32, 71.81, 68.22, 67.57, 58.15 percent of formulation F2, F3, F1, F4, F5, F6 respectively. Formulation F2 containing ethyl cellulose (1%) showed the maximum release after 6 hours. Stability

studies of microspheres at pH 1.1, 3.5 and SGF were conducted and found to be stable (Yang et al. 2021). FT-IR studies showed that there was no interaction between the drug and polymers.

Figure 3: The *in vitro* drug release profile

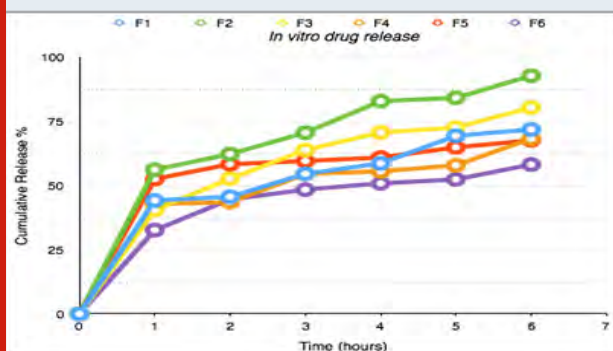


Table 4. Stability Studies of floating microspheres

Medium	Initial Size	Final Size
pH1.1	256.02±2.5446	258.12±2.5446
pH3.5	256.02±2.5446	260.42±2.5446
SGF	256.02±2.5446	258.68±2.5446

Results are shown as Mean±Standard Deviation, (n=3).

CONCLUSION

The current Amethopterin formulation research was carried out in an effort to develop a floating drug delivery system composed of a floating multiple-unit system. By emulsion solvent evaporation, novel floating hollow microspheres were effectively developed for the sustained and stomach-specific action of Amethopterin. As a result of its low density, this multiparticulate drug delivery method demonstrated excellent flotation characteristics. From *in vitro* drug release studies, it can be concluded that, by changing the ratio of polymer and solvent, drug release can be controlled. The results of all the physiochemical tests of all formulations were found to be satisfactory. *In vitro* float ability studies revealed that most of the microspheres (54.56 % to 96.28 %) were floatable. The *in vitro* drug release was found to be in range of 58.15 % to 92.71 % at the end of 6 hours. The proposed solution, which combines good buoyant ability with an appropriate drug release pattern, may be beneficial in terms of improved Amethopterin bioavailability. The findings of the present study presented that these floating microspheres can be selected for the development of gastro retentive drug delivery system of Amethopterin for potential therapeutic use.

ACKNOWLEDGEMENTS

The authors received no direct funding for this research. Moreover, authors acknowledge with gratitude the Department of Pharmacy, GLA University, Mathura, Uttar Pradesh for providing the necessary infrastructure and facilities to conduct this research work.

Conflict of Interests: Authors declare no conflict of interests to disclose.

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Agricultural Communication

Development of Technology Transfer in Agriculture During the COVID-19 Pandemic in Russia

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ABSTRACT

In an ever-changing environment, strategic decisions are made when implementing changes in the technology transfer process. Currently, it is important to implement effective measures to stimulate the development of technology transfer in the agro-industrial complex in modern conditions; to develop ways to stimulate the demand of agricultural enterprises for modern technologies, including by optimizing prices for them. Insufficient demand for innovative technologies also arises due to possible risks. In this regard, it is necessary to develop ways to reduce risks in the process of technology transfer in agriculture. The authors investigate the impact of the COVID-19 pandemic on technology transfer and the way the coronavirus forced people to change their habits and isolate themselves from each other. During this period of time, the role of digital technologies has increased and technology transfer has come to help build new ties between inventors and consumers of scientific achievements. The authors tried to consider the scenario of technology transfer development in agriculture by proposing an algorithm for implementing the technology transfer process in the country's agriculture. Methods of benchmarking, comparative analysis and a systematic approach were used to process information obtained from electronic databases, online publications, the legal reference system, and Internet information resources. As indicated in the article, an important task today is to organize effective interaction between participants in the technology transfer process, including in the virtual space on the Internet sites. For the effective functioning of the technology transfer market in agriculture and its infrastructure, it is necessary to regularly monitor the market situation in order to meet the interests of both buyers and sellers as much as possible.

KEY WORDS: CORONAVIRUS, DIGITAL ECONOMY, ISOLATION, PANDEMIC, TECHNOLOGY TRANSFER ALGORITHM.

INTRODUCTION

The first wave of the COVID-19 pandemic showed that the development of science, innovation and digital technologies is not just an abstractly declared political priority in the countries that are long-standing technological leaders. People around the world, who previously got used to the achievements of science and technology and no longer noticed their advantages, today, in isolation, personally feel their importance for each person. As the history of global economic crises or epidemics shows, each of them "clears" the field and opens up new opportunities for long-term innovative growth. It is important today to lay the foundation for the advanced development of science and

innovation, to increase its contribution to economic growth and the well-being of citizens, to increase the efficiency and achieve a new quality of the economy and the agricultural sector through the introduction of digital technologies, to ensure equal and effective opportunities for realizing the creative and entrepreneurial potential of citizens in the digital environment (Kostin and Khomchenko 2021).

And this conductor will be a technology transfer that will unite science and the real sector of the economy, and its most important segment is agriculture. The emergence and spread of COVID-19 contribute to changing of both the social and the economic life of mankind. The positive consequences of the pandemic may include the acceleration of the introduction of digital technologies that provide wide information access to users through the use of innovative tools. Within the restrictions imposed by governments, entrepreneurs and ordinary consumers are actively developing digital solutions

Article Information:*Corresponding Author: ukolovang@sgau.ru

Received 19/08/2021 Accepted after revision 25/12/2021

Published: 31st December 2021 Pp- 1544-1549

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.27>

to continue their activities in a remote format (Accounts Chamber of the Russian Federation 2020).

Digitalization contributes to the transition to the online environment of all branches of the agro-industrial complex, allows people to make online purchases, exchange information about innovations. This trend is developing due to the urgent need and to the created material base for the widespread use of digital technologies (Accounts Chamber of the Russian Federation 2020). In addition, the active study of the functioning of the transfer mechanism in the context of a pandemic continues. Solovyova (2019) investigated the conditions of interaction between the institutional structures of the Russian Federation and a number of other countries based on the analysis of indicators of innovative development. She justified the need to reduce the raw material orientation of economic development and increase the share of exported products of innovative developments (Solovyova 2019). Researchers Kostin and Khomchenko (2021) proposed a systematized concept of technology transfer, the impact of the pandemic on the development of technology transfer in different countries and regions was analyzed on a qualitative and quantitative level (Kostin and Khomchenko 2021).

The purpose of the study is to build an algorithm for the implementation of the technology transfer process in agriculture, taking into account the likely scenario of the development of the transfer in a pandemic. In accordance with the purpose of the study, the following main tasks are to be solved: to generalize and to systematize theoretical provisions on the formation and development of technology transfer mechanisms; to clarify the factors of the coronavirus pandemic affecting the effectiveness of technology transfer; to analyze the indicators of the functioning of technology transfer in the field of intellectual property in a number of countries during the pandemic; to formulate the stages of the implementation of the technology transfer process in agriculture in the conditions of the coronavirus pandemic and in the post-pandemic period; to scientifically substantiate the recommendations of the effective functioning of the technology transfer market in agriculture (Solovyova 2019; Kostin and Khomchenko 2021). The working hypothesis to substantiate the scenario for the development of technology transfer in agriculture in the context of the coronavirus pandemic is based on the development of an algorithm for the transfer implementation process.

MATERIAL AND METHODS

The methodological basis of scientific research consists of methods of analysis and synthesis of economic information, the method of deduction and induction, the causal method and other methods of data processing – they were used in the study and assessment of the current state of innovative development of the analyzed countries in order to identify trends and patterns of technology transfer development. Indicators of the functioning of technology transfer in the field of intellectual property for 2017-2019 in a number of countries (China, USA, Japan, Germany, France, Russia) were selected as the information base of the study according to the principle of priority of innovative economic

development (World Intellectual Property Organization 2019; World Intellectual Property Organization 2020; World Intellectual Property Organization 2021).

Methods of benchmarking, comparative analysis and a systematic approach were used to process information obtained from electronic databases, online publications, the legal reference system, and Internet information resources. To test the hypothesis, methods of generalization and synthesis were used in order to increase the efficiency of the transmission mechanism, to identify the connections between its elements. To develop an algorithm for technology transfer, methods of constructing graphic images were used. The selected methods allow us to evaluate the transfer of technologies, and to develop mechanisms for implementing and improving the efficiency of this process.

RESULTS AND DISCUSSION

The principles of technology transfer organization are disclosed in the works of many scientists. The works of various authors are devoted to the mechanisms of transfer of intellectual property from the university environment to the real sector of the economy. The methodological and scientific-practical basis for studying the mechanisms of technology transfer were monographs, scientific articles and other scientific works of authoritative scientists-economists and researchers. In the work of the above-mentioned authors, the significance and principles of the organization of technology transfer are revealed, recommendations for stimulating technology transfer are indicated, ways of organizing the activities of technology transfer centers are proposed, as well as mechanisms for the formation of additional subjects of innovation infrastructure (Schumpeter 1982; Butler 2011; Nishizawa 2011; Somaya 2011; Shore and McLauchlan 2012; Luksha and Yanovsky 2012; Correa 2013; Gaponenko 2013; Stolyarov, Sazhina and Kudina 2014; Rossinskaya and Pleshko 2014; Cardamone 2015; Litvinov 2015; Rozhdestvensky 2015; Ruposov 2016; Ugnich 2016; Likholetov and Terebova 2017; Allard 2017; Falko 2017; Porter 2017; Gerasimenko 2018; Kashirin 2018; Gavrilyuk, Voronov and Leontiev 2018; Ukolova et al. 2020). Solovyova (2019) analyzed the indicators of innovative development of a number of countries and, based on the generalization of the experience of functioning, made a conclusion about the need for innovative export orientation (Solovyova 2019). Russian researchers Kostin and Khomchenko (2021) systematized the concept of technology transfer in the context of a pandemic (Kostin and Khomchenko 2021).

Reports of foreign and Russian scientists explore different points of view on the implementation of technology transfer, but it is worth noting the lack of a priority algorithm for its implementation in agriculture. This circumstance required the need for further development of implementation mechanisms and ways to improve the efficiency of technology transfer, as well as the development of practical recommendations for improving existing forms of technology transfer in Russia's agriculture. The slowdown in economic growth around the world was observed in

2019, and at the beginning of 2020 it has increased due to the coronavirus pandemic (Solovyova 2019).

The large-scale decline in economic activity due to the closure of many leading economies affected all countries, disrupting the usual way of life. Conferences, seminars, and exhibitions of scientific achievements were suspended. The gap between developers and consumers of scientific

achievements leads to missed opportunities for farmers. Regulators and governments were forced to urgently apply a wide range of support measures, to carry out these activities online. At the same time, the root causes of the current crisis lie outside the economic or financial sphere, which makes it unlike the previous ones. This crisis caused fear, fright, a threat to their lives. And this imposed a certain communication between people. This is confirmed by the data presented in Table 1 (Solovyova 2019).

Table 1. The answers to the question How do you assess the threat from the new coronavirus and the response to it?

Reaction to the coronavirus is hypertrophied and seems more dangerous than the virus itself	369 (43%)
Threat of the virus is still underestimated in the world — this explains the pandemic nature of the spread of the disease	277 (32%)
Response to the coronavirus is adequate to the threat posed by the COVID-19 pandemic	211 (25%)

Table 2. Indicators of the functioning of technology transfer in the field of intellectual property for 2017-2019

Indicators	Russia	USA	Germany	France	China	Japan
	2019 (World Intellectual Property Organization, 2021)					
number of patent applications for inventions	35 511	621 453	67 434	15 869	1 400 661	307 969
number of applications for utility models	10 136	-	11 668	454	2 268 190	5 241
number of applications for the creation of industrial designs	10 928	49 848	44 097	37 404	711 617	32 176
	2018 (World Intellectual Property Organization, 2020)					
number of patent applications for inventions	37 957	597 141	67 898	16 222	1 542 002	313 567
number of applications for utility models	9 747	-	12 307	608	2 072 311	5 388
number of applications for the creation of industrial designs	8 943	47 137	44 460	12 495	708 799	31 468
	2017 (World Intellectual Property Organization, 2019)					
number of patent applications for inventions	36 883	606 956	67 712	16 247	1 381 594	318 479
number of applications for utility models	10 643	-	13 301	428	1 687 593	6 105
number of applications for the creation of industrial designs	7 390	45 881	45 803	12 132	628 658	32 457

The uncertainty is supplemented by the fact that traditional relations in the economy and in society are fundamentally changing, the nature of labour relations is beginning to change, the remote access mode is being practiced more and more. As of mid-2021, the probability of a third wave of the pandemic is quite high, while its duration and scale are still unclear, and the prospects for further development of the countries' economies are becoming less certain, and

the scale and mechanisms for stabilizing the situation are becoming more diverse and non-standard (Solovyova 2019; Ukolova et al. 2020).

As it has been repeatedly stated in the media, digitalization in the post-quarantine world will gain unprecedented momentum. "The pandemic is likely to accelerate the digital revolution, all its components: 5G communications,

enterprise robotics, artificial intelligence and e-government technologies, digital commerce and payments," says Jeffrey Sachs. And here it is impossible to do without the help of the state, its support. The state is more interested in the development of technology transfer than anyone else. In the leading countries of the world, such as the United States, China, Japan, and the European Union, technology transfer is the basis for building the economy of the future, based on the active use of artificial intelligence, robotics, information and communication technologies, reducing the intensity of human labour use and, as a result, increasing the productivity and efficiency of economic entities of national economies as a whole. Here it is worth mentioning the statement of Virginia Marie "Jinni" Rometti "The only way to survive is continuous transformation" (Golovanov 2020).

In the conditions of a pandemic, it is impossible to draw an unambiguous conclusion about the increase in the participation of information resources in creating the value of high-tech products, the introduction of innovations based on the accumulation of additive technologies (Table 2).

Analyzing the impact of the COVID-19 pandemic on technology transfer, we can note:

- in RF on indicator 'the number of Russian patent applications for inventions' in 2019 we come across a decrease of 6.4% compared to 2018 and 3.7% compared to 2017;

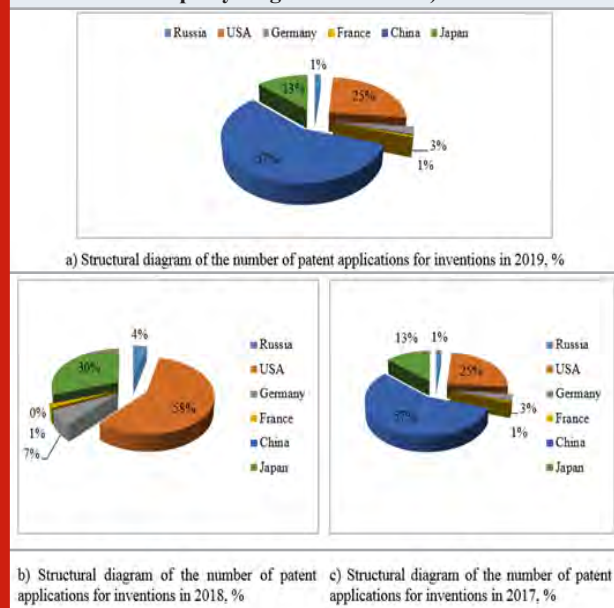
- indicator 'the number of applications for utility models' in 2019 revealed an increase of 4% compared to 2018 and a decrease of 4.8% compared to 2017; the indicator of the number of applications for the establishment of industrial designs has increased by 22.2% and 37.8% to 2018 and 2017, respectively.

According to studies of US indicators, it was found that the number of patent applications for inventions in 2019 increased by 4.1% and 2.4% compared to the data of 2018-2017. According to the indicator 'number of applications for utility models', there is no data, the increase in the indicator- 'number of applications for the creation of industrial designs' was 5.8% and 8.6%, respectively, compared to the data of 2018 and 2017. The change in the studied indicators in Germany revealed a negative trend: in 2019, the number of patent applications for inventions decreased by 0.7% and 0.4% compared to 2018 and 2017. The number of applications for utility models decreased by 5.2% and 12.3% relative to the study period. According to the number of applications for the creation of industrial designs, the decrease was 0.8% and 3.7%. In France, the situation is ambiguous regarding the change in innovation activity in 2019: the number of patent applications for inventions decreased by 2.2% and 2.3% compared to 2018 and 2017. In terms of the number of applications for utility models, a decrease of 25.3% compared to the data of 2018 and an increase of 6.1% compared to 2017; the number of applications for the creation of industrial designs increased almost 3 times compared to the study period (Porter 2017;

Gerasimenko 2018; Kashirin 2018; Gavriluk, Voronov and Leontiev 2018; Ukolova et al. 2020).

In China, the indicators are consistently high, however, according to the indicator, the number of domestic patent applications for inventions decreased by 9.2% compared to 2018 and a slight increase (1.4%) compared to 2017 data. According to the indicator, the number of applications for utility models in 2019 showed an increase of 9.5% compared to 2018 and by 34.4% compared to 2017. The number of applications for the creation of industrial designs increased by 0.4% and 13.2% compared to the data of 2018 and 2017, respectively. The study of changes in Japan's indicators revealed: a decrease in the number of domestic patent applications for inventions in 2019 by 1.8% and 3.3% compared to the data of 2018-2017. According to the indicator-the number of applications for utility models fell by 2.7% and 14.2%, the change in the indicator – the number of applications for the creation of industrial designs, respectively, by 2.2% (an increase compared to 2018) and 0.9% (a decrease compared to 2017). Information about the changes taking place during the pandemic is shown in Figure 1.

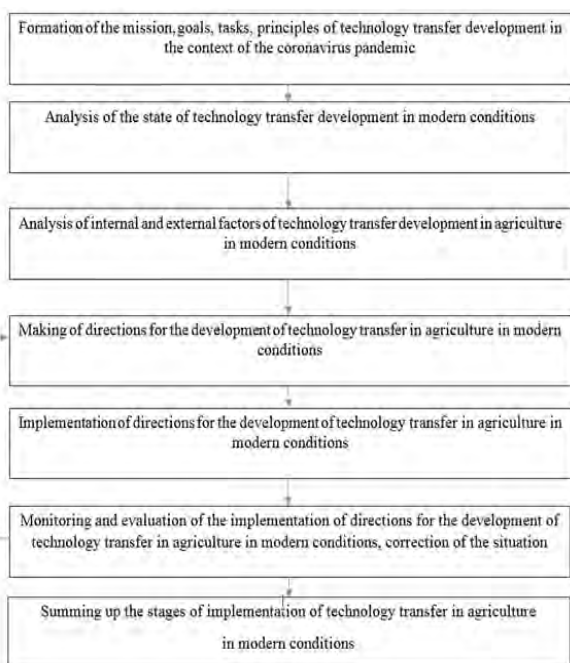
Figure 1: Comparative indicators of technology transfer during the COVID-19 pandemic and before its onset (World Intellectual Property Organization 2021)



The change in the share of the number of patent applications for inventions of the countries selected by us as analyzed allowed us to establish that China occupies a leading position in 2019 and in 2017, the second place in 2019 and in 2017 belongs to the United States (25%), in 2018 the United States was the leader (58%). Japan is on the 3rd place in 2019 and in 2017 (13%), in 2018 an increase of up to 30% was noted. In 2019, Germany accounts for 3%, Russia and France for 1% each. In 2018, Germany accounted for 7%, Russia-4%. In 2017 Germany accounted for 3%, Russia-1%.

Thus, in the conditions of the COVID-19 pandemic, the formation of intensive market interaction of economic entities of the United States was noted, in other countries (Russia, Germany, France, Japan, China), a slight decrease in innovation activity was recorded. It is important to implement measures to expand intra- and interregional cooperation in the field of technology transfer in agriculture in order to expand the customer base (potential buyers of innovative technologies from other regions). An important task in modern conditions is the organization of effective interaction between participants in the technology transfer process, including in the virtual space on the Internet (Figure 2). At the first stage, described in this algorithm, the mission, goals, tasks, and principles for the development of technology transfer in agriculture in the conditions of the coronavirus pandemic and in the post-pandemic period are formulated.

Figure 2: Algorithm for implementing the technology transfer process in agriculture



At the second stage, the state of development of technology transfer in agriculture in modern conditions is analyzed. Special attention is paid to the period of the pandemic and the peculiarities of the development of technology transfer in this period of time. At the third stage, the internal and external factors of the development of technology transfer in agriculture in modern conditions are analyzed. At the same time, special attention is also paid to the period of the pandemic and the influence of external (including international) factors. At the fourth stage, the development of directions for the development of technology transfer in agriculture in modern conditions is carried out, taking into account risks and uncertainties. At the fifth stage, the directions for the development of technology transfer in the agricultural sector of the economy are being implemented, taking into account the influence of external and internal environmental factors, possible risks and uncertainties.

Next the implementation of the directions of technology transfer development in agriculture in modern conditions is monitored and evaluated, and the situation is adjusted. After that, the directions of development of technology transfer in agriculture are adjusted and their implementation is carried out. At the last stage, the results of the stages of the implementation of technology transfer in agriculture in modern conditions are summarized. The generalization of the research results of foreign and domestic scientists on the consideration of the principles of the organization and mechanisms of technology transfer served as the basis for the development of an algorithm (Kashirin 2018; Gavrilyuk, Voronov and Leontiev 2018; Ukolova et al. 2020).

This has helped with the implementation of the technology transfer process universal for the agricultural production industry. The algorithm is based on the development and implementation of effective development directions formed taking into account the current state of agricultural production changing under the influence of external and internal factors. An analysis of the current state of the innovation environment and the functioning of the transfer mechanism of a set of leading countries (China, the United States, Japan, Germany, France) allowed us to conclude that the positive dynamics due to the impact of the COVID-19 pandemic was noted only for the United States. In other countries, including Russia, there was a slight slowdown in innovation activity, probably caused by an increase in the influence of uncertainty and risk factors in new, non-standard conditions for market conditions (Ukolova et al. 2020).

CONCLUSION

The findings of the present study conclude about the impact of the COVID-19 pandemic on the slowdown or progressive development of the transfer mechanism can be made at the end of 2021, when it is possible to evaluate the extraordinary ways developed by countries to overcome the negative consequences of the pandemic, due to the individual characteristics of the functioning of the countries under consideration. The optimal technology transfer mechanism considered by us for application in the field of agricultural production should be built taking into account changing market conditions, the consequences caused by the COVID-19 pandemic, natural and climatic factors (fundamental for agricultural production) and the experience of countries that have overcome the impact of the pandemic (the United States) in modern economic conditions.

ACKNOWLEDGEMENTS

The reported study was funded by RFBR, project number 20-010-00324.

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Microbiological Communication

Microbial Contamination Analysis of Mobile Phones from Certain Users of Chattogram, Bangladesh

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ABSTRACT

Mobile phone is an essential part of everyday life in modern days. Mobile phones act as vehicles for transmitting pathogenic bacteria due to lack of awareness and widespread use. This study aimed to investigate the bacterial contamination of mobile phones of different categories people at Chattogram city, Bangladesh. In our study, swab samples from 40 mobile phones of hospital patients, students, businessmen, and fishermen were collected for the isolation, identification of mobile phone associated bacteria, and their antibiogram. In this study, total viable count (TVC) was performed by the pour plate method and total coliform count (TCC) by the most probable number (MPN) method. Besides these, five selective media were used to isolate pathogenic bacteria from mobile phones and then identified. Antibiotic sensitivity assay was performed by disc diffusion method with 10 different antibiotics. Mobile phones of hospital patients (20165 cfu/ml) and students (1578 cfu/ml) showed the highest and lowest TVC respectively. Coliform bacteria were detected from the mobile phones of 100% hospital patients, 90% from both businessmen, and fishermen but only 30% from students. *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were found the most prevalent bacteria but *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Micrococcus* sp., *Bacillus* sp., *E. coli*, *Salmonella* sp., *Citrobacter* sp., *Serratia* sp., *Proteus* sp., and *Enterobacter* sp. were also detected. Almost all the isolates were highly resistant to ampicillin, amoxicillin, rifampin, erythromycin and sensitive to ciprofloxacin, gentamicin, azithromycin, and tetracycline. Our findings ensure, mobile phones act as a significant source of pathogenic organisms for humans and can serve as a channel for cross-transmission of microorganisms. So, washing hands before and after handling food and also personal hygiene is very important.

KEY WORDS: COLIFORM, KLEBSIELLA PNEUMONIAE, PSEUDOMONAS AERUGINOSA.

INTRODUCTION

A mobile phone is a device that may make and receive calls over a communication system whilst touring a good geographical area. It does so by connecting to a cellular network provided by a portable operator, allowing access to the general public telephone network (Suganya and Sumathy 2012). In every place and situation, it's used for communication by different groups of individuals. For sending and receiving messages, photos, and videos mobile phone provide several additional services like SMS, email, internet, and MMS (Roy et al. 2013). It may also be used for business purposes, within the medical field, and in online banking and finance.

Mobile phone is a potential carrier of a variety of microorganisms (Dave and Shende 2015, Olsen et al. 2020). Research has been shown that mobile phones may well be a health hazard with ten thousand microbes living on each square of the phone (Kilic et al. 2009). People carried their mobile phones in hospitals, toilets, kitchens, etc. as a result they became loaded with thousands of microorganisms (Bhoonderowa et al. 2014, Bhardwaj et al. 2020). After handling fish, meat, and animals selected groups of individuals don't wash their hands, as a result, contamination of mobile phones occurred. Moreover, mobile phones may be contaminated with *E. coli* and other enterobacteriaceae by hands washing water (Rather 2009).

Certain microbial species promptly colonized on the human body surface because it's constantly in contact with environmental microorganisms (Prescott et al. 2005). *Staphylococcus aureus* is generally present on the skin and causes pimples, boils etc. Besides, it also causes other

Article Information:*Corresponding Author: zobaid@cu.ac.bd

Received 15/08/2021 Accepted after revision 22/09/2021

Published: 31st December 2021 Pp- 1550-1556

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.28>

diseases like pneumonia and meningitis (Roy et al. 2013; Jamalludeen 2020). Irrational use of antibiotics is one of the most significant factors for the presence of emerging multi-drug resistant microorganisms everywhere. Poor drug quality and inadequate doses are also major reasons for the emergence of multi-drug resistant bacteria especially in developing countries like Bangladesh (Okeke et al. 1999). If this case continues, no dose or levels of antibiotics are going to be effective against bacterial diseases (Debnath et al. 2017). Antibiotic-resistant bacteria prompt high morbidity and mortality, patients need to stay a long time in hospital and causes higher treatment expenses (Olu-Taiwo et al. 2020).

In this research, an attempt was made to detect and characterize mobile phone associated bacterial pathogens among four categories of people living in Chattogram city, Bangladesh using microbiological techniques. Furthermore, the antibiotic susceptibility pattern of the isolates was also determined.

MATERIAL AND METHODS

The study was carried out in Chattogram city which is known as a seaport city and financial center in Bangladesh. A total of 40 mobile phone samples were collected from 4 categories of people living in Chattogram city such as 10 from students, 10 from fishermen, 10 from hospital patients, and 10 from businessmen. Sterile cotton swab was first moistened with normal saline and scratched over both surfaces of the tested mobile phones. The cotton ends of these swabs were cut off and soaked in 50 ml of sterile peptone water. The sample bottles were then preserved in an ice-fitted (4-8°C) basket to restrict bacterial growth and within 4-5 hours of the collection carried to the laboratory and preserved at 4°C until analysis.

Live microbial load on mobile phones can able to estimate by this method and the count represents the number of colony-forming units (cfu/ml) of the sample. TVC was conducted by means of the serial dilution agar plating method (Marzan et al. 2017). In this method, 1 ml of peptone water containing sample was inoculated into 9 ml of distilled water to get 10-1 dilution and then serially made up to 10-6 dilutions. One ml sample from each dilution was dispensed in the sterilized petriplate and then melted nutrient agar (about 45°C) was poured in each petriplate, mixed uniformly by rotated clockwise and anti-clockwise, and then allowed to solidify. After incubation at 37°C for 24 hours only 30-300 colonies containing plates were allowed for counting. The TVC was calculated according to the following formula:

$$\text{cfu/ml} = (\text{Number of colonies}) / (\text{volume plated} \times \text{dilution factor})$$

The presence of total coliform bacteria was assayed through the MPN technique according to the standard method (Clesceri et al. 1996). This test was carried out in three stages like presumptive, confirmed, and completed test. In presumptive test, for each sample, 3 sets of MacConkey broth containing screw cap tubes were prepared and in

each tube Durham's tube were used for the detection of gas formation by coliform bacteria. All the test tubes were incubated at 37°C for 48 hours for gas formation. For confirm test, brilliant green lactose bile broth containing test tubes were inoculated with positive presumptive test samples and incubated at 37 °C for 48 hours for gas formation. Complete test was performed for all the positive confirmed test samples. The positive samples were streaked on Eosin methylene blue containing plates and incubated for 24 hours at 37°C. Then selected nucleated colonies (with or without metallic sheen) were transferred to Lauryl tryptose broth tube and nutrient agar slant and then incubated at 37°C for 24-48 hours. After incubation, formation of gas, and in agar culture presence of Gram-negative, non-spore-forming, rod-shaped bacteria make sure the presence of coliform bacteria.

Five selective media were used to check microbial contamination of mobile phones. These were: Eosin Methylene Blue (EMB) agar (*E. coli*) and other coliform groups of bacteria, Xylose Lysine Deoxycholate (XLD) agar (*Salmonella* sp.), *Salmonella-Shigella* (SS) agar (*Shigella* sp.), Cetrimide agar (*Pseudomonas* sp.), and Mannitol salt agar (*Staphylococcus* sp.). Selective media were streaked by gas positive sample of MPN and after incubation, the grown colonies were transferred to nutrient agar slants and preserved for further microscopic, biochemical characterization, and antimicrobial susceptibility tests.

Kirby-Bauer method was used to determine the sensitivity and resistance of different antibiotics in-vitro (Bauer et al. 1996). This method can rapidly determine the efficacy of an antibiotic by the diameter of the zone of inhibition. A suspension of pure bacterial culture was prepared in peptone broth and incubated. The broth was spread by sterile cotton swab homogeneously on the solidified Mueller-Hinton agar plate and then the antibiotic discs were placed. The plates were then kept at freeze for 30 minutes and then incubated for 24 hours. The used antibiotic discs were: gentamicin (10 µg), azithromycin (15 µg), ciprofloxacin (5 µg), ampicillin (10 µg), tetracycline (30 µg), erythromycin (15 µg), amoxicillin (15 µg), rifampicin (5 µg), chloramphenicol (30 µg), ceftriaxone (30 µg). After incubation, the diameters of the zone of inhibition were measured.

RESULTS AND DISCUSSION

All the 40 samples were examined randomly and found 100% of mobile phones were contaminated with microbiota. The average cfu/ml was calculated and the highest average cfu/ml was observed in the mobile phones of hospital patients (20,165 cfu/ml) followed by fishermen (18,600 cfu/ml), businessmen (15,870 cfu/ml) and the lowest in students (1578 cfu/ml) mobile phones (Figure 1). This result indicated that students are handled their mobile phones carefully and kept them clean as a result carry comparatively less microorganisms, on the other hand, hospital patients should aware of the cleanliness of their mobile phones because of the highest TVC found on their mobile phones. Otherwise, nosocomial infections will be spread by mobile phones. The results are in accordance with other findings who found that 99% healthcare workers

phones were contaminated with pathogenic microbes and multi-drug resistant bacteria (Bhat et al. 2011; Huffman et al. 2020, Olsen et al. 2020; Simmonds et al. 2020). A study was conducted in Kashmir on which the highest TVC was found in animal handlers and lowest in veterinary surgeon's mobile phones (Roy et al. 2013).

Total coliform count includes aerobic and facultative anaerobic, non-spore forming, Gram-negative bacilli capable of growing with the fermentation of lactose and production of acid within 24 hours at 35-37°C. The presence of coliforms in collected samples indicates that mobile phones were contaminated with pathogenic organisms. These happen because people carry their phones in the toilet, kitchen, etc., and are not aware of the hygienic conditions of mobile phones. Thus, mobile phones become loaded with coliforms and other types of bacteria.

Figure 1: Average cfu/ml of mobile phones of different user groups. The presented values are the mean and standard error of mean. N=40 (10 mobiles for each group).

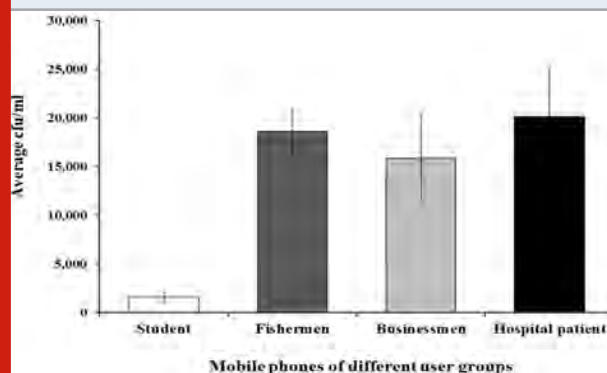


Table 1. Results of MPN test for estimation of total coliform count according to WHO guidelines.

Mobile phones of different groups	Sample No.	MPN index / 100 ml	Average (%) in each groups	Mobile phones of different groups	Sample No.	MPN index / 100 ml	Average (%) in each groups
Students	MM1	150	30%	Businessmen	MM21	28	90%
	MM2	23			MM22	28	
	MM3	0			MM23	21	
	MM4	0			MM24	1100	
	MM5	0			MM25	210	
	MM6	1100			MM26	460	
	MM7	0			MM27	23	
	MM8	0			MM28	93	
	MM9	0			MM29	20	
	MM10	0			MM30	0	
Fishermen	MM11	>2400	90%	Hospital patients	MM31	15	100%
	MM12	210			MM32	75	
	MM13	0			MM33	7	
	MM14	1100			MM34	7	
	MM15	7			MM35	150	
	MM16	>2400			MM36	460	
	MM17	120			MM37	210	
	MM18	64			MM38	150	
	MM19	460			MM39	15	
	MM20	150			MM40	460	

For total coliforms, the values ranged from 0 to 2400 MPN per 100 ml. In total, 77.5% of mobile phones of four different categories of people were found contaminated with coliform bacteria which indicated the poor hygienic condition of the mobile phones of the study area. These mobile phones can act as a carrier for coliform transmission. Some of these mobile phones can also be a cause of nosocomial infections. 22.5% of mobile phones were not contaminated with coliform which indicates a relatively good hygienic condition of these mobile phones.

In our result, the mobile phones collected from students were found less contaminated with coliforms (30%). On the contrary, the highest percentage of coliform contamination (100%) was found among mobile phones of hospital patients and 90% from businessmen and fishermen (Table 1). The presence of Gram-negative rod, *Enterobacter aerogens*, a

number of coliform, indicates the possibility of the presence of fecal contamination on mobile phones (Dave and Shende 2015). Our data revealed that students are relatively more aware of the maintenance of hygienic conditions of mobile phones and hospital patients should handle their mobile phones carefully.

To check the mobile phones contamination, bacteria were isolated, identified, and then confirmed by using microscopic, cultural, and biochemical tests (Table 2). After 24 hours of incubation at 37°C, dark colonies with a green metallic sheen and dark colonies without green metallic sheen on EMB agar, colonies that appeared as red or entirely black were on XLD agar, colorless colonies and pink-colored colonies on SS agar, yellow and pink colored colonies on mannitol salt agar plates were picked and purified. The purified isolates were then subjected to

Gram staining along with different morphological and biochemical tests to identify the bacterial strain. Compared with the standard description given in “Bergey’s Manual of Determinative Bacteriology” bacterial isolates were identified up to species level (Buchanan and Gibbons 1974).

From our study, a total of 12 different types of bacteria were isolated from 40 samples collected from students, fishermen, businessmen, hospital patients. We found average percentage of *Klebsiella pneumoniae* (75%) and *Pseudomonas aeruginosa* (58%) were the most prominent bacteria from all types of mobiles followed by *Micrococcus*

sp. (35%), *Staphylococcus aureus* (25%), *Salmonella* sp. (25%), *Bacillus* sp. (10%), *Proteus* sp. (8%), *Staphylococcus epidermidis* (5%), *Serratia* sp. (5%), *Citrobacter* (3%), *E. coli* (3%), and *Enterobacter* sp. (3%) (Table 3). The study is in accordance with the findings of others in which *S. aureus*, *P. aeruginosa*, *E. coli*, *S. typhi*, and *S. epidermidis* were isolated on mobile phones of healthcare workers in Bangladesh (Debnath et al. 2017). From the mobile phones of dentists 52.52% *Pseudomonas* sp. were detected (Lee and Lee 2019) and a higher percentage of *Klebsiella* sp. was identified from mobile phones of healthcare university students (Olu-Taiwo et al. 2021) which supports our results.

Table 2. Microscopic and biochemical properties of the bacteria isolated from drinking water sample of different schools in Chattogram city, Bangladesh

Bacterial Isolates	Shape	Gram stain	Indole	Citrate	Catalase	TSI				Oxidase	MR	VP	Nitrate R.	Urease
						Butt	Slant	H ₂ S	Gas					
<i>E. coli</i>	Rods	-	+	+	+	A	A	-	+	-	+	+	+	+
<i>K. pneumoniae</i>	Rods	-	-	+	+	A	A	-	+	-	-	+	+	+
<i>P. aeruginosa</i>	Rods	-	-	+	+	K	K	-	-	+	-	-	+	-
<i>S. aureus</i>	Cocci	+	-	+	+	A	A	-	-	-	+	+	+	+
<i>S. epidermidis</i>	Cocci	+	-	-	+	A	A	-	-	-	-	+	+	+
<i>Bacillus</i> sp.	Rods	+	-	+	+	NC	A	-	-	-	-	+	+	-
<i>Salmonella</i> sp.	Rods	-	-	+	+	A	K	+	+	-	+	-	+	-
<i>Proteus</i> sp.	Rods	-	+	-	+	A	K	+	+	-	+	-	+	+
<i>Citrobacter</i> sp.	Rods	-	-	+	+	A	A	+	+	-	+	-	+	-
<i>Enterobacter</i> sp.	Rods	-	-	+	+	A	A	+	-	-	-	+	+	-
<i>Micrococcus</i> sp.	Cocci	+	-	-	+	NC	K	-	-	+	-	+	+	+
<i>Serratia</i> sp.	Rods	-	-	+	+	A	K	-	-	-	-	+	+	+

TSI= Triple sugar iron test, A=Acidic (Yellow), K= Alkaline (Red), NC= No change, MR= Methyl red, VP= Voges-Proskauer, + = Positive/Present, - =Negative/Absent, R= Reductase, K- *Klebsiella*, S- *Staphylococcus*, P- *Pseudomonas*, E- *Escherichia*

In the mobile phones of all groups, *Klebsiella pneumoniae* was found with higher percentage, and the highest prevalence was found in fishermen, businessmen, and hospital patients (80%) and lowest in students (60%) (Table 3). *Pseudomonas aeruginosa* was also isolated from mobile phones of all groups with the highest prevalence in fishermen, businessmen, and hospital patients (60%) and lowest in students (50%). It has been proven that many pathogens particularly *P. aeruginosa* remain viable for months on inert surfaces (Kramer et al. 2006). The presence of *Pseudomonas* sp., *K. pneumoniae*, and *E. coli* on mobile phones need serious attention from the public as this organism is associated with hospital infections and may serve as a vehicle for the spread of nosocomial infections (Karabay et al. 2007).

Highest percentage of *S. aureus* was identified from hospital patients (50%) and lowest from fishermen (10%) mobile phones. *S. aureus* is a normal flora of humans, carried on hands, nose, mouth, skin, clothes, bed linen, and other human environments (Melnick and Edward 2004). In our study, *S. epidermidis* was only isolated from the

mobile phones of fishermen (20%) whereas *Serratia* sp., *Enterobacter* sp., *Salmonella* sp., *Proteus* sp., *Citrobacter* sp. and *E. coli* was completely absent in the mobile phones of students.

E. coli is an indicator organism, so its presence on mobile phone indicated that other enterobacteriaceae might be present on mobile phones (Karabay et al. 2007; Tambekar et al. 2008). The highest percentage of *Micrococcus* sp. (60%) and *Salmonella* sp. (60%) was isolated from student's and hospital patient's mobile phones respectively (Table 3). The observations of the present study coincide with the findings of others (Roy et al. 2013). The mobile phones of students of Cape Coast University showed high levels of bacterial contamination like *Bacillus* sp. and *Pseudomonas* sp. (Tagoe et al. 2011). From the mobile phone of health workers, marketers, food vendors, lecturers, students different pathogens like *S. aureus*, *Enterococcus faecalis*, *P. aeruginosa*, *E. coli*, *Klebsiella* sp., *Serratia* sp., *Proteus vulgaris*, and *Bacillus* sp. were frequently isolated (Akinyemi et al. 2009; Kilic et al. 2009; Famurewa and

David 2009; Al-Abdalall 2010; Singh et al. 2010; Gashaw et al. 2014; Olu-Taiwo et al. 2021).

Antibiotic sensitivity assay of 12 different isolates was performed by disc diffusion method, using 10 different types of antibiotics and the results are presented (Table 4). From

the results of the antibiotic sensitivity test, it is observed that most of the organisms were found highly resistant to ampicillin, amoxicillin, erythromycin, and rifampin. On the contrary, most of the organisms were found highly sensitive to ciprofloxacin, gentamicin, azithromycin, and tetracycline.

Table 3. Comparison of percentages of isolated bacteria among samples collected from mobile phones of students, fishermen, businessmen, hospital patients

Isolated Bacteria	Students (%)	Fishermen (%)	Businessmen (%)	Hospital patients (%)	Average (%)
<i>K. pneumoniae</i>	60	80	80	80	75
<i>P. aeruginosa</i>	50	60	60	60	58
<i>S. aureus</i>	20	10	20	50	25
<i>S. epidermidis</i>	0	20	0	0	5
<i>Micrococcus</i> sp.	60	30	40	10	35
<i>Bacillus</i> sp.	10	20	10	0	10
<i>Serratia</i> sp.	0	0	10	10	5
<i>Enterobacter</i> sp.	0	0	10	0	3
<i>Salmonella</i> sp.	0	30	10	60	25
<i>Proteus</i> sp.	0	10	0	20	8
<i>Citrobacter</i> sp.	0	10	0	0	3
<i>E. coli</i>	0	0	10	0	3

Table 4. Antibiotic sensitivity profile of different bacteria from drinking water samples

Bacterial isolates	AMP (10µ)	AMX (10µ)	ERY (15µg)	RMP (5 µg)	TET (30µg)	CRO (30µg)	CAP (30µ)	AZM (15µg)	CIP (5µg)	GEN (10µg)
<i>K. pneumoniae</i>	R	R	R	R	S	S	S	S	S	S
<i>S. aureus</i>	R	R	I	R	S	I	S	S	S	S
<i>P. aeruginosa</i>	R	R	R	R	R	I	R	S	S	S
<i>S. epidermidis</i>	R	R	R	R	I	I	I	I	S	S
<i>E. coli</i>	R	R	R	R	S	R	S	S	S	S
<i>Enterobacter</i> sp.	R	R	R	R	S	I	R	S	S	S
<i>Serratia</i> sp.	R	R	R	R	S	R	S	S	S	S
<i>Citrobacter</i> sp.	S	S	R	R	S	I	I	S	S	S
<i>Micrococcus</i> sp.	R	R	R	R	I	I	R	I	S	S
<i>Bacillus</i> sp.	R	R	R	R	S	I	I	S	S	S
<i>Salmonella</i> sp.	R	I	R	R	S	S	S	S	S	S
<i>Proteus</i> sp.	S	S	S	S	S	S	S	S	S	S

S-sensitive; R-resistant; I-intermediate; AMX-amoxicillin, AMP –ampicillin, ERY-erythromycin, RMP-rifampin, TET-tetracycline, CRO- ceftriaxon, CAP- chloramphenicol, AZM-azithromycin, CIP-ciprofloxacin, GEN-gentamicin, K- *Klebsiella*, S- *Staphylococcus*, P- *Pseudomonas*, E- *Escherichia*

In Bangladesh, for treatment purposes antibiotics are randomly used which are available in any pharmacy. Due to indiscriminate use (misuse, overuse, self-medication) of antibiotics, and not completing the dose suggested by physicians, the antibiotic-resistant bacteria was developed. By genetic recombination, resistant strains might be

developed against antimicrobial agents (Buxton and Fraser 1977). In Kashmir, most of the pathogens like *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *E. faecalis*, and *S. aureus* had already developed resistance to the most commonly used antibiotics including ampicillin. In the valley, multi-drug resistant pneumonia and typhoid are common (Ahmad

2008). *S. aureus*, *E. coli*, and *K. pneumoniae* was found highly resistant to ampicillin by others which supports our results (Olu-Taiwo et al. 2021). Antibiotic drug resistance bacteria may cause high treatment failures, increased healthcare costs, and also increase morbidity and mortality (Brady et al. 2009; Neidell et al. 2012). So, our results revealed that pathogenic bacteria can harbor on mobile phones and some of the isolated bacteria become multi-drug resistant which will help us to select the most suitable antibiotics to fight against these organisms.

CONCLUSION

Mobile phone act as a vehicle for transmitting infectious agents. In the present study, coliform and other bacteria were highly present in the mobile phones of hospital patients which are alarming for the hospital patients and their visitors. On the contrary, students mobile phones were found less contaminated. In all the mobile phones, *K. pneumoniae* and *P. aeruginosa* were present with a huge percentage. Most of the organisms were found highly resistant to ampicillin, amoxicillin, erythromycin and rifampin and highly sensitive to ciprofloxacin, gentamicin, azithromycin, and tetracycline. There are no restrictions for using mobile phones, as a result, pathogens carried by mobile phones cause diarrhea, skin infections, pneumonia, and meningitides. So, we should be aware of limiting mobile phone usage as it has a high risk for spreading infectious agents. Mobile phone users should follow and adopt cost-effective and simple hygienic measures for a safe and healthy life.

ACKNOWLEDGEMENTS

This research was done in the laboratory of the Department of Microbiology, University of Chittagong, Bangladesh. The authors become grateful to the Department of Microbiology.

Authors contribution: MZA designed the project, supervised, contributed in experiments and prepared the manuscript draft, editing and then reviewed the final manuscript. SMM carried out the laboratory experiments and contributed in manuscript draft. Both the authors read and approved the final manuscript.

Conflict of Interest: The authors declare that they have no conflict of interests.

Submission Declaration: The work has not been published previously, approved by another author and responsible authorities where the work was carried out. If accepted, it will not be published elsewhere in the same form, in English or in any other language, without the written consent of the copyright-holder.

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Agricultural Communication

Role of Growth-Promoting Bacteria as Biocontrol Agent Against Root Knot Nematode of Tomato

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ABSTRACT

There are significant losses which have occurred in crops due to the infestation of plant parasitic nematode which are known as hidden enemy due to their presence in rhizosphere and their infection site on the roots. Synthetic nematicidal control is an effective strategy to combat this biotic stress but an inappropriate and deficient application of chemical pesticides have an adverse effect on soil micro-flora and fauna. Due to the environmental and regulatory pressure, use of potential biocontrol agents is the new approach for nematode management by the farming community. For this study, four potential rhizobacteria from different habitats BHU1, BHU2, BHU3 and BHU4 were assessed for their antagonistic activities against *Meloidogyne incognita* infecting tomato plant. These were characterized on the basis of their morphological and biochemical activities. In vitro screening of bacterial isolates was conducted in a 25-microwell plate by addition of second stage juvenile (J2) of *M. incognita* with nematode application. Among four bacteria, three potential antagonistic bacteria were able to kill nematode within 24 hours. Mortality percentage of J2 *M. incognita* observed in sterile distilled water selected bacterial isolates ranged from 23.33 to 100% in 3h to 24h periods. Moreover, all bacterial isolates except BHU2 isolate were found positive for production of extracellular enzymes like catalase, oxidase, chitinase, amylase and gelatinase which favour effective biopesticide activity of bacteria. Further selected isolates of bacteria associated with tomato have shown a great potential as biocontrol agents against root-knot nematode in tomato during pot experiment. Based on the fact stated above, the current research focused on plant growth promoting rhizobacteria based nematodes biocontrol strategies with direct and indirect mechanism of PGPR for nematode management.

KEY WORDS: BIOCONTROL, KNOT-ROOT, LYCOPERSICON ESCULENTUM, MELOIDOGYNE INCOGNITA, PGPR.

INTRODUCTION

Rhizosphere is one of the most crucial zones where plant and microbes interact symbiotically. In this aspect, plants mainly benefit from microbial consortium for acquiring nutrients as a vital mineral and in return providing habitat to micro-organism. Root-knot nematodes (RKNs) are mainly involved in causing damage to vegetable crop and estimated loss upto 21.3% to agricultural products annually. *M. incognita* is highly destructive plant parasitic nematode

with rapid multiplication in seasonal vegetable crop likes tomato, brinjal, okra, spinach etc (Razavi et al. 2017; Kumar et al. 2020).

M. incognita penetrate epidermis of root and produced several hydrolytic enzymes from stylet that degrade cell wall of plant root in order to enter through plant and not only caused diseases but also restricted water and minerals uptake of plants, due to hindering biochemical, physiological and morphological status of plants. Farmers recognized insect pests and other constraints as production problems but overlooked plant parasitic nematodes due to its indirect effect on the plants. Nematode diseases are difficult to control when it enter in the root system hence pre adaptive measures are more successful to control them rather than post adaptive

Article Information:*Corresponding Author: rakeshsingh@bhu.ac.in

Received 15/10/2021 Accepted after revision 13/12/2021

Published: 31st December 2021 Pp- 1557-1563

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.29>

management practices. Plant parasitic nematodes not only cause damage individually but form disease-complexes with other microorganism and increased the crop losses (Etesami and Adl 2020).

In rhizospheres several beneficial micro-organisms *viz* bacteria fungi actinomycetes etc are found which control the nematode population naturally. In natural rhizospheric zone many plant growth promoting bacteria having very good growth promotion activities and potential biocontrol ability have been found. Which have direct mechanism for plant growth with mineral solubilization, phytohormone production while indirect mechanism with production of hydrogen cyanide, siderophore, antibiotic production and also have induced systemic resistance activities. Rhizobacteria are reported to produce diverse range of bioactive chemical which promote growth of plant and provide protection against many phytopathogen including *M. incognita* (Khanna et al. 2019; Liu et al. 2019; Etesami and Adl 2020).

Tomato is one of the most susceptible vegetables against root knots, nematodes mostly causing 30 to 40 % yield losses in tropical regions, and also it has been also found that *M. incognita* is responsible in deteriorating the quality of fruit and yield of tomatoes, (El-Shawadfy 2021). Among the various microorganisms, plant growth promoting rhizobacteria have attracted considerable attention for their usefulness in biofertilizer and biological control ability. Hence the current research work was focused on their biofertilizer ability as well as on the eco-friendly management of *M. incognita* by plant growth promoting traits for enhancing plant defense mechanism of tomato seedlings, raised under nematode infested soil as previously reported by El-Ashry et al. (2020) and Zao et al. (2021).

MATERIAL AND METHODS

Rhizospheric soil samples were collected from agricultural field of rice, tomato, oat and barley growing at four districts *viz* Varanasi, Mirzapur, Chunar and Jaunpur of Uttar Pradesh in India. Collected soil samples were kept in sterilized plastic bags at 4°C. Serial dilution technique was used for screening of bacterial colonies on King's B medium. Serial diluted plates were incubated at 30°C for 48-72 hours (h) and bacterial colonies were isolated, maintained as pure cultures on King's B media. For the morphological and biochemical characterization of growth promoting bacteria, isolated bacteria were identified firstly by macroscopic observation i.e., gram reaction, colony morphology, pigmentation, mobility and cell shape followed by several biochemical and enzymatic test *viz* Catalase test, Oxidase, Chitinase, Amylase and Gelatinase (Hayward 1960; Blazevic and Ederer 1975; Collins 1980; Fadden 1980; Somasegaran and Hoben 1994; Kaur et al. 2012).

Qualitative assay of phosphate solubilizing activity of bacteria was measured by Pikovaskaya on Pikovaskaya's Agar Medium and clear transparent zone around the bacterial colony confirmed phosphate solubilization activity within 24 to 72 h (Pikovaskaya 1948). Indole acetic acid (IAA) Production was detected as described by Bric et

al. (1991). Appearance of pink colour in broth by adding of Salkowaski reagent indicated IAA production. For the quantification of IAA, absorbance was taken at 540 nm by using UV/visible spectrophotometer (Bric et al. 1991).

HCN production was evaluated on Whatman filter paper No.1 soaked with picric acid (0.05%) solution in 2% sodium carbonate and placed in the test tube sealed with parafilm and incubated. A colour change of the filter paper from deep yellow to reddish-brown colour at 30°C for 48-72 h indicated HCN production and quantified (Baker et al. 1987). Siderophore production of bacterial isolates was detected by observing orange halo zone around the bacterial colony on chrome azurol S agar media after 72 h of growth. Quantification of siderophore was calculated in µg/ml (Schwyn and Neilands 1987).

For the preparation and screening of bacterial strains against *M. incognita*, nematode inoculum was prepared from large numbers of egg masses by hand-picked using a sterilized forceps from heavily infected tomato roots. These egg masses were washed in distilled water and placed in 10-cm diameter 15 mesh sieves. The hatched juveniles were collected and incubated in a micro-well plate containing 3 ml sterile distilled water for at 25°C. For the screening of bacterial isolates having ability to kill *M. incognita in vitro*, four bacterial isolates were screened for their nematode killing ability against *M. incognita* in microwell plate (25 wells). Heavy cell suspension for each bacterial isolates was prepared (1×10^6 cfu/ml) in sterile distilled water using a UV/Visual spectrophotometer. Each well contained 100 (J_2) of *M. incognita* in 1ml of sterile distilled water with 10µl/ml of bacterial suspension. Mortality of *M. incognita* was evaluated at 3, 6, 12 and 24 h time interval.

For the pot experiment, all the four promising bacteria were inoculated on tomato seeds (variety Kashi Abhiman) by seed biopriming. Seeds were soaked with bacteria containing 10^9 CFU/ml for overnight. Bioprimed seeds were sown in pots containing 1 kg sterilized soil followed by 2000 J_2 population of *M. incognita* in each pot. Subsequently single plants per pot was maintained at 60% water holding capacity and plants were uprooted after 21 days. Seed germination and vigour index were observed by towel method. Leaf area was calculated by using following formula (Yashida et al. 1972; International Seed Testing Association 1993). The experiment was performed in completely randomized block design. Statistical significance between the treatments was compared by least significant difference test at $P < 0.05$ probability level on different growth promoting parameters using SPSS16.0 software.

RESULTS AND DISCUSSION

Four potential bacteria *viz* BHU1, BHU2, BHU3 and BHU4 were isolated from different rhizospheric locations (Table1) and their morphological characteristics of rhizobacterial isolates BHU1, BHU2, BHU3 and BHU4 showed variation in colony, shape, colour, pigmentation, colony surface and margin. Bacterial strain was identified on the basis of morphological appearance of bacterial colony and their

biochemical performance which is mention in table 1. BHU1 showed green pigmentation while BHU3 isolates

showed yellow pigmentation on YEMA agar media with rough and shiny surface (Table2).

Table 1. Isolation of bacteria from rhizospheric soil from different locations

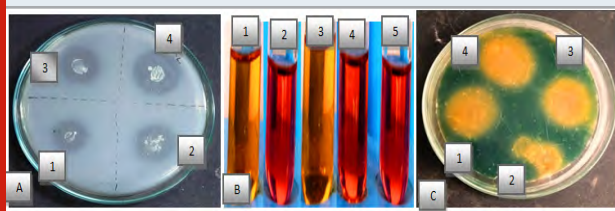
S.No.	strains	Location	Rhizospheric soil associated with Crops
1	BHU1	Varanasi (25.3176 N, 82.9739 E)	Rice (<i>Oryza sativa</i>)
2	BHU2	Mirzapur (25.32N, 83.33E)	Tomato (<i>Lycopersicon esculentum</i>)
3	BHU3	Chunar (25.1037 N, 82.8721 E)	Oat (<i>Avena sativa</i>)
4	BHU4	Jaunpur(24.240N, 82.70E)	Barley (<i>Hordeum vulgare</i>)

Table 2. Morphological characterization of selected isolate

Ss. No.	Bacteria	Strain	Gram stain	Colony Shape	Colony Colour	Pigmentation	Colony Surface	Colony Margin
1	<i>Pseudomonas fluorescens</i>	BHU1	-ve	Round	Yellow	Green	Smooth	Entire
2	<i>Enterobacter</i> spp	BHU2	-ve	Biconcave	White	No pigmentation	Rough	Entire
3	<i>Bacillus</i> spp	BHU3	-ve	Circular	Creamy white	Yellow	Rough	Undulate
4	<i>Burkholderia</i> spp	BHU4	-ve	Circular	Yellow	No pigmentation	Rough	Entire

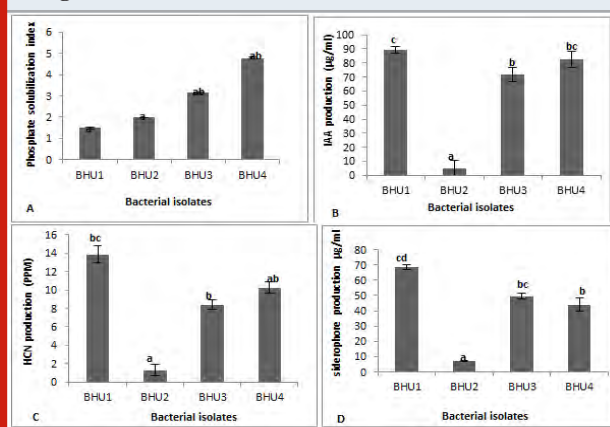
Bacterial strain shows various type of biochemical characterization like phosphate solubilization, indole acetic acid, HCN and siderophore production (Table 3). Selected bacteria showed phosphate solubilization activities on Pikovaskaya's media by forming clear transparent zone around the bacterial colony. Among four isolates highest phosphorus was solubilized by BHU4 strain (Figure1&2A). Phosphorus is a major nutrient required for plant growth and development but plants are unable to utilize phosphate because 95-99% phosphate found in the soil in insoluble form. Making phosphorous in available form for plants is an important trait for PGPR selection (Shahzad et al. 2008).

Figure 1: Biochemical characterization of PGPR Phosphate solubilization (A), IAA production (B), Siderophore (C)



Some PGPR have been reported to produce citrate, lactate, succinate activity that dissolve the mineral phosphates as a result of anion exchange or chelation of Fe and Al ions associated with phosphate. This leads to an increase in the soluble P in the rhizosphere resulting its more uptake by the plants. IAA is important phytohormones which stimulatory effect on plant growth under biotic stress condition. Among four bacteria, three bacteria produced IAA in tryptophan yeast broth. Highest IAA production was recorded by BHU1 (89µg/ml) followed by BHU4 (82 µg/ml) and minimum IAA production by BHU2 (5 µg/ml) production (Figure 1&2B) (Shahzad et al. 2008).

Figure 2: Biochemical characterization of growth promoting bacteria (A) P-solubilization, (B) IAA production, (C) HCN production and (D) Siderophore production. Significant of variance ($p < 0.05$) has been analyzed by Duncan Multiple Range Test.



HCN producing bacteria (BHU1, BHU3 and BHU4) showed antagonistic activities against phytopathogen three bacteria produced HCN with highest HCN production 13ppm by BHU1 (Figure 2C). HCN production is a type of volatile organic compounds (VOCs) toxic for phytopathogenic nematodes and provides protection from various biotic stresses. As the plants exposed to nematode infection grow weak in their defense system hence shielding them by HCN producing bacteria which can be helpful in overcoming the chances of pathogenic infection. Siderophore production was confirmed by the change of CAS broth colour to brown colour (Fig. 1C). Three bacteria (BHU1, BHU3 and BHU4) were observed producing siderophore and highest siderophore production was observed by BHU1

(68µg/ml) (Figure 2D). Siderophores are low molecular weight iron chelating compound which restrict the growth of phytopathogen, indirectly by depriving fungal pathogens






and plant parasitic nematodes from iron uptake and supported plant growth by iron supply resulting in increased dry biomass of plant and rootlet elongation (Cheng et al. 2017; Sahebani and Gholamrezaee 2021).

Table 3. Extracellular enzyme production by selected bacteria

S. no	strains	Catalase	Oxidase	Chitinase	Amylase	Gelatinase
1	BHU1	+++	+	+	+	+
2	BHU2	+	-	++	-	-
3	BHU3	++	++	+	++	++
4	BHU4	+++	+++	+++	+++	+++

(-) no production, (+) weak, (++) moderate and (+++) strong

Table 4. Mechanism of antagonistic bacterial infection inside the body of *M. incognita*

S.No.	Treatment	Microscopic study of nematode	Note
1	Control	 (J ₂)/ 100 ml of sterile water	Nematodes were alive and move freely
2	BHU1	 (J ₂)/ 100 ml of sterile water + 5 ml of bacterial suspension (1x10 ⁶ cfu)	Nematodes' body regions were lysed.
3	BHU2	 (J ₂)/ 100 ml of sterile water + 5 ml of bacterial suspension (1x10 ⁶ cfu)	Nematodes were alive and move freely
4	BHU3	 (J ₂)/ 100 ml of sterile water + 5 ml of bacterial suspension (1x10 ⁶ cfu)	Nematode was dead with an intact body
5	BHU4	 (J ₂)/ 100 ml of sterile water + 5 ml of bacterial suspension (1x10 ⁶ cfu)	Nematode was dead with an intact body.

Extracellular enzyme activities are important parameter for selection of growth promoting bacteria and also favour strong evidence for improve defense mechanism. Selected bacteria were showing positive catalase activity, maximum catalase production was observed by BHU1 and BHU4. The oxidase test used to identify bacteria that produce cytochrome C oxidase, an enzyme of the bacterial electron transport chain. The reagent N, N-dimethyl-p-

phenylenediamine is a dark-blue to maroon color when oxidized and become colorless when reduced. BHU4 was observed strongly positive for oxidase test. Chitinase enzyme serves various functions such as antagonistic activity against chitin-containing pests and in nutrient cycling. BHU4 strain produced chitin followed by BHU2. Gelatin hydrolysis test is used to detect the ability of an organism to produce gelatinase which is a type of proteolytic enzyme that liquefy gelatin hydrolysis of gelatin indicates the presence of gelatinase (Subedi et al. 2020).

Maximum gelatin was produced by BHU4 bacteria followed by BHU2 (Table3). Three bacterial isolates BHU1, BHU3 and BHU4 had greater efficiency to produced lytic enzyme like catalase, oxidase, chitinase amylase and gelatinase. Calalase and oxidase enzyme reduce the oxidative damage while α -amylase enzyme in the aleurone layer helping in hydrolyzing the endosperm starch into sugars, which provide the energy for vigour index parameter. Other important mechanisms include production of extracellular lytic enzymes, such as CHI, β -1,3-GLU and protease, which lyse chitin and glucan (present in the cell wall of fungi) and protein in the nematode cuticle (Lee et al. 2015; Gupta et al. 2017; Subedi et al. 2020; Sahebani and Gholamrezaee 2021).

When these bacteria were examine for their antagonistic properties against *M. incognita* three bacteria BHU1, BHU3 and BHU4 kill nematode but BHU2 did not show ability to kill it (table 4 and 5). Bacteria BHU1 and BHU3 kill *M. incognita* 100% and 93% respectively after 12 hours. The bacterial mechanism of infection caused the lysis of nematode *M. incognita* particularly on the oesophageal region (BHU1) and intact body (BHU3) which is mention in table 4 and 5. In many studies it has been found that growth promoting microbes produced various types of secondary metabolites and volatile organic compound (VOC) at infected region. Antagonistic bacteria tend to prohibit the invasion of RKNs into the roots and maintain the balance of nutrients and mineral status in plants moreover, growth promoting bacteria also control the gall formation and maintain the vascular integrity in order to boost the physiology and biochemistry of the plant. Consequently, the most vital mechanism to prevent gall formation through

PGPR's by production of nematicidal compound like hydrogen sulfide, ammonia, butyric acid and degradation of nematode attractants of root exudates. Furthermore, they

bind to the root surface and produced hydrolytic enzyme that can kill the nematode (Safni et al. 2018; Sahebani and Gholamrezaee 2021).

Table 5. Mortality percentage of *M. incognita* hours after inoculation

S.No.	Treatments	Mortality percentage of <i>M. incognita</i> hours after inoculation			
		3 h	6 h	12 h	24 h
1	Control	0±0a	0±0a	0±0a	0±0 ^a
2	BHU1	33.33±3.9 ^b	66.66±4.5 ^c	100±0 ^d	100±0 ^b
3	BHU2	0±0 ^a	0±0a	0±0 ^a	0±0 ^a
4	BHU3	23.33±2.8 ^b	46.66±3.4 ^b	93.3±6.9 ^{cd}	100±0 ^b
5	BHU4	23.33±2.8 ^{ab}	56.66±4.8 ^{bc}	76.66±6.4 ^b	100±0 ^b

Values are mean of three replicates SE. Significant of variance ($p < 0.05$) is analyzed by Duncan multiple range test

Multiple Range Test: Biopriming tomato seed with bacteria showed higher root length, shoot length, vigour index and reduced gall/ knot formation after 21 days of seed germination. In the current study, it has been found that *M. incognita* cause root knot disease in tomato seedling resulted in declined root length, shoot length, fresh weight and dry weight, leaf area, leaf number of tomato seedlings. This reduction is due to the formation of root knot in the root leads to the reduction in the growth promotion due to disruption of root system of tomato plant and inhibition of nutrient uptake from the root system (Safni et al. 2018; Sahebani and Gholamrezaee 2021).

Biopriming tomato seed with bacteria showed higher root length, shoot length, vigour index and reduced gall/

knot formation after 21 days of seed germination. Plants inoculated with BHU1, BHU2, BHU3 and BHU4 strain exhibited significant increase in root and shoot length compared to the un-inoculated control. Maximum root length and shoot length of tomato plant was observed by BHU1 strain followed by BHU4 bioprimed bacteria as compare to control plant i.e. 475% root length increment and enhanced shoot length by 6.74cm. Highest fresh root weight as compare to untreated plant infested with *M. incognita* and shoot weight was enhanced upto 2.36g by BHU1 strain over the control plant (Figure 3). Similarly seed germination and viour index reduced in tomato crop under *M. incognita* infection which is a type of biotic stress (Khanna et al. 2019; Sahebani and Gholamrezaee 2021).

Table 6. Effect of selected isolates in tomato plants under nematode infection

S. No.	Treatment	Root length (cm)	Shoot length (cm)	Root Weight (g)	Shoot weight (g)	Root Dry weight (g)	Shoot dry weight (g)	Leaf area (cm ²)	No. of leaf	Germination (%)	Vigour index	Number of gall (knot)/ root
1	Control	4.63±0.1 ^a	3.28±0.2 ^b	0.08±0.0 ^a	0.39±0.0 ^a	0.01±0.0 ^a	0.030±0.0 ^a	2.36±0.03 ^a	12±0.57 ^a	42.22±13.5 ^a	335.1±28 ^a	51±2.6 ^b
2	BHU1	26.65±0.4 ^d	10.02±0.1 ^{bc}	0.41±0.0 ^c	2.36±0.09 ^d	0.04±0.0 ^c	0.17±0.0 ^{ab}	5.30±0.1 ^d	20±0.57 ^d	100±0.0 ^d	3880.66±9.13 ^b	0±0.0 ^a
3	BHU2	9.46±0.2 ^a	4.91±0.1 ^a	0.25±0.02 ^b	0.77±0.02 ^b	0.02±0.0 ^a	0.08±0.0 ^{ab}	2.77±0.03 ^{ab}	14.66±0.33 ^{ab}	57.77±8.8 ^b	837.5±52 ^c	63±6.5 ^{ab}
4	BHU3	14.30±0.3 ^{ab}	8.21±0.1 ^b	0.38±0.02 ^{ab}	1.26±0.0 ^{cd}	0.03±0.0 ^{ab}	0.07±0.0 ^b	4.57±0.05 ^{cd}	16.33±0.08 ^b	80±0.0 ^{cd}	1836±19 ^{cd}	0±0.0 ^a
5	BHU4	22.14±0.6 ^c	8.97±0.2 ^b	0.38±0.03 ^{ab}	1.76±0.1 ^c	0.03±0.0 ^{ab}	0.015±0.0 ^b	5.29±0.09 ^c	18.66±0.8 ^{bc}	93.33±6.6 ^d	2956±28.23 ^d	0±0.0 ^a

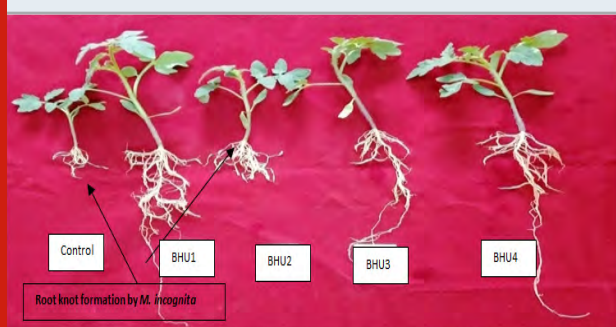
Values are mean of three replicates SE. Significant of variance ($p < 0.05$) is analyzed by Duncan Multiple test range

Growth promoting attributes like nutrient mobilization and hormone production maintained even under stressed condition by BHU1, helped in enhancing the root growth, nutrient and water uptake and augmenting the dry weight.

Highest root and shoot dry weight were observed by BHU1 and BHU3 strain as compare to control plant. High phosphate solubilizing activity of BHU1 more than 4.8cm zone of clearance must have favored the plant growth

directly and depicted as enhancement of fresh and dry root weight and shoot weight respectively and the leaf area had significantly increased and the treated plant with BHU1 and BHU4 was observed to impart more leaf area with 124% increment as compare to control plant. Biopriming of seed with selected bacteria significantly enhanced percentage of seed germination ranged from 57-100%. Maximum vigour index 3880 as compare to untreated plant was observed in BHU1 treated seed which is a very important parameter for promotion of plant growth (Table 6). While almost similar type of result was also observed in tomato by bacterial induction of resistance in tomato against root-knot nematode *M. javanica* with biocontrol agents (Zhai et al. 2018; Dey and Raghuwanshi 2020; Mazrou et al. 2020).

Figure 3: Effect of selected isolates in tomato plants under nematode infection.



CONCLUSION

The finding of the present study suggests that BHU1 bacterial strain have a very good potential to augment plant growth activity and potential biological control managing root knot nematodes in tomato and in future leads to incorporate compatible other microorganism enhancing their positive attributes to replace chemical application with promising biocontrol agent. While studying the rhizospheric microorganism and their interaction with plant under biotic stresses there are chances to recognized some microorganism which can enhance directly or indirectly defense immunity in the plant. The performance of individual bacterial strain will always lead to screen potential microorganism for harnessing them to mitigate losses occurred due to biotic stresses.

ACKNOWLEDGEMENTS

This study was financially supported by Banaras Hindu University, Varanasi, Uttar Pradesh, India.

Conflict of Interests: Authors have no conflict of interests to disclose.

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Toxicological Communication

On the Phytotoxicity of Waste-Water from Textile Industry on Selected Crops Seed Germination and its Treatment Using Bacteria and Zinc Oxide Nanoparticles

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ABSTRACT

Industrialization plays a major role for the economic development of any nation. In spite of various positive aspects of industrialization, the foremost negative aspect is pollution by discharge of more waste water in to the environment. So, the aim of the present work is to analyse the phytotoxic effect of textile industry waste water on seed germination and treating the waste water using Bacterial Species and Zinc Oxide Nanoparticle. Physico-chemical parameters of dyeing industry waste water such as color, temperature, pH, conductivity, turbidity, total dissolved solid, total hardness, COD, BOD, oil and greases, chloride, sodium, potassium, chromium, copper, total alkalinity and zinc were analysed as per standard methods. To confirm the harmfulness, an investigation was made to study the degree of toxicity of dyeing industry effluent on seed germination and growth of *Sorghum bicolor* (white sorghum) *Vigna unguiculata* (cow pea) were selected for this study and it was placed in soil containing pots and watering with untreated industry waste water and treated waste water. The waste water was treated by two methods, one by bacterial degradation, using *Pseudomonas fluorescens* and the secondly, by chemical oxidation, using zinc oxide nanoparticles. After treatment their efficiency was tested with above two plants. All the above said parameters were found to be high in untreated waste water. There was a gradual decrease in the percentage of seed germination and seedling growth due to higher concentration of effluent, when compared with control. In this comparative study, dye degradation (methyl orange) degradation by the chemical oxidation using zinc oxide nanoparticle was more effective and faster than the biological oxidation of bacterial species.

KEY WORDS: DYE DEGRADATION, INDUSTRIALIZATION, MICROORGANISM, NANOPARTICLE, PHYTOTOXICITY.

INTRODUCTION

Water contamination is a current environmental concern that has caught a lot of attention throughout the world. Textile dyeing and finishing industry effluents are the major source of water contamination. The textile industry faces a challenge in using large quantities of dyes and auxiliaries that are required for current textile processes due to substantial changes in client demands (Mamatha and Beena 2018). Factories discharged a wide range of pollutants, many of which contain chemical elements in higher concentrations to harm the surface and ground water supplies (Khan and Malik 2018; Bharagava et al. 2020).

The waste water treatment system in Indian companies was substantially established to fulfil the waste water discharge regulations, but only 10% of the waste water is now treated, and the rest of the untreated water discharged into neighbouring water bodies. The major industrial pollutants are industrial effluents, which contain organic and inorganic chemicals, acids, alkalies, suspended particles, and other recalcitrant compounds (Mehta and Bhardwaj 2012). However, dumping of untreated effluents into the environment affects the ecological niches of living organisms (Bharagava et al. 2020). Municipalities from government sector, even private entities face a serious of difficulty with wastewater disposal, particularly in large urban areas with limited space for land-based treatment and disposal. But still, they are taking corrective steps to make safer (Naveenraj et al. 2021).

The textile mills daily discharging millions of liters of untreated effluents loaded with synthetic dyes into public

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Received 15/10/2021 Accepted after revision 17/12/2021

Published: 31st December 2021 Pp- 1564-1570

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.30>

wastewater stream that eventually drain into water bodies. This can lead to acute toxicity of aquatic ecosystem. This also impacts water quality by changing the pH and increasing the Biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) (Imtiazuddin and Tiki 2020). Natural dyes come from a variety of sources, including vegetables, plants, minerals, insects, and animals. But synthetic dyes are made from organic molecules; they may be produced in a consistent manner. These dyes will linger in the environment for a long time if they are not properly treated (Olukanni et al. 2006). Among other dyes, Azo dyes are the greatest group of colorants in terms of number and production volume, accounting for 60-70 percent of all organic dyes produced worldwide (Carliell et al. 1998; Bafana et al. 2011; Imtiazuddin and Tiki 2020).

The success of azo dyes is owing to their ease and low cost of synthesis when compared to natural dyes, as well as their structural variety, high molar extinction coefficient, medium to high light and moisture fastness qualities. They're employed in a variety of industries, including textiles, pharmaceuticals, and cosmetics, as well as food, paper, leather and paints industries (Chung et al. 1992; Mansour et al. 2007; Seesuriyachan et al. 2007). Azo dyes have an azo bond ($-N=N-$) in their basic structure, which is difficult to breakdown naturally. Depending on the number of azo groups, this class of dye is divided into mono azo, di azo, tris azo, and poly azo dyes (Engel et al. 2008). Reactive azo dye is resistant due to their stability, xenobiotic nature, and it is not completely destroyed by standard treatments such as light, chemicals, or activated sludge. (Grekova-Vasileva et al. 2009; Imtiazuddin and Tiki 2020). Hence in the present study focused on treatment of textile industry waste water containing Azo dyes by using bacteria and Nanoparticles.

Waste water, on the other hand, is a resource that can be used for productive purposes because it includes nutrients that can be used in agriculture and aquaculture as well. As a result, wastewater can be viewed as a resource on one hand and a problem on the other. The use of industrial and domestic sewage effluents for irrigation has recently become a popular method of repurposing waste water, owing to the availability of significant amounts of N, P, K, and Ca, as well as other critical nutrients (Niroula 2003, Ali et al 2020).

Its repurposing could have a positive impact on the rural community. Another advantage of reusing wastewater for irrigation is water conservation; due to water shortage, industrial effluents are employed as a source of irrigation for crops; however, indiscriminate effluent uses, ignores the fact that untreated effluent can have severe effects on crop development and quality. Therefore, it is necessary to study the impact of these effluents on crop system before they are recommended and used for irrigation (Hari et al. 1994; Thamizhiniyan et al. 2009). The present investigation has been carried out to study the degree of toxicity of dyeing industry effluent on seed germination and development of *Sorghum bicolor* (white sorghum) *Vigna unguiculata* (cow pea). These crops we are commonly cultivating in agriculture land.

MATERIAL AND METHODS

The effluent samples were collected from dyeing industry in plastic container located at Srivilluputhur, Tamil Nadu (Fig-1 20L water sample). After collection, the effluent was immediately transported to the laboratory for analysis. Physico-chemical parameters such as colour, temperature, pH, conductivity, turbidity, total dissolved solid, total hardness, COD, BOD, copper, chloride; chromium, sodium, total alkalinity, potassium and zinc were analyzed as per the standard methods (APHA 2012).

The zinc oxide nanoparticle was obtained by the precipitation method. In a beaker 7.4g of zinc nitrate was taken in 50ml of water. In another beaker 2.65g of Sodium carbonate was taken in 50ml of water. Then sodium carbonate was added drop by drop to the solution of zinc nitrate. This mixture was stirred nearly 15-20min to get mixed together. Then it was filtered using the Whatman filter paper to filter the precipitate. The precipitate thus formed was washed with the water for 2 to 3 times to remove the minute particles. After washing with water, the precipitate was again washed with ethanol to remove the moisture present. Then it was dried in a hot air oven for 90-100 degree for overnight period of 4-5 hrs. Thus the moisture was fully removed and obtained a powdered substance. Finally powdered substance was kept in a muffle furnace at 300°C for 2hrs (Fig -2).

For the characterization of zinc oxide nanoparticles, four main methods were considered.

- FT-IR (Fourier transform infrared spectroscopy)
- XRD (x-ray diffraction)
- SEM (scanning electron microscope)
- UV-DRS (UV-Vis diffuse reflectance spectroscopy)

The methyl orange was degraded by using the zinc oxide nanoparticle. The zinc oxide nanoparticle was taken as a catalyst for the degradation process. 250 ml of textile dyeing waste water was taken into a conical flask with the addition of 0.5g catalyst zinc oxide nanoparticle. This was kept under the sunlight for 24hrs and the stirring done after each 15min which it undergoes with the photo catalytic reaction. The zinc oxide nanoparticle added to the waste water degrades and decolorizes the compounds present in it. Then the absorbance of degraded waste water was measured by using UV-visible spectrophotometer. The degradation of methyl orange was done by bacteria. Bacterial species was cultured (*Pseudomonas fluorescens*) using minimal media. Then the cultured bacteria were added in to the waste water taken in a different concentration as 1, 2, 3, 4, 5µl in 5ml. Thus it was kept in an incubator for overnight and the absorbance was measured for initial, 3rd and 6th day concentration. From the absorbance efficiency of degradation was noted.

To study the effect of untreated and treated waste water on seed germination and growth, the healthy and uniform seeds of *Sorghum bicolor* (White sorghum) and *Vigna unguiculata* (cow pea) were selected and surface sterilization done with 0.1% HgCl₂ and thoroughly washed with distilled water to avoid surface contamination. Germination experiments were carried out (Fig-3) in two set of soil containing pots (one pot for white sorghum and other pot for cow pea).

One set irrigated with untreated waste water and another set of pots irrigated with treated industrial waste water. The germination was observed and recorded after 48 hours.

RESULTS AND DISCUSSION

It is important to study all features of the textile effluent to improve environmental performance and also to sustain considerable quality of the individual companies. Below are the images of nanoparticles collected with the help of precipitation method and seed germination done to initiate the experiment.

The Physico- Chemical parameters of textile effluent have been analysed and the experimental results were compared with standard BIS limits. The results of the analysis are presented in table -1.

Effect of effluents on seed germination: Experiment on *Sorghum bicolor* and *Vigna unguiculata* showed (Fig -4 a, b) an inhibitory effect of untreated industrial effluents on seed germination and its early growth.

Figure 1: Waste water collected from industry



Waste water treated with Zinc oxide nanoparticle (Fig -5a, b) and bacterial species (Fig -6a, b) used for irrigating the seed containing pots (*Sorghum bicolor* and *Vigna unguiculata*).

Figure 2: Nanoparticle obtained from precipitation method



The colour of the effluent was key issue in textile industry, and it was widely recognized as a important pollutant in wastewater. In fact, even at low concentrations, colour in effluent is easily visible to human eyes, and no one likes the appearance of unclean water (Ibrahim et al. 1996; Wijetunga

et al. 2010). The effluent selected for the present study was very dark brown to black color, with observed value 70 hazen, it was higher than value of BIS (25 hazen). This value indicates that the effluent was highly colored due to presence of different dyes, colour producing compounds, metals, pH of the effluent, temperature during dyeing and bleaching of fabrics. Color reduces aquatic life's photosynthetic activity and has an impact on other metrics such as DO, BOD and others. In general, traditional treatment methods have a hard time to removing colours (Forgacs et al. 2004; Przystaś et al. 2012; Hubbe et al. 2012; Imtiazuddin and Tiki 2020).

Figure 3: Seed germination study



Table 1. The Physico-chemical parameters of dyeing industry effluent

Parameters	Observed value	BIS Value
Colour , Hazen	70	25
Temperature	29.1	50
pH@25°C	6.75	5.5-9
Conductivity, mS/cm	2822	300
Turbidity, NTU	266	10
Total dissolved solid , mg/ L	3404	2,100
Total hardness , mg/L as CaCO ₃	188	500
Chemical oxygen demand, mg/L	300	250
Biological oxygen demand, mg/L	128	100
Oil and greases, mg/L	16	10
Chloride , mg/L as Cl	616	600
Sodium, mg/L	234	200
Potassium, mg/L	43.8	12
Chromium , µg/L	BDL(DL1:0.5)	100
Copper , µg/L	0.047mg/L as cu	3000
Total alkalinity	138 mg/L as CaCO ₃	
P-Alkalinity	nil mg /l as CaCO ₃	
Zinc, µg/L	BDL(DL1:0.02) mg/l as Zn	5000

The waste water temperature observed to be 29.1°C. The acidic and basic nature of the effluent can be identified by pH value and also it determines the presence or absence of

various ionic species of the textile effluent. The pH value of the dyeing industry effluent was found to be little acidic pH 6.75 but it was within the permissible limit prescribed by BIS (pH 5.5-9). Although no health-based guideline was proposed for pH but sometimes, eye irritation and other skin disorders are associated with values of pH greater than 11, but in our result found within the BIS permissible limit. Joshi and Kumar (2011) observed the pH of textile effluent ranged from 7.6-7.9 whereas another study reported the pH of dye industry effluent ranged between 8.2 and 9.0 (Joshi and Kumar 2011; Ahmad et al. 2012; Farid et al. 2012; Imtiazuddin and Tiki 2020).

Figure 4: Seeds containing pots irrigated with untreated waste water

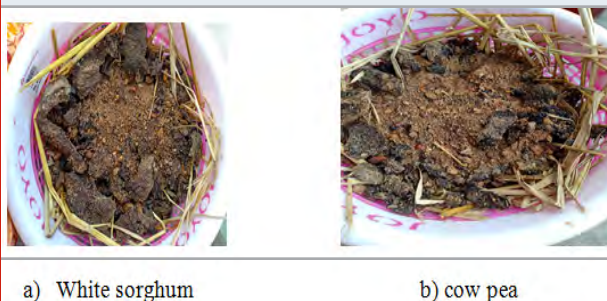


Figure 5: Seed containing pot irrigated with Zinc oxide nanoparticle treated waste water



Figure 6: Plants irrigated with *Pseudomonas fluorescens* treated waste water



Electrical Conductivity is an important physical parameter to measure the sodium hazard of water quality. The Electrical Conductivity found in the effluent (2822 mS/cm) was greater than that of the permissible limit of BIS (300 mS/cm) this may be due to the continuous discharge of the chemicals and salts used along with dyes in the industries. Our results in accordance with Saravanan et al. (2012) they reported that electrical conductivity of tannery industry effluent was above the accepted limits (Saravanan

et al. 2012). Turbidity is to measure the degree, in which the water loses its transparency due to the presence of suspended particulates. High intensity of scattered lights it means that higher turbidity. The value of turbidity was recorded as 266 NTU which have been found to be much higher than the BIS limit (10 NTU). This may be due to more colour, more total suspended solids and oily scum incorporate with colloidal matter increases the turbidity which gives bad appearance and foul smell. Thus, turbidity should be measured and treated carefully before disposal (Elango 2017; World Health Organization 2017; Bharagava et al. 2020).

Inorganic salts (bicarbonates, chlorides, and sulphates of calcium, magnesium, potassium and sodium) and minor amount of soluble organic matter make up Total Dissolved Solids (TDS). As a result, TDS is equal to the total number of cations and anions in water. Although TDS is not generally regarded a main pollutant, it is utilised as an indicator of aesthetic features of drinking water as well as an aggregate signal of the presence of a wide range of chemical pollutants. The value of TDS ranges from 2320 to 9840, indicating the presence of high amount of inorganic salts. From this study we observed the TDS was about 3404 mg/L, it was higher than the BIS value (2100mg/L) (Islam and Guha 2013). The hardness of the analysed water sample was 188 mg/L as CaCO₃. The maximum limit of total hardness recommended by WHO and American Public Health Association (APHA) are 500 and 250 mg/L respectively. Samples evaluated possess hardness within the permissible limits according to APHA and WHO. Low hardness value may be due to water softening steps carried out in textile industries. Our findings are similar to study report published in the past. (Prasad 2011; Kumari et al. 2011; Joshi and Shrivastava 2012; Bharagava et al. 2020).

Chemical Oxygen Demand recorded in the effluent was 300 mg/L⁻¹ exceeded the permissible limit prescribed by BIS (250 mg/L⁻¹). Saravanan et al. (2012) recorded that chemical oxygen demand of tannery industry effluent was above the acceptable limits. Increases in COD can be due to huge amount of industrial wastes such as detergents, softeners, non-biodegradable dyeing chemicals, formaldehyde based dye fixing agents etc. Higher concentration of COD in water implies toxic conditions and the presence of biologically resistant organic substances (Saravanan et al. 2012; Elango 2017; Bharagava et al. 2020).

The excess BOD due to the presence of more organic contaminants of textile effluents in to a water body. The low or nil BOD shows good quality water, whereas a high BOD indicates the water is highly contaminated and not suggested for drinking purposes. The experimental data of present work shows 128 mg/L BOD. It was found to be higher than the permissible limit of BIS. Increased level of BOD leads to microbial oxygen demand causes reducing DO which may induce hypoxia conditions with subsequent adverse effects on aquatic biota (Goel 1997). Oil and grease value was recorded as 16 mg/L which was quite higher than BIS limits (10 mg/L). Presence of oil and grease in water bodies leads to the formation of oil layer, which causes significant environmental problems like it

prevent the transfer of oxygen from atmosphere to water medium as a result reduces the amount of dissolved oxygen (DO) at the bottom of the water bodies (Central Pollution Control Board 1990; Bharagava et al. 2020).

In the present study the amount of chloride was recorded as 616mg/L as Cl which exceeds the BIS limits (600 mg/L). Mainly high chloride content in water destroys the microorganisms which are necessary to maintain food chains in aquatic life and it was found to be favour the EC, TDS, TSS, alkalinity and sulphate (United States Environmental Protection Agency 1999). The studies of Sajani and Muthukkaruppan (2011) revealed that sugar mills effluent contains high amount of chloride (United States Environmental Protection Agency 1999; Samuel and Muthukkaruppan 2011). Sodium recorded in the effluent was 234 mg/L, this level was higher than the value set by BIS is 200 mg/L. Potassium content recorded in the effluent was 43.8 mg/L found to be higher than the permissible limit of BIS (12 mg/L). But other compounds like copper, chromium, alkalinity and zinc levels are found to be trace value (BDL). Our results are accordance with Falah and Hussein (2013) who observed that no significant changes were observed in the concentration of zinc, iron, cadmium, copper, nickel, cobalt and manganese before and after treatment of real and simulated textile industrial wastewater. Moreover, the concentrations of most of these metals were very low or non-detective (Falah Hussein 2013; Naveenraj et al. 2021).

Effect of effluents on seed germination: Experiment on *Sorghum bicolor* and *Vigna unguiculata* showed (Fig -4 a, b) an inhibitory effect of untreated industrial effluents on seed germination and its early growth. Inhibition of seed germination may be due to greater number of dissolved solids that increases the salinity and conductivity of the absorbed solute by seed before germination moreover higher salt content also changes the osmotic potential outside the seed thereby reducing the amount of water absorbed by the seed which results in retardation of seed germination (Mehta and Bhardwaj 2012; Naveenraj et al. 2021).

Similar result reported by David Noel and Rajan (2015), was made investigation to study the degree of toxicity of dyeing industry effluent on seed germination and early growth of Lady's finger. Germination experiments were carried out in sterilized petri dishes containing 25, 50, 75 and 100% concentrations of untreated dyeing industry effluent. The germination percentage, growth parameters like plumule and radicle length, relative toxicity, percentage of phyto toxicity and tolerance index on the seed germination in response to dyeing effluent at various concentrations were also calculated. There was a gradual decrease in the percentage of seed germination and seedling growth with higher concentration of effluent. Suppression of germination at higher concentrations of effluent may be due to high levels of total dissolved solids which enhance the salinity and conductivity of the solute absorbed by the seeds before germination (Lav and Jyoti 2012; David Noel and Rajan 2015; Naveenraj et al. 2021).

Waste water treated with Zinc oxide nanoparticle and

bacterial species used for irrigating the seed containing pots (*Sorghum bicolor* and *Vigna unguiculata*). By this comparative study of both processes, the dye degradation by the chemical oxidation of zinc oxide nanoparticle was more effective and faster than the biological oxidation of bacterial species (Fig -5a, b). The zinc oxide nanoparticle has an Advanced Oxidation Process (AOP). The zinc oxide nanoparticle also has a property of photo catalytic activity thus it possesses the higher seed germination and growth, when compared with waste water treated with *Pseudomonas fluorescens* (Fig - 6 a, b). The research finding of Hussein (2013) reported that Zinc oxide was most effective catalyst with all types of dyes the extent of decolourization reaches 100 %. The extent of decolourization was also 100 % when anatase was used as photocatalyst but that need more time than zinc oxide was used (Falah Hussein 2013; Naveenraj et al. 2021).

CONCLUSION

The findings of present study suggest that the physico-chemical parameters were relatively high in dyeing industry effluent and it was toxic to plants, severely affected the seed germination and seedling growth. The waste water treated by chemical oxidation of zinc oxide nanoparticle was more effective and fast than the biological oxidation of a bacterial species in seed germination and growth. The study further concluded that the plant *Sorghum bicolor* was more suitable to grow when compare to *Vulgna unguiculata*. The future will concentrate on cost analysis of the treatment techniques and also on treatment of complex dyes. In Tamil Nadu, particularly in Tirupur, Erode, Karur, Srivilluputhur and other districts, the textile industries are growing very fast due to its several advantages but on the other hand it is one of the root causes for environmental pollution.

ACKNOWLEDGEMENTS

The study was financially supported by the Aarupadai Veedu Institute of Technology, Vinayaka Missions Research Foundation and Presidency College, University of Madras, Chennai.

Conflict of Interests: Authors declare no conflict of interests to disclose.

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Biotechnological Communication

Biodegradation of Basic Yellow Auramine- O Dye using *Staphylococcus* sp. Isolated from Textile Industry Effluent

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ABSTRACT

Due to the increased use of synthetic dyes in various industries, there is an increased disposal of wastewater containing harmful dyes. These, in turn, have affected plants, animals, and humans. The physical and chemical methods of dye decolorization have failed to degrade the synthetic dyes in industrial effluents completely. The microbial decolorization is better due to its versatility, dynamic metabolism, and potential machinery of enzymes. This study aimed to degrade basic yellow dye auramine O by bacteria isolated from textile industry effluent. In this regard, five bacterial strains were isolated and screened from a soil sample taken from textile industry effluent. The initial physical and biochemical characterization of the bacterial isolates 1 and 2 indicated catalase test-positive, starch test-negative, motility agar test-negative, gram staining test-positive, and morphology-*Bacillus*. The bacterial isolates 3, 4, and 5 indicated oxidase test-negative, urease test-positive, gram staining test-negative, and morphology-*Staphylococcus*. All the isolates were further subjected to a screening test, where isolate 5 showed maximum dye decolorization of 98.9% in 96 h. The biodegradation of dye was optimized for different values of initial pH (4-10), inoculum size (2% -10%), initial dye concentration (50 mgL⁻¹ to 400 mgL⁻¹), carbon source (glucose, fructose, xylose, starch and lactose) and nitrogen source (peptone, ammonium sulphate, yeast extract, ammonium nitrate and urea). Maximum dye decolorization was observed for initial dye concentration of 200 mgL⁻¹, initial pH of 6, inoculum size of 10%, yeast extract as nitrogen source, and glucose as carbon source. Therefore, dye degradation by bacteria can be used as a potential method for auramine O dye treatment.

KEY WORDS: AURAMINE O DYE, BACTERIA, BIODEGRADATION, DECOLORIZATION, TEXTILE INDUSTRY EFFLUENT.

INTRODUCTION

Dyes are an essential component in different industries like paper, food, leather, textile, paint, and cosmetics. Roughly around 25 dye groups are present based on the chemical structure of chromophore. Many dyes are grouped as textile dyes which are used to dye different fabrics (Shet et al. 2013; Hombalimath et al. 2012; Shet et al. 2016; Kulkarni et al. 2017; Abe et al. 2019; Benkhaya et al. 2020). Disposal of industrial- effluents to the water bodies causes water pollution. The environmental pollution can cause health risks to all the living things on the earth (Varjani 2017; Varjani and Upasani 2017; Bencheqroun et al. 2019; Shet et al. 2021; Achappa et al. 2021). The dyes used in textile industry

causes artistic damage and stops the light diffusion in water, leading to a decreased level of dissolved oxygen and thus affecting the aquatic life's photosynthesis rate (Ajaz et al. 2020; Achappa et al. 2020). Among different dyes, Auramine O is a diarylmethane dye that is used as a fluorescent stain in paper, leather, textile, and paint industries. Various methods are used to remove the auramine O dye from textile effluents: physical, chemical, Physico-chemical, and biological (Cao et al. 2019; Varjani and Upasani 2019; Nakkeeran et al. 2020; Achappa et al. 2021; Shet et al. 2021).

The biological method has many advantages like; it is simple, cheap, and environmentally friendly process. The large numbers of microbes present are maintained easily and require less preparation (Kulkarni et al. 2017; Crini and Lichtfouse 2018). Different microbes such as fungi, algae, bacteria, and yeast decolorize different dyes (Roy et al. 2018; Shet et al. 2020; Bhavikatti et al. 2020). Textile

Article Information:*Corresponding Author: anilrshet@gmail.com

Received 25/08/2021 Accepted after revision 15/11/2021

Published: 31st December 2021 Pp- 1571-1576

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.31>

wastewater treatment can be done using pure or mixed microbial cultures (Tochhawng et al. 2019; Patil et al. 2020). Different factors like nutrients, soluble salts, initial pH, heavy metals, incubation temperature, dye structure, etc., affect the dye decolorization (Al-Amrani et al. 2014). There are different reports available which showed different dye decolorization using microbes (Varjani and Upasani 2016; Kiayi et al. 2019; Pratiwi et al. 2019; Achappa et al. 2021; Shet et al. 2021). In the Present study, biodegradation of basic yellow dye auramine O using bacteria has been reported. The effect of inoculum size, initial pH, carbon source, initial dye concentration, nitrogen source, and incubation temperature has been examined and the results obtained are discussed.

MATERIAL AND METHODS

Media components were obtained from Himedia laboratories Pvt. Ltd. All the analytical grade chemicals were purchased from S.R.L. Pvt. Ltd. U.V. Visible Spectrophotometer (Make: Metler Toledo) was used for the analysis of the samples. The samples were collected from different disposal points of the local textile industry in the form of soil samples. Sterile plastic bottles and spatula were used to collect the samples. It was then stored in the laboratory at 4°C. The soil samples were cultured on sterile nutrient agar plates, and 5 isolates were screened and transferred to sterile slants and preserved.

For screening, Bushnell and Has mineral medium was prepared using a standard protocol. The broth pH was adjusted to 7. The dye of concentration 100 mgL⁻¹ was added to the test tubes. This was distributed in test tubes and autoclaved to maintain sterility. The isolates were added to separate test tubes and incubated. Initial studies were done to decide on the operating environment such as initial pH (7), substrate concentration (100 mgL⁻¹), and incubation temperature (30 °C). Percentage dye decolorization was determined by taking 1.5 mL aliquots from time to time from different test tubes. Then the sample was centrifuged at 5,000 rpm for 15 minutes to take out the cells. The supernatant was then measured for its absorbance at 370 nm using UV-visible spectrophotometer. The percentage dye decolorization was determined by using the mathematical expression: % Dye decolorization = $(C - T/C) \times 100$. Where C = Initial absorbance, T = Final absorbance.

Standard gram staining and microscopic observations were carried out to characterize the isolates. The isolates were transferred on the slide with a sterile inoculating loop. It was allowed to dry. A drop of violet stain was put on the culture and waited for 30 seconds, then washed under running water carefully. A drop of iodine was added and waited for 30 seconds to allow it to dry. Then safranin stain was added and waited for 30 seconds till it dried and washed under running water carefully. The slide was then observed under a microscope [100X] with immersion oil. Biochemical characterization like oxidase test, catalase test, urease test, starch hydrolysis test, and motility agar test were conducted on the isolates and recorded. Different media and process parameters were optimized by One Factor At a Time (OFAT) to get the highest decolorization efficiency of auramine O

dye by the selected bacterial isolates. The experiments were performed in triplicates. The effect of different pH (4, 6, 8, and 10) was observed for the dye decolorization efficiency of the selected bacterial isolate.

The Bushnell and "Hass" medium was spiked with auramine O dye of concentration 100 mgL⁻¹ and was incubated at a temperature of 30°C. The initial value of pH was set using 0.01 M HCl / 0.01 M NaOH solutions. Percentage dye decolorization was determined. The effect of three different initial dye concentration (50, 200, and 400 mgL⁻¹) of Auramine O dye was examined on the dye decolorization efficiency. The inoculum was added to 10 mL medium, maintained at 1:50 inoculum to broth ratio and incubation temperature of 30°C. Percentage dye decolorization was determined by taking 1.5 mL aliquots from time to time from different test tubes. The samples were centrifuged at 5,000 rpm for 15 minutes to take out the cells. The supernatants were then measured for its absorbance at 370 nm using UV- visible spectrophotometer, and % decolorization was calculated. Abiotic decolorization was checked using an un-inoculated blank.

Different inoculum sizes (2% and 10%) were used to examine the effect on the dye degrading efficiency of the selected bacterial isolates. The Bushnell and "Hass" medium was spiked with auramine O dye of concentration 100 mgL⁻¹ and was incubated at a temperature of 30°C. Percentage dye decolorization was determined. To analyze the effect of media components on the bacterial dye degradation, five different carbon sources: fructose, glucose, xylose, starch, and lactose, and five different nitrogen sources: peptone, ammonium sulphate, yeast extract, urea and ammonium nitrate were used (4 gL⁻¹). The Bushnell and "Hass" medium was spiked with auramine O dye of concentration 100 mgL⁻¹ and was incubated at a temperature of 30°C. Decolorization of the dye was determined.

RESULTS AND DISCUSSION

Physical and Biochemical characterization: The soil samples were collected from local textile industry effluent where auramine O dye was used for dyeing purposes. The organisms were cultured on agar slants, and they were identified by gram staining. The biochemical and microscopic characterization of the bacterial isolates are shown in table 1 and 2 respectively. The biochemical tests and gram staining indicated that the culture was a mixed culture that contained both gram-positive and gram-negative organisms. Some of them were rod-shaped, and some of them were round-shaped. Bacterial isolates 1 and 2 showed catalase test- positive, starch test- negative, motility agar test- negative, and the gram staining test showed that they were gram-positive strains and *bacillus* morphology. The bacterial isolates 3, 4 and 5 were *staphylococcus* as they were gram-negative, oxidase test negative, and urease test positive.

Biodegradation of Auramine O dye: All the five bacterial isolates showed effective ability in decolorizing auramine O dye. Degradation started in few hours, and after 96 h, the average percentage of dye removal by *Bacillus* spp.

was 96% and by *staphylococcus* spp. was 98%. No color decrease was observed in a sterile control. At 24 h, isolate 5 showed a higher percentage of dye decolorization when compared to other bacterial isolates. For all the bacterial isolates, the % dye decolorization was observed to increase with time. At 96 h, isolate 5 showed the maximum dye decolorization of 98.9%, as shown in figure 1. Since isolate 5 showed maximum dye decolorization capacity, it was considered for further optimization studies.

Table 1. Biochemical characterization of the bacterial isolates

Test	Bacterial Isolates				
	1	2	3	4	5
Gram staining	(+) ve	(+) ve	(-) ve	(-) ve	(-) ve
Catalase test	(+) ve	(+) ve	-NA-	-NA-	-NA-
Motility agar	(-) ve	(-) ve	(-) ve	(-) ve	(-) ve
Starch agar	(-) ve	(-) ve	-NA-	-NA-	-NA-
Urease	-NA-	-NA-	(-) ve	(-) ve	(-) ve
Oxidase	-NA-	-NA-	(+) ve	(+) ve	(+) ve

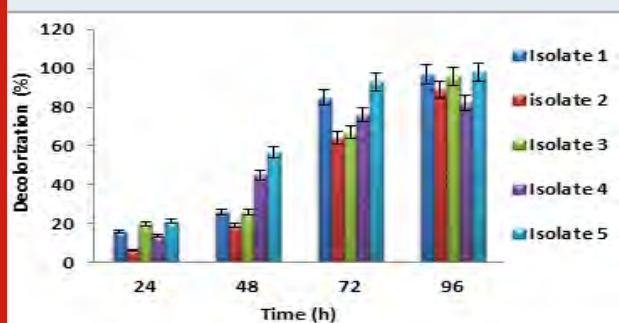
Bacterial oxido-reductive enzymes were responsible for the textile dye decolorization. The bacterial metabolism enabled the use of complex xenobiotic compounds of the dyes and breaks them down into less complex metabolites. The bacteria obtained from actual effluent disposal sites have the enzymes activated, which assisted the decolorization of dye (Radia and Romana, 2019; Ajaz et al. 2020; Shet et al. 2021).

Effect of initial pH: pH is a vital variable for bacterial growth and an important factor for wastewater treatment. Depending on the dye type, pH may be acidic, alkaline, or neutral. Dye decolorization rate may change with the change in pH of the wastewater. Dye decolorization can be improved by modifying the wastewater pH to support bacteria degrading the dye or by choosing the microbes that can grow at the pollutant's pH (Al-Amrani et al. 2014; Varjani and Upasani 2017). In this study, as shown in (figure 2), maximum dye decolorization was seen for pH of 6, followed by pH 8. Highest dye decolorization at an initial pH of 8 to 10 using *Lysinibacillus boronitolerans* CMGS-2 was reported in the previous studies (Basutkar and Shivannavar 2019). Decolorization of malachite green of 98% was obtained by $\text{RuO}_2\text{-TiO}_2$ and Ti mesh electrodes coated with Pt at an initial pH of 4.5 (Singh et al. 2016; Achappa et al. 2021).

Table 2. Microscopic characterization of the bacterial isolates

Property	Bacterial Isolates				
	1	2	3	4	5
Elevation	Flat	Flat	Flat	Flat	Flat
Margin	Irregular	Irregular	Irregular	Irregular	Irregular
Opacity	Opaque	Opaque	Opaque	Opaque	Opaque
Colour	Pale	Pale	Pale	Pale	Pale
Surface	Moist, shiny	Moist, shiny	Moist, shiny	Moist, shiny	Moist, shiny
Gram staining	(+) ve	(+) ve	(-) ve	(-) ve	(-) ve
Shape of vegetative cell	Rod	Rod	Coccus	Coccus	Coccus

Figure 1: Percentage dye decolorization by different bacterial isolates



Effect of initial dye concentration: Initial dye concentration affects the dye decolorization. The enzymes produced by dye degrading bacteria may not identify a low concentration of dye. But the high concentration of dye is toxic to bacteria and affects dye decolorization by blocking active sites of

enzyme. Similarly, simple structured and low molecular weight dyes are easy to degrade and complex structured and high molecular weight dyes are hard to degrade. The dye decolorization decreased with the increase in initial dye concentration (Liu et al. 2016; Li et al. 2019). In (figure 3), the maximum dye concentration of 400 mg/L showed less dye decolorization when compared to 200 mg/L and 50 mg/L. Similar result trend was reported in the previous studies (Patil et al. 2016; Barathi et al. 2020).

Effect of inoculum size: Inoculum size plays a crucial role in the dye decolorization present in industrial effluents. Larger inoculum size increased dye decolorization while smaller inoculum size showed lesser dye decolorization (Kisand et al. 2001). The same trend was observed in the decolorization of auramine O dye, as shown in figure 4. It was observed that dye decolorization was more for 10% inoculum size when compared to 2% inoculum size. Similar result trend was reported in the previous studies (Roy et al. 2018; Aktar et al. 2019; Shet et al. 2020).

Effect of nitrogen and carbon sources: Nitrogen and carbon supplements are required by the microorganisms for rapid decolorization of dyes (Varjani and Upasani 2019). Organic sources such as yeast extract, peptone and the combination of complex organic sources and carbohydrates have been reported for high and rapid dye decolorization rates by both pure and mixed cultures (Kisand et al. 2001; Nakkeeran et al. 2020).

Figure 2: Percentage dye decolorization for different initial pH

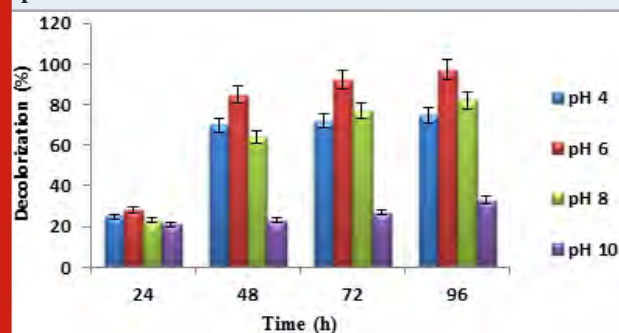


Figure 3: Percentage dye decolorization for different initial dye concentration

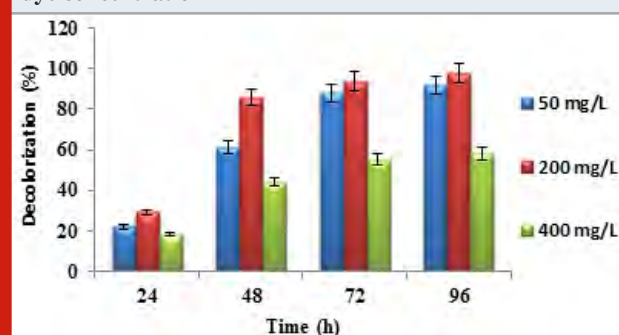
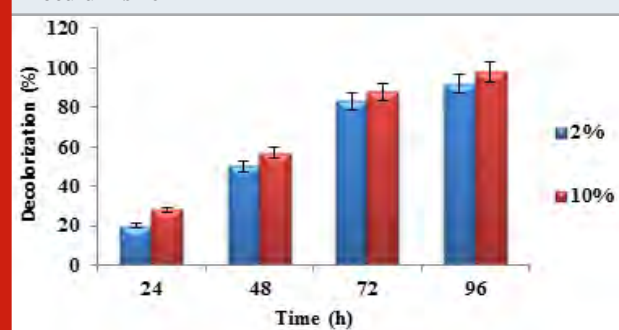


Figure 4: Percentage dye decolorization for different inoculum size



In figure 5, it was observed that nitrogen source peptone favoured maximum dye decolorization efficiency followed by yeast extract. In the case of carbon source, glucose favoured maximum dye decolorization followed by lactose as shown in figure 6. Similar results were reported in the previous studies (Patil et al. 2016; Barathi et al. 2020). For the microbial metabolism of dyes and dyes intermediates,

the easily available and effective carbon source is glucose (Khan et al. 2012; Achappa et al. 2021).

Figure 5: Percentage dye decolorization for different nitrogen sources

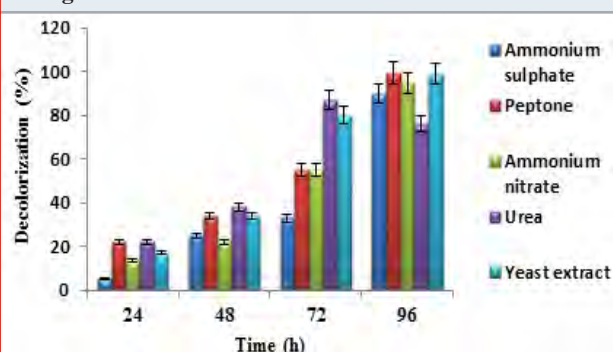
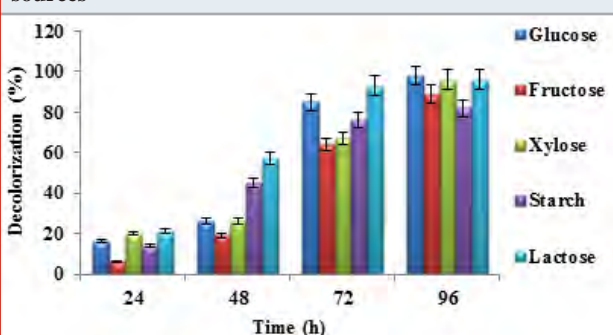


Figure 6: Percentage dye decolorization for different carbon sources



CONCLUSION

The findings of the present study deal with degradation of auramine O dye present in textile industry wastewater before disposal to water bodies which otherwise affects the environment. Thus, the degradation of dye present in the industrial effluent is essential and mandatory. The biological treatment method offers the prospective benefits when compared to physical and chemical treatment methods. The potential bacterial isolates were isolated and screened from the effluents of the textile industry. The gram staining indicated that the culture is a mixed culture that contained both gram-positive and gram-negative organisms. The physical and biochemical tests of the culture confirmed *bacillus* and *staphylococcus* species. Optimization studies showed that initial pH, inoculum size, nitrogen source, carbon source, and initial dye concentration significantly affected the degradation potential of bacteria. Therefore, bacterial strains can be used successfully for the degradation of textile dye basic yellow auramine O.

ACKNOWLEDGEMENTS

This study was supported by the Department of Biotechnology, K. L. E. Technological University, Hubballi, Karnataka, India. Authors thank the department for providing lab facilities to carry out this research work. Moreover, Anil R. Shet and Laxmikant R. Patil

have designed the experimental work and executed the experiments. Anil R. Shet, Laxmikant R. Patil, Veeranna S. Hombalimath, and Santosh A. Kadapure have carried out data analysis and interpretation. Anil R. Shet has written the manuscript. Sharanappa Achappa and Shivalingasari V. Desai have done the grammar check, plagiarism check and proof reading. The manuscript has been approved by all the authors.

Conflict of interests: Authors declare no conflicts of interests to disclose.

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Biomedical Communication

Glutamate Elicits Therapeutic Responses in Light-Induced Sleep-Deprived Zebrafish, *Danio rerio*

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ABSTRACT

Sleep deprivation disrupts most neurotransmitters, which can lead to adverse behavioural changes and other psychiatric illnesses. Many neurotransmitter systems, including dopamine (DA), serotonin (5-HT), norepinephrine (N.E.) and GABA, have been implicated in the pathophysiology of mood disorders. The precise significance of sleep deprivation (S.D.) changes in the neurotransmitter levels and the mechanism underlying behavioural alterations is unknown. According to research, sleep deprivation (S.D.) has a major effect on an individual's quality of life and ability to perform essential physiological functions. As a result, we wanted to confirm the levels of neurotransmitters and behavioural modifications in zebrafish after 24, 48, and 72 hours of sleep deprivation and glutamate treatment on the sleep-deprived groups. The T-maze test was used to assess learning and memory alterations in zebrafish. We used the Novel Tank Test (NTT) and Light and Dark Test (LDT) to examine the anxiety-like behaviour. The spectrofluorimetric method was used to determine the quantities of DA, 5-HT, N.E. and GABA. From this study, it is evident that 72h sleep-deprived fish had a loss of learning and memory via T-maze test and also the anxiety levels were very high in the sleep-deprived group than the other groups. The groups that received glutamate after sleep deprivation showed betterment in the behavioural response. Also, the levels of neurotransmitters were increased in the glutamate treated groups than the sleep-deprived groups. Our findings indicate that sleep loss dramatically impairs behavioural responses and disrupts most neurotransmitter concentrations. When sleep-deprived fish were given glutamate, their behaviour and neurotransmitter levels were nearly identical to those of the control group. This study will have a greater impact on sleep deprivation therapy and pave the way for using the neurotransmitters as external therapeutic agents in treating sleep deprivation and other behavioural changes related to sleep deprivation. It has been suggested that zebrafish is an excellent testing subject for loss of sleep on cognition and that it may also be an efficient model for unravelling the pathways that underpin learning and memory formation.

KEY WORDS: ANXIETY, BEHAVIOR, GLUTAMATE, LEARNING AND MEMORY, SLEEP DEPRIVATION.

INTRODUCTION

Over the last few decades, *Danio rerio* has grown in popularity as an animal model in hereditaries and developmental biology. It has also expanded prominence in behavioural studies converging on memory retention (American Psychiatric Association 1994). This little fish exhibits various sleep-like properties, such as circadian control, periods of dormancy followed by an elevated arousal verge, preference for resting sites, and sleep rebound homeostasis (Merikangas et al. 2011; Jansen et al. 2011). When combined with the zebrafish's daily circadian cycle, this species is a relevant model for sleep-related studies

(Dilsaver 2011). Sleep is a common occurrence in most species, including humans, and has been observed to be an evolutionarily conserved phenomenon (Cantero et al. 2003; McNamara et al. 2008). Sleep is critical for the learning process and memory consolidation, although its mechanisms and functions remain unknown (Besedovsky et al. 2019).

Interrupted sleep has been linked to poor attention and health-related difficulties, both of which have been verified by experimentation (Vogel et al. 1999; Hublin et al. 2001; Rajaratnam 2001; Buzsaki et al. 2002; National Health Interview Survey et al. 2005; Harvey et al. 2008; Centers for Disease Control and Prevention et al. 2011). According to research, sleep deprivation (S.D.) has a major effect on an individual's quality of life and ability to perform essential physiological functions (Praag et al. 1975; Moore et al. 1975; Serra et al. 1979; Bannerman et al. 2004; Derry et

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Received 25/09/2021 Accepted after revision 28/12/2021

Published: 31st December 2021 Pp- 1577-1583

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.32>

al. 2006; Vadodaria et al. 2017). Sleep deprivation leads to poor memory acquisition. The sleep stage has a substantial effect on memory consolidation. Slow-wave sleep, for example, is required for the maintenance of memories dependent on the hippocampus, such as declarative and spatial memories, which are more severely affected than other types of memories by sleep deprivation (Graeff et al. 1996; Schildkraut and Massat 2000; Bredy et al. 2013; Shields et al. 2015; Besedovsky et al. 2019).

Sleep deprivation has a noticeable effect on neurotransmitter levels, resulting in detrimental behavioural changes and other health consequences. S.D. has been associated with a variety of neurodegenerative and behavioural disorders. Diminished sleep can be a positive trigger for manic episodes (Berns et al. 2003). Numerous neurotransmitter systems have been linked to behavioural changes, including dopamine (DA), serotonin (5-HT), and norepinephrine (N.E.). Dopamine (DA) has been implicated in the pathophysiology of manic episodes since the (1970s), and alterations in dopaminergic neurotransmission have been linked to neurobiological abnormalities (Zachmann et al. 1966; Barbose et al. 2011). Clinical findings have established a link between dopamine and manic episodes for many years. Five-hydroxytryptamine (5-HT) is a neurotransmitter that has remained mostly unaltered throughout evolution. It regulates various physiological processes and behaviours, including cardiovascular control, pain sensitivity, eating, reproduction, cognition, impulsivity, aggression, and mood (Weber et al. 2018).

5-HT appears to boost wakefulness and prevent sleep through activating neurotransmitters such as ACh and noradrenaline. Norepinephrine (N.E.) has long been associated with depression and manic episodes (Decker et al. 2000; Machado et al. 2004). Glutamate is the most important excitatory neurotransmitter (Seigel et al. 1984; Berns et al. 2003; Armani et al. 2012). Glutamate has a deleterious impact on brain physiology in circumstances of overexcitation (Tamilselvan et al. 2017). Astrocytes are responsible for 90% of glutamate uptake, highlighting some of the essential characteristics of tripartite synapse integrity and brain function (Brady et al. 2013; Stewart et al. 2019).

The interaction of glutamate with particular membrane receptors is responsible for many neurological activities such as cognition, memory, movement, and sensation; however, excessive extracellular glutamate accumulation contributes to the progression of most neurodegenerative illnesses (Derry et al. 2006). The precise process by which S.D. impacts neurotransmitter levels and emerging repercussions, resulting in behavioural alterations in fish, is unknown. Thus, we attempted to confirm neurotransmitter alterations in zebrafish following 24, 48, and 72 hours of S.D. and S.D. induced behaviour changes (Stewart et al. 2019). The purpose of this study was to employ spectrofluorimetric methods to compare the effects of sleep deprivation on neurotransmitter levels in the brains of sleep-deprived, control, and glutamate-treated fishes. In addition, we looked into the behavioural circuits of zebrafish that had been subjected to sleep deprivation for several hours.

Furthermore, to scrutinize the impacts of sleep-inducing medications, we investigated the behavioural reaction of sleep-deprived fish exposed to glutamate. Therefore, we hypothesized that (1) sleep deprivation impairs memory formation and changes neurotransmitter levels in zebrafish, and (2) glutamate promotes sleep by synthesizing GABA and mitigates the S.D. effects.

MATERIAL AND METHODS

For zebrafish and conditions for housing, male zebrafish at four months of age were obtained from Prince Aquarium and housed in 50-L tanks with proper housing conditions and disinfection. A standard process comprised monitoring oxygen and pH levels while maintaining the temperature at 28°C and the hardness of the water. The 12:12 light-dark cycle, with (ZT) set to 06 am–06 pm were maintained for zebrafish. Zebrafish were fed blood worms and commercial meals twice daily. There were no animal ethical issues involved to carry out this research.

For sleep settings and drug administration, the major Zeitgeber for circadian rhythms is the light-dark cycle was used. The control group cycle was maintained at 12L:12D light: dark. Previous studies have established that the extended light phase in zebrafish may cause S.D. (Vetter et al. 2015). The cycle was extended, and the light was given to the fish during the dark period to get a more extended light phase. Sleep deprivation was maintained for extended hours, maintaining a light phase throughout the study period. The present study was conducted for 10 days, consisting of three days in a row of sleep deprivation and 7 days of treatment with glutamate. Six fishes in a group were used in the current investigation (n=6).

Group 1: Control ; Group 2: 24h Total SD; Group 3: 48h Total SD; Group 4: 72h Total SD ; Group 5: 24h Total SD + Glutamate (150mg/L) ; Group 6: 48h Total SD + Glutamate (150mg/L); Group 7: 72h Total SD + Glutamate (150mg/L).

For behavioural analysis, learning and memory test in a T-Maze was used. The T-maze is a multi-species operational activity that is widely used to measure memory. T-maze was used to study learning and memory activities in zebrafish. It was constructed from a clear acrylic glass sheet (shown in Fig.1(a)). The maze measurements were 50 cm x 10 cm x 10 cm long arm, 20 cm x 10 cm x 10 cm short arm and 10 cm x 10 cm start box at the stem base. Green and red sleeves covered the two short sides on the left and right. The maze was filled with water to a depth of 6 cm, and the water temperature was kept constant at 28°C during the experiment. Transfer latency (T.L.) was calculated when it took the fish to reach the deeper chamber.

Novel tank test was conducted for the study using rectangular glass aquaria (L×W×H: 24×8×20 cm) filled with water from the same source and having the same properties as the experimental aquaria as shown in Fig.1(b). Canon EOS 1500D 24.1 DSLR camera mounted on a tripod in front of the test aquarium was used to video the fish for a total of five minutes. Following the discharge of the fish

into the NTT, the operator ensured that no human activity interfered with the experiment. We did not undertake video analysis during the first 60 seconds of the inquiry because it was a period designed to acclimatize the fish to the stress of transfer and the new surroundings. Three portions were created in the test tank: the top, the middle, and the bottom. The time spent in each part of the tank was recorded (Harvey et al. 2008).

The light/dark preference test, as opposed to the novel tank test, examines zebrafish exploratory behaviour under a motivational conflict of both light and dark sleeves, as shown in Fig.1(c). Furthermore, the effects of glutamate are prevalent in this examination. In brief, 30 minutes after drug treatment, the animals were transferred to the central compartment of a black and white tank (15 cm × 10 cm × 45 cm H × W × L) for a 3-min acclimation period, after which the doors that delimit this compartment were removed, and the animal was free to explore the apparatus for 5 minutes.

The brain tissue examination was conducted with frozen fish brains which were first cut into slices (about 1-mm thick) using a microtome. Tissue pieces were placed in a pre-cooled microhomogenizer sealed with a glass cork. For extraction 0.1 mL HCl-n-butanol (0.85 mL 37 percent HCl in 1 L n-butanol for spectroscopy) was used. In a glass homogenizer, the sample was centrifuged for 10 minutes at 2000 g. It was necessary to separate the supernatant phase (0.08 ml) and transfer it to an Eppendorf tube with a volume of 1.5 ml that contained 0.2 ml heptane (for the spectroscopy) and 0.025 ml of 0.1 M HCl. After 10 minutes of vigorous shaking, the organic phase formed on the inorganic phase was removed. The aqueous phase was used to estimate neurotransmitters: 5-HT, N.A., DA, and GABA (Schlumpf et al. 1974).

For the estimation of Norepinephrine and Dopamine, the Trihydroxyindole approach was shrunk in size by a factor of two for this assay. A total of 0.02 ml HCl phase was added to 0.02 ml 0.4 M HCl and 0.01 ml EDTA/sodium acetate buffer (pH 6.9) (to the mixture 0.01 ml iodine solution was added for oxidation). 0.01 ml Na₂SO₃ was added to 5 M NaOH, the chemical was allowed to react for 2 minutes before being tested. Afterwards, 0.01 mL of acetic acid was added to the solution, and it was then heated for 6 minutes at 100°C. On reaching room temperature, emission spectra were collected in the microcuvette with a spectrofluorophotometer for dopamine at 485 nm and noradrenaline at 375 nm.

For the serotonin assay, changes in reagent and solvent quantities were required to achieve the desired fluorescence yield while using smaller vials. For serotonin determination, the O-phthalaldehyde technique was used. To 0.025 ml of the tissue extract, OPT reagent and HCl was added. Fluorophore development involved boiling the mixture at 100 °C for 10 minutes. At equilibrium, emission spectra or intensity was measured in a spectrofluorophotometer at 470 nm.

The estimation of GABA content was measured using the following experimental procedure (Lowe et al. 1958). 0.1

ml of tissue extract, 0.2 ml of 0.14 M ninhydrin solution in 0.5 M carbonate–bicarbonate buffer (pH 9.95), and copper tartrate reagent were added to the reaction tube. Spectra of fluorescence emission were obtained after 10 minutes of boiling the mixture in the water bath. For the statistical analysis, the mean and standard deviation of the mean was used to represent all of the data. Comparing the outcomes of the different groups was done using one-way ANOVA followed by a DMRT test for comparisons between the groups. In all groups, a p-value of less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

T-Maze for learning and memory: The fish in our study subjected to light-induced S.D. performed significantly worse behaviour when compared to the control and treatment groups. A previous survey of avoidance learning after S.D. showed that zebrafish could learn, create memories, and reactivate memories associated with the stimuli. Studies found that fish that had not slept for 24 hours could still respond and learn the association between red and green sleeves in the T-maze the same way the control group did. Due to poor memory, 48h and 72h S.D. fish showed difficulties distinguishing between red and green sleeves (Detke et al. 2015).

Time spent in the green and red arm of T-maze: The total amount of ±SD time to the green and red arm of the maze by the sleep-deprived and sleep-deprived + glutamate treated groups have been graphically represented in (fig 2a and fig 2c), respectively.

T-maze for learning and memory using the number of entries in each arm as a parameter: Number of entries into the green arm and red arm: The total number of ±SD entries to the green and red arm of the maze by the sleep-deprived and sleep-deprived + glutamate treated groups has been graphically represented in (fig 2b and fig 2d), respectively.

NTT for checking anxiety-induced behaviour changes by using time spent in each zone as a parameter: We selected the novel-tank and light-dark tests to examine anxiety-like behaviours in the current investigation. Zebrafish tend to linger near the sides and bottom of the tank during the novel-tank test when first introduced to a novel setting. This is referred to as thigmotaxis in rodents. In zebrafish, typical vertical exploration activities are gradual and likely to intensify over time. 72h S.D. fishes displayed delayed latency to access the upper half of the tank, decreased time spent in the upper half of the tank, and higher frequency of erratic movements and freezing bouts in the novel tank-test, which has been associated with greater anxiety-like behaviour (American Psychiatric Association 1994; Dilsaver et al. 2011) which was less noticeable in 24h and 48h S.D. fishes. Our findings show that fish exposed to light caused S.D. had considerably fewer exploratory behaviours when examined using the novel-tank test, as demonstrated by fewer transitions to the upper half of the tank compared to the glutamate-treated groups. This finding suggests that S.D. affects the fish, possibly impacting the behaviours

observed in this testing paradigm. The novel-tank test results indicate that S.D. affects exploratory behaviour, as evidenced by more minor exploratory transitions to the upper compartment in the novel-tank test (Besedovsky et al. 2019).

Figure 1: (a) T-maze set up to assess the learning and memory in the fish group (b) Novel Tank Test to assess the anxiety-like behaviour in the zebrafish (c) Light and Dark test to assess the exploratory behaviour of zebrafish.

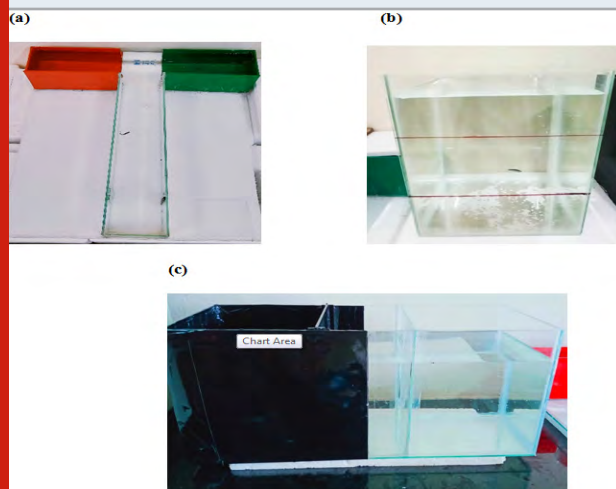


Figure 2: T-maze test (a) Graphs showing the amount of time spent in the red and green arm by the control and S.D fish groups, (b) Total average Number of entries to the red and green arm by the control and S.D fish groups, (c) Showing the total amount of time spent in the red and green arm by the control and S.D + glutamate groups and (d) Total average Number of entries to the red and green arm by the control and S.D+ glutamate groups. Values are expressed as mean \pm SD, and $p < 0.05$ is considered significant in all groups.

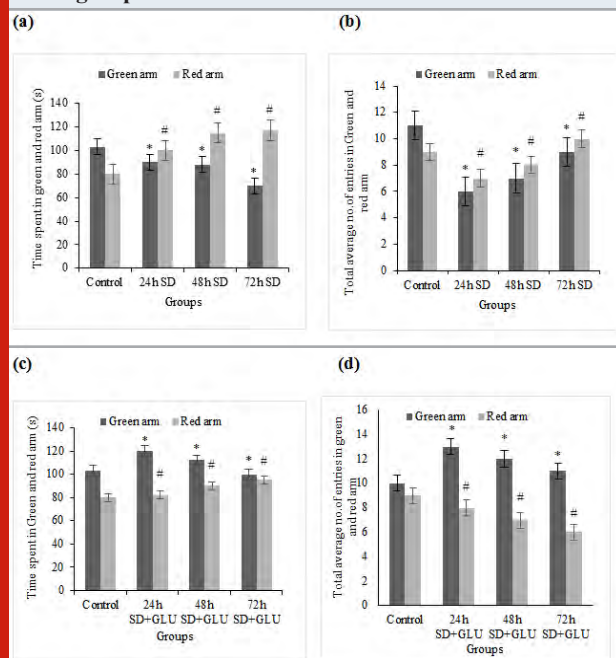
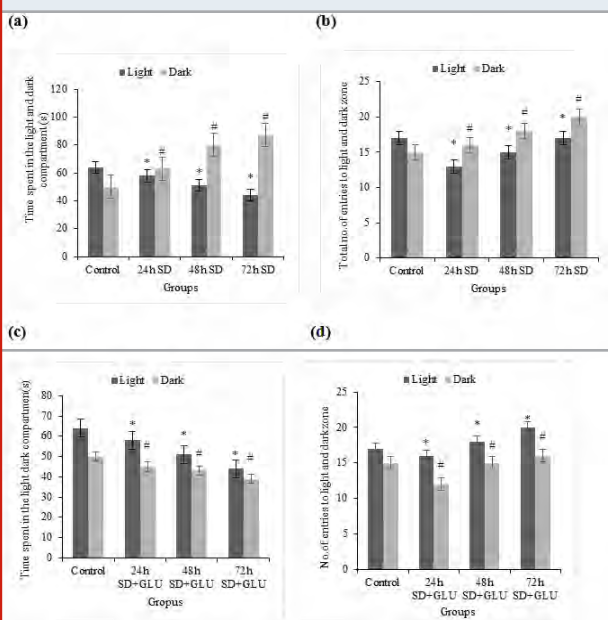


Figure 3: Light and dark test (a) Graphs showing the amount of time spent in the light and the dark compartment by the control and S.D fish groups, (b) Total average Number of entries to the light and dark zone by the control and S.D fish groups, (c) Showing the total amount of time spent in the light and the dark compartment by the control and S.D + glutamate groups and (d) Total average Number of entries to the light and dark zone by the control and S.D+ glutamate groups. Values are expressed as mean \pm SD, and $p < 0.05$ are considered significant in all groups.



Time spent in the top and bottom zone of the tank: The total time spent in the top and bottom zone of the tank \pm SD by sleep-deprived and sleep-deprived + glutamate treated zebrafish groups has been graphically represented in fig 4a and 4c, respectively.

NTT for checking anxiety-induced behaviour changes by using the number of entries to each zone as a parameter:

Number of entries towards the top and bottom zone of the tank: The Total number of entries to the top and bottom zone of the tank \pm SD by sleep-deprived and sleep-deprived + glutamate treated zebrafish groups have been graphically represented in fig 4b and 4d, respectively.

LDT for assessing anxiety-like behaviour in zebrafish by using time spent in light and dark zone as a parameter:

Scototaxic adults are known to exist in zebrafish. As a result, more entries into the white compartment imply more exploratory activity in the light and dark test, whereas more time spent in the black chamber shows a stronger predisposition toward anxiety-like behaviour. Ambient light levels can influence the light-dark test outcome, with fish exhibiting higher levels of white avoidance in brightly lit situations versus dark environments. Like the novel-tank test, the light-dark test showed altered behaviour in both the 48h and 72h S.D. groups compared to the control and 24h S.D. group. The light and dark examination revealed

that the 24h S.D. group was not significantly affected. In the the glutamate treated groups reached the light compartment

nearly twice as much as untreated groups (Besedovsky et al. 2019).

Figure 4: Novel tank test (a) Graphs showing the amount of time spent in the top and the bottom zones by the control and S.D fish groups, (b) Total average Number of entries to the top and the bottom zones by the control and S.D fish groups, (c) Showing the total amount of time spent in the top and the bottom zones by control and S.D + glutamate groups and (d) Total average Number of entries to the top and the bottom zones by the control and S.D+ glutamate groups. Values are expressed as mean \pm SD, and $p < 0.05$ are considered significant in all groups.

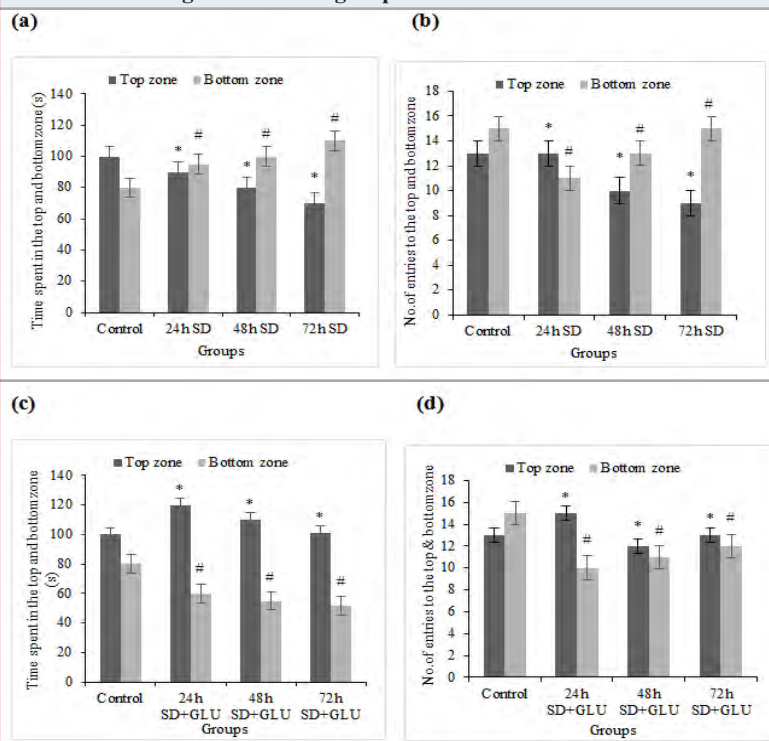


Table 1. Concentration of dopamine, serotonin, norepinephrine and GABA levels (ng/mg) in control, sleep deprived and glutamate treated fishes

Groups	Dopamine	Serotonin	GABA	Norepinephrine
Control	0.169 \pm 0.003	0.191 \pm 0.004	0.236 \pm 0.004	0.201 \pm 0.002
24h SD	0.156 \pm 0.004	0.185 \pm 0.003	0.185 \pm 0.005	0.175 \pm 0.003
48h SD	0.142 \pm 0.004	0.174 \pm 0.004	0.142 \pm 0.003	0.152 \pm 0.004
72h SD	0.137 \pm 0.005	0.152 \pm 0.006	0.112 \pm 0.004	0.102 \pm 0.005
24h SD+GLU	0.152 \pm 0.003	0.181 \pm 0.003	0.182 \pm 0.002	0.174 \pm 0.003
48h SD+GLU	0.169 \pm 0.004	0.176 \pm 0.004	0.198 \pm 0.003	0.169 \pm 0.004
72h SD+GLU	0.182 \pm 0.005	0.189 \pm 0.006	0.206 \pm 0.002	0.183 \pm 0.005

With $n = 6$ in each group, values are expressed as mean S.D. One-way ANOVA was used to compare group means, which was followed by the DMRT. $P < 0.05$ was deemed significant when compared to the control group.

Time spent in the light and dark zone: The total time spent in the light and dark zone of the tank \pm SD by sleep deprived and sleep-deprived + glutamate treated zebrafish

groups has been graphically represented in fig 3a and 3c, respectively.

LDT for assessing anxiety-like behaviour in zebrafish by using the total number of crossings between the compartment as a parameter: Total number of entries to the light and dark zone: The Total number of entries to the light and dark zone of the tank \pm SD by sleep deprived and sleep-deprived + glutamate treated zebrafish groups have been graphically represented in fig 3b and 3d, respectively.

Effects of S.D. and glutamate treatment on neurotransmitters levels in the brain: The effects of S.D. and SD+ glutamate treatment on zebrafish have been summarized in Table 1.

CONCLUSION

The findings of the present study suggests that zebrafish learning and memory were impaired due to sleep deprivation. Notably, when S.D. fish were exposed to glutamate treatment their behavioural routine was comparable to the control group's performance. As a result of our research, we identified a positive relationship between behavioural scores and neurotransmitter levels in the brain, indicating that S.D. generates anxiety-like behaviour that interferes with learning and memory. After three nights of sleep deprivation and treatment with glutamate, we identified a substantial learning impairment and improvement in zebrafish. Future studies may evaluate various glutamate dosages and exposure regimens, the traumatic effects of S.D. on hormones, and the effect of S.D. on prolonged memory development. Researchers can also explore how S.D. impacts mental health and whether alternative medications can treat sleep disorders.

Conflict of Interests: Authors declare no conflicts of interests to disclose

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Biotechnological Communication

Bovine Anti-leukemic Measures for Improving Live-Stock Farms in Belgorod and Kemerovo Regions of Russia

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ABSTRACT

Bovine leukemia remains one of the most urgent viral diseases in veterinary medicine, and potentially dangerous for humans. The strategy of combating it is aimed at improving the existing measures and full recovery of agricultural enterprises. The quality of animal products is a priority in the field of food safety. The aim of the research was to develop effective antileukemic measures for the improvement of livestock farms in the Belgorod and Kemerovo regions. The proposed antileukemic measures are to increase the frequency of serological studies from 6-th months to the 2-3th months among animals in areas with poor leukemia in cattle, as well as to increase the sensitivity of immunodiffusion test (AGID) due to highly centrifugation of the tested samples and an increase in the temperature of incubation samples close to the physiological norm for animals. The improved technique of staging an immunodiffusion reaction (AGID) allows detecting an average of 12% more infected animals with BLV in comparison with the approved method of staging a serological reaction AGID. Intensive introduction of PCR diagnostics to identify the causative agent of bovine leukemia in young calves, after the neonatal age period, will allow detecting early infection of animals and adjusting the program of antileukemia measures in disadvantaged farms. The introduction of PCR diagnostics in calves in the postnatal period of development in permanently dysfunctional livestock farms will contribute to the recovery of young animals from cattle leukemia in dysfunctional farms. The proposed antileukemia measures for the improvement of livestock farms in the Belgorod and Kemerovo regions made it possible to develop effective preventive measures for disadvantaged farms, improving the epizootic situation in the regions. So, in 2021, it was possible to completely improve the permanently dysfunctional economy of the LLC «Pobeda» in the Belgorod region.

KEY WORDS: ANTILEUKEMIC MEASURES DIAGNOSTICS, BOVINE LEUKEMIA VIRUS, ENZOOTIC BOVINE LEUKOSIS, POLYMERASE-CHAIN-REACTION, REHABILITATION OF DISADVANTAGED FARMS.

INTRODUCTION

Protection of animal and human health is the main task of antiepidemic and antiepidemic services in different countries of the world. Bovine leukemia (EBL) is one of the most pressing infectious viral diseases in animals. This is a slow-moving infectious disease of a tumor nature, which causes enormous economic and economic damage to livestock farms, and also poses a potential medical and social danger to humans (Frie et al. 2016; Stepanova 2016; Sviridenko 2017; Mishchenko et al. 2018; Murakami et al. 2019; Donnik et al. 2021; Kovalenko et al. 2020; Kovalenko

et al. 2021). Modern approaches to recovery from bovine leukemia require modernization in laboratory diagnostics of this disease, thereby preventing the spread of viral infection (DeBrogniez et al. 2015; Gulyukin et al. 2019). The effectiveness of antileukemia measures depends on the improvement of laboratory diagnostics methods and earlier detection of infected animals with the bovine leukemia virus during the incubation period, as well as strict observance of veterinary and sanitary measures to form sustainable welfare for bovine leukemia (Gulyukin et al. 2015; Donnik et al. 2015; Valikhov 2018; Donnik et al. 2021).

In recent years, there has been a directed process to improve the health of dysfunctional farms for bovine leukemia through constant laboratory monitoring and isolation of identified infected individuals (Donnik 2011). The basic test for detecting infected EBL cows is the immune

Article Information:

Received 15/09/2021 Accepted after revision 19/12/2021

Published: 31st December 2021 Pp- 1584-1590

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.33>

diffusion reaction (AGID), and the final diagnosis is made after hematological studies (Donnik et al. 2013). Modern diagnostics of bovine leukemia is based on the identification of the causative agent of the disease, which requires molecular genetic studies (PCR). The molecular genetic method is highly sensitive and reliable, which opens up new opportunities for the study of this disease. The aim of the research was to develop effective antileukemic measures for the improvement of livestock farms in the Belgorod and Kemerovo regions (Mishchenko et al. 2018; Murakami et al. 2019; Donnik et al. 2021).

MATERIAL AND METHODS

The studies were performed in the Belgorod State Agricultural University named after V.Ya. Gorin "and dairy farms of the Belgorod region – LLC «MMF Nezhegol» (dairy farm No. 1), JSC «Orlik» (dairy farm No. 2), CJSC «Voskhod» (dairy farm No. 3) and LLC «Pobeda» (dairy farm No. 4) in the period 2020-2021 years (Donnik et al. 2021). The object of research is blood serum and stabilized blood of cattle from farms with poor leukemia in cattle. Blood samples for research were taken from the caudal vein with disposable instruments into sterile tubes. Diagnosis of animals with leukemia in cattle is carried out by studying the hematological parameters of the blood of animals that previously reacted positively in the reaction of immunodiffusion. As an additional method to the main diagnostic tests carried out - serological and hematological studies, PCR diagnostics using the test-system «Leukemia» was used on young cows after the 14th day of rearing up to six months of age, obtained from AGID (+) cows in LLC «Pobeda» (dairy farm No. 4). The laboratory studies of cow samples were carried out according to unified methods (Order of the Ministry of Agriculture and Food of the Russian Federation of May 11, 1999 N 359) and the protocol of serological studies (Donnik et al. 2021).

Using an automatic hematological analyzer "URIT-3020", the leukocyte formula and the calculation of the content of certain types of leukocytes were determined. Microscopic assessment of stained smears according to Leishman was determined on Labor microscopes, 60 × /0.75 with a built-in camera; for molecular genetic studies (PCR), the Leukemia test system was used according to the instructions for its use. For statistical data processing, mathematical models were used on a personal computer (Murakami et al. 2019).

RESULTS AND DISCUSSION

The antileukemia measures carried out on the territory of the Belgorod and Kemerovo regions began, first of all, with epizootological monitoring and accounting of all stationary dysfunctional farms, while laboratory diagnostics always plays a special role. The developed improved formulation of the immunodiffusion reaction (AGID) and an increase in the frequency of sampling from the studied animals helped to more effectively identify infected animals and carry out comprehensive health measures on the disadvantaged territory of farms. The proposed antileukon measures are to increase the frequency of serological studies carried out per year among animals in unfavorable areas for bovine

leukemia and the time of the beginning of specific studies, in the postnatal period of the calf. Improvement in the diagnosis of bovine leukemia consists in increasing the sensitivity of the AGID due to the highly centrifugation of the tested samples and increasing the temperature of the temperature control of the samples *in vitro*, bringing it closer to the physiological norm for animals *in vivo* (Kovalenko et al. 2021).

In the experimental part of the work, during the development of an improved laboratory technique for the formulation of the AGID reaction, 1251,0 samples of cattle blood serum from were taken from a leukemia-dysfunctional farm - LLC «Pobeda» (dairy farm No. 4). They were investigated using various methods of high-speed centrifugation and temperature conditions close to the physiological parameters of the animal in order to identify the maximum number of additional AGID (+) positive bovine heads that previously gave a negative reaction of immune diffusion in the standard formulation of AGID (Kovalenko et al. 2020; Kovalenko et al. 2021; Donnik et al. 2021). The proposed antileukon measures are in the Improved method of staging the immunodiffusion reaction, which makes it possible to identify, on average, 12% more infected BLV animals in comparison with the approved method of staging a serological reaction. The use of the proposed method for improving the diagnosis of leukemia in cattle made it possible to identify cows infected with leukemia at earlier stages of the incubation period, which are in the initial period of the production of antileukemic antibodies, which made it possible to reduce the time spent by these animals in the herd and contributed to the acceleration of health measures without significant material and time costs (Donnik et al. 2021).

In the complex of antileukemic measures with a goal the recovery of dysfunctional farms, we proposed to reduce the frequency of serological tests from 6 to 3 months, and, if possible, laboratory tests can be carried out more often, based on specific cases. Since a six-month study period can contribute to the re-infection of AGID -negative animals with infected individuals in the early stages of the development of the leukemia process, in which antibodies to BLV were not previously detected in the immunodiffusion reaction (AGID) (Kovalenko et al. 2020; Donnik et al. 2021).

The antileukemic measures used consisted in the fact that in addition to the existing objective informational tests to determine the infection (using the immunodiffusion reaction) and the morbidity of animals from among the infected, an automatic hematological analyzer can also be used as an additional tool, the data of which make it possible to determine the severity of the course leukemic process. So, in dairy farm No. 4 antileukemic measures were worked out to improve the economy as following: step-by-step identification of sick animals; determination of the area of prevalence of the pathogen; study of the level of cow leukemia virus infection on the farm; establishment of ways and mechanisms of transmission of the bovine leukemia virus in a particular farm; formation of schemes for isolated rearing of young animals; development of

technological charts for the repair of livestock by uninfected young animals. So, on the dairy farm No.2 at the beginning of 2020 (01/01/2020) the level of infection was 31.7% (there were 3526 animals in total), and in June (01/06/2020) no positively responding animals were detected in the AGID, thanks to the implementation of isolating preventive veterinary and sanitary measures aimed at improving the animal population (Fig. 1) (Kovalenko et al. 2020; Donnik et al. 2021).

Figure 1: The results of the use of anti-leukemia measures in improving the economy in Dairy farm No.2

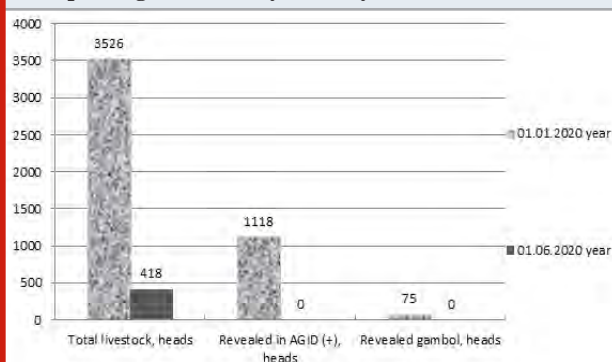
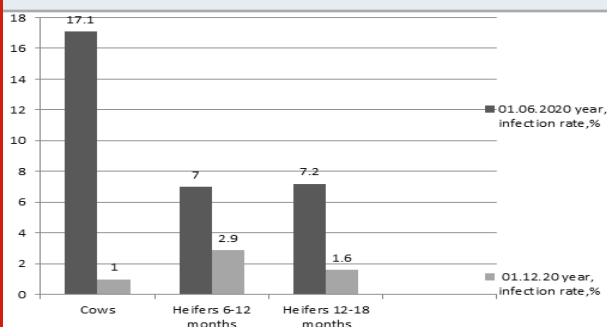


Figure 2: The level of infection of animals by BLV in Dairy farm No.4

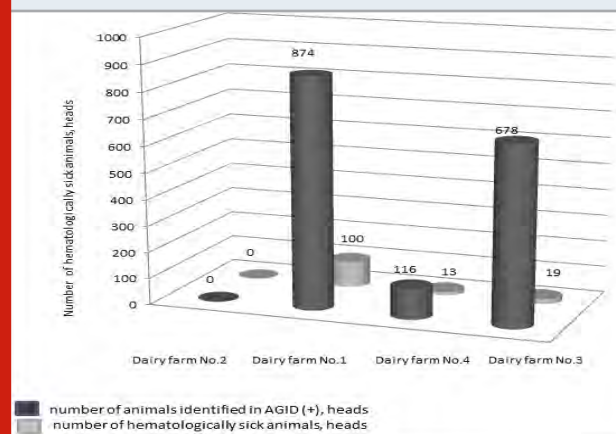


In January 2020, in four dysfunctional livestock farms (EBL) of the Belgorod region with a population of 6605 heads, a positive reaction in the AGID, 3343 heads, which were 50.6% of the total number of livestock, and among them, hematologically sick individuals of 219 heads were identified (6.55%). Since the livestock farm of «Dairy farm No.4» was unfavorable for leukemia in cattle for many years, where the level of infection of the livestock with BLV infection significantly exceeded 30% infection of the herd, it was decided to use this farm as a model. According to the improved method of early detection of animals infected with the leukemia virus in cattle, blood sampling for serological studies should be carried out not every six months, but every 2-3 months. So, in Dairy farm No.4, by the end of 2020 the application of the developed technique for early diagnosis of leukemia made it possible to increase the detectability of infected livestock in the immunodiffusion reaction (AGID), in comparison with the beginning of the study period, 01.01.2020, from 1668 to 1741 heads, or 6.35% (Donnik et al. 2021).

At the same time, there were only 22 animals with hematologically sick cattle leukemia at the end of 2020, which in percentage terms was 1.26%. Thus, as a result of laboratory studies of samples from infected cows, a direct dependence of the concentration of the desired antibodies in the tested sera and the influence of the temperature factor of incubation on the absolute binding of antibodies to antigens was established under conditions of the immunodiffusion reaction more physiologically close to *in vivo*. This provided an increase in the sensitivity of ACID and made it possible to detect, on average, 20.25% more animals infected with the leukemia virus, compared with the staging of a standard AGID (Kovalenko et al. 2021; Donnik et al. 2021).

The result of PCR studies carried out in the beginning 2020, the presence of BLV was found in 16 samples (11.11%) from 114 calves (blood samples). Taking into account these results, this test was used in studies conducted in May – June 2020. As a result, in May, the presence of BLV was detected in 14 out of 50 blood samples from calves aged 15 days to 3 months (28% of the total number of examined individuals), in June - in 2 out of 36 blood samples of animals from 15 days to 1.5 months (5.5%). In general, in 2020, the presence of the BLV was detected in 32 calves under 6 months of age out of 230 individuals born from AGID-positive cows, which amounted to 13.9%. All animals, in whose blood BLV proviral DNA was detected, were removed from the general herd and fed in a separate slaughter house after gaining the appropriate weight (Donnik et al. 2021).

Figure 3: Detection rate of hematologically sick individuals in relation to the total number of cattle leukemia virus infected



Antileukemia measures, consisting in the application of the developed diagnosis of EBL, contributed to the recovery from leukemia infection of the cattle of Dairy farm No.2, and in two other previously dysfunctional leukemia farms of Dairy farm No.3 and Dairy farm No.4 of the Belgorod region to reduce the level of infection by November 2020 from 56.4% and 84.1% to 41.4% and 11.5%, respectively. The developed approach to the diagnosis of leukemia in cattle during health-improving measures made it possible to completely recover from leukemic infection the number of cattle of Dairy farm No.2 and in the other two previously unsuccessful farms of Dairy farm No.3 and Dairy farm

No.4 of the Belgorod region to reduce the level of infection by November 2020 from 56.4% and 84.1% to 41.4% and 11.5%, respectively (Donnik et al. 2021).

Additional serological studies (AGID) carried out within six months, 01.06.-01.12.2020, in the dysfunctional farm of Dairy farm No.4 showed that the level of infection in cows of all sex and age groups, as well as heifers 6 ... 12 and 12 ... 18 months, decreased to 1%, respectively, 2.9% and 1.6% (Fig. 2). In general, in this farm, the infection rate of animals with bovine leukemia decreased from 84.1% at the beginning of 2020 to 1.6% at the end of 2020, which indicates the effectiveness of the health-improving measures taken.

The results of a serological study of the blood serum of the Dairy farm No.4 livestock (828 heads), carried out twice in February and April 2021, showed that there were no animals that responded positively in the AGID. Additional PCR studies of 21 samples from calves from 15 days of age in February 2021 also did not reveal the genomic material of the causative agent of bovine leukemia. In addition, to study the severity of the pathological process, we used an automatic hematology analyzer URIT-3020, which allows, in addition to quantitative study of the

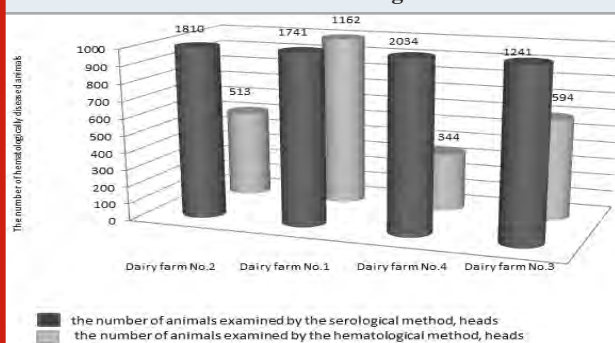
content of the leukocyte pool (the number of leukocytes), to detect the content of granulocytes (basophils, eosinophils, neutrophils), lymphocytes and monocytes.

According to the results of the conducted hematological studies of 2613 heads of cattle during 2020, the presence of 132 hematological individuals in permanently dysfunctional farms was established. So, in LLC "Nezhegol" 100 hematologically sick cows were identified among 874 RID (+) heads, in LLC "Pobeda" 13 hematologically heads among - 116 heads; at Voskhod CJSC there are 19 hematologically sick players among - 678 heads, which in percentage terms was 11.4%, respectively; 11.2%; 2.8% (fig. 3). So, in Dairy farm No.1 there were identified 100 hematologically sick among 874 AGID (+) heads, in Dairy farm No.4 - 13 hematologically sick among 116 heads; at Dairy farm No.3 there are - 19 hematologically sick players among - 678 heads, which in percentage terms amounted to 11.4%, respectively; 11.2%; 2.8% (Fig. 3). Using the standard technique for determining the number of leukocytes using the Goryaev camera, it was found that among 1668 serologically positive animals in dysfunctional breeding farms of the Belgorod region, only 132 hematologically patients were identified, which amounted to 7.9% (Table 1).

Table 1. Statistical data of hematological studies in permanently dysfunctional farms in the Belgorod region

Name farms	Investigated serological method, heads	Investigated by hematological method, heads	Revealed hematologically sick animals, heads	Rented hematologically sick animals, heads	Remaining hematologically sick animals on 30.11.20r., heads
Dairy farm No.1	1741	1162	100	78	22
Dairy farm No.2	1810	513	0	0	0
Dairy farm No.3	1241	594	19	39	0
Dairy farm No.4	2034	344	13	16	0
TOTAL	6826	2613	132	133	22

Figure 4: The ratio of the number of hematologically studied animals to the total number of serological studies



Based on the "leukemia key", the status of cattle in stationary dysfunctional farms was determined: up to 12 thousand / μ l (healthy animals), and over 12 thousand / μ l infected and sick animals (from 12 to 20 thousand / μ l - the

initial stage of the hematological stage of leukemia; from 20 to 30 thousand / μ l - the middle stage of the hematological stage of leukemia; over 30 thousand / μ l - the transition to the tumor stage of leukemia. In general, for all unfavorable stationary farms of the Belgorod region 2613 heads were examined by hematological method among seropositive individuals in AGID, and 6826 animals were subjected to serological research, which amounted to 38.2%; at the same time, 132 hematologically sick heads were identified and 133 (100%) were handed over for slaughter, and at the end of this year there were 22 heads left (Table 1).

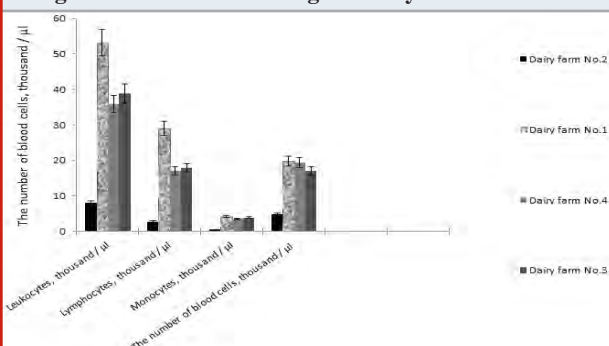
It is also worth noting that the ratio of the number of hematologically studied animals to the total number of serological tests (AGID) in stationary dysfunctional livestock agricultural enterprises of the Belgorod region - Dairy farm No.2; Dairy farm No.1; Dairy farm No.4 and Dairy farm No.3 were as follows: 513 heads by 1810 heads (28.3%); 1162 heads to 1741 heads (66.7%); 344 heads

by 2034 heads (16.91%) and 594 heads to 1241 heads. (47.56). (fig. 4).

Assessing the effectiveness of standard hematological studies, it should be noted that the excretion rate of hematologically sick animals among the total number of seropositive animals in stationary cattle leukemic cattle breeding enterprises of the region ranges from 0.05% to 1.53%. The standard method for determining the number of leukocytes in the studied blood samples does not reveal the depth of development of persistent leukocytosis in infected animals. For this purpose, hematological studies were additionally carried out to identify the formed elements of the leukocyte pool (granulocytes, monocytes and lymphocytes) in cattle blood samples using an automatic hematological analyzer URIT-3020.

Thus, it was found that the greatest damage to the hematopoietic system of animals was in the farm of Dairy farm No.1 with a total infection rate of more than 80%: the average number of leukocytes in 1 μ l of tested blood samples was 53.1 ± 2.3 thousand / μ l, lymphocytes 29.1 ± 1.7 thousand / μ l, monocytes 4.2 ± 0.4 thousand / μ l, granulocytes 19.8 ± 2.0 thousand / μ l (Fig. 5).

Figure 5: Results of hematological studies at hematologically sick livestock of livestock enterprises in the Belgorod region using an automatic hematological analyzer URIT-3020.

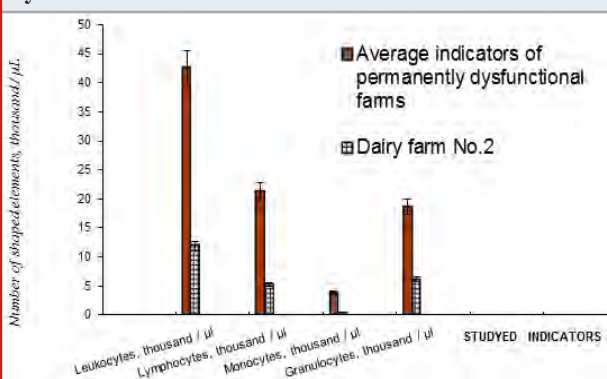


Further, according to the degree of infection (more than 40%) and the development of the leukemic process of the cattle population, the stationary dysfunctional economy of Dairy farm No.3, where the indicators of the content of leukocytes, lymphocytes, monocytes and granulocytes on average among sick animals were 38.9 ± 3.0 , respectively. thousand / μ l, 17.9 ± 3.3 thousand / μ l, 3.9 ± 0.7 thousand / μ l and 17.1 ± 1.3 thousand / μ l (Fig. 5). In Dairy farm No.4, the indicators of the content of leukocytes, monocytes, lymphocytes and granulocytes on average among sick animals were, respectively, 35.8 ± 3.1 thousand / μ l, 17.1 ± 2.3 thousand / μ l, 3.5 ± 0.8 thousand / μ l and 19.4 ± 1.5 thousand / μ l (Fig. 5).

In general, for all stationary dysfunctional farms among infected animals BLV, an average of leukocytes, lymphocytes, monocytes, and granulocytes, respectively, 42.6 ± 2.9 / thousand / μ l, 21.3 ± 1.9 thousand / μ l, 8 ± 0.5 thousand / μ l, 18.7 ± 1.8 thousand / μ l (Fig. 5). In Dairy farm No.2 (free from leukemic infection at the end of the year)

no hematologically sick individuals were found, therefore the indicators of the content of leukocytes, monocytes, lymphocytes and granulocytes were practically close to the reference ones and amounted to 11.9 ± 1.3 thousand / μ l, respectively, $5, 3 \pm 1.4$ thousand / μ l, 0.5 ± 0.9 thousand / μ l, and 6.1 ± 0.8 thousand / μ l. (Fig. 6).

Figure 6: Average hematological indicators in stationary dysfunctional livestock farms



The data of hematological parameters for the leukocyte pool indicate the severity of the development of the leukemic process and make it possible, in addition to the existing tests, to characterize the qualitative changes in the pools of the agranulocyte and granulocyte series. The unified method for counting the number of formed elements - a hematological test using a Goryaev chamber makes it possible to determine quantitative indicators of the content of leukocytes in 1 μ l, but does not make it possible to determine the differentiated composition of blood leukocytes (granulocytes and agranulocytes), which is especially important for determining the stage of development of the leukemic process during complex health-improving antileukemic measures. Studies conducted over several decades of the spread of leukemia in cattle, led to the conclusion that not all breeds are equally susceptible to the leukemia virus.

The most susceptible were black-and-white breeds with an influx of Dutch, German and Danish blood (Forlettia et al. 2013). The bovine lymphocyte antigen (BoLA) system is the main constituent of the bovine histocompatibility complex. The genes of the histocompatibility complex encode highly polymorphic cell surface molecules, which are antigenic peptides of T cells (Forlettia et al. 2013), thereby playing a decisive role in the immune response to foreign agents. In cattle, three DRB genes in the composition of BoLA were identified in the histocompatibility complex (Valikhov 2018; Murakami et al. 2019; Kovalenko et al. 2020).

However, only the DRB3 gene is the main pronounced locus, since DRB1 is a pseudogene, and the DRB2 gene, as shown by Northern blot analysis, is not expressed in bovine leukocytes or is expressed at a very low level. The DRB3 gene (class II gene), which encodes the b-chain of the histocompatibility complex, is the only functional DRB gene actively transcribed in cattle. Exon 2 of the BoLA DRB3 gene has a high level of polymorphism and

is represented by more than 100 different alleles described to date (Úsuga-Monroy et al. 2016). When introducing innovative methods of molecular genetics into the breeding process of improving domestic populations of dairy cattle, it is necessary, first of all, to investigate the molecular genetic basis that determines the emergence and formation of certain characteristics of an organism, including susceptibility and resistance to infectious diseases, in particular to the bovine leukemia virus. In this case, it is necessary to take into account the specialization in the productivity of certain populations of livestock, since the incidence of leukemia among dairy cattle is significantly higher than that of beef cattle.

Therefore, within the framework of the research of scientists of the Kuzbass Agricultural Academy, the task was to determine the resistance to the leukemia virus in black-and-white cattle by the BOLA-DRB3 gene and to identify animals that carry not only generally recognized alleles of resistance to leukemia, but also alleles that are specific genetic markers of resistance to leukemia virus. The object of research was the animals of the Kemerovo population of the black-and-white breed. Studied 187 animals: 122 of them are sick and 65 healthy. In studies, the greatest polymorphism was noted for the alleles of the BOLA-DRB3 gene, attributed by the majority of researchers to neutral - 47 variants were identified. In total, the frequency of these alleles was 43.6%. Generally neutral allelic variants are less than 5% of the frequency each. At the same time, it should be noted that the most common alleles are 0601 - 7.8% and 1801 - 7.2%. The analysis of the obtained results of the study showed that the frequency of occurrence of alleles 1801 and 0601 in the groups of patients with leukemia and healthy animals differed significantly. The frequency of the 0601 allele in healthy animals is 11.7 times higher ($P < 0.01$) than in the group of sick animals. The frequency of the 1801 allele in healthy animals is 8.3 times higher ($P < 0.05$) than in the group of sick animals (Murakami et al. 2019; Kovalenko et al. 2020).

Since the data on the number of leukocytes in AGID (-) negative animals have physiological normative indicators, it can be said with confidence that the data on the quantitative ratio of leukocyte elements (lymphocytes, monocytes and granulocytes) could be used when working out the technique in the case of stable reliable results to characterize the course of the leukemia process, using it as a test for early diagnosis of infected animals BLV and at the final stage of health-improving anti-leukemia measures. The method of early diagnosis of bovine leukemia developed by us made it possible to speed up the process of rehabilitation of permanently disadvantaged farms of LLC «POBEDA» during the year and so thanks the full implementation of the plan of preventive health improvement (anti-leukemia) measures developed on the territory of the Belgorod region in 2021 year. For the implementation of health-improving measures, the following stages were proposed for identifying animals with leukemia:

- conducting epizootological studies and dividing the herd into infected AGID (+) and conditionally healthy AGID (-);

isolation of infected animals indoors;

- placement of livestock infected with the leukemia virus on separately located farms, which contain exclusively infected animals, in accordance with the instructions for combating bovine leukemia, taking into account their routes of movement both on the territory of the corresponding departments and inside livestock areas, separate milking, separate service personnel and the use of technical means (Kovalenko et al. 2020).

The developed antileukemia measures help to effectively carry out the process of rehabilitation of disadvantaged livestock farms by increasing the frequency of serological studies and increasing the sensitivity of the immunodiffusion reaction AGID. The use of the proposed methods helps to detect infected cattle in the early incubation period and prevent further spread of the pathogen through the separation of animals in the system of general veterinary and sanitary antiepidemiological measures. This reduces the time spent by infected animals in the herd, contributing to the acceleration of antileukemia health measures without significant material and time costs. An additional effect is provided by the use of molecular genetic tests that allow identifying the causative agent of BLV in young calves in the post-neonatal period of their development (Kovalenko et al. 2020).

CONCLUSION

The findings of the present study proposed antileukemia measures for the improvement of livestock farms in the Belgorod and Kemerovo regions made it possible to develop effective preventive measures for disadvantaged farms, improving the epizootic situation in these regions. The use of the developed antileukemia measures made it possible in 2021 year to completely improve the number of animals from BLV in one of the inpatient dysfunctional livestock farms of the Belgorod region.

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Pharmaceutical Communication

***In vitro* Microrhizome Induction and Essential Oil Production from Aromatic Ginger *Kaempferia galanga* L. An Economically Important Medicinal Herb**

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ABSTRACT

Kaempferia galanga L. or 'aromatic ginger' is a stem-less herb in Zingiberaceae having different pharmacological properties like antioxidant, antimicrobial, nematocidal, vasorelaxant and wound healing activity. The plant is generally a vegetatively propagated annual herb; its conservation using conventional methods takes more time to get sufficient amount of planting materials for commercial cultivation. Micropropagation by *in vitro* methods helps to overcome the present demand for this high sought medicinal and aromatic species. At present the concern on *in vitro* propagation is directed to rhizome or storage organ induction for productive acclimatization and to reduce the injury during transportation. Microrhizomes are the small rhizomes developed in *in vitro* conditions and its induction is an effective biotechnological tool for the production of quality planting materials as they are genetically stable and disease free. The present study is discussing the role of silver nitrate (AgNO₃) along with sucrose in *in vitro* microrhizome induction in *K. galanga* for the first time. MS medium fortified with 2.0 mg l⁻¹ AgNO₃ along with 6% (w/v) sucrose produced maximum amount of microrhizomes i.e., 4.52±0.11 g after 3 months that increased to 5.70±0.20 g in six months of harvesting. Here we also reports the comparative analysis of chemical constituents in the essential oil of *in vivo* rhizomes and *in vitro* microrhizome through GC-MS analysis that further reveals the superior characteristics of the microrhizomes in terms of the bioactive components ethyl p-methoxy cinnamate and ethyl cinnamate, the esters that contribute the nematocidal, antituberculosis, anti-inflammatory, antifungal and larvicidal properties to the oil. This protocol for *in vitro* microrhizome induction can be used for the commercial production of rhizomes and essential oil in *K. galanga* and the outcome of this study can be further used for mass production of pathogen-free microrhizomes and conservation for its sustainable utilization of the species.

KEY WORDS: AGNO₃, ESSENTIAL OIL, KAEMPFERIA GALANGA, MICRORHIZOME, SUCROSE.

INTRODUCTION

Kaempferia galanga L. is an endangered medicinal plant of Zingiberaceae that is extinct in the wild, but available under cultivation mainly in South East Asia and China for its aromatic rhizome. The plant is economically important as the main ingredient of several ayurvedic preparations

such as Dasamularishta, Valiya rasnadi kasaya, *Kaccoradi churna*, Asanaeladi taila, Valya narayana taila and is used for the healing of rheumatism (Rastogi and Mehrotra 1993; Sivarajan and Balachandran 1994; Kareem 1997). The rhizome extract contains n-pentadecane, ethyl p-methoxy cinnamate, ethyl cinnamate, camphene, borneol, cineol, p-methoxy styrene, kaempferol and kaempferide (Tewtrakul 2005). The rhizome has stimulatory, expectorant, carminative and diuretic medicinal properties generally. It possesses a camphoraceous odour and the decoction prepared from the rhizome is used for dyspepsia, headache and malaria. *K. galanga* essential oil (galangal oil) is having high market

Article Information:*Corresponding Author: preethahemanth@yahoo.com

Received 15/09/2021 Accepted after revision 18/12/2021

Published: 31st December 2021 Pp- 1591-1599

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.34>

value (Rs 2000/- for 10 ml, Silky scents L.L.C) (Alsalmi et al. 2020).

Essential oil compounds were analyzed earlier in its rhizome by many workers and the plant contains 2.4 to 3.9% volatile oil (Rao et al. 2009; Raina et al. 2015; Sunitha et al. 2018; Alsalmi et al. 2020). Ethyl p-methoxy cinnamate present was found to exhibit anticancer activity which amount to 30.6% and its vasorelaxation effect was also reported (Srivastava et al. 2019). The species is conventionally propagated by rhizomes and there is no seed setting by natural means. In such circumstances plant tissue culture based micropropagation is the most efficient propagation method for quality planting material production. However, in the rhizomatous species, the micro clones raised through *in vitro* cloning are not suitable for direct commercial planting, as they need at least two generations in green house to form sufficient quantity of minirhizome for planting in the field. *In vitro* microrhizome production can overcome this hurdle and can be exploited for the utilization of commercial product of interest thereby overcoming the delay in the utilization of micropropagated plantlets (Alsalmi et al. 2020; Srivastava et al. 2021).

The chief advantage of these microrhizomes is that they are disease free can be directly transferred to the field without any acclimatization procedure and can be transported internationally as they do not require any special measures as well as quarantine (Regunath and Shameena 2013). Induction of vegetative storage organs under *in vitro* conditions their application in planting material production, *in vitro* conservation and germplasm exchange has been reported in many Zingiberaceous species (Raina et al. 2014; Swarnathilalaka et al. 2016; Nguyen et al. 2020). Microrhizome production can be done in any seasons in sterile conditions and they have advantages of easily storage, transport and can be used in germplasm conservation. Taking into consideration of these merits, the present study was undertaken with an effort to establish an efficient system for microrhizome induction in *K. galanga* (Mohamed et al. 2014; Zahid et al. 2021).

Here an attempt has been made to develop a convenient and effective method for high frequency *in vitro* micro rhizome induction in using different concentrations of sucrose and AgNO_3 supplementation to the medium followed by comparison of chemical constituents in the essential oil of *in vivo* rhizomes and *in vitro* microrhizomes as there exist some lacuna regarding this aspect in the targeted species. The system established will be highly beneficial for mass production of quality planting materials and its economic utilization as well as germplasm exchange thereby providing another means of conservation in this species.

MATERIAL AND METHODS

Rhizomes of *K. galanga* L. collected from Kundara, Kollam District, Kerala, India (JNTBGRI Herbarium Voucher number TBG 60677) were used as the plant material for the *in vitro* shoot culture establishment in the present study. Fresh rhizomes with axillary buds collected from the field-grown plants were thoroughly washed under running

tap water, outer scales were removed and washed in 5% Teepol (v/v) for 20 minutes, again washed in running tap water and treated with 0.2% bavistin (fungicide) for 10 minutes. After several rinses in distilled water, they were subjected to sterilization with 0.1% (w/v) HgCl_2 for 8-10 minutes followed by 4-5 rinses in sterile distilled water, then inoculated aseptically in MS (Murashige and Skoog) medium (Murashige and Skoog 1962) containing 0.5 mg l^{-1} BA. The initiated shoots were further subcultured to fresh MS medium augmented with 3.0 mg l^{-1} BA and 0.5 mg l^{-1} NAA for stimulating the shoot multiplication (Preetha et al. 2012). For microrhizome induction, *in vitro* shoots were trimmed to 1-1.5 cm length bearing the rhizomatous base and then inoculated to different media treatments.

To study the effect of sucrose on *in vitro* microrhizome induction, varying concentrations of sucrose (3, 6, 9 and 12%) along with 3.0 mg l^{-1} BA and 0.5 mg l^{-1} NAA was checked. To examine the effect of different concentration of AgNO_3 and sucrose in *in vitro* microrhizome induction, the *in vitro* shoots established in 0.3 mg l^{-1} BA and 0.5 mg l^{-1} NAA were subcultured to the fresh medium of the same PGR composition augmented with different concentration of silver nitrate (1.0 mg l^{-1} and 2.0 mg l^{-1}) and 3, 6 and 9% sucrose (w/v). Here two different concentrations of AgNO_3 and three different concentrations of sucrose were used. The different treatments were $1T_1$ ($1.0 \text{ mg l}^{-1} \text{AgNO}_3 + 3\%$ sucrose), $1T_2$ ($1.0 \text{ mg l}^{-1} \text{AgNO}_3 + 6\%$ sucrose), $1T_3$ ($1.0 \text{ mg l}^{-1} \text{AgNO}_3 + 9\%$ sucrose), $2T_1$ ($2.0 \text{ mg l}^{-1} \text{AgNO}_3 + 3\%$ sucrose), $2T_2$ ($2.0 \text{ mg l}^{-1} \text{AgNO}_3 + 6\%$ sucrose), $2T_3$ ($2.0 \text{ mg l}^{-1} \text{AgNO}_3 + 9\%$ sucrose) respectively. The response of the cultures were periodically noted for up to six months. Bulging of basal portion of the stem was taken as the indication of microrhizome induction.

Experiments were carried out in triplicates with at least ten explants per treatment. The results were taken after 3 and 6 months respectively. Morphogenic response of plants regarding the number of shoots, number of shoots, length of shoots, number of leaves per shoots during these periods and the amount of microrhizomes produced were recorded and statistically analysed by one way analysis of variance (ANOVA) and the means were compared by Duncan's multiple range test $p \leq 0.05$ using the computer software SPSS/ PC + version 4.0 (SPSS Inc., Chicago, USA).

Twenty five grams each of microrhizomes and *in vivo* rhizomes from the field were subjected to hydro-distillation using a modified Clevenger-type glass apparatus for 4 h. The oil samples were separated by using di-ethyl ether and anhydrous sodium thio sulphate. The GC-MS analysis was done on a Hewlett Packard 6890 gas chromatograph fitted with a cross-linked 5% phenyl methyl siloxane HP-5MS capillary column (30m x 0.32mm, film thickness 0.25 mm) coupled with a 5973 series selective mass detector. 1.0 ml of the essential oil was injected. Helium was used as the carrier gas at 1.4 ml/min. Constant flow mode, with injector temperature 220°C and oven temperature 60°C to 246°C (3°C/min). Mass spectra at electron impact (EI+) mode were taken at 70 Ev. The oil constituents were identified by MS library search (WILEY 275), comparison of the relative retention indices were calculated with respect to

homologous of n-alkanes (C6-C30, Aldrich Chem.Co.Inc) and by comparison of mass spectrum reported in the literature (Dool and Kratz 1963; Adams 2007).

RESULTS AND DISCUSSION

Effect of sucrose on Morphogenic response of shoot culture: In the present experiment the morphogenic response of *K. galanga* in different concentration of sucrose (3, 6, 9 and 12%) (w/v) were recorded after 3 months as well as 6 months of inoculation. The mean number of shoot was 4.33 ± 0.31 in 3% (w/v) sucrose (control) which increased to 9.33 ± 0.33 with the increase of concentration of sucrose to 9% (w/v). There after it decreased 6.67 ± 0.30 in 12% (w/v) sucrose. The results agree with the findings in *Z. officinale*, where maximum number of shoots were noticed in MS medium supplemented with 8% (w/v) sucrose while in *Kaempferia parviflora* MS medium fortified with

6 % (w/v) sucrose produced maximum number (8.5) of shoots (Mehaboob et al. 2019; Labrooy et al. 2020). Here, in *K. galanga*, maximum shoot length (4.51 ± 0.11 cm) was observed in 6% (w/v) sucrose level. Shoot length reduced considerably as the concentration of sucrose increased further. Very short shoots (1.67 ± 0.07) were noticed in MS medium with 12% (w/v) sucrose (Table 1). Regarding the number of leaves per shoots, maximum number of leaves were recorded in 6% (w/v) sucrose and this parameter remained almost same in the rest of the treatments. Maximum leaf area (15.17 ± 0.17) was observed in the highest sucrose concentration tested, i.e., 12% (w/v) and the leaves were not much expanded in 6 and 9 % (w/v) sucrose levels. However comparatively large leaves were seen in the control i.e., 3% (w/v) sucrose. Similar trend of morphogenic response was exhibited in six months old cultures also (Fig. 1a).

Table 1. Morphogenic Response of *K. galanga* in Sucrose Treatments

Sucrose (%)	Number of Shoots		Length of Shoots (cm)		Leaf Number Per Shoot		Leaf Area (cm ²)	
	3 months	6 months	3 months	6 months	3 months	6 months	3 months	6 months
3	4.30 ± 0.3^c	5.67 ± 0.32^d	2.80 ± 0.09^c	3.59 ± 0.07^b	3.40 ± 0.25^b	4.83 ± 0.21^b	11.75 ± 0.25^b	12.00 ± 0.57^b
6	6.30 ± 0.32^b	7.33 ± 0.30^c	4.51 ± 0.11^a	4.52 ± 0.08^a	4.00 ± 0.41^a	5.37 ± 0.37^a	8.00 ± 0.20^d	8.50 ± 0.12^d
9	9.33 ± 0.33^a	11.33 ± 0.33^a	3.53 ± 0.06^b	3.67 ± 0.11^b	3.70 ± 0.21^b	3.92 ± 0.22^c	8.67 ± 0.33^c	9.33 ± 0.66^c
12	6.67 ± 0.30^b	8.33 ± 0.34^b	1.67 ± 0.07^d	2.54 ± 0.08^c	2.67 ± 0.21^c	2.67 ± 0.16^d	15.17 ± 0.17^a	16.00 ± 0.50^a

*Data represents mean values of ten replicates repeated thrice, recorded after 3 and 6 months of culture. Mean values followed by the same letter in the superscript in a column do not differ significantly based on ANOVA and t-test at $p \leq 0.05$

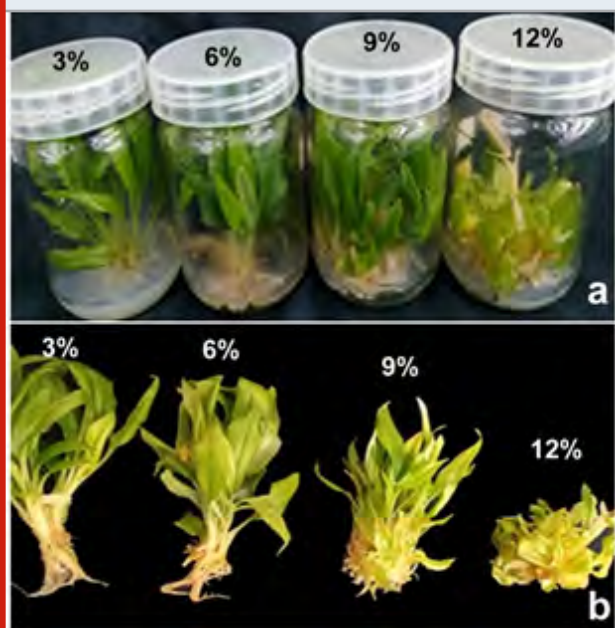
Table 2. Effect of Sucrose on Microrrhizome induction in *K. galanga*

Sucrose (%)	Weight of Plant (g)		Weight of Plant Without Leaf (g)		Weight of Microrrhizome (g)	
	3 months	6 months	3 months	6 months	3 months	6 months
3	5.83 ± 0.08^d	11.45 ± 0.09^d	1.01 ± 0.00^c	2.52 ± 0.06^c	0.57 ± 0.01^c	0.07 ± 0.03^d
6	15.23 ± 0.11^b	13.34 ± 0.07^c	4.31 ± 0.09^b	3.67 ± 0.02^b	2.17 ± 0.05^b	2.58 ± 0.01^c
9	18.69 ± 0.45^a	20.71 ± 0.13^a	7.26 ± 0.14^a	10.26 ± 0.05^b	2.93 ± 0.08^a	3.94 ± 0.03^b
12	11.95 ± 0.06^c	19.48 ± 0.43^b	4.43 ± 0.06^b	10.74 ± 0.10^a	1.93 ± 0.03^a	3.01 ± 0.04^a

*Data represents mean values of ten replicates repeated thrice, recorded after 3 and 6 months of culture. Mean values followed by the same letter in the superscript do not differ significantly based on ANOVA and t-test at $p \leq 0.05$

Effect of Sucrose on Microrrhizome Induction: The present experiment has analysed the effect of different concentration of sucrose in inducing microrrhizomes in *K. galanga*. In some previous studies on Zingibers, certain specific concentration of sucrose was effective in microrrhizome induction such as 6-8% (w/v) sucrose was better for *Curcuma longa*, while 6% (w/v) sucrose was most effective in *Curcuma aromatica* and *Curcuma zedoaria* (Shirgurkar et al. 2001; Nayak 2000; Anisuzzaman et al. 2008; Zahid et al. 2021). In *K. galanga*, more amount of

rhizomes were observed in 6 and 9% (w/v) sucrose that produced 2.17 ± 0.05 g and 2.93 ± 0.08 g microrrhizomes after three months (Table 2). However, the amount of microrrhizomes increased predominantly in 6 months old cultures and maximum quantity was observed in 9% (w/v) sucrose which produced 3.94 g microrrhizomes, while in 12% (w/v) sucrose 3.01 ± 0.04 g microrrhizomes were recorded (Fig. 1b).

Figure 1: Microrhizome induction in *K. galanga* after six months in sucrose treatments

Maximum plant weight was noticed in 9% (w/v) sucrose (Table 2). Similarly in *Curcuma longa* MS medium supplemented with 60-90g sucrose helped in microrhizome induction while in *Curcuma amada* maximum response was obtained in MS medium supplemented with 80 g^l of sucrose where 76.2% explants formed microrhizomes but

the control failed to induce microrhizome (Sanghamitra 2002; Pramila et al. 2011). Also, the role of sucrose in *in vitro* microrhizome induction in another rhizomatous herb *Accorus calamus* was reported in previous studies (Devi et al. 2012). Agreeing with this, in *Zingiber* species maximum microrhizome induction was noticed in 8% (w/v) and 10% (w/v) sucrose and medium containing 6% (w/v) and 12% (w/v) sucrose failed to produce microrhizomes (Tombisana and Singh 2015; Zahid et al. 2021).

A healthy and maximum microrhizome production was reported recently in ginger that was obtained in the MS medium with 0.5 mg^l⁻¹ BA, 0.5 mg^l⁻¹ IAA and 8% (w/v) sucrose under 8-hour photoperiod (Mehaboob et al. 2019). Our result pattern is concomitant with the findings in *Zingiber officinale*, where microrhizomes were induced when MS medium was supplemented with 4.5 to 9% (w/v) sucrose. All these findings further substantiates that high concentration of sucrose treatments have a profound effect in microrhizome evoking in *K. galanga*, when compared to control (3% (w/v) sucrose). High rate of microrhizome induction with increasing concentration of sucrose may be due to the presence of high carbon energy in sucrose because rhizomes mostly store carbohydrate. Very earlier Bhat et al. (1994) suggested that sucrose might act as an energy source and an osmoticum in inducing rhizome formation. Reports of Chirangini et al. (2005) also supports our findings that rhizomes serve as a sink where assimilates are uploaded and in an *in vitro* culture system assimilates provided as sucrose may have been transported to the stem for rhizome formation (Bhat et al. 1994; Chirangini et al. 2005; Mehaboob et al. 2019; Zahid et al. 2021).

Table 3. Morphogenic response of *K. galanga* in AgNO₃ and sucrose supplementation

Treatments	Number of Shoots		Length of Shoots (cm)		Leaf Number Per Shoot		Leaf Area (cm ²)	
	3 months	6 months	3 months	6 month	3 month	6 month	3 month	6 month
1T1	7.61±0.24 ^c	8.41±0.36 ^c	4.26±0.20 ^b	5.00±0.23 ^b	5.41±0.18 ^a	6.24±0.17 ^a	6.55±0.31 ^c	7.5±0.14 ^c
1T2	8.40±0.24 ^b	9.52±0.34 ^b	5.02±0.24 ^a	6.32±0.20 ^a	4.22±0.29 ^b	5.81±0.25 ^b	7.76±0.14 ^b	8.2±0.31 ^b
1T3	9.00±0.36 ^a	10.83±0.24 ^a	3.23±0.06 ^c	4.22±0.06 ^c	3.75±0.18 ^c	4.23±0.19 ^c	8.26±0.14 ^a	9.6±0.42 ^a
2T1	9.52±0.28 ^a	12.22±0.03 ^a	6.60±0.27 ^a	6.95±0.039 ^a	5.00±0.21 ^a	6.32±0.67 ^a	8.36±0.20 ^b	9.0±0.02 ^b
2T2	7.61±0.24 ^b	10.41±0.01 ^b	4.75±0.27 ^c	5.01±0.12 ^b	4.33±0.28 ^b	5.35±0.04 ^b	11.80±0.55 ^a	12.7±0.43 ^a
2T3	4.63±0.12 ^c	Stunted growth	4.16±0.21 ^c	Stunted growth	3.42±0.34 ^c	Stunted growth	7.40±0.21 ^c	Stunted growth
Control	4.31±0.31 ^d	5.60 ±0.32	2.84±0.09 ^d	3.63±0.07 ^d	3.41±0.25 ^d	4.86±0.21 ^d	11.8 ± 0.25 ^a	12.0±0.05 ^a

*Data represents mean values of ten replicates repeated thrice, recorded after 3 and 6 months of culture. Mean values followed by the same letter in the superscript do not differ significantly based on ANOVA and t-test at p ≤ 0.05.

Effect of Sucrose and AgNO₃ on Morphogenic Response of Shoot Cultures: The second set of experiments of the present study has analyzed the morphogenic responses in terms of different concentration of silver nitrate and sucrose supplementation during *in vitro* culturing of *K. galanga*. AgNO₃ is a salt of silver and it is commonly used as an anti-ethylene compound in plant tissue culture (Sarropoulo et al. 2016). Significant variation was noticed among different parameters analysed in different period of data collection here also. Among the various treatments, 2T₁ evoked the

production of maximum number of shoots (9.5±0.29) after 3 months of culturing than the control (4.3±0.31) and these shoots were comparatively elongated also. In MS medium augmented with 1.0 mg^l⁻¹ AgNO₃ and varying concentration of sucrose (Treatments 1T₁, 1T₂, 1T₃), the mean number of shoots as well as leaf area exhibited a linear increase with the increase in the concentration of sucrose in the nutrient medium both in 3 months and 6 months of observation (Table 3). A gradual increase in the mean shoot length (4.26±0.20 cm to 5.02±0.24 cm) was noticed

as the concentration of sucrose was increased to 6% (w/v) which suddenly dropped to lower values (3.23 ± 0.06) when the sucrose concentration was 9% (w/v) upon 3 months of

culture. This trend repeated after 6 months of observation also. Effect of AgNO_3 on shoot multiplication was already reported in *Sphaeranthus indicus* and in *Moringa oleifera* (Harathi et al. 2016; Drisya et al. 2019).

Table 4. Effect of sucrose and AgNO_3 on Microrhizome Induction in *K. galanga*

Treatments	Weight of Plant (g)		Weight of Plant Without Leaf (g)		Weight of Microrhizome (g)	
	3 months	6 months	3 months	6 months	3 months	6 months
1T ₁	7.83 ± 0.12^c	10.25 ± 0.09^c	2.64 ± 0.035^c	3.21 ± 0.09^c	1.57 ± 0.05^c	2.35 ± 0.03^c
1T ₂	9.90 ± 0.15^b	12.57 ± 0.09^b	3.18 ± 0.09^b	4.12 ± 0.02^b	2.22 ± 0.01^b	3.54 ± 0.04^b
1T ₃	10.66 ± 0.09^a	14.56 ± 0.07^a	4.40 ± 0.08^a	5.57 ± 0.05^a	3.24 ± 0.06^a	4.66 ± 0.01^a
2T ₁	16.70 ± 0.11^b	18.78 ± 0.10^b	5.75 ± 0.07^b	7.68 ± 0.05^b	2.65 ± 0.06^b	3.01 ± 0.05^b
2T ₂	17.09 ± 0.12^a	19.68 ± 0.16^a	7.47 ± 0.17^a	9.63 ± 0.02^a	4.53 ± 0.11^a	5.70 ± 0.20^a
2T ₃	11.10 ± 0.13^c	Stunted growth	4.87 ± 0.05^c	Stunted growth	2.23 ± 0.01^b	Stunted growth
Control	3.09 ± 0.09^d	3.42 ± 0.097^d	1.99 ± 0.08^d	2.00 ± 0.07^d	0.98 ± 0.02^d	1.08 ± 0.01^d

*Data represents mean values of ten replicates repeated thrice, recorded after 3 and 6 months of culture. Mean values followed by the same letter in the superscript do not differ significantly based on ANOVA and t-test at $p \leq 0.05$.

Figure 2: Microrhizome induction in *K. galanga* shoots in sucrose and AgNO_3



The mean number of leaves per shoots gradually decreased with regard to the increase in the concentration of sucrose in both periods of data recording and the leaf area exhibited linear increase in the values but were having comparatively lesser area than control (Table 3). While the level of AgNO_3 was increased to 2.0 mg l^{-1} with varying concentration of sucrose (Treatments 2T₁, 2T₂, 2T₃), a linear reduction in the mean number of shoots and mean length of shoots was noticed. Though 2T₁ executed maximum shoot production i.e., 9.52 ± 0.29 after 3 months and 12.22 ± 0.04 after 6 months, it significantly reduced to 7.6 ± 0.24 and 10.4 ± 0.02

after 3 months and 6 months respectively as the sucrose concentration was elevated to 6% (w/v). Further increase in the concentration of sucrose to 9% (w/v) i.e., treatment 2T₃ produced a significant decrease in the mean number of shoots after 3 months (4.63 ± 0.12 shoots) (Fig. 2a) and as the culture period was extended to 6 months, the shoots become stunted. Similar trend was apparent with the mean length of shoots and mean number of leaves per shoot (Table 3). Statistically significant leaf area was observed in 2T₂ and control, while in 2T₁, the leaves were less expanded and in 2T₃ the shoots were stunted and died in six months, hence insufficient to calculate the leaf area (Table 3) (Drisya et al. 2019).

It has been experimentally proved that AgNO_3 reduces ethylene production by inhibiting amino cyclopropane-1 carboxylic acid (ACC), present in ethylene biosynthetic pathway (Kumar et al. 2009). Ethylene hormone attaches to its receptors in the presence of copper ions. It has been proved that silver ions could be substituted by copper ions because of similarity in size and thus blocks the receptors and prevent the response from ethylene (Kumar et al. 2016). In addition to the inhibitory effect of silver ions on ethylene and growth stimulation, nitrate in AgNO_3 as the main source of nitrogen and its interference in the structure of amino acids and nucleic acids, is one of the growth factors in plants leading to longitudinal growth of roots and shoots and increased leaf area as observed in *K. galanga* ((Sun et al. 2017). At the same time AgNO_3 increases the production of polyamines, having a common precursor (S-adenosyl methionine) with ethylene. The metabolism of polyamines is related to the production of NO, which is an essential signaling component for plant growth (Pal et al. 2015; Agurla et al. 2017; Mohd et al. 2018). This substantiates the necessity of an in depth study on the role of polyamines

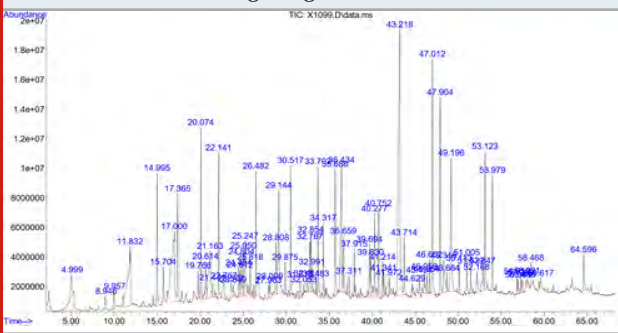
in plant growth and development and the effects on plant signaling substances which would further provide a concrete

evidence for the stimulatory effect of AgNO_3 on plant growth responses as observed in the present study.

Table 5. Essential oil components in *in vivo* rhizome of *K. galanga*

Peak number	Retention time	Abundance %	Compound	Common name	Chemical formula
53	43.218	12.219	n-Hexadecanoic acid	Palmitic acid	$\text{C}_{16}\text{H}_{32}\text{O}_2$
8	17.00	6.447, 2.508	n-Decanoic acid	Capric acid	$\text{CH}_3(\text{CH}_2)_8\text{COOH}$
5	11.832	4.468	Octanoic Acid	Caprylic Acid	$\text{C}_8\text{H}_{16}\text{O}_2$
29	29.144	3.082	Dodecanoic acid	Lauric acid	$\text{C}_{12}\text{H}_{24}\text{O}_2$
64	49.196	3.948	Octadecanoic acid	Stearic acid	$\text{C}_{18}\text{H}_{36}\text{O}_2$
42	36.434	3.924	Tetradecanoic acid	Myristic acid	$\text{C}_{14}\text{H}_{28}\text{O}_2$
60	47.012	3.920	2(3H)-Furanone, 5-dodecylidihydro	Gamma palmitolactone	$\text{C}_{16}\text{H}_{30}\text{O}_2$
61	47.904	3.255	RH-Pyran-2-one, tetrahydro-6-nonyl	δ -tetradecalactone	$\text{C}_{14}\text{H}_{26}\text{O}_2$
41	35.686	2.132	2-Propenoic acid,3 (4-methoxyphenyl)-ethyl ester	Ethyl p-methoxy cinnamate	$\text{C}_{12}\text{H}_{14}\text{O}_3$
21	24.804	0.795	2-propenoic acid , 3-phenyl,ethyl ester	Ethyl cinnamate	$\text{C}_{11}\text{H}_{12}\text{O}_2$

Figure 3: GC-MS -Chromatogram of *In vivo* microrhizome oil of *K. galanga*



Effect of AgNO_3 and Sucrose on Microrhizome Induction: To study the effect of AgNO_3 on microrhizome induction in *K. galanga* MS medium supplemented different concentration sucrose with AgNO_3 (1.0 and 2.0 mg l^{-1}) or without AgNO_3 were tested. Results were taken after three months and six months as in the previous experiment. MS medium supplemented 1.0 mg l^{-1} AgNO_3 along with 9% (w/v) sucrose (1T₃) produced 3.24 ± 0.07 g microrhizomes in three months of culture and after six months the weight of rhizomes increased to 4.66 ± 0.01 g (Table 4). These cultures showed a significant increase in number of shoots, weight of plant and amount of rhizomes. Weight of the plant recorded was 10.66 ± 0.09 g after three months and it was raised to 14.56 ± 0.07 g after six months.

MS medium containing 2.0 mg l^{-1} AgNO_3 along with 6% (w/v) sucrose produced 4.53 ± 0.11 g *in vitro* rhizomes in three months of culture and the amount increased significantly after 6 months (5.70 ± 0.20 g) (Fig. 2b, Table 4). Similarly, in ginger plantlets microrhizomes size was

improved in MS medium supplemented with 1.9 mg l^{-1} AgNO_3 and 80 g l^{-1} sucrose (Nguyen et al. 2020). In the present study, MS medium fortified with 3% (w/v) sucrose, but without AgNO_3 (control) produced least number of shoots and microrhizomes. Here it is very clear that AgNO_3 has a significant promoting effect on microrhizome induction. The positive effects of AgNO_3 on microrhizome induction can be attributed to the binding of Ag^{2+} cations to ethylene receptors at the cell membrane thereby interfering with the typical inhibitory effect of ethylene on organ size's elongation and determination as established in ginger (Nguyen et al. 2020).

However, in our experiment, higher concentration of AgNO_3 (2.0 mg l^{-1}) along with 9% (w/v) sucrose (2T₃ treatment) showed an inhibitory effect on *in vitro* microrhizome induction in *K. galanga*. Ethylene inhibitory effect of AgNO_3 was reported in many other works. Similar inhibitory effect of AgNO_3 in concentrations higher than 11 μM is already reported in two species of ginger. Here we have discussed the combined effect of sucrose and AgNO_3 on microrhizome induction in *K. galanga*. In the light of the findings established here it can be concluded that sucrose and AgNO_3 played an important role in *in vitro* microrhizome induction in *K. galanga* (Ticona and Oropeza 2013; Singh et al. 2013; Moniuszko 2015; Nguyen et al. 2020).

GCMS Analysis of Essential oils from *in vivo* rhizomes and Microrhizomes: Essential oils collected from *in vivo* rhizomes and *in vitro* microrhizomes of *K. galanga* were analysed by GC-MS and the composition of both rhizome oils are shown in Tables 5 and 6 respectively. As per GC-MS analysis, there were 79 components present in *in vivo* rhizome oil and most of the compounds are

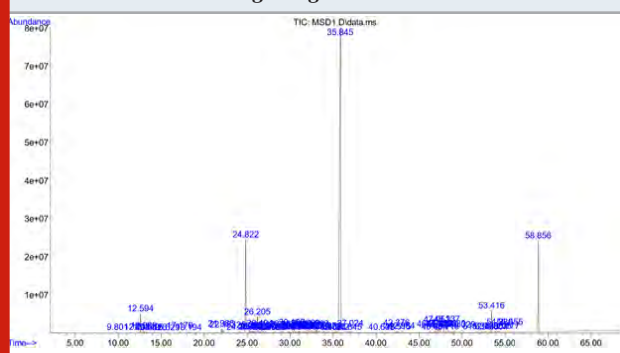
present in lower amounts (Fig. 3). Some of the major compounds and their reported activities are represented in Table 5. Palmitic acid (12.219%) was detected as the predominant compound in *in vivo* rhizome oil. In addition to this, capric acid (8.955 %), caprylic acid (4.468 %), lauric acid (3.082 %), myristic acid (3.924 %), stearic acid (3.948%), ethyl-p-methoxy cinnamate (2.132%) and ethyl cinnamate (0.795%) are also detected in *in vivo* rhizome

oil sample. In *in vitro* microrrhizome oil, there were 74 compounds upon by GC-MS analysis. Ethyl-p-methoxy cinnamate (58.088%) was detected as most abundant component. Remaining compounds present in *in vitro* rhizome oils were ethyl cinnamate (10.155%), octahydro-4a (2H)-naphthalinyl methanol (2.045%), borneol (1.349%), pentadecane (1.482%), α -cadinol (0.729%) and retinol (0.501%) (Table 6).

Table 6. Essential oil components in *in vitro* microrrhizome of *K. galanga*

Peak number	Retention time	Abundance %	Compound	Common name	Chemical formula
48	35.845	58.088	2-Propenoic acid,3 (4-methoxyphenyl)-ethyl ester	Ethyl p-methoxy cinnamate	$C_{12}H_{14}O_3$
14,15	24.822, 25.290	9.774.0.381	2-propenoic acid , 3-phenyl,ethyl ester	Ethyl cinnamate	$C_{11}H_{12}O_2$
3	12.231	1.349	Borneol	Borneol	$C_{10}H_{18}O$
19	26.205	1.482	Pentadecane	Alkane hydrocarbon	$C_{15}H_{32}$
37	31.980	0.729	α -Cadinol	Cadinane sesquiterpenoid	$C_{15}H_{26}O$
30	30.187	0.633	12-Oxybicyclo[9.1.0]dodeca-3, 7diene,1,5,5,8-tetramethyl	Humulene epoxide 2	$C_{15}H_{24}O$
59	47.323	0.501	Retinol	Retinol	$C_{20}H_{30}O$
s40	32.517	0.413	Caryophyllene	Bicyclic sesquiterpene	$C_{15}H_{24}$

Figure 4: GC-MS -Chromatogram of *In vitro* microrrhizome oil of *K. galanga*



Great variation was noticed in the case of chemical constituents in *in vivo* rhizome and *in vitro* microrrhizome as per GC-MS analysis. Based on previous reports ethyl-p-methoxy cinnamate and ethyl cinnamate were the predominant bioactive compounds in the essential oils of *K. galanga* and their presence was detected in both samples analyzed here. The percentage of ethyl-p-methoxy cinnamate and ethyl cinnamate was comparatively higher in *in vitro* microrrhizome (Fig. 4) than the *in vivo* rhizome, which was the control sample. Most of the compounds found in the essential extracted here have been reported to exhibit significant biological activities. The major among them viz. ethyl cinnamate and ethyl-p-methoxycinnamate are esters which contribute the nematocidal, anticancer, antituberculosis, anti-inflammatory, antifungal and larvicidal properties to the oil (Liu et al. 2010; Muhammad

et al. 2012). According to Ajay (2014) monoterpenes and sesquiterpenes were found in the essential oil of *K. galanga* rhizomes which also may contribute the flavour and fragrance properties to the oil. Anti-cancer activity of *K. galanga* was due to the presence of the compound ethyl p methoxi cinnamate and vassorelaxation effect of ethyl p- methoxy cinnamate in *K. galanga* was also reported in previous studies (Srivastava et al. 2019; Srivastava et al. 2021). In our experiment a good percentage (58%) of ethylp methoxy cinnamate was detected from microrrhizome oil sample.

CONCLUSION

The findings of the present study reports the technology for microrrhizome induction using sucrose and $AgNO_3$ in *K. galanga* which can be effectively used to produce quality planting material at affordable price for commercial purpose. In GC-MS analysis, there were 79 components present in *in vivo* rhizome oil and on par with these 74 components were detected in microrrhizome oil. The findings established here offers the development of a novel method for the extraction of volatile oil from microrrhizomes which can be further scaled up by bioreactor technology. This protocol for microrrhizome induction can be used for the commercial production of rhizomes and essential oil in *K. galanga* and thus ensuring the conservation and sustainable utilization of this species.

ACKNOWLEDGEMENTS

The study was financially supported by DST-SERB,

Government of India under the TARE scheme and also we thank the Director, JNTBGRI, Palode, Thiruvananthapuram for granting the facilities for GCMS analysis..

Conflict of Interests: Authors declare no conflict of interests to disclose.

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Microbiological Communication

Determination of Molecular Weight and Antimicrobial Activities of a Purified Bacteriocin from *Lactiplantibacillus plantarum* MDP 5

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ABSTRACT

Nowadays, unlimited use of antibiotics and preservatives have become a big concern regarding the human health which turn the interest of biotech industries into their research on biologically active molecules from probiotic microbes, since they are nontoxic and suitable for many safer applications. On this background, the present investigation focused on the characterization of a bacteriocin from a *Lactobacillus* strain. The characterization of a bacteriocin was done using a *Lactiplantibacillus plantarum* MDP 5 isolated from the local markets of Puducherry and production was performed under standardized cultural conditions. The study on the maximum recovery of bacteriocin using ammonium sulphate precipitation method revealed that the 60% saturation rate evidenced highest activity of 6500 AU/ml with 4.113g/L dry weight followed by the purification was done with RP-HPLC method using C18 column. The purified bacteriocin revealed a novel molecular weight of 22 kDa with the help of SDS-PAGE which has not been reported from *Lactobacillus* species. Further, the purified bacteriocin evidenced appreciable antimicrobial activities against all the tested human bacterial pathogens of this study. The highest antimicrobial activity was recorded against *Escherichia coli* MTCC 443 followed by *Staphylococcus aureus* MTCC 96, *Vibrio parahaemolyticus* MTCC 451, *Enterococcus faecalis* MTCC 9845, *Pseudomonas aeruginosa* MTCC 741 and *Klebsiella pneumoniae* MTCC 109 in the concentrations of 8AU/ml, 16AU/ml, 32AU/ml, 64AU/ml, 256AU/ml and 512AU/ml, respectively. From the overall observation, this study explored a novel bacteriocin purified from a probiotic bacterium represented potential antimicrobial activities against many human pathogens which suggesting its possible use for the safe therapeutic applications.

KEY WORDS: ANTIMICROBIAL, BACTERIOCIN, LACTIPLANTIBACILLUS PLANTARUM, MOLECULAR WEIGHT, PURIFICATION.

INTRODUCTION

Rapidly increasing microbial resistance to antibiotics is becoming a global threat which needs an immediate attention to resolve this massive issue on behalf of the whole mankind (Carlet et al. 2012; Balan 2012). Antibiotic resistance is not a new phenomenon and are naturally common which has been known since the discovery of Penicillin however the overwhelming resistance of microbes can be directly linked to its improper use through self-medication,

excessive medical prescription, prolonged use of practice in veterinary applications, etc., over the past several years globally (Mathur et al. 2018). Further, there are more complications relating to the overuse of antibiotics such as killing of normal, beneficial and indigenous microbiota as well as environmental contamination etc. which are the root cause for many prolonged ecological issues. Hence, it is our significant urgency to develop an alternative method or substance to overcome such global crisis (Balan et al. 2019; Ng et al. 2020; Dai et al. 2021).

In the last two decades, antimicrobial peptides especially bacteriocin are in the frontline of research regarding the alternatives to the existing antibiotics but have never been

Article Information:*Corresponding Author: drsumathimicro@gmail.com

Received 25/10/2021 Accepted after revision 24/12/2021

Published: 31st December 2021 Pp- 1600-1606

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.35>

substantially improved to the commercially available formats (Cavera et al. 2015). Bacteriocin are known as ribosomally synthesized proteinaceous compounds which can inhibit the growth of closely related microbes through various mechanisms. The bioactive properties of bacteriocin are first classified during 1925 however their role on biomedical arena have been recently reported (Inglis et al. 2013). Many extensive studies have been carried out till date by US Food and Drug Administration (FDA) on bacteriocin regarding their applications as a potential food preservative. Till 2012, 62 genera and 162 species are considered as safe microbial cultures for many fermentation purposes which warrant the research on antibiotics from the probiotic and prebiotic strains for their safe use on human health (Kareem and Razavi, 2020; Trejo-González et al. 2021).

Bacteriocin produced by lactic acid bacteria have been recognized as potential food preservative, in contrast their use in the field of pharmaceuticals is still in the underdeveloped stage (Hassan et al. 2020; Negash and Tsehai 2020). Despite the fact, bacteriocin are well known for the antimicrobial properties, they were less concentrated for their use as a therapeutic agent (Arthur et al. 2014). The recent trends on the search for potential alternatives in place of antibiotics enhance the studies on safe bioactive compounds from lactic acid bacteria, hence the present investigation characterize a bacteriocin compound from a *Lactobacillus* species. To accomplish the stated aim of this study, the bacteriocin produced from the *Lactiplantibacillus plantarum* MDP 5 was precipitated, purified and characterized their molecular weight and antimicrobial activities against a panel of various pathogenic bacteria.

MATERIAL AND METHODS

To analyse the microorganism and culture conditions: the bacteriocin producing bacterium, *Lactiplantibacillus plantarum* MDP 5 was used in this study for the molecular weight determination and antimicrobial activities against a panel of pathogenic bacterial strains. *L. plantarum* MDP 5 was originally isolated from the milk and dairy products collected from the local markets of Karaikal, Puducherry, India. This strain was molecular identified using 16S rRNA partial sequence method and the sequence was submitted in the NCBI GenBank with the accession number MW301154.1. The following cultural conditions were used in this study for the production of bacteriocin were 2% fructose, 1% peptone, 2% inoculum at 108 CFU/ml, pH 7, 35°C temperature, 200 rpm agitation, 0.1% MgSO₄, 0.1% FeSO₄ and 0.1% KHPO₄.

The bacteriocin precipitation was carried out under different concentrations of ammonium sulphate ranging from 10 to 100% saturation rate at 0°C for an overnight period (Green and Hughens 1955). After 60hrs incubation, the cultured broth was centrifuged at 3000rpm for 20min. and the cell free supernatant was collected and added with different saturation rates of ammonium sulphate for the precipitation of bacteriocin. The precipitated compounds were collected under centrifugation at 10,000rpm for 20 min. The collected precipitates were dialyzed against dialysis membrane

with the pH 7 phosphate buffer solution (PBS) for 24h with intermediate changes of PBS to remove the salt and the final solution was subjected to lyophilization. The final lyophilized samples were assayed for bacteriocin bioassay and the dry weight of the total crude sample was also taken into account for selection of best percentage ammonium sulphate saturation rate for the maximum recovery of bacteriocin. The sample showed the highest recovery was used for the purification using the column chromatography.

To study the bacteriocin bioassay, the production of bacteriocin was evaluated by microtiter plate assay method (Kang and Lee 2005). The assay was performed in 96-well flat bottom polystyrene microtitre plates with lids (Tarsons, India). In this assay, well plates were filled with 100 µL of serially diluted bacteriocin samples followed by the addition of 20 µL indicator strain, *V. cholerae* MTCC 3906 at a concentration of 105 CFU/ml assessed using optical density at 620nm and 80 µL of 2.5x concentrated tryptone soy broth (TSB). The growth control wells have 100 µL of phosphate buffer (pH 7), 20 µL of indicator strain and 80 µL of 2.5x concentrated tryptone soya broth (TSB). The susceptibility control well plate has 100 µL of 4mg/ml streptomycin containing phosphate buffer (pH 7), 20 µL of indicator strain and 80 µL of 2.5x concentrated tryptone soya broth (TSB). After 6 hrs incubation, the well plates were covered with lids and incubated at 37 °C for 48 hrs. After incubation, the absorbance of the well broth was recorded at 620 nm for each well using a microplate reader (Biotek Elx808, WI, USA), where the assays were carried out in triplicate. The growth inhibition percentages of the tested pathogens were calculated as follows:

$$\% \text{ Growth inhibition} = [(1 - (A_s/A_c))] \times 100$$

where A_s represents the absorbance of the well with test samples and A_c represents the absorbance of the control well (without any added bioactive sample). Further, one arbitrary unit (AU) of bacteriocin activity was defined as the reciprocal of the highest dilution of supernatant causing 50% growth inhibition of indicator strain.

For the bacteriocin purification, the lyophilized sample was dissolved in 5ml distilled water, filtered using a 0.2µm syringe filter and the purification was done using a Reverse Phase (RP) - High Pressure Liquid Chromatography (HPLC). RP-HPLC was performed with a Waters 600 HPLC system (Waters, USA) equipped with an Xterra Prep RP18 OBD column (Waters, USA; 5 µL, 18 × 100 mm) held at 40°C. The solvent system consisted of distilled water (solvent A) and acetonitrile (solvent B). The compounds were eluted at a flow rate of 4ml/min with a linear gradient from the mixture A:B (100:0, vol/vol) to A:B (0:100, vol/vol) in 12min. The absorbances of the eluted fractions were measured at 210 nm (Mani et al. 2016). All the collected fractions were dried under rotary evaporation and stored at - 20°C. All the collected individual fractions were studied using bacteriocin bioassay and the fraction showed the appreciable activity was studied for the molecular weight of the purified bacteriocin using SDS PAGE.

The molecular weight of bacteriocin was identified with the help of SDS-PAGE (Hames 1998). A volume of 30 μ l SDS gel loading buffer and samples of purified and crude bacteriocin were individually taken in one ml tube which were heated for 3 min at 60°C. Assemblies were fixed in an electrophoresis apparatus and 15 μ l of purified and crude bacteriocin and protein markers in the range between 10–44 kDa were loaded in various wells. The gel was run at 50 V and stained for the identification protein bands using Coomassie brilliant blue.

For the antimicrobial activities of bacteriocin, a battery of six different pathogenic bacterium were procured from MTCC (Microbial Type Culture Collection), CSIR-Institute of Microbial Technology, Chandigarh, India, for the antimicrobial study of the purified bacteriocin. The pathogenic bacterial strains were *Vibrio parahaemolyticus* MTCC 451, *Escherichia coli* MTCC 443, *Staphylococcus aureus* MTCC 96, *Enterococcus faecalis* MTCC 9845, *Klebsiella pneumoniae* MTCC 109 and *Pseudomonas aeruginosa* MTCC 741. These strains were cultured on tryptic soy broth (TSB) at 37°C overnight and the inoculum of each strain was adjusted to a concentration of 108 CFU/ml. The assay was carried out in a 96-well flat bottom polystyrene microtitre plates with lids (Tarsons, India). An aliquot of 250 μ l TSB were added in the well plates prepared with different concentrations of purified bacteriocin ranging from 8 AU mL⁻¹ to 512 AU mL⁻¹ and wells without any added bacteriocin concentration were used as negative and growth control. All the wells except the negative control were inoculated with 2.5 μ l of the prepared test bacterial strains. The inoculated well plates were covered with lids and incubated at 37°C for 48hrs. After incubation, the absorbance was measured at 600 nm for each well using a microplate reader (Biotek Elx808, WI, USA). The growth inhibition percentage of the tested microorganism was calculated as follows (Balan et al. 2016):

$$\% \text{ Growth inhibition} = [(1 - (Ac/Ao)) \times 100]$$

where Ac represents the absorbance of the well with known bacteriocin concentration c and Ao represents the absorbance of the control well (without bacteriocin). Confocal laser scanning microscopy (CLSM) was used to visualize the bacterial cell biomass in the microtiter well plates after antimicrobial assay performed using bacteriocin at different concentrations. A loopful of broth culture after the antimicrobial treatment was smeared on glass microscopic slides and fixed with 2% (v/v) glutaraldehyde in PBS for 15min. Excess fixative was removed by washing the smears with PBS for 15min. The smears were stained for bacterial biomass with 0.01% (w/v) acridine orange in PBS for 15min, which was followed by washing with PBS for 30min to remove excess dyes. The stained films were visualized in situ by CLSM with an Olympus LSMGB200 CLSM (Olympus Optical Co. Ltd., Tokyo, Japan). The CLSM used an argon ion laser at 488 nm for excitation and a 605–632 nm band-pass filter for emission. Images were captured and processed using Olympus LSMGB200 CLSM bundled programs (Rice et al. 2005).

RESULTS AND DISCUSSION

Ammonium sulphate precipitation of bacteriocin:

Industrial interest on bioactive molecules like bacteriocin from Lactic acid bacterium is becoming more because of its safer applications in the many arenas (Kranjec et al. 2021). The production of bacteriocin in this study was performed using a *L. plantarum* MDP 5 under standardized cultural conditions. After incubation, the cultured broth was centrifuge separated for the cell free supernatant. The supernatant was divided into ten equal proportions of 100ml and various percentage saturation rates of ammonium sulphate were used for the bacteriocin precipitation. After an overnight incubation, protein precipitates were separated using centrifugation methods and evaluated for the bacteriocin activity as well as crude protein content. Among the studied ten different saturation rates, 60% ammonium sulphate revealed highest 6500AU/ml bacteriocin activity followed by 70%, 80% and 90% ammonium sulphate with 6400AU/ml, 6100AU/ml and 5600AU/ml bacteriocin activities, respectively (Table 1) (Kranjec et al. 2021).

Table 1. Crude bacteriocin precipitation using various saturation rates of ammonium sulphate

Saturation rate of Ammonium Sulphate (%)	Dry weight of crude protein (g/L)	Bacteriocin activity (AU/mg)
10	2.782	2200
20	3.127	3100
30	3.473	3900
40	3.786	4500
50	3.902	5200
60	4.113	6500
70	4.390	6400
80	4.782	6100
90	5.351	5600
100	5.672	5000

Figure 1: Appreciable precipitation of bacteriocin using 60% ammonium sulphate from cell free supernatant of *L. plantarum* MDP 5 cultured broth



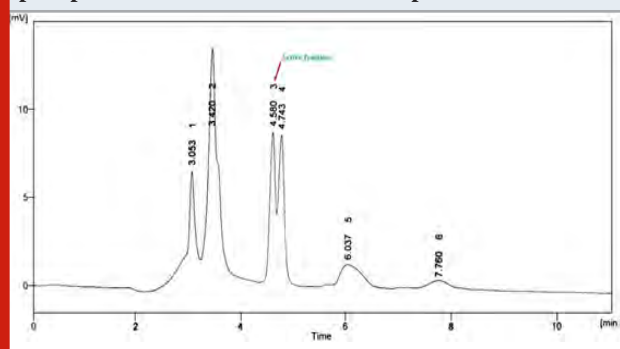
The increasing percentage saturation rates of ammonium sulphate enhance the quantity of protein precipitation however reduced the bacteriocin activity. The 60% ammonium sulphate concentration which have showed highest bacteriocin activity recorded 4.113 g of protein

per litre of cultured broth. The loss of activity may be due to the denaturation of desired compound at the higher concentration of ammonium sulphate (Balan et al. 2012; Balan et al. 2013). Further, the loss of bacteriocin activity was observed from 70% ammonium sulphate hence the 60% saturation rate was taken into account for the best recovery of bacteriocin in this study (Fig. 1). Similar to this study, different percentage saturation rates of ammonium sulphate were performed for the precipitation of bacteriocin produced by *Lactobacillus pentosus* ZFM94 however revealed highest recovery at 40% ammonium sulphate (Dai et al. 2021).

Purification and molecular weight of bacteriocin:

Followed by precipitation of bacteriocin, purification was performed using RP-HPLC method. Among the collected fractions during purification procedure, six fractions revealed the presence of organic compounds, further, bacteriocin activity was evidenced only at the third fraction as shown in the figure 2 and this fraction was dried using rotary vacuum evaporation for the further analysis. The purified fraction revealed a total dry weight of 0.576 g from one litre of cultured broth and revealed the recovery rate of 90.3 % from crude precipitated form. The same RP-HPLC method using C_{18} column was used in the recent studies for the purified of two bioactive molecules namely Aneurinifactin and Staphylosan from a marine bacterium and marine yeast (Balan et al. 2017; Balan et al. 2019; Dai et al. 2021).

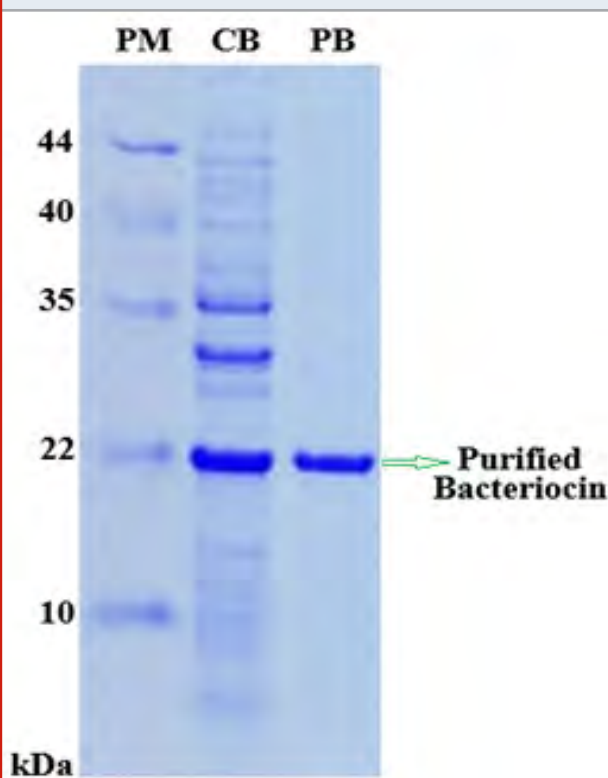
Figure 2: HPLC chromatogram of crude bacteriocin precipitated from 60% ammonium sulphate



The purity and the molecular weight of the purified bacteriocin was analysed with the help of SDS-PAGE. Results revealed that the purified fraction has only one molecule that is bacteriocin with a molecular mass of 22 kDa (Fig. 3). The purified bacteriocin recorded 5,030 AU activity per milligram whereas the crude precipitated form reported only 780 AU activity per milligram. Further, the purified bacteriocin evidenced 5.03 AU of bacteriocin activity per μ g and a single AU of bacteriocin activity was recorded from 0.199 μ g of purified bacteriocin. According to Kareem and Razavi (2020) and Moradi et al. (2021), molecular weight of most bacteriocin purified from lactic acid bacteria were reported earlier within 10 kDa. Till date, the highest molecular weight of *Lactobacillus bacteriocin* was purified from a *L. helveticus* which was about 15 kDa. Based on the above observations, the purified bacteriocin

from this study is a novel bioactive compound (Kareem and Razavi 2020; Hassan et al. 2020; Moradi et al. 2021).

Figure 3: Molecular weight determination of the purified bacteriocin using SDS PAGE in which the lane "PM" represents the protein molecular weight marker, lane "CB" represents the crude bacteriocin obtained after precipitation procedure and the lane "PB" represents the purified bacteriocin after RP-HPLC



Antimicrobial activities of the purified bacteriocin:

The antimicrobial activities of the purified bacteriocin were evaluated with the help of microdilution method using a series of double dilution concentrations from 8 AU/ml to 512 AU/ml. Interestingly, all the six human bacterial pathogens tested in this study revealed strong susceptibility against this purified bacteriocin and all the pathogens revealed their complete growth inhibition within these tested concentrations. The strongest antimicrobial activity was recorded against *Escherichia coli* MTCC 443 at 8 AU/ml followed by *Staphylococcus aureus* MTCC 96, *Vibrio parahaemolyticus* MTCC 451, *Enterococcus faecalis* MTCC 9845, *Pseudomonas aeruginosa* MTCC 741 and *Klebsiella pneumoniae* MTCC 109 in the concentrations of 16 AU/ml, 32 AU/ml, 64 AU/ml, 256 AU/ml and 512 AU/ml, respectively (Table 2) (Hassan et al. 2020; Moradi et al. 2021).

Furthermore, the results revealed that this purified bacteriocin was activity against both gram positive and negative bacteria since *Staphylococcus aureus* MTCC 96 and *Enterococcus faecalis* MTCC 9845 were gram positive bacteria and rest of the strains were gram negative

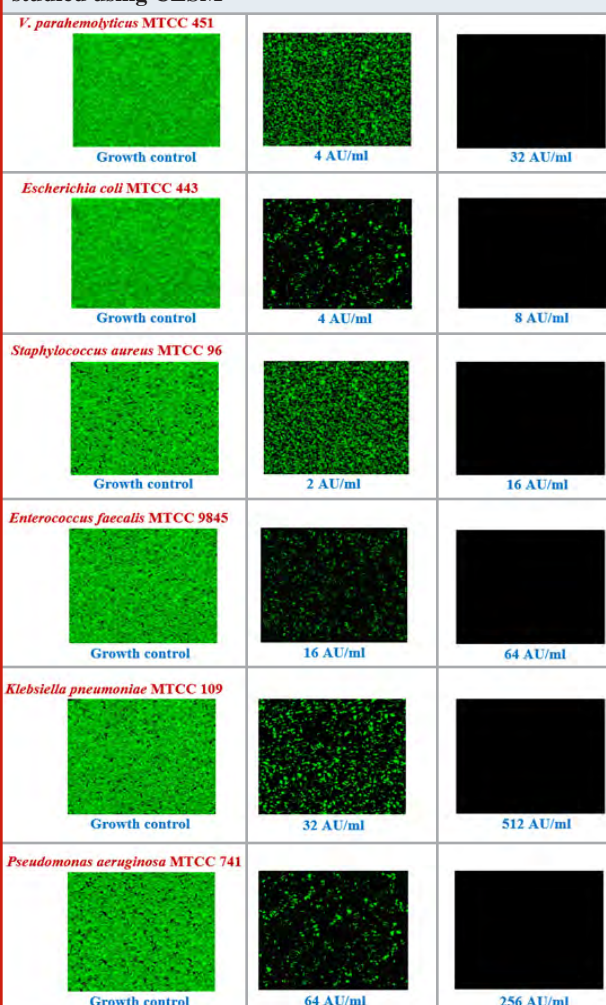
bacteria. CLSM was used to study the bacterial biomass of the treated well plates at different concentrations of the purified bacteriocin. The microscopic photographs at all the significant concentrations were illustrated in the figure 4. Further, all the growth control well plates showed dense biomass of bacterial cells, the concentrations at

which bacteriocin revealed completion growth inhibition showed no cell and bacteriocin activity dependent cell biomass was observed on the rest of the photographs shown which illustrated the microscopy representation of the antimicrobial activity (Hassan et al. 2020; Moradi et al. 2021).

Table 2. Antimicrobial activities of the purified bacteriocin at various concentration against different pathogenic bacteria, all the experimental values are expressed as mean \pm standard deviation, (n=3) and “*” represents the repeated values of the last column

Test bacterial pathogen	Arbitrary unit per millilitre (AU/ml) of bacteriocin required to inhibit the percentage growth of human pathogens						
	8	16	32	64	128	256	512
<i>Vibrio parahaemolyticus</i> MTCC 451	73 \pm 2.3	91 \pm 3.7	100 \pm 0	*	*	*	*
<i>Escherichia coli</i> MTCC 443	100 \pm 0	*	*	*	*	*	*
<i>Staphylococcus aureus</i> MTCC 96	88 \pm 3.3	100 \pm 0	*	*	*	*	*
<i>Enterococcus faecalis</i> MTCC 9845	55 \pm 2.3	76 \pm 3.3	93 \pm 2.7	100 \pm 0	*	*	*
<i>Klebsiella pneumoniae</i> MTCC 109	37 \pm 1.7	49 \pm 2.3	61 \pm 2.7	72 \pm 3.3	83 \pm 3.7	93 \pm 4.3	100 \pm 0
<i>Pseudomonas aeruginosa</i> MTCC 741	45 \pm 2	56 \pm 2.3	69 \pm 3.3	82 \pm 3.7	96 \pm 4	100 \pm 0	*

Figure 4: Antimicrobial activities of the purified bacteriocin studied using CLSM



Similarly, bacteriocin obtained from *Lactobacillus plantarum* zrx03 procured from infant's feces revealed broad antimicrobial activity against *Bacillus subtilis* CICC 10002, *Bacillus anthracis* CICC 20443, *Escherichia coli* JM109 ATCC 67387, *Salmonella* CMCC 541 and *Staphylococcus aureus* ATCC 25923 (Lei et al. 2020). Likewise, bacteriocin of a *Lactobacillus* species procured from yoghurt showed appreciable antimicrobial activity against *Acinetobacter baumannii* and *Staphylococcus aureus*. The above studies signify the antimicrobial activity of the purified bacteriocin from this investigation (Hassan et al. 2020).

CONCLUSION

The findings of the present study produced bacteriocin using a probiotic *L. plantarum* MDP 5 which was ammonium sulphate precipitated with 60% saturation rate and further purified using RP-HPLC procedure. SDS-PAGE revealed the novel molecular weight of this purified bacteriocin having 22 kDa. Furthermore, the purified bacteriocin evidenced appreciable antimicrobial activity against all the tested six bacterial pathogens. All the above studies proved that this bacteriocin can possibly be used as a safe and potential antimicrobial agent in the field of pharmaceutical applications.

ACKNOWLEDGEMENTS

The study was financially supported by the Department of Microbiology, Faculty of Science, Annamalai University, Chidambaram, Tamil Nadu, India. Moreover, authors thank the department for providing lab facilities and supporting the research work.

Conflict of Interests: Authors declare no conflict of interest to disclose.

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Biotechnological Communication

Comparative Plasmid-Mediated Molecular Resistance Status of Diarrheic *Escherichia coli* Isolates from Human and Goat Kids

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ABSTRACT

Globally antibiotic resistance has become a major concern, which warrants the real time monitoring for resistance in very common pathogenic organisms. *E. coli* is normal micro flora in humans, but sometimes it can be pathogenic. For the observation and increment of antimicrobial resistance among pathogen, *E. coli* has been one of the important pathogens. It is present everywhere in fecal, water, food etc., if resistant *E. coli* will present in the environment that it can be transferrable anywhere through water, fecal food, animals and humans. This is very dangerous to living beings. This study was designed on status of antibiotic resistance in *E. coli* isolates in human kids and animal kids, both. Newborns are affected more because of poor or lack of immune system. In this study, fecal materials were used as sample material collected from goat kids (0-3 months) and human children (up to 3 years) residing in same local area. Fifteen fecal samples were collected from human children (up to 3 years) and goat kids (0-3 months) in each case to study the risk of transmission of resistance in *E. coli* isolates. PCR was conducted on genomic DNA isolates for the presence of *uspA* gene of *E. coli*. Multiplex PCR were conducted on plasmid DNA isolates for the resistance specific genes. Molecular resistance results in goat kids isolates showed resistance to antibiotics with tetracycline, sulphonamide, gentamycin, streptomycin and cephalothin to the level of 93.33, 53.33, 46.66, 13.33 & 6.66% respectively, whereas, human *E. coli* isolates were showed the highest resistance to sulphonamide, Tetracycline and β -lactams were as 53.33, 46.66 and 13.33% respectively but no resistance with gentamycin and streptomycin. Here, we concluded that humans and animals both were refractory to the various groups of antibiotics. This study will help in making the strategy for prevention or reduction of resistance in public

KEY WORDS: *E. COLI*, GENOMIC DNA, PLASMID DNA, RESISTANCE.

INTRODUCTION

Childhood diarrhea is a major public health problem and second leading cause of high mortality below 5 year of age children's. Nearly 1.7 billion cases of childhood diarrheal diseases have been reported every year, killed around 525,000 five year age children, rating for 8% of all deaths worldwide. The deaths from diarrhea mostly occur in children below 2 years of age. Diarrheal diseases have a detrimental impact on child growth and cognitive development. Increased risks of malnutrition in children are associated with diarrheal diseases. The prevalence of the disease remains at an alarming rate where infants and children are unmoving at

risk of death and other complications, while over the past two decades the decrease in the episodes of childhood diarrhea is observable globally (Paul 2020).

E. coli is Gram-negative, non-spore forming, facultative anaerobic bacteria belongs to the Enterobacteriaceae family. Natural gastrointestinal flora part of the humans and warm blooded animals forms from it (Aijuka and Buys 2019). *E. coli* association in animals such as goats has major significance for infection in humans, particularly rearing goats as backyard farming. Infection to humans can transmit through animals as their infected and diseased meat or between handling procedures or through ingestion by the consumer (Zerabruk et al. 2019; Paul 2020).

Also, during slaughtering via fecal contamination, *E. coli* presence in animal feces allows the entry in the food chain

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Received 21/10/2021 Accepted after revision 19/12/2021

Published: 31st December 2021 Pp- 1607-1612

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.36>

resistant bacteria to antibiotics is present everywhere in surroundings and greatly increased their obstructive effect. Most *E. coli* are harmless commensal organisms (Zerabruk et al. 2019). *E. coli* are an opportunistic pathogen that can endure well in aquatic systems and are exceptionally proficient at horizontal gene transfer, which is believed to be the vector for antibiotic-resistance dissemination (Maeusli et al. 2020). The study was aimed to molecular resistance typing of *E. coli* isolates both in young age human children and in goat kids (1) Frequency of single resistant gene in both (2) Frequency of one or more resistant gene (MDR) in both and (3) which group of antibiotics was showed more resistance.

MATERIAL AND METHODS

Fecal samples were collected in goat kid's belonging to 0-3 month age group from rectum with the help of cotton swab (HI media, India) and from human child up to 3 years directly without touching the surface. Human samples were collected with the consent of parents/guardians. After collection samples were directly stored in the ice box for transportation. If possible, fresh samples were processed for identification in the laboratory or if not possible then immediately samples were stored at -70° C until the use.

For identification of bacteria, fecal samples were directly streaked on enriched media brain heart infusion solid media for growth from which a single colony used for further experiments such as lactose fermentation on differential media, streaking on selective media (eosin methylene blue media) for confirmation. To hold *E. coli* isolates for further process, all isolates were maintained and stored at a temperature of -70°C. Staining was performed from gram staining kit (BD & BBL Difco, USA) as per the method described in the previous studies (Hedge et al. 2013). Biochemical tests were performed from the Himedia kit (HI media, Mumbai, India) and Sigma kits (Japan). DNA from a cultural broth was isolated as per Yang et al., 2008. Isolated DNA was stored at -70° C until their use.

Polymerase chain reaction (PCR) test was performed for the usp A gene of *E. coli* in Peq lab thermo cycler. The reaction mixture (25µl) were prepared as 22.5 µl Invitrogen master mix (accuprime), 1µl DNA template, and .75 µl of each primer forward and reverse. PCR conditions were as 95°C (5 minute); 30 cycles of 95°C (1 minute), 58 °C (30 seconds) 72°C (1 minute); and a final extension at 72°C (5 minutes) stored at 4°C for infinite. PCR product was seen in the Gel doc machine with the help of gel electrophoresis. 1.3 % agarose gel containing ethidium bromide was prepared in a casting tray. After solidification, PCR products were filled in the well with 6x gel loading dye (Thermo scientific), then run on the power supply at 60 volts for 1 hour in a gel tank filled with TAE buffer.

Fifteen human and fifteen goat kids *E. coli* isolates processed for Plasmid DNA isolation whereas, a single colony was inoculated in Luria bertani broth and incubate at 37°C for 3-4 hour. After incubation culture broth centrifuge at 1500 rpm for 5 minutes and supernatant discarded. Pellet was washed with PBS 2-3 times and processed for DNA

isolation as per the GSure Mini Kit protocol (GCC, New Delhi). Isolated DNA was stored in -70° C until the use. Polymerase chain reaction (PCR) tests were performed in Peqstar 96 x universal gradient thermocycler (PEQLAB Biotechnologie GmbH, Germany). The reaction mixture (25µl) was prepared as 10µl emerald master mix (Takara, New Delhi), 1 µl DNA template and 1 µl of each primer forward and reverse and the remaining amount (12 µl) of nuclease - free water. PCR conditions for Cep were as 95°C for 5 min.; 30 cycles of 95°C for 1 min, 55°C for 30 sec respectively 72°C for 1 min.; and a final extension at 72°C for 5 min stored at 4°C for infinite time. PCR products were seen in the gel doc machine with the help of gel electrophoresis. 1.3 % agarose gel was prepared in a casting tray.

After solidification, PCR products were filled in the well with 6x gel loading dye (Thermo Scientific, US). Run on the power supply at 60 volts for 1 hour in the gel tank filled with TAE buffer. For multiplex PCR, the reaction mixture was prepared as listed above, and in reaction mixture added all four genes forward and reverse primer in the same tube and decrease the amount of water. PCR conditions for (Gentamycin- Gen), (Tetracycline - Tet), (Sulphonamide - Sul) and (Streptomycin - Strp) were as 95°C (5 minute); 30 cycles of 95°C (1 minute), 53.5°C (30 second) respectively 72°C (1 minute); and final extension at 72°C (5 minute) stored at 4°C for an infinite time. Results were seen as per the previous method.

Figure 1: Microscopic result showing pink colour (gram-ve) rod shaped bacteria (*E. coli*)



RESULTS AND DISCUSSION

E. coli were characterized based on cultural, biochemical, and molecular methods in the laboratory. Observed findings on bacteria were as gram - negative and rod - shaped (Fig.1), Catalase - positive, oxidase negative and IMViC tests such as (+, +, - and-) (Fig. 2), Pale yellow colonies on Brain heart infusion agar, smooth pink colonies on Mac Conkey agar and green metallic sheen on EMB were observed. (Fig. 3, 4 & 5). The primers were used in the study for confirmation and resistance as described in the previous studies respectively procured from Eurofins (Table-1) (Momtaz et al. 2012; Mishra et al. 2019). Different genes such as gen, strp, cep, tet, and sul resistant genes from the *E. coli*

were successfully amplified using species - specific primers in animals and as well as in humans. PCR Amplification resulted in a single amplicon of 286,447,462,577& 822-bp as illustrated (Fig. 7, 8, 9 & 10) (Momtaz et al. 2012; Rubab and Oh 2021).

Figure 2: Bacteria showing biochemical Test positive or negative













1	2	3	4	5	6	7	8	9	10	11	12
											
Positive No. and their property						Negative No. and their property					
2. Lysine utilization						1. Citrate utilization					
3. Ornithine utilization						4. Urease					
6. Nitrate Reduction						5. Phenylalanine deamination					
8. Glucose						7. H ₂ S production					
10. Lactose						9. Adonitol					
11. Arabinose						12. Sorbitol					

Figure 3: *E. coli* showing pale yellow colonies on BHI media



Figure 6: PCR amplification of *uspA* gene; Lane M-DNA Marker, Lane 1- Positive control, Lane 2-Positive PCR product

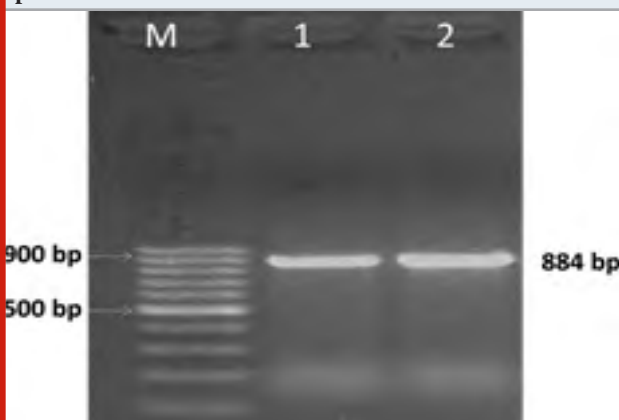


Figure 4: *E. coli* showing lactose fermentation on MCA media

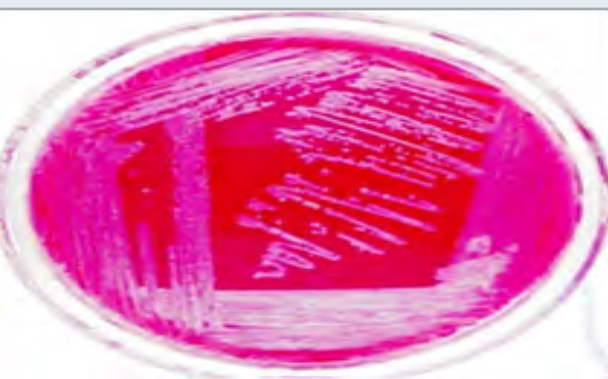
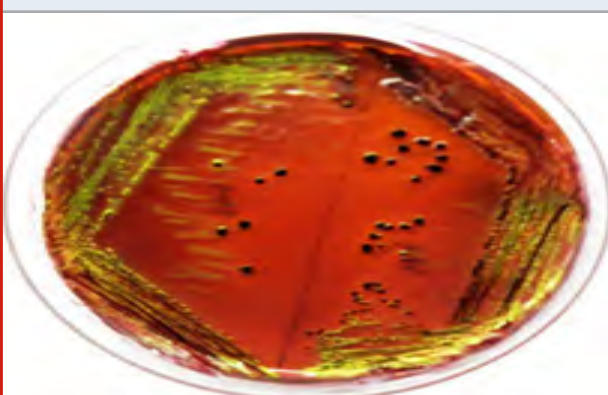


Figure 5: *E. coli* showing metallic sheen on EMB Media



In our results, it was observed that genes associated with resistance to gentamycin, streptomycin sulphonamide, cephalothin, and tetracycline observed in 46.66, 13.33, 53.33, 6.66%, and 93.33% samples respectively in goat kids, Tetracycline, cephalothin, and sulphonamide resistance genes were observed as 26.66, 13.33, and 53.33% respectively in human kid samples and no gene resistance to gentamycin and streptomycin was recorded in humans' samples (Table 2, 3 & 4 and graph 1, 2 & 3).

Our results were similar in goat kids except for streptomycin where a high prevalence of tetracycline (36 to 97%), sulfathiazole (50 to 100%), and streptomycin (53 to 100%) resistance was detected in chickens with similar to previous studies (Smith et al. 2007). In a study have reported that tetracycline and sulphonamide resistance in *E. coli* from slaughtered commercial chickens were 52.6, 47.36 % respectively. They also reported amino glycosides and β -lactams resistance in chickens were nil (Momtaz et al. 2012; Rubab and Oh 2021).

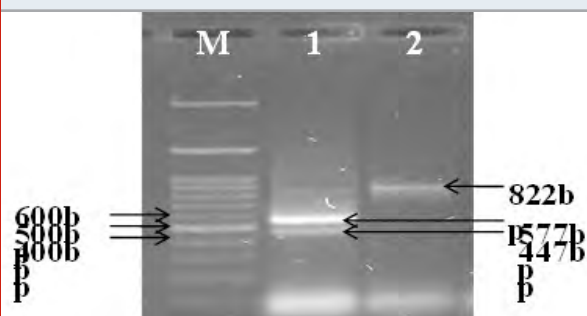
In study, frequency of streptomycin, tetracycline, gentamycin, and sulphonamide genes were in 96.10%, 85.06%, 54.54%, and 40.25%, respectively in *E. coli* from pediatric patients, the variation in this study may be attributed to the level of exposure to antibiotics in goat kid (Heidary et al. 2013). Study reported allow frequency of occurrence of genes of tetracycline, aminoglycosides and other β -lactamases genes perform well as signs of emerging

resistance in children, unlike the sulphonamide resistance (Singh et al. 2019). The study reported tetracycline, sull (for sulphonamide), streptomycin resistance followed by 77.17%, 45.94% and 34.65% from *E. coli* isolate from chicken meat (Rahman et al. 2020).

Table 1. The primers used in the study for molecular and resistance gene confirmation of *E. coli*

Primer (Gene)	Primer sequence	Product length (bp)
UPEC (usp A)	(F)CCGATACGCT GCCAATCAGT (R)ACGCAGACC GTAGGCCAGAT	884
β -lactams (Cep)	(F)TGGCCAGAAC TGACAGGCAAA (R)TTTCTCCTGAA CGTGGCTGGC	462
Gentamycin (Gen)	(F)CTTCAGGATG GCAAGTTGGT (R)TCATCTCGT TCTCCGCTCAT	286
Streptomycin (Strp)	(F)TATCCAGC TAAGCGCGAACT (R)ATTTGCCGACT ACCTTGGTC	447
Tetracycline (Tet)	(F)GGTTCACCTC GAACGACGTCA (R)CTGTCCGACA AGTTGCATGA	577
Sulphonamide (Sul)	(F)TTCGGCATTC TGAATCTCAC (R) ATGATCTAA CCCTCGGTCTC	822

Figure 7: PCR amplification of strp, sul and tet gene; Lane M-DNA Marker Lane1 -Positive PCR product (strp and tet) Lane 2-Positive PCR product (sul)



In a study reported that STEC isolates from different sources show the highest presence of ampC genes with a frequency of 47%. The detection rates tet (A) were 35%, respectively.

Figure 8: PCR amplification of cep gene; Lane M-DNA Marker Lane1, 2 & 4 -Positive PCR product (cep) Lane 3-Negative PCR product (cep)

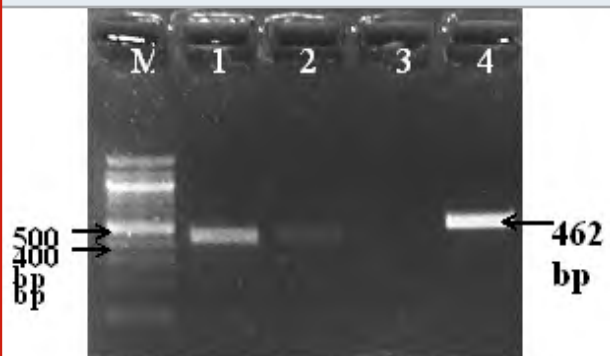


Figure 9: PCR amplification of Gen gene; Lane M-DNA Marker Lane 1-Negative PCR product, Lane2-Negative PCR product, Lane3-Positive PCR product Lane4- Positive control

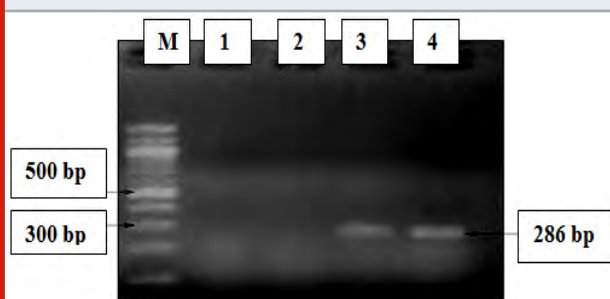


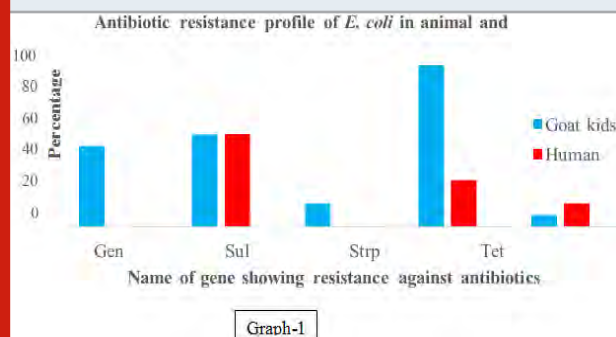
Figure 10: PCR amplification of sul and tet gene; Lane M-DNA Marker Lane 1 -Positive control (Sul), Lane2-Negative control (sul), Lane 3-Positive PCR product (tet) Lane 4&5-Positive PCR product



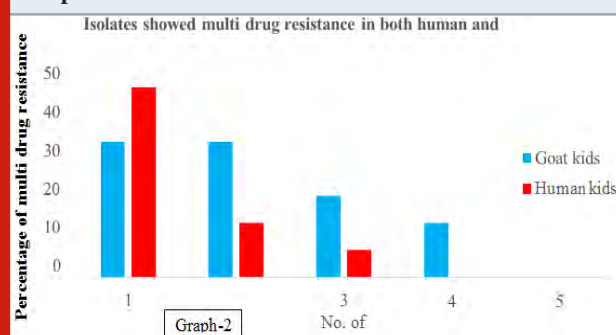
Nearly 74.5% of those isolates were resistant to all of the tested antibiotic resistance genes. In our results, in case of goat kid 100 percent while in case of human kids 33.33% *E. coli* isolate were resistant to all of the tested antibiotic resistance genes (Rubab and Oh 2021). In our results 26.66 % *E. coli* isolates from goat kids were showed multi drug resistance to group of antibiotics as Aminoglycoside, Sulphonamide and Tetracycline.

Table 2. Frequency of resistant antibiotic genes was present in goat kids and human kids.

Group of antibiotics	Gene	No. of tested	No. of resistant isolates (Percentage)	
			Goat kids	Human kids
Aminoglycoside	Gen		7 (46.66)	0 (0)
	Strp		2 (13.33)	0(0)
Sulphonamide	Sul	15-15 each	8 (53.33)	8 (53.33)
β -lactams	Cep		1 (6.66)	2 (13.33)
Tetracycline	Tet		14 (93.33)	4 (26.66)

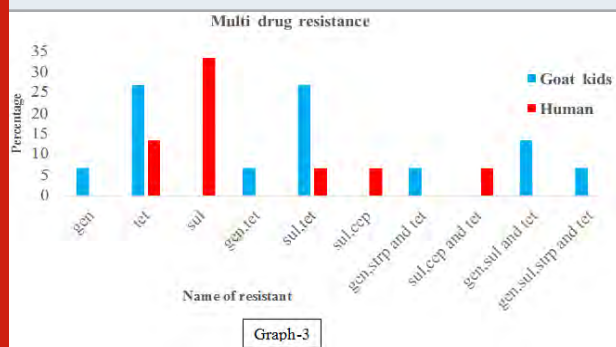
Graph 1**Table 3. Frequency of multi drug resistant antibiotic genes was present in goat kids and young age children's.**

Resistant to Genes	No. of tested	No. of resistant isolates (Percentage)	
		Goat kids	Human kids
Only 1		5 (33.33)	7 (46.66)
2		5(33.33)	2 (13.33)
3		3 (20.00)	1 (6.66)
4	15-15 each	2 (13.33)	0(0)
5		0 (0)	0(0)

Graph 2**Table 4. Name of multi drug resistant antibiotic genes was present in goat kids and young age children's.**

Group of antibiotics	Name of exactly resistant Genes	No.of tested	No. of resistant isolates (%)	
			Goat kids	Human kids
Aminoglycoside (AG)	Gen		1 (6.66)	0
Tetracycline (T)	Tet		4(26.66)	2 (13.33)
Sulphonamide (Sulk)	Sul		0	5
AG and Tetracycline	Gen, Tet		1(6.66)	0
Sul and T	Sul, Tet		4 (26.66)	1(6.66)
Sul and β -lactams	Sul, Cep		0	1(6.66)
AG and Tetracycline	Gen, Strp and Tet		1(6.66)	0
Sul, β -lactams and Tetracycline	Sul, Cep and Tet		0	1(6.66)
AG, Sulphonamide and Tetracycline	Gen, Sul and Tet	(15-15)	2(13.33)	0
AG, Sul, β -lactams and Tetracycline	Gen, Sul, Cep and Tet		1(6.66)	0
AG, Sulphonamide and Tetracycline	Gen, Sul, Strp and Tet		1(6.66)	0

Graph 3



CONCLUSION

The findings of the present study suggests that the antibiotic resistance is observed in *E. coli* isolated from both humans and goat kids. Sulphonamide group of antibiotics showed resistance in both. In goat kids, tetracycline showed the highest resistance and sulphonamide in humans. In our study observed that *E. coli* isolated from goat kids showed resistance to amino glycoside, tetracycline, β – lactams, and sulphonamide group of antibiotics but *E. coli* isolated from human kids showed resistance to tetracycline, β – lactams, and sulphonamide group of antibiotics. Goat isolates showed resistance to four groups of antibiotics, while human isolate showed resistance to three groups of antibiotics.

ACKNOWLEDGEMENTS

This work was financially supported by Indian Council of Agriculture Research, New Delhi under AINP-NM Project.

Conflict of Interests: Authors declare no conflict of interests to disclose.

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Biotechnological Communication

Biosurfactant-Producing Bacteria Isolated from Oil Contaminated Soil and its Media Optimization for Enzyme Production

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Oil bio-degradation mechanism by microorganisms is requested for an effective microbial remediation of soil contamination by oil spills. The current examination pointed the identification of a biosurfactant producing bacteria for biosurfactant production from oil contaminated sites from Tamil Nadu. The biosurfactant testing screening methods were used to screen the potent strains and sequencing studies were used for *Pseudomonas* species identification. The bacterial isolate BS17 subjected to be the potent enzyme (Protease, Lipase and Esterase) producer. Among the tested production media, the ground nut oil cake was identified to be the optimum media for protease (0.47069 Unit/ml), lipase (9 Unit/ml) and esterase activity (3.891 Unit/ml) for bacterial isolate BS17. The bacterial isolate BS17 showed greatest lipase (15 Unit/mL) protease (0.8067 Unit/mL) and esterase (4.756 Unit/mL) enzyme activity at pH 9.0. At 35 °C bacterial isolate BS17 showed greatest enzyme action in protease (1.2772 Unit/mL), lipase (17 Unit/mL) and esterase (5.2972 Unit/mL) enzyme activity. At 48hrs of incubation period bacterial isolate BS17 showed most extreme enzyme activity in protease (3.361 Unit/mL), lipase (28 Unit/mL) and esterase (8.918 Unit/mL). The sequence of BS17 was deposited in NCBI and Accession number was received [MT337593.1]. Statistical analysis with the minimum significant difference (LSD) test of ANOVA was carried out to determine the oil degradation efficiency. This paper demonstrated the isolated *P. aeruginosa* (BS17) crude oil biodegradation from oil contaminated land soil sample. Strain BS17 was proved as potent bio-surfactant producer using crude oil by utilizing carbon and energy source in oil degradation mechanism.

KEY WORDS: ESTERASE, HYDROCARBONS, LIPASE, PROTEASE, *P. AERUGINOSA*, SEQUENCING.**INTRODUCTION**

Soils contaminated by petrol hydrocarbons can influence soil well-being. Also, increased focuses on the consequences for human health well-being. They can kill soil microorganisms, decreasing their number and bio-activity. Soil organisms help make supplements accessible to plants. 'Reducing microorganisms' numbers or action additionally influences plants. Some petrol hydrocarbons can be taken up by plants and represent a danger to grazing animals, wildlife, and plant-eating bugs. With a significant level of petroleum contamination, seeds can't grow. At times, even develop

plants can't grow any longer. Some oil-based goods can extremely clog the soil and so it affects the penetration of water into the soil. Polycyclic aromatic hydrocarbons (PAHs) are harmful impurities in different conditions since it has cancer-causing and mutagenic impacts (Silva et al. 2020; Patel et al. 2020).

The biodegradation of PAHs can be considered on one hand to be a piece of the typical cycles of the carbon cycle, and then again as the expulsion of man-made poisons from the climate. The utilization of microorganisms for biodegradation of PAH-contaminated soil and other source of petroleum contaminated environments. Among toxins of hydrocarbons, other petroleum source (alkanes and aromatic compounds combination) is reported as soil hydrocarbon substance leakage from ship tanks or re-rented

Article Information:*Corresponding Author: chithumicros@gmail.com

Received 25/09/2021 Accepted after revision 22/12/2021

Published: 31st December 2021 Pp- 1613-1619

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.37>

in unintentional leakage accident. Hydrocarbons are for the most part unmanageable and impervious to microbial attack because of their low dissolvability and bioavailability. In this way, these mixtures are profoundly industrious in the climate and are bio-accumulated in microorganisms (Azadi et al. 2020).

The accomplishment of bio-degradation is reliant upon the microbial capacity to degrade the hydrocarbons and their growth and metabolic growth kinetics. Higher rates of hydrocarbon degradation are regularly accomplished with a bacterial enhancement consortium confined from the climate that necessities bio-restoration. To get efficient diesel oil-degrading bacterial cultures, information on the variety of the microbial local area presents in polluted soil with diesel oil, their metabolic highlights, and their ability to degrade crude oil. The important properties of hydrocarbon-degrading bacterial species are the production of bio-surfactants (Habib et al. 2020). These organisms produce small surfactant atoms that decrease surface tension. Bacterial consortia often reported as efficient degrader of hydrocarbons and emulsifying compound production. The application of bio-surfactants activating and eliminating the impurities by solubilization and emulsification in waste treatment (Mujumdar et al. 2019; Lawniczak et al. 2020).

There are various studies reported on bacterial bio-surfactants, but the potency of activity is based on their compound chemical nature. A *Pseudomonas* species was known for biosurfactant production. Microorganisms produce biosurfactants as biofilm which associates with an interface and modifies the surface properties like wettability and different properties. A bacterium isolated from sea water polluted with oil, *Pseudomonas aeruginosa* has proved the capacity to breakdown hydrocarbons following 28 days of incubation. The percentage of degradation by the above species has been studied because of bio-surfactant production. Bioremediation is the main technique that has been acknowledged treatment by utilizing native microbial colonies. Certain bio-surfactant-producing microorganisms can degrade a few classes of hydrocarbons. Bio-surfactant characterization is classified based on the microbes' type, compounds nature, the polar groups of compounds.

Bio-surfactants are reported as one of the non-toxic substances, as they are one of the good choices in cosmetic, food, and pharmaceutical industries (Lawniczak et al. 2020). In this work, we have collected oil spilled soil samples from various locations and bacteria cultures were isolated to test the efficiency of bio-surfactant production. In recent research papers, the bacteria belong to 79 genera are reported as petroleum hydrocarbon degraders which includes *Pseudomonas* have been observed for bio-degradation of crude oil (Ma et al. 2021). Our study reported that isolated strain is a potent strain to facilitate enzyme production in a large-scale up process. Moreover, this strain is proved with higher efficiency in bio-surfactant production.

MATERIAL AND METHODS

General microbiological and laboratory techniques for the

readiness of media, bacteria isolation, and stock culture were maintained. Land oil spilled soil was collected from distinct districts of Tamil Nadu. The examples were collected at a profundity of 5cm from the outside of the dirt. By following serial dilution and streak plate method, the bacterial colonies were isolated and slanted cultures was prepared to maintained the stock culture for further examination. The isolated bacterial colonies were inoculated in nutrient broth with the addition of 0.1 gram of oil (engine oil, petrol, or diesel) and incubated the culture for 2 days. After incubation, the culture was centrifuged for 12 min at 4500 rpm speed to remove the bacterial cells. After centrifugation, the cleared layer of supernatant was carefully transferred into a new tube and used the same for further characterization studied. A 10 µl of the crude bio-surfactant was spotted on a thin layer chromatography plate in mobile phase of chloroform, water, and chloroform (CHCl_3 : H_2O : CH_3OH) in the ratio of 70:0.5:10 (v/v/v) as a mobile phase. The developing reagent (ninhydrin) was utilized to visualize lipid-based bio-surfactant (red spot) and anthrone reagent to visualize glycol-based lipid bio-surfactant (yellow spot). Rf values were then calculated (Rani et al. 2020).

Drop collapse test was examined to screen the bio-surfactant-producing strain. It relies upon the rule that a dropped of fluid containing a bio-surfactant would fall totally over the oil surface. 4µl of mineral oil was added to a cleared glass slide and incubated the slide at room condition and afterward, 6 µl of supernatant was applied to the glass slide with oil covered, and size of each dropped was seen. The outcome was viewed as sure when the width of the dropped was expanded by 1mm from that which was formed by deionized watered which was taken as the negative controlled (Rani et al. 2020).

For the emulsification stability test, E24 of bacterial cultures was dictated by the addition of 3 ml oil to a similar measured of culture tube, following 3 min shaking in vortexer and kept undisturbed for 1 day (Rani et al. 2020). The percentage of emulsification capacity was determined:

Emulsification stability % = (Total Height of the emulsified layer / height of total liquid column) × 100

For the lipolytic activity, about 1 gm of agar and gelatin was weighed and dissolved in 100 ml of deionized watered and autoclaved. About 25 ml of the above mixture was poured into a sterile petri plate and wells was cut and the supernatant containing bio-surfactant was added into the wells with a positive controlled under aseptic conditions and incubated at room temperature for one day. Following that the plates was developed with 15% mercuric chloride prepared in 6M HCL solution and the mean diameter was noted. Lipase-producing bacterial strains formed a zone of cleared hollow (hydrolysis) on the tributyrin agar medium. The color zone was estimated after 24 hours of incubation at 37°C. The lipolytic activity of each bacterial culture was determined by estimating the diameter of the zones. The strain with the highest zone was utilized for additional examination. The media utilized contained peptone 1 gm, NaCl 0.5 gm, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.01 gm, agar 1.8 gm, pH 7.

4 for 100 ml media quantity, and sterilized. Tween 80 was included at a final working concentration of 1% (v/v). Then the isolates was spread on the plate, incubated for 24 hours, and the formation of color halos was noted. The strain with the biggest color halo zone was utilized for additional investigation (Guan et al. 2020).

For protease activity, 500 µl of casein (1 %), and 450 µl of 0.2 mol/l phosphate buffer (pH 7.0) was added to 350 µl of crude enzyme extract. At 60 °C the reaction content was incubated for 10 min. The reaction was stopped by the 2 ml of 10 % trichloroacetic acid addition. Then the tube was centrifuged at 7000 x g for 12 minutes and to the separated supernatant, 6 ml of 0.3 mol/l Na₂CO₃, 2 ml of folin and ciocalteau's phenol reagent was added. Followed by 30 min incubation and measured at 660 nm utilizing tyrosine as a standard, the absorbance of the blue color formed (Habib et al. 2020).

The free lipase activity was examined by spectrophotometrically by following the method with some modifications ((Kumar et al. 2020). The reaction mixture contains 0.05M phosphate buffer (pH 8.0) 3 ml, 0.8 mM p-NPP (0.1 ml), 0.2 ml of lipase and incubated at room temperature. And by adding 1 ml of ethanol, the reaction was stopped. A controlled was studied without the addition of enzyme. Absorbance was measured at 410 nm using UV spectrophotometer. One international unit (IU) of lipase action was measured when the enzyme catalyzing and the released of 1 µmol of p-nitrophenol per min from p-NPP (Abi Habib et al. 2020). For esterase activity, the assay solution contained 15 ml of 1% (v/v) tributyrin (substrate), 1% solution of gum acacia, 600µl calcium chloride (2%) and 1.5 ml of NaCl (1M). The whole reaction mixture was studied by titration 10mM NaOH (10mM) and the esterase activity was calculated.

Esterase activity = $\frac{\text{Vol. of consumed NaOH (ml)} \times \text{NaOH Molarity}}{\text{Esterase volume (ml)} \times \text{Time in min}}$

Esterase volume (ml) × Time in min

One unit of esterase activity was concluded as the amount of enzyme that released 1 µmol fatty acid min⁻¹ at 30 °C with pH 7 under the test conditions (Sani et al. 2021). For the media optimization and to induce the maximum enzymatic activity of protease, lipase, and esterase, the parameters of the following factors like media, pH, temperature, incubation period, carbon source, and nitrogen source was optimized based on a one-factor at a time method. The bacterial isolates were cultured on different substrates (4% w/v) such as coconut oil cake, cottonseed oil cake, groundnut oil cake, gingelly oil cake, soya bean oil cake, rice bran, wheat bran, and the studied was compared with nutrient broth medium at 37 °C with pH of 7.5 and incubated for 48 hours. The cultured broth was then centrifuged at 12,000 rpm for 15 minutes. The cleared supernatant was collected and used as a crude enzyme for studying the enzymatic activity and total protein content. The effect of pH on enzymatic activity was examined with various pH 4 - 12.

The ideal pH for maximum enzyme activity was noted by changing the pH of the medium. The effect of temperature

on enzymatic activity was examined with different temperatures such as 25 °C, 30 °C, 35 °C, 40 °C, 45 °C, and 50 °C. The ideal temperature for maximum enzyme activity was chosen by changing the temperature of the medium. The effect of the incubation period on enzymatic activity was studied at different time intervals that include 12 hours, 24 hours, 36 hours, 48 hours, 60 hours, and 72 hours. The ideal incubation period for the highest enzymatic activity was recorded by changing the incubation period of the medium (Al-Dhabi et al. 2020).

For morphology study and molecular identification, the most effective bacterial culture was distinguished by standard biochemical tests and morphological examination by microscope. Genetic identification of strain in which the genomic DNA of the bacteria was extracted utilizing the standard procedure. Then the 16s rDNA was amplified used PCR by utilizing universal primer, 968F (AACGCGAAGAACCTTAC) and 1541R (AAGGAGGTGATCCAGCCGCA) (Fadeev et al. 2021). Polymerase chain reaction (PCR) was performed in a 30 µl volume of master mixed (sigma aldrich, USA) and 10 ng of genomic DNA. PCR amplicons was further separated on 1 % of agarose gel and PCR amplicons (16s rRNA gene) was purified used pcr clean-up kit (genelute™). PCR amplicons was sequenced by a single by-pass sequencing method. The consensus sequence of 16s rDNA was utilized for basic local alignment searched tool (BLAST) examination against the data set in the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). Sequence alignment was done by utilizing Clustal w and by dictated by the neighbor-joining technique the phylogenetic tree was constructed used Mega 5 software (Modi et al. 2019). The curated FASTA sequence was submitted to Genbank and the accession number was acquired.

RESULTS AND DISCUSSION

All the dirt soil samples were gathered at a profundity of 5cm from the land surface. The gathered soil samples had an identification of dark tone because of consistent oil spillage. The gathered oil spilled soil samples in sterile polythene sacks were stored at 4°C aseptically and utilized for additional exploration studied. Most of the strains was belongs to *Pseudomonas* genus, especially *P. aeruginosa* species, which widely reported for crude oil degradation (Shweta et al. 2021).

Figure 1: Thin Layer Chromatography: TLC analysis of purified bs produced two spots in the TLC plate with an R_f value of 0.89. The bio-surfactant fraction after centrifugation showed a positive result with Molish reagent and vapor of iodine, representing the lipid moieties presence and carbohydrate moieties. The TLC analysis determined the presence of the glycolipid nature of extracted bio-surfactant.

Oil spreading and Drop collapse test: Among the other selected bacterial isolates, the broth culture of bacterial isolate BS17 was noted as the highest oil spreading efficiency (38 mm) followed by bacterial isolate 45 (36 mm). The bacterial isolates 40 (7 mm), 41 (9 mm), 70

(4 mm), 62 (8 mm) were observed as the lowest zone formation. The remaining bacterial isolates observed have no zone of displacement which is recorded as an absence of bio-surfactant production. The bacterial isolate BS17 was observed as the highest bio-surfactant activity in the drop collapse test (Rani et al. 2020).

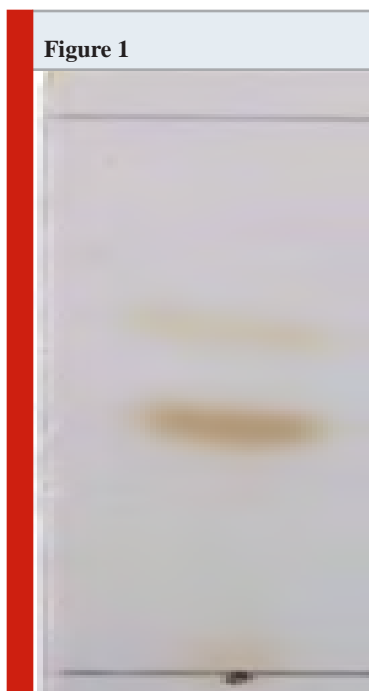
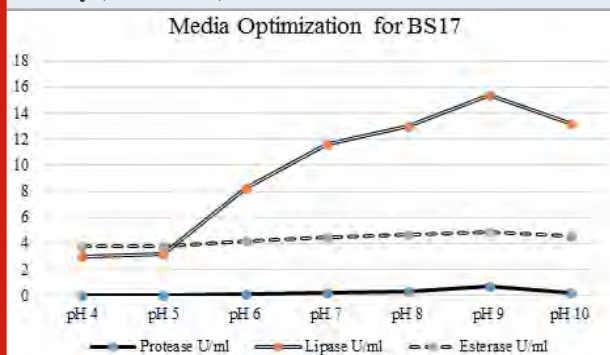


Figure 1

Figure 2: Among the tested production media, the groundnut oil cake was found have been the optimum media for protease (0. 47069 u/ml), lipase (9 u/ml), and esterase activity (3. 891 u/ml) for bacterial isolate BS17.



Emulsification stability test: The effectiveness of biosurfactants in supplying oil to bacterial cells was investigated by measured the emulsifying activity (E24 index) of the biosurfactant. The screening was studied by adding 2 ml of gasoline to 1 ml of the supernatant and stirring vigorously and keeping overnight. The emulsification activity was calculated as a percentage. Among the fifty bacterial isolates screened, the emulsifying activity was found have been maximal in bacterial isolate BS17 (55 %), followed by bacterial isolate BS56 (48 %). The lowest leveled of emulsion activity (0. 3 %) was observed in bacterial BS38, BS32, and BS45 (Rani et al. 2020).

Figure 3: The investigation of the impact of various pH uncovers that enzyme production was extraordinarily affected by fluctuating pH of the production media. The bacterial isolate BS17 showed greatest lipase (15 U/ml) protease (0. 8067 U/ml) and esterase (4. 756 U/ml) enzyme activity at pH 9. 0. At pH 4. 0, 5. 0 and 12. 0 the bacterial isolate 17 recorded total shortfall of protease activity and low lipase activity (2 U/ml, 3 U/ml and 3 u/ml) individually and esterase action (3. 676 U/ml, 3. 837/U/ml and 4. 162 u/ml). As there was an increment in the pH esteem from pH 4. 0 to 9. 0 there was a significant increase in lipase, protease, and esterase production in bacterial isolate BS17. The protein content was also recorded high up to pH 9. 0 after which there was a rapid decrease in the protein content of bacterial isolate BS17 which relates to the decrease in enzyme production (Hou et al. 2021).

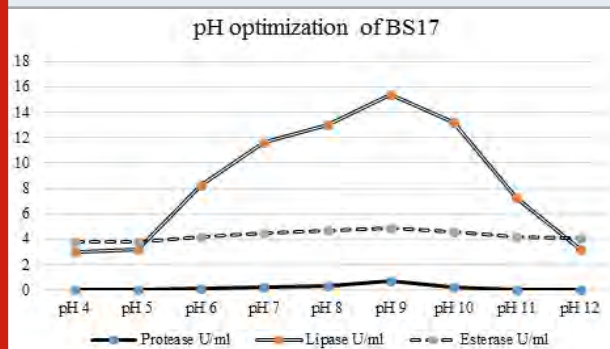
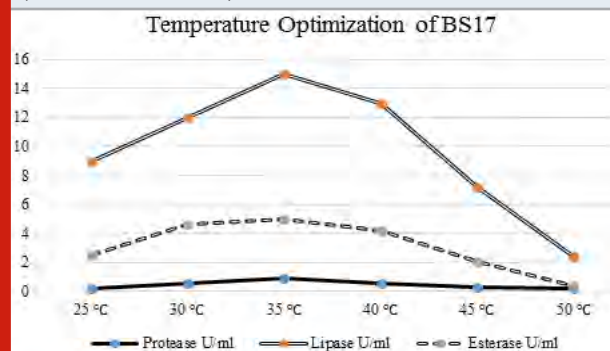


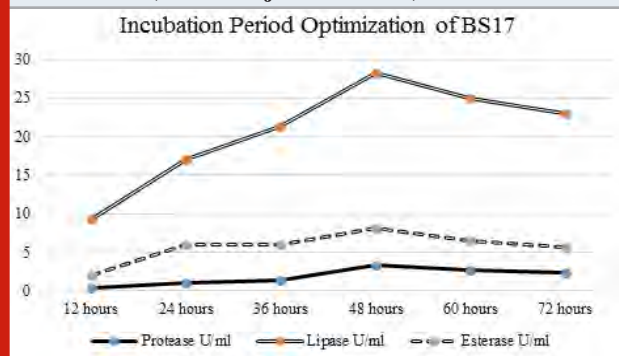
Figure 4: Optimization of temperature for promoting the enzyme activity. At 35 °C bacterial isolate BS17 showed the greatest enzyme action in protease (1. 2772 U/ml), lipase (17 U/ml), and esterase (5. 2972 U/ml) enzyme activity. There was an increase in chemical creation in protease, lipase, and esterase action when the temperature increments from 25 °C to 35 °C. Continuously there was a decline in a compound movement when the temperature ascends from 40 °C to 50 °C. The protein content additionally showed a significant increase up to temperature 35 °C after which there was a decrease in the protein content of bacterial isolate BS17 (Benabda et al. 2019).



Extraction of protease, lipase & esterase enzymes from supernatant containing bio-surfactant: From the past outcomes concerning the fundamental screening of potent bacterial isolates for the production of biosurfactants by

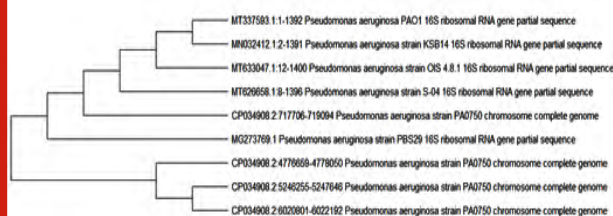
oil spread test, drop collapse method, and emulsification activity test. Based on the result we got, 31 bacterial isolates was separated to assess the lipolytic, proteolytic, and esterolytic activities by estimating the hydrolysis of tributyrin, gelatin, and tween 80 around the bacterial colonies. Out of the 31 bacterial isolates BS27 bacterial isolates was observed for lipolytic activity and isolate BS17 was identified have been the best lipase producer. While 24 bacterial isolates showed esterolytic activity and 25 bacterial isolates showed proteolytic action with bacterial isolate BS17 was showed have been the best esterolytic and proteolytic production. The bacterial isolates BS17, BS34, and BS47 showed the best lipolytic activity with no response of protease and esterase activity. Despite the reports that these enzymes were highly demanded for various applications in biotechnology industries (Al-Ghanayem et al. 2020).

Figure 5: At 48hrs of the incubation period, bacterial isolate BS17 showed the most extreme enzyme activity in protease (3. 361 U/ml), lipase (28 U/ml), and esterase (8. 918 U/ml). There was an increment in enzyme catalysis in protease, lipase, and esterase activity when the incubation time frame increases from 12 hrs to 48 hrs. There was a critical lessening in enzyme production of protease, lipase, and esterase when the incubation time frame ascends from 60 hrs to 72 hrs. Least protein content in protease (0. 4033 U/ml), lipase (9 u/ml) and esterase (2. 972 U/ml) action was recorded as 12 hrs incubation period. The protein content likewise showed a huge increase up to 48 hrs incubation period after which there was a significant reduction in the protein content of isolate BS17 (Chimbekujwo et al. 2020).



Oil Degradation by extracted bio-surfactant: The measured of oil in contaminated soil samples before microbial treatment and after treatment with the particular bio-surfactants was assessed utilizing the gravimetric technique. In the supernatant of bacterial isolate BS56, the leveled of oil degradation percentage was 88. 75 %. This was supposed have been the highest degradation percentage among the screened 17 bacterial isolates. Followed that, the bacterial isolate BS17 showed an oil degradation percentage of 87. 4 1%. In the supernatant of bacterial isolate BS39, the degradation of oil percentage was 79. 30 %. This was recorded as the least oil degradation percentage (Christova et al. 2019).

Figure 6: The potent isolate BS17 has shown maximum response in all screening tests. The sequenced 16s rRNA was curated and the phylogenetic tree was constructed used Mega5 software to align the closely related species. From the analysis, the BS17 was closely related to *Pseudomonas aeruginosa*. The 16s rRNA sequence of BS17 was submitted and the NCBI Genbank accession number was received [MT337593.1].



Optimization of media for promoting the enzyme activity: Production media plays a crucial role in the production of microbial enzymes. Different media such as coconut oil, cottonseed oil cake, groundnut oil cake, gingelly oil, soyabean oil cake, rice bran, nutrient broth, wheat bran was tested to studied the effect of media on maximum enzyme production for protease, lipase, and esterase activity. From the previous data we got, the bacterial isolate BS17 and isolates was selected for their ability to produced lipase, protease, and esterase in higher production. The bacterial isolates BS17 and was subjected have been the potent producer and was chosen for further optimization studied (Al-Dhabi et al. 2020).

Optimization of pH for promoting the enzyme activity: The role of pH was one of the significant variable parameters for microbial growth in the production medium. As the pH assumes a significant part in all the organic cycles, so the enzyme production was tested at various pH liked 4 - 12.

Optimization of incubation period on enzyme activity: Incubation time assumes a significant part in enzyme production. To examine the impact of the incubation period on protease, lipase, and esterase production, different incubation periods, such as 12 hrs, 24 hrs, 36 hrs, 48 hrs, 60 hrs, and 72 hrs was utilized.

Molecular identification of potent bio-surfactant producing bacterial isolates by Phylogenetic tree construction:

Statistical analysis: Results and all the experiments were represented as standard deviation (SD) and \pm mean. ANOVA table was used to determine the significant differences statistically ($p < 0.05$) by used SPSS 16.0 (IBM Corp, USA) (Mishra et al. 2019).

CONCLUSION

The findings of the present study suggests that the biodegradation of crude petroleum by *P. aeruginosa* BS17 isolates from hydrocarbon-contaminated soil. Strain BS17

was identifies to be an effective raw petroleum degrader and could potent biosurfactant utilizing raw petroleum as the sole carbon and fuel source throughout degradation. The biosurfactant produces by BS17 has high surface action and displays superb emulsification action against various hydrocarbon substrates. Every one of these positive properties works with the strain as a proficient bacterial biosurfactant in different ecological applications, especially in the remediation of unrefines petroleum pollutes areas.

Conflict of Interests: Authors have no conflict of interests to disclose.

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Agricultural Communication

Implementation of Innovation Development Models in Agrarian Sphere of Economy at the North Caucasian Federal District, Russia

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ABSTRACT

The present paper aims to analyse the problems of the innovation development component in the agricultural economy of the North Caucasian Federal District of the Russian Federation in terms of the operational settings of agro-economic systems, facilities of economic entities, organisational and economic mechanisms, managerial function and prioritisation of innovation directions. The current aspects of innovation-driven development of the regional agroindustrial complex are discussed. Priorities of innovation development in agriculture and related industries of the agroindustrial complex of the North Caucasian Federal District are proposed, based on the technological reequipment of agroindustrial enterprises, instituting the innovation-driven mechanism of agroindustrial operations, building up the information, people and financial support of the innovation development system of the agroindustrial complex, ensuring proper economic conditions for implementing innovation programmes and projects, state financing of innovation activities, broad attraction of investment and improving solvency. The practical implementation of innovations should rely on a built system of regional innovation-driven development, clear and structured iterations in adopting all groups of innovations in agroindustrial sectors based on centralised vertical alignment between all branches of government and horizontal cross-departmental cooperation. A crucial regulatory role in this process should be played by government structures across economic and education sectors closely tied with agroindustrial production. Effective implementation of innovation policies will depend on ensuring proper economic conditions for putting forward innovation programmes and projects, on the availability of state funding to support innovation activities, attracting investment in the innovation sphere, advancing entrepreneurship and commercialisation of innovation projects. The identification of factors inhibiting the adoption of innovation in the agro-economy of the North Caucasian Federal District and current trends in innovation activities of enterprises and operations suggests a conclusion concerning the need for a systemic approach to innovation in the region taking into account national interests and makes the basis for further proposals of scientifically-based directions of economic transformations and coordination of efforts between all government structures engaged in this process and responsible for solving these tasks. The findings of this paper can be used by the control bodies of the regional agroindustrial complex, by the students of agricultural colleges and other public and private entities of the North Caucasian Federal District for better economic transformations and coordination.

KEY WORDS: AGROINDUSTRIAL COMPLEX, FACTORS OF INNOVATION, INNOVATION-DRIVEN TRANSFORMATION, REGIONAL AGRICULTURAL INNOVATION.

INTRODUCTION

In the current setting, the problem of transforming agricultural enterprises to a new level of economic management is

pursued, and the task is to develop, adopt and implement innovation-led approaches to technologies, organisation and management of agricultural production and related sectors of the agro-economy. It is crucial to make sure that the ongoing transformations are maintained continuously and progressively to achieve the indicative targets adopted and applicable as the basis of government programmes. The

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Received 21/10/2021 Accepted after revision 13/12/2021

Published: 31st December 2021 Pp- 1620-1626

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.38>

effectiveness of innovation activities depends on a complex of elements (Kornilova et al. 2021).

A successful transformation of the agro-economy depends on the ability to understand the existing problems that can get in the way in using state-provided funding and hamper the region's competitiveness. Therefore, it is crucial to analyse the current situation with innovation in the agro-economy through the lens of several interrelated problems, the causative relations and potential lines of development. The aspects of innovation processes and innovation development are covered in the previous works (Kanygin 1974; Santo 1990; Bulakh and Fliaster 1991; Zhebrovskii 1992; Samkaev and Strizhkov 2007; Neudakhin 2007). The trends observed in innovation processes in agroproduction are discussed in previous studies (Ushachev, Sandu and Ogloblin 2007). The reviewed works by foreign authors provide input to identify the main theoretical approaches to innovation processes in general and with regard to regional agro-economies in particular (Bakytzhan et al. 2020; Polushkina et al. 2020; Kornilova et al. 2021).

Numerous approaches currently exist in arranging the operation of regional agro-economies, specifically, the adoption of advanced equipment, intensification of production, technological innovation, implementation of scientific results in production and business-related and socioeconomic dimensions of innovation. These include upgrading the institutional environment and transformation of state support, as well as new methods of production management in the agro-economy. The transition to an effective innovation economy integrates a systemic approach, modernisation of the methods and forms of management of the resource potential across the levels of the innovation system and a new level of cooperation between different parts of the value chain. The existing approaches are based on setting the foundation of innovation requirements and analysing the regional innovation potential as needed for innovation development (Bakytzhan et al. 2020).

The observed requirements primarily reflect some of the specifics of the agro-economy:

- beyond being profitable, innovation in the agroindustrial complex should also contribute to food security of the country and region, which stipulates the long-term nature of scientific research that eventually may or may not lead to further implementation (Fedotenkov 2014; Iunusova 2018).
- considerable time lag between the implementation of innovation and economic results (1-7 years).
- wide product ranges maintained by production enterprises, reflecting high production risks in agroindustrial sectors (Kurbanov 2019).
- extreme dependence on natural and weather conditions substantiating the breeding of specific varieties for the area and cultivating crops under cover.

- low levels of cooperation and the lack of machine tractor stations result in a situation when specialist vehicles are unavailable for most small and medium-sized enterprises, leading to lower economic results in production and even further unaffordability of the equipment (Polushkina et al. 2020).

- the system for implementing scientific results in production in the agro-economy was destroyed and never restored until now, which has led to the extremely low rate of innovation in most medium-sized and small enterprises. Only the biggest regional producing enterprises usually have access to effective innovations (Reznichenko and Andreev 2016). The discussion of the regional aspects of innovation will focus on the definition of innovation as a sequence of activities to create and improve agricultural goods, to advance technologies and management systems based on the integration of scientific results or production expertise (Ushachev 2007; Dokholyan 2019; Polushkina et al. 2020).

At the regional level and the agroindustrial economy, innovation development would also involve the development of an underlying system of economic relations and conditions. Innovation requirements can be external, originating from regional strategic development plans, or internal, shaped within agroindustrial enterprises and their interactions and interrelations (Kazikhanov 2016; Polushkina et al. 2020; Kornilova et al. 2021). The main problem of our study is the identification of regional innovation requirements and the available potential, including innovation infrastructure, people potential (research institutions, HEIs, etc.), the degree of wear-and-tear of fixed capital, operation margins of the enterprises, adoption rates of information innovation, organisational and management aspects of innovation.

MATERIAL AND METHODS

To solve the set problems, we conducted a mixed type study comprising several stages, including description, explanation, assessment, comparison and analysis of relationships. The object was the North Caucasian Federal District consisting of the Republic of Dagestan, the Republic of Ingushetia, the Kabardino-Balkarian Republic, the Karachayevo-Circassian Republic, the Republic of North Ossetia-Alania, the Chechen Republic and the Stavropol Territory. The description part of our study was addressed by plain gathering of data from documents. The main sources for document review were research works by Russian and international researchers and statistical materials. One of the main goals we focused on was to capture data to further aggregate them.

RESULTS AND DISCUSSION

The figures in Table 1 suggest that spending on the adoption of information and communication technologies has been rising in the North Caucasian Federal District over the last three years. However, there are significant severalfold differences among the constituent federal entities in terms of the amounts spent on informatisation. When specific items of spending are considered, the maintained trend is

toward channeling the most funding toward purchases of equipment and paying telecommunication bills, while only a small fraction of total spending comes into employee trainings, which showed a downside trend between 2017

and 2018 (38.4 million rubles in 2017 vs. 19.7 million rubles in 2018). No further statistical data is provided. Therefore, we should note that all economic sectors and specifically in rural areas do not realise the innovation potential of the existing staff (Gokhberg et al. 2020).

Table 1. Spending on the adoption of information and communication technologies (million rubles) (Federal State Statistics Service, 2020)

Region	Spending on ICT in 2017, total	Spending on ICT in 2018, total	Spending on ICT in 2019, total
North Caucasian Federal District	8,832.7	10,635.9	13,802.3
Republic of Daghestan	905.9	1,367.0	1,404.3
Republic of Ingushetia	379.7	521.5	306.9
Kabardino-Balkarian Republic	515.3	596.7	572.0
Karachayevo-Circassian Republic	515.3	598.6	651.5
Republic of North Ossetia-Alania	770.0	819.2	1,088.0
Chechen Republic	1,423.0	1,411.4	2,298.9
Stavropol Territory	4,323.6	5,321.4	7,480.6

Table 2. Aggregate level of innovation activities of enterprises, % (Gokhberg, et al. 2019; Gokhberg et al. 2020)

Russian Federation	9.5	8.4	12.8	9.1
North Caucasian Federal District	6.2	2.9	4.4	1.7
Republic of Dagestan	6.7	2.5	2.2	0.5
Republic of Ingushetia	–	–	10.5	0.8
Kabardino-Balkarian Republic	8.3	2.4	10.3	3.9
Karachayevo-Circassian Republic	4.3	0.8	6.5	5.7
Republic of North Ossetia-Alania	7.7	3.8	9.6	1.6
Chechen Republic	0.8	0.3	0.2	0.2
Stavropol Territory	7.2	4.9	7.9	5.1

As can be seen from the table, the level of innovation activity shows unstable growth on average for the region, however, it shows a steady downward trend in the Republic of Daghestan, and for some regions, no comparable figures are available. Moreover, the level of innovation activity is very low even when all types of economic activities are considered, so one may assume that the figure would be even lower in agriculture. The figures in Table 3 show that innovation resources in the North Caucasian Federal District are primarily focused on technological innovation (much as in the majority of other regions (except the Far Eastern Federal District). The data indirectly confirms the high rate of wear-and-tear of capital assets in production and the insufficiency of modern machinery, mechanisms and technologies.

Table 3. Enterprises putting forward technological, marketing or organisational innovation in 2017, % (Gokhberg et al. 2019; Gokhberg et al. 2020)

	Relative share of organisations conducting specific types of innovations, as percentage of the total		
	technological	marketing	organisational
Central Federal District	3.8	0.5	1.1
Northwestern Federal District	2.9	1.4	1.1
Southern Federal District	4.6	0.1	0.7
North Caucasian Federal District	0.9	0.7	0.2
Volga Federal District	2.7	0.6	0.7
Ural Federal District	3.4	0.3	1.2
Siberian Federal District	3.1	0.3	0.8
Far Eastern Federal District	1.2	0.4	1.6

No regional figures on innovation activities across the types of economic activities are provided, therefore, consider the nationwide Russian figures for agriculture. Considering the data from Table 4, one can see that the vast majority

of agricultural and food enterprises spend their available resources to purchase machinery and equipment, software and research. Employee trainings were provided by 2.9% of enterprises in agriculture and 7% of enterprises in the food

industry. Given the fact that the count of enterprises does not include private subsistent farms producing a significant share of products in the regions of the North Caucasian

Federal District (68.7% of potatoes, 66.2% of vegetables, 32.8% of cattle and poultry for slaughter, 68.4% of milk), therefore, the respective percentage is actually very small (Gokhberg et al. 2020).

Table 4. Relative share of enterprises in the RF conducting technological innovation by the types of economic activities in 2018, % of the total (Gokhberg, et al. 2019; Gokhberg et al. 2020).

	Enterprises conducting							
	Research and development	Purchases of machinery and equipment	Purchases of new technologies	Purchases of software	Engineering	Employee training	Marketing research	Other, incl. design
Agriculture	8.1	78.1	2.4	9.0	7.6	2.9	0.5	11.9
Food production	18.9	70.4	3.7	7.8	9.3	7.0	6.7	21.1
Beverage production	15.9	59.1	9.1	13.6	13.6	6.8	9.1	56.8

The data in Table 5 shows that the degree of wear-and-tear of fixed assets in agricultural spheres is significant, which has led to a massive trend of fleet upgrading among enterprises. When the numbers of researchers by branches of science are considered, the share of researchers in the field of agricultural sciences equals 13% of the total, which

is next to the lowest figure among seven types of research. In (2019), internal spending on scientific research and development in agricultural sciences equaled 17% of the total amount. The predominant share comes into natural sciences and engineering disciplines (28% and 24% respectively).

Table 5. Fixed asset condition in agriculture, forestry, fishing and fish farming as of the end of 2019, % (Federal State Statistics Service 2020)

	RF	Central Federal District	Northwestern Federal District	Southern Federal District	Volga Federal District	Ural Federal District	Siberian Federal District	Far Eastern Federal District	North Caucasian Federal District
Degree of wear -and-tear	41.4	39.0	43.5	42.9	41.2	38.5	46.3	47.5	40.6
Completely worn out	10	8.6	12.1	11.6	10.2	9.2	12.6	10	9.2

The data in Table 6 shows that agricultural enterprises in the region have remained profitable on average over the last 15 years, however, a downward trend or sharp fluctuations have been observed in the most recent years in profitability figures. Moreover, regional averages are primarily driven by the biggest constituent entity, the Stavropol Territory. The profitability of agricultural enterprises has also shown a downward trend, as can be seen from Table 7 (almost 10% over the past five years).

The above data in Table 7 indicates that agricultural enterprises in the region have generally remained profitable. However, a downward trend has been observed since 2015, with negative indicators in nearly all entities of the North Caucasian Federal District, except the Stavropol Territory and the Karachayevo-Circassian Republic. Based on the data from Tables 6 and 7, the question remains how strong is the role of state support of producers in maintaining the levels.

According to Table 8, agricultural enterprises in nearly all constituent entities (except the Republic of Ingushetia and the Kabardino-Balkarian Republic) have increased investment in fixed capital. However, the primary direction is to rebuild fixed capital due to its considerable natural wear-and-tear, as mentioned above, thus, the ability to renew the active component of fixed assets makes the basis for the adoption of new technology on the way to innovation in the agroindustrial complex (Gokhberg et al. 2019).

Meanwhile, when government funding is limited, the role of the state could be in ensuring a favourable investment climate through the development of infrastructure, efforts to provide consultancy and education services for producers with the use of information technology, employee training and continued education and by adopting new methods and forms of economic support for agricultural producers implementing and advancing innovations in their operations. The list of factors inhibiting innovation cited by industry leaders was topped by economic factors, specifically, deficits

of own funding sources and high costs of innovations, insufficient state support; internal factors came second, including, poor innovation potential of the enterprises and

skills shortage, as well as uncertainty of economic benefits from innovations (Gokhberg et al. 2020).

Table 6. Balanced financial result (profit minus loss) of enterprises, million rubles (Federal State Statistics Service 2020).

	Plant farming						Animal farming					
	2005	2010	2015	2017	2018	2019	2005	2010	2015	2017	2018	2019
North Caucasian Federal District	1,176	3,743	16,820	11,071	13,943	10,030	91	515	2,707	2,610	3,399	823
Republic of Daghestan	41	-5	68	172	178	169	4	39	24	144	51	-14
Republic of Ingushetia	-50	-21	-12	-25	4	-54	-3	-1	0.1	0.4	0.4	–
Kabardino-Balkarian Republic	-157	-57	135	235	202	114	-17	-8	201	103	53	51
Karachayevo-Circassian Republic	39	-175	219	445	437	383	-90	39	10	11	14	34
Republic of North Ossetia-Alania	-84	-48	0.2	0.3	-59	76	-10	-33	-0.0	–	–	9
Chechen Republic	...	-235	-58	-81	-15	2	...	-4	-10	-0.2	-3	-11
Stavropol Territory	1,387	4,284	16,468	10,325	13,196	9,340	207	483	2,482	2,352	3,284	754

Table 7. Profitability of sales, goods (works, services), %, among enterprises (Federal State Statistics Service 2020).

	in plant farming						in animal farming					
	2005	2010	2015	2017	2018	2019	2005	2010	2015	2017	2018	2019
North Caucasian Federal District	10.5	16.6	36.6	21.2	23.2	18.9	5.3	7.0	14.8	13.9	17.4	9.2
Republic of Daghestan	4.0	0.7	5.6	8.6	8.7	12.8	-1.4	-9.1	-1.3	5.1	5.6	-4.1
Republic of Ingushetia	-37.5	-29.3	-11.7	10.4	13.8	-14.2	-11.5	28.7	9.8	19.8	12.3	–
Kabardino-Balkarian Republic	-4.2	4.7	10.9	7.5	12.9	17.5	-1.1	3.0	12.8	11.1	10.4	10.2
Karachayevo-Circassian Republic	10.0	-7.5	22.2	19.6	11.7	14.2	-13.4	6.7	9.7	7.6	5.0	3.8
Republic of North Ossetia-Alania	-20.7	-21.8	3.2	-61.5	-48.6	14.9	-7.8	22.5	–	–	–	-6.5
Chechen Republic	...	-29.2	-9.9	-11.5	-12.0	-11.3	...	-15.8	-28.8	-20.6	-2.6	-44.7
Stavropol Territory	12.9	19.7	39.7	23.0	25.3	19.5	9.4	10.1	15.4	14.7	18.2	9.9

The above analysis of economic indicators of the agroindustrial complex of the North Caucasian Federal District suggests that innovation activity levels of most enterprises are low. They lack capabilities to adopt new technologies in production, which is attributed by enterprise leaders to high initial costs and insufficient support from the state in agriculture. While on average across the agroindustrial complex of the North Caucasian Federal District, operations are breaking even, the levels of profitability in many constituent federal subjects have been declining and the averages are primarily driven by the biggest constituent entity, the Stavropol Territory.

In this context, enterprises face operating obsolete or undifferentiated technical equipment, using extensive technologies or technological facilities that are morally or physically outdated. Moreover, the already limited free cash is primarily used to purchase machinery and equipment and auxiliary items (such as software).

Research, design, marketing and organisational innovations only constitute a minor percentage of the total use. A crucial component for advancing ahead in the way of innovation development of the agroindustrial complex is the development of infrastructure of innovation activities

and making sure that the combination of facilities, legislative and other instruments are in place to provide for information, expert, marketing, financial, people and

other forms of support of innovation operations (Gokhberg et al. 2020).

Table 8. Investment in fixed capital by the types of economic activities (excluding small businesses; million rubles) (Geometr Russia, 20018; Federal State Statistics Service, 2020).

	2017			2018			2019		
	Total	Incl.		Total	Incl.		Total	Incl.	
		Agriculture, forestry, fishing and fish farming	Informatisation and communication activities		Agriculture, forestry, fishing and fish farming	Informatisation and communication activities		Agriculture, forestry, fishing and fish farming	Informatisation and communication activities
North Caucasian Federal District	190,560	24,070	8,004	227,652	24,535	9,286	268,066	33,238	10,731
Republic of Dagestan	23,138.7	1,199.8	1,405.8	29,744.8	20,37.0	1,684.1	36,036.9	3,451.1	2,263.0
Republic of Ingushetia	9,624.9	220.2	274.1	11,785.5	4.3	344.9	15,066.6	-	989.7
Kabardino-Balkarian Republic	15,714.8	2,575.0	656.2	16,123.5	1,926.9	776.4	20,034.7	1,148.4	1,316.3
Karachayevo-Circassian Republic	12,633.8	549.8	354.5	15,728.6	935.1	430.0	17,460.2	-	604.8
Republic of North Ossetia-Alania	16,653.8	7.5	705.1	18,825.2	17.8	859.6	21,101.7	1064.4	1,257.0
Chechen Republic	42,698.7	4,974.4	1,331.8	51,328.0	2,484.8	1,302.0	55,166.3	2,296.6	1,261.6
Stavropol Territory	70,095.1	14,543.4	3,276.8	84,116.0	17,128.9	3,889.3	103,200.0	24,499.5	3,038.6

Given the identified factors inhibiting innovation processes, the following directions of innovation development arguably appear as the most relevant in the current economic settings: technological reequipment of enterprises in the agroindustrial complex; adoption of effective resource-saving technologies; storage and processing of agricultural products; melioration efforts for soil improvement and preventing soil degradation, maintaining farming systems, forming and maintaining agroecosystems and agrolandscapes; given favorable environmental settings, a promising direction is presumably the development of organic production, fishery and fish farming; enhancing the economic mechanism of maintaining the innovation ways of the agroindustrial complex; building up information, people and financial support to maintain innovation development in the agroindustrial complex (Baliyants et al. 2019; Bashirova 2019).

The efficient adoption of innovation will significantly depend on ensuring stable economic and legal settings and state funding for innovation activities. The financial rehabilitation of agricultural enterprises would require a new approach to the economic component of production operations and improvement of solvency. This implies balancing the mechanisms of purchases of agricultural products and establishing long-term storages (Gokhberg et al. 2020).

CONCLUSION

The findings of the present study suggests that a steady development of agricultural production depends on

overcoming the factors inhibiting innovation activities in the sectors of the agroindustrial complex; solving the problem of effective resource management would help to progressively address the issues of providing competitive food supply in the region and country. The innovation-driven development of the North Caucasian agroecology is contingent on the availability of effective mechanisms for developing and spreading innovation and innovation management, which creates relevance for building regional innovation systems. Focusing on competitiveness of the agroindustrial production of the North Caucasian Federal District would help to improve the rankings of its constituent regions both at the national and international level, which will benefit the overall perceptions of the mesoregion.

ACKNOWLEDGEMENTS

The study was financially supported by the RFBR, project No. 20-010-00965A.

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Pharmaceutical Communication

Evaluation of Antioxidant and Antidiabetic Potential of Polyherbal Drug Made with Three Herbs: An *In vitro* Cell Culture Model

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ABSTRACT

In diabetes, the postprandial phase is characterized by a rapid and large increase in blood glucose levels, and the possibility that the postprandial “hyperglycemic spikes” may be relevant to the onset of cardiovascular complications has recently received much attention. Medicinal use of herbal medicine in the treatment and prevention of diseases including diabetes has a long history compared to conventional medicine. These plants have no side effects and many existing medicines are derived from the plants. Hence, the current investigation was planned to make a poly herbal drug (PHD) through *Punica granatum* (fruits), *Illicium verum* (flowers) and *Nyctanthes arbor* (leaves) and assess their antioxidant and antidiabetic activities *in vitro* and in HepG2 cell line. The respective plant methanolic extracts and PHD are exposed to phytochemical assessment and to discriminate the bioactive factors by Gas Chromatography–Mass Spectrometry. We evaluated the antioxidant properties 2, 2-diphenyl-1-picrylhydrazyl scavenging, hydrogen peroxide scavenging, thiobarbituric acid reactive substances and total antioxidant activity of individual plant extracts and the PHD. At the same time, *In vitro* and cell culture approaches were used to assess the anti-diabetic activity. The PHD has a higher concentration of secondary metabolites than individual plant extracts, according to our findings. On the other hand, we also notice that PHD demonstrated higher antioxidant capability and considerable *in vitro* glucose-lowering effects along with noteworthy inhibition of α -amylase, glucosidase, lipase, dipeptidyl peptidase-IV, collagenase and protein glycation in HepG2 cell line. In conclusion, this study clearly demonstrated the significant antioxidant and antidiabetic activities of the PHD. Hence, PHD may be used as a potential source in the management of diabetes, hyperglycemia and the related state of oxidative stress.

KEY WORDS: ANTIOXIDANTS, FREE RADICALS, MEDICINAL PLANTS, PHYTOCHEMICALS, POSTPRANDIAL DIABETES.

INTRODUCTION

Diabetes mellitus (DM) is a multifaceted metabolic disorder attributable to a complete deficiency of insulin or comparative be short of insulin as an outcome of defection in insulin production, action or a combination of both. The clinical feature of DM is glucose intolerance, which results in hyperglycemia. Type 1 DM (T1DM) and type 2 DM (T2DM) are the two main types of DM. T1DM, which is linked with full or near-total insulin insufficiency and

autoimmune destruction of pancreatic β -cells, affects around 5-10% of people with DM. T2DM, on the other hand, has evolved into a virulent illness marked by β -cell dysfunction, various degrees of insulin resistance, and increased hepatic glucose production (American Diabetes Association 2015). Nearly 425 million persons have DM; by 2045, this number is expected to rise to 629 million. 79 percent of adults with DM live in low- and middle-income countries, 1 of every 2 (212 million) people with DM is undiagnosed, and 352 million people are at risk of developing T2DM (IDF 2018). In most developing countries, such as India, DM is widespread (Uddand Rao et al. 2019; Cole and Florez 2020).

Article Information:*Corresponding Author: sarabioc@gmail.com

Received 25/10/2021 Accepted after revision 15/12/2021

Published: 31st December 2021 Pp- 1627-1635

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.39>

According to statistics, India has the world's highest number of DM patients and has been termed the "diabetes capital of the world". At the same time, the limitation of the accessible treatments and also the harmful side effects, the look for new medication continues to be directed against DM (Abate and Chandalia 2007; Parim et al. 2019). The researchers have an interest in looking out new medication to treat DM because of impending hypoglycemic activity and negligible side effects of healing plants (Kavishankar et al. 2011; Cole and Florez 2020). One technique of a therapy to reduce hyperglycemia, the inhibition of carbohydrate hydrolyzing enzymes in the digestive organs, slows down and reduces the digestion and absorption of ingested carbohydrates, especially after a meal. As a result, these inhibitors may be able to minimize the postprandial rise in blood glucose levels (Narkhede, 2011; Gurung et al. 2020). Consequently, natural inhibitors derived from dietary plants have a reduced inhibitory impact on carbohydrate hydrolyzing enzyme activities and can be employed as a possible postprandial hyperglycemia treatment with little adverse effects (Uddand Rao et al., 2020).

On the other hand, oxidative stress plays a crucial function in DM physiopathology; thus, the interest of using natural antioxidants as beneficial tools exists (Uddand Rao et al. 2018). There is a rising interest in using therapeutic plants and their phytoconstituents as natural sources because of their well-known capability to scavenge free radicals. Plants are effective sources of natural antioxidant molecules with a variety of pharmacological activities and few or no side effects that protect human health from a variety of diseases (Omari et al. 2019). The idea of poly herbal drug (PHD) is well known in the therapeutic system. Plant formulations and concentrated concentrations of plants are employed as a contrast to individual ones in traditional Indian drug procedures. The Ayurvedic herbals are set up in a variety of measuring structures in India's traditional helpful framework called Ayurveda, with the most of them including PHD (Parasuraman et al. 2014; Hill-Briggs et al. 2020).

Since PHD is a result of nature, they are modestly less expensive, environment-friendly, and promptly introduced than allopathic medications (Parasuraman et al. 2014; Hill-Briggs et al. 2020). Because of the rapid rise in DM, safe and efficient treatments are required, and one potential strategy is to minimize postprandial hyperglycemia by delaying glucose absorption by inhibiting carbohydrate-hydrolyzing enzymes and scavenging free radicals (Uddand Rao et al. 2020). There was no scientific data available about the PHD made with *Punica granatum* (fruits), *Illicium verum* (flowers) and *Nyctanthes arbor* (Leaves) and their antioxidant and anti-diabetic properties so far. Therefore, in this study we made an effort to prepare PHD with three herbs as mentioned above and evaluated their antioxidant and antidiabetic activities through *in vitro* and cell culture models.

MATERIAL AND METHODS

For the sample collection, The *P. granatum* fruits were taken from a local garden in Erode, Tamil Nadu, the *I.*

verum flowers was taken from the neighborhood garden, Tiruchengode, Namakkal, Tamil Nadu and *N. arbor* (Leaves) was procured from local garden, Salem, Tamil Nadu, India. The plants *P. granatum* (Voucher No: BSI/SRC/5/23/2020/Tech/510), *I. verum* (Voucher No: BSI/SRC/5/23/2020/Tech./509) and *N. arbor* (Voucher No: BSI/SRC/5/23/2020/TECH/511) were authenticated by the Southern Regional Centre, Botanical Survey of India, TNAU Campus, Coimbatore, Tamilnadu, India. For sample preparation, the *P. granatum* (fruits), *I. verum* (flowers), and *N. arbor* (leaves) were rinsed with cleaned water and on a fresh sheet, air-dried for seven days at room temperature. The dry plant materials were made of powder and extraction was carried out with the help of soxhlet apparatus in the presence of methanol. Then the samples were evaporated to waterlessness at decreased pressure at 40°C by rotary evaporator (Superfit, Precision Scientific, India). A PHD was made through extracts of *P. granatum*, *I. verum* and *N. arbor*. A variety of concentrations and combinations were tried and a last valuable formulation with equal volume of each one plant extracts (1:1:1 ratio) was fixed for experiments.

For better formulation stability during shelf life, several additives such as Tween-80, Sodium CMC, sweetening agent (Sodium saccharin), and flavouring agent (Lemon oil) were incorporated. Preliminary phytochemical screening was done by Kokate's techniques to test the plant individual extracts and PHD for the presence of a range of phytoconstituents (Kokate 1986). For quantitative estimation of phytochemicals, we used the individual concentrates and PHD to quantify secondary metabolites based on the elemental phytochemical examination results; for example, total phenolic content, total flavonoids, and total tannins were measured in accordance with the guidelines (Folin and Ciocalteu 1927; Singleton and Rossi 1965; Ordóñez et al. 2006).

GC-MS (Gas Chromatography-Mass Spectrophotometry) analysis, the plant individual extracts and PHD were subjected to identify bioactive compounds by GC-MS as described in the previous studies (Uddand Rao et al. 2020). Analysis of the mass spectrum the compounds were identified using GC-MS and the National Institute of Standards and Technology (NIST) database. For evaluation of antioxidant activity, plant individual extracts and PHD was determined using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, Hydrogen peroxide (H₂O₂) scavenging assay, total antioxidant activity as determined by the phosphomolybdate assay proposed in the previous studies and Thiobarbituric acid reactive substances (TBARS) assay (Ruch et al. 1989; Shimada et al. 1992; Kikuzaki and Nakatani 1993; Prieto et al. 1999). For assessment of *in vitro* antidiabetic effects, the inhibitory activities of α -amylase, α -glucosidase (Becerra-Jimenez et al. 2008) and glucose diffusion inhibition were measured as per customary protocols.

All experiments were performed in triplicate, with results expressed as a percentage of inhibition (Gallagher et al. 2003; Thalapaneni et al. 2008). For determination of antidiabetic activity by cell culture model, the HepG2 cell

culture was incubated at 37°C in a humidified environment with 5% CO₂, and growth media, consisting of RPMI 1640 media supplemented with 10% foetal calf serum, was replaced every 2-3 days. Venter et al. (2008) described a technique for determining glucose consumption in HepG2 cells (Venter et al. 2008).

The difference between the cell-free and cell-containing wells was used to compute the amount of glucose utilized. The percentage of glucose intake was calculated in contrast to the untreated controls. For Assay of metabolic enzymes inhibition potential of PHD in HepG2 cell line, the inhibition of α-amylase, α-glucosidase, lipase, dipeptidyl peptidase-4 (DPP-IV), and collagenase were measured as per respective protocols (Sancheti and Seo 2010; Lewis and Liu 2012; Odeyemi 2015; Idowu et al. 2018). The percentage of inhibitory activity was calculated by using the following formulae: % Inhibition = $(1 - A/B) \times 100$, A = Absorbance of the untreated (Control), B = Absorbance of the test well.

For protein glycation assay, the protein glycation assay was performed according to the methodology described by Idowu et al. (2018). The studies were done three times, and the percentage of inhibition was estimated using the formula below: % Inhibition = $(1 - A/B) \times 100$

A = Fluorescence of test well, B = Fluorescence of negative control. For Statistical analysis, the investigational data were presented as the average of three replicates' standard deviations (SD). Wherever relevant, the results be treated to a unidirectional analysis of variance (ANOVA) and the significant difference ($p < 0.05$, 0.01) between means was decided by the least significant difference (LSD) using Statistical Package for Social Sciences (SPSS) version 15.0 for Windows.

RESULTS AND DISCUSSION

DM is a metabolic disorder characterized by hyperglycemia and glucose intolerance, as well as abnormalities in insulin production and insulin action to increase glucose uptake. The high prevalence of morbidity and death, as well as higher health-care costs for management and treatment, make this condition a global burden. Herbal medications are made from a variety of plant sources, either in its natural state or after processing with various ingredients. Despite the fact that a variety of oral hypoglycemic medications, as well as insulin, are used to treat DM, there is a revived curiosity among patients in using natural medicines with anti-diabetic properties (Brahmanaidu et al. 2017; Vadivel et al. 2018; Unuofin and Lebelo 2020). A large number of conservative or native medications are also driving the cutting-edge prescription arrangement. In comparison to a single herb, the PHD offers a greater and broader therapeutic potential, particularly for DM (Petchi et al. 2014; Williams et al. 2020).

In this study, we attempted to make PHD from *P. granatum* (fruits), *I. verum* (flowers), and *N. arbor* (leaves) and evaluated their antioxidant and anti-diabetic properties. Preliminary phytochemical screening: The methanolic extract of PHD contained secondary metabolites such as

alkaloids, carbohydrates, phenols, flavonoids, amino acids, proteins, terpenoids, Saponins and Tannins. Quantitative estimation of phytochemicals: The quantification of secondary metabolites such as phenolics, flavonoids, and tannins in *P. granatum*, *I. verum*, *N. arbor*, and the PHD is shown in Figure 1. PHD has the highest concentration of phenolics (Fig. 1A), flavonoids (Fig. 1B), and tannins (Fig. 1C) when compared to individual plant extracts. The levels of phenolics were found to be larger than flavonoids and tannins, according to these calculations. As shown in figure 1A-C, the individual extracts of *P. granatum*, *I. verum*, and *N. arbor* extracts showed a noticeable quantity of these secondary metabolites. Since DM may pose a hazard to our populace, scientists all around the world are scouring phytochemicals for natural active agents (Pimple et al. 2011; Chan et al. 2021).

Figure 1: Quantitative estimation of secondary metabolites such as (A) phenolics, (B) flavonoids, and (C) tannins present in the *P. granatum*, *I. verum*, *N. arbor* and the PHD. Values are mean \pm SD, n=3. Values are significant at * $P < 0.05$, ** $P < 0.01$.

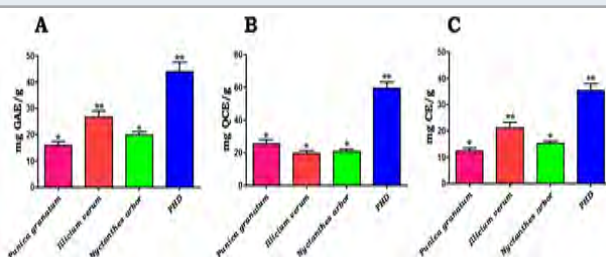
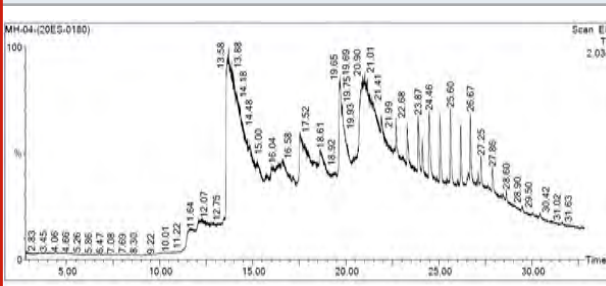


Figure 2: GC-MS chromatogram of the methanolic extract of PHD.



The antioxidant efficacies of phenolic compounds have been shown to be robust, and they play an important role in the management of chronic diseases like DM. Flavonoids are polyhydroxy polyphenolic chemicals with a wide range of applications in herbal medicine. Flavonoids, which are divided into flavanols, flavones, and flavanones, have a variety of therapeutic benefits, including anti-diabetic qualities (Roman et al. 1995; Adefegha et al. 2013). GC-MS analysis: GC-MS analysis of the PHD (Table 1, Fig 2) revealed that several compounds as exposed tables 1. Antioxidant activity: For antioxidant activity, when compared to the plant extracts individually, PHD had the DPPH radical scavenging activity is greatest at doses ranging between 100µg/mL to 1000µg/mL (Fig. 3A). Similarly, PHD demonstrated H₂O₂ decomposition activity in a dose-

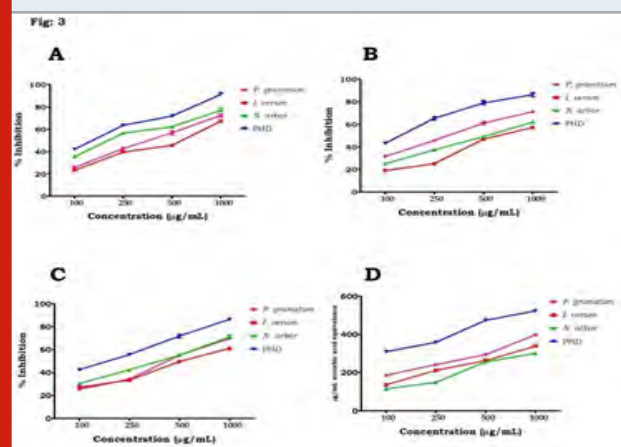
dependent manner, with the maximum activity reported at 1000 µg/mL. At the same time, Individual plant extracts were shown to have moderate H₂O₂ scavenging capabilities when compared to PHD (Fig. 3B). Figure 3C shows the % inhibition of *P. granatum*, *I. verum*, *N. arbour*, and PHD at various doses using the TBARS test. When compared

to separate plant extracts, our results clearly demonstrated that the PHD might possibly prevent lipid peroxidation in a dose-dependent manner. Phosphomolybdate test was used to investigate the total antioxidant capabilities of *P. granatum*, *I. verum*, *N. arbour*, and PHD at various doses, and the findings revealed dose-dependent antioxidant activity (Fig. 3D) (Zhang et al. 2020).

Table 1. Compounds identified in the methanolic extract of PHD with GC-MS

RT	Name of the Compound	Molecular Formula	Molecular Weight	Peak Area %
11.662	1,2-Cyclohexanedione	C ₆ H ₈ O ₂	112	1.197
12.252	Cyclohexanone, 2-Methyl-	C ₇ H ₁₂ O	112	0.977
12.307	Cyclohexanone, 2-Methyl-	C ₇ H ₁₂ O	112	1.051
12.882	5-Trans-Methyl-1r,3-Cis-Cyclohexanediol	C ₇ H ₁₄ O ₂	130	0.844
13.713	Benzene, 1-Methoxy-4-(1-Propenyl)-	C ₁₀ H ₁₂ O	148	27.781
16.619	Benzene, (1-Methoxyethyl)-	C ₉ H ₁₂ O	136	12.014
17.534	Phenol, 4-(2-Propenyl)-, Acetate	C ₁₁ H ₁₂ O ₂	176	8.983
18.615	2-Cyclopenten-1-One, 2-(2-Butenyl)-4-Hydroxy-3-Methyl-, (Z)-	C ₁₀ H ₁₄ O ₂	166	4.328
19.690	N-Hexadecanoic Acid	C ₁₆ H ₃₂ O	256	8.856
21.011	Dodecanoic Acid, 9-Decen-1-Yl Ester	C ₂₂ H ₄₂ O ₂	338	17.146
21.901	3-(5-Benzoyloxy-3-Methylpent-3-Enyl)-2,2-Dimethyloxirane	C ₁₇ H ₂₄ O ₂	260	17.146
22.681	Oxalic Acid, Isohexyl Nonyl Ester	C ₁₇ H ₃₂ O ₄	300	3.813
23.291	Octane, 3,4,5,6-Tetramethyl-	C ₁₂ H ₂₆	170	2.387
23.867	Octadecane, 3-Ethyl-5-(2-Ethylbutyl)-	C ₂₆ H ₅₄	366	1.183
24.097	Cyclopropyl 2-(5'-Methyl-2'-Furyl) Cyclopropyl Ketone	C ₁₂ H ₁₄ O ₂	190	0.974
24.462	Hexatriacontane	C ₃₆ H ₇₄	506	1.595
25.042	Heptacosane	C ₂₇ H ₅₆	380	0.883
25.602	Ethanol, 2-(Octadecyloxy)-	C ₂₀ H ₄₂ O ₂	314	0.836

Figure 3: (A) DPPH radical scavenging activity, (B) H₂O₂ scavenging activities, (C) TBARS and (D) total antioxidant capacities of *P. granatum*, *I. verum*, *N. arbor* and PHD at different concentrations, Values are mean ± SD, n=3.



When compared to individual plant extracts, the PHD demonstrated much higher activity. Free radicals, such as reactive oxygen species (ROS), have been linked to DM, obesity, inflammation, cardiovascular disease, atherosclerosis, ageing, and cancer (Meriga et al. 2017; Brahmanaidu et al. 2017). It's critical to strike stability between the pace of free radical creation and exclusion. Excessive cell radical formation is typically harmful; in any event, oxidative cellular stress occurs when there is a significant increase in excessively radical formation or a reduction in radical clearance from the cell (Valko et al. 2007; Li et al. 2019).

According to research and scientific data, oxidative stress is linked to the onset and development of DM (Rösen et al. 2001). Using DPPH radical and H₂O₂ scavenging action, TBARS test, and total antioxidant activity, we evaluated the antioxidant properties of PHD and individual plant extracts in this study. DPPH is a stable, nitrogen-focused free radical that is transformed to diphenylpicryl hydrazine

after absorbing hydrogen from the polyphenolic extract's antioxidants (Vladimir et al. 2011). The drop in DPPH seen by the PHD was caused by either a hydrogen atom exchange or an electron exchange. Additionally, phenolic compounds and flavonoids are abundant hydrogen benefactors, making them excellent antioxidants (Michalak 2006; Yaribeygi et al. 2020).

H₂O₂ is a mild oxidising agent that may inactivate a few enzymes directly, often by oxidising crucial thiol groups. H₂O₂ may quickly permeate cell membranes, and once inside, it interacts with Fe²⁺ and perhaps Cu²⁺ ions to produce hydroxyl radicals, which are harmful to cells. Because of their hydrogen-giving and scavenging capacities, secondary metabolic products may act as free radical scavengers (Beyhan et al. 2010). PHD had a substantially greater overall antioxidant capacity than individual plant extracts, according to our research. The total phenolic content and antioxidative activity have been linked in several studies. Natural antioxidants' efficiency is determined in part by the chemical composition and chemical structures of the extract's active components (Hossain et al. 2015; Jiao et al. 2019).

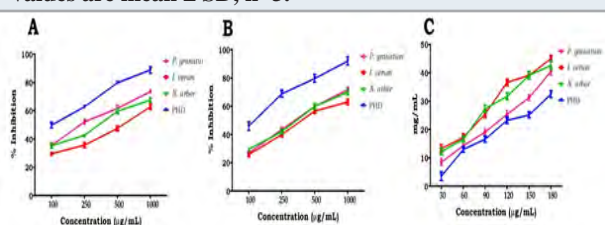
We observed high quantities of total phenolic content, flavonoids, tannins, and bioactive components in PHD using GC-MS throughout this investigation. PHD's high antioxidant action might be attributed to these components. *In vitro* antidiabetic activity of PHD: the current research found a dose-dependent increase in % inhibitory efficacy against the α -amylase (Fig. 4A) and α -glucosidase enzymes (Fig. 4B). The PHD demonstrated considerable inhibitory action ($p < 0.05$) against these enzymes, with the maximum dosage of 1000g/mL having the maximum inhibitory action. The individual extracts of *P. granatum*, *I. verum* and *N. arbor* did not demonstrate substantial inhibitory activity against α -glucosidase and α -amylase as compared to PHD.

The current study used a variety of biochemical and cell-based tests to determine the probable mechanism(s) of *P. granatum*, *I. verum*, *N. arbour*, and PHD extracts antidiabetic effects. Glucose absorption inhibition activity: When compared to metformin, the results of this study on glucose uptake in HepG2 cells indicated that PHD increased glucose uptake in HepG2 cells. This suggests that the PHD works similarly to metformin in that it increases glucose absorption in the liver. Metformin can have a hypoglycemic effect by activating the AMP activated protein kinase in the liver, which can lead to a variety of pharmacologic effects, such as glucose inhibition, lipid synthesis inhibition, and enhanced hepatic insulin sensitivity (Viollet et al. 2012). Phytochemicals such as phenols, terpenoids, flavonoids, and flavanols have been shown to limit glucose release from the liver while also increasing glucose absorption in the hepatic cells, therefore altering the intracellular signalling pathway (Hanhineva et al. 2010; Peng et al. 2019).

Effect of PHD on glucose utilization in HepG2: when compared to the control, PHD had the greatest potential for preventing the flow of glucose molecules over the dialysis membrane (Fig. 4C). In comparison to PHD, individual

plant extracts were less efficient at stopping glucose molecules from diffusing. Figure 5A shows the findings for glucose uptake in HepG2 cells in the presence of the plant extract at concentrations of 25, 50 and 100 μ g/mL. When compared to the untreated control, the PHD generated a significant ($p < 0.05$) greater increase in glucose absorption in HepG2 cells at all concentrations in a concentration-dependent manner, tests were conducted. Phytochemicals have beneficial effects through a variety of pathways, including glucose and lipid metabolism modulation, insulin secretion, cell stimulation, the NF- κ B signalling pathway, inhibition of gluconeogenic enzymes, and ROS protection. Insulin deficiency has an impact on glucose, protein, and fat metabolism, as well as water and electrolyte balance (Frier et al. 2006; Saeedi et al. 2020).

Figure 4: *In vitro* anti-diabetic activities of individual plant extracts and PHD, (A) α -amylase inhibitory activity, (B) α -glucosidase inhibitory activity and (C) glucose diffusion, Values are mean \pm SD, n=3.



A high postprandial blood glucose response is connected to micro- and macrovascular issues in people with DM, and is more strongly linked to cardiovascular disease risk than fasting blood glucose. α -glucosidase enzymes in the intestinal lumen and the brush border membrane convert starch and oligosaccharides to monosaccharides before absorption, which is essential for carbohydrate digestion. Inhibiting the activity of these digestive enzymes was thought to cause a delay in the decomposition of starch and oligosaccharides, leading to a decrease in glucose absorption and, as a result, a decrease in postprandial blood glucose levels rising (Puls et al. 1977; Lachin et al. 2021).

Effect of PHD on α -amylase in HepG2: when compared to various plant extracts, the results showed that PHD had the most significant impact on α -amylase at all doses tested (Fig. 5B). Both PHD and individual extracts had a considerable impact on α -amylase at the maximum concentration (100 μ g/ml) tested. α -glucosidase inhibition potential of PHD in HepG2: figure 5C depicts that PHD demonstrated that a highest significant effect on α -glucosidase at all the tested concentrations and highest at 100 μ g/ml than the individual plant extracts tested. However, as positive controls, EGCG was somewhat more efficacious than the extract and untreated control in the respective experiments. Lipase inhibition assay in HepG2: PHD exhibited significant inhibition against pancreatic lipase in a concentration dependent manner (Fig. 6A). On the other hand, individual plant extracts demonstrated less lipase inhibition activity when compared to PHD and orlistat. This shows that PHD (100 μ g/ml) may have anti-diabetic properties through processes involving lipase inhibition (Lachin et al. 2021).

Dipeptidyl peptidase-4 inhibition assay in HepG2: the PHD exhibited highest significant inhibition against Dipeptidyl peptidase-4 at all the tested concentrations as similar like standard diprotin A (Fig. 6B). When compared to the untreated control and individual plant extracts, there was a high significant inhibition of the PHD. Collagenase inhibition activity of PHD in HepG2: In this study, the extracts of *P. granatum*, *I. verum*, *N. arbor* exhibited moderate inhibition activity of collagenase when compared to PHD (Fig. 6C). On the other hand, PHD demonstrated that the highest activity at 100 µg/ml concentration similar like EDTA.

Figure 5: Effect of PHD and individual extracts on (A) glucose utilization, (B) α -amylase inhibition and (C) α -glucosidase inhibition in HepG2 cell line. Values are mean \pm SD, n=3. Values are significant at *P<0.05, **P<0.01.

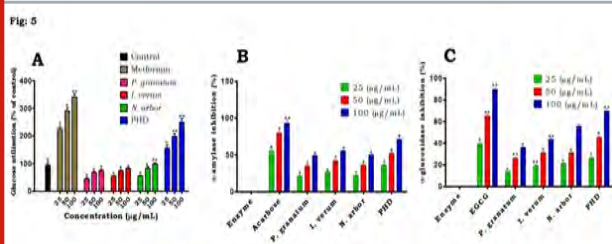
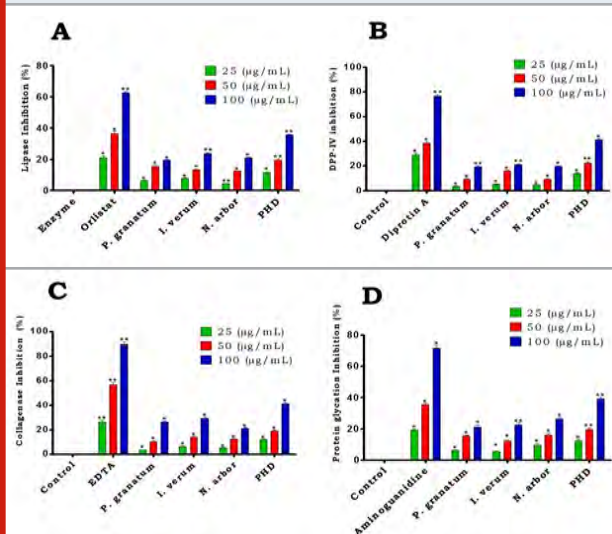


Figure 6: Effect of PHD and individual extracts on (A) Lipase inhibition, (B) DPP-IV inhibition, (C) Collagenase inhibition and (D) Protein glycation in HepG2 cell line. Values are mean \pm SD, n=3. Values are significant at *P<0.05, **P<0.01.



Protein glycation assay in HepG2: the findings demonstrated that the PHD extract inhibited protein glycation in a dose-dependent manner at all concentrations tested (Fig. 6D). At the same time, individual plant extracts demonstrated only moderate activity when compared to PHD and aminoguanidine. The inhibition of lipase and collagenase enzymes by secondary plant chemicals has the potential to manage postprandial hyperglycemia and hyperlipidemia, while inhibiting enzyme catalysis will

reduce the number of monosaccharides and fatty acids accessible for absorption (Villa-Rodriguez et al. 2018). Pancreatic lipase is accountable for the hydrolysis of dietary lipids and its inhibition outcome in reduced fat absorption, contributing to DM (Conforti et al. 2012). Anti-diabetic medicines are used to treat or manage DM, and their mechanisms of action are known. Lipase and DPP-IV enzyme inhibition are among them (Saeedi et al. 2020).

When compared to the individual positive controls, our results showed that the extract inhibited lipase and DPP-IV significantly. This suggests that the anti-diabetic mechanism of PHD is as a result of through the inhibition of these enzymes. On the other hand, protein glycation has been established in studies to be one of the outcomes of abnormally high blood glucose in DM individuals (Ulrich and Cerami 2001; McKay et al. 2019). Protein glycation is a reversible process in which a reducing sugar and a free amino group of a protein form adducts that yield glycation products over time. These responses play a crucial role in the aetiology of DM complications. When compared to relevant standards, at the concentrations investigated, the PHD showed significant suppression of collagenase and protein glycation. However, this is the first study to look at both the PHD's protein glycation and anticollagenase properties (Kiho et al. 2000; Eckel et al. 2021).

CONCLUSION

The findings of the present study demonstrated that the PHD has contained abundant phytoconstituents and showed a significant free radical scavenging activity and glucose lowering effects *in vitro*. On the other hand, PHD exhibited noteworthy inhibition of α -amylase, α -glucosidase, lipase, DPP-IV, collagenase and protein glycation in HepG2 cell line. Hence the results obtained from this recommended that PHD may be considered for the treatment of postprandial glycemia in people with type 2 diabetes mellitus. However, further preclinical studies are crucial in advanced animal models to validate its antioxidant and anti-diabetic activities.

ACKNOWLEDGEMENTS

P. Chandrasekaran and V. V. Sathibabu Uddand Rao are equally contributed to this study. Authors thank the management of K.S. Rangasamy College of Arts and Science (Autonomous), Tiruchengode, Namakkal District, Tamilnadu, India for providing necessary facilities to carry out this study.

Conflict of Interests: Authors declare no conflicts of interests to disclose.

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Biomedical Communication

Prevalence of Violation Among Nurses Working in Public Health Facilities in Saudi Arabia

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King Saud University, Riyadh, Saudi Arabia.**ABSTRACT**

Workplace violence (WPV) is more likely to affect those who work in community services, notably nurses. WPV is generally recognized as a workplace health hazard and has been a significant concern in a number of countries. Limited studies have been conducted in the global countries and this study was designed as a cross-sectional study from Saudi Arabia to document the WPV among the nurses. The aim of this study was to enroll the consequences obtain during the WPV among the nurses in Saudi Arabia. In this cross-sectional study, we have enrolled 550 nurses based on the signed of informed consent form and filled questionnaire. The study results indicated that 77.6% of nurses were violated at work. The 90.4% of the majority violations were classified as verbal abuse. The relatives of the patients violated 66.7% of the nurses, and the occurrence occurred during the evening shifts of the working hours between 2.30-10.30pm. The most violations occurred in the patient room, accounting for 46.8% of all violations, with the severe workload accounting for 89.2% of all violations. However, among the violations, 21.4% of the nurses were reported to senior management. In conclusion, this study confirms the nurses who have participated in this were mentally strong in handling the WPV during their job. Further studies are required for looking at the factors that increase the chance of a repeat event and those that decrease it, as well as development of an intervention program for the emotional load that accompanies WPV. To prevent workplace violence in healthcare settings, it is equally vital to implement policies that supplement the existing call to action.

KEY WORDS: NURSES, HEALTHCARE WORKERS, WORKPLACE VIOLATION, ABUSE.**INTRODUCTION**

One of the top worldwide health issues that has recently gotten more attention is the mistreatment of nurses in the workplace. Workplace violence is defined by the World Health Organization (WHO) as any occurrence where workers are abused, intimidated, or assaulted due to their work-related activities, such as commuting to and from work. Many national organizations and other relevant organizations, including the National Council on Workplace Violence (WPV), consider both physical and psychological trauma, including attacks, verbal abuse, bullying, and both sexual and racial harassment, to be workplace violence (Stephens, 2019). WPV is defined as a pattern of episodes in which employees are abused, threatened, or assaulted at work, with explicit or implicit threats to their safety, well-being, or health (Anderson and Stamper, 2001). For certain registered nurses, over 25% of incidents of patient

or family member physical violence result in injuries, but over 50% result in exposure to verbal abuse or bullying (Al-Qadi, 2021). Other professionals who work in mental and emergency facilities can also be among the casualties (Bordignon and Monteiro, 2021).

This description encompasses all instances, from small assaults to intentional murder (Hegney et al., 2003). The vast majority of WPV research have been dedicated to healthcare workers and nurses, whereas nursing students worldwide have received a lack of attention. The vast majority of academic time in nursing programs is spent in clinical settings. Younger age, less clinical abilities, and less communication skills put them at an elevated risk of WPV. Students at risk of violence may be victims in both clinical and academic environments (Normohammadi et al., 2021).

The International Council of Nurses (ICN) acknowledged WPV as a significant issue in nursing and asked that priority be given to concerns of clinical competence and WPV treatment. The frequency of WPV in the Saudi population was estimated to be between 50-86% (Alkorashy and Al

Article Information:*Corresponding Author: malmomani@ksu.edu.sa

Received 25/10/2021 Accepted after revision 17/12/2021

Published: 31st December 2021 Pp- 1636-1641

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.40>

Moalad, 2016). The WPV varies among countries, with the UK documenting 19.6%, while China and Korea confirm 77.1% and 78.6%, respectively (Liao et al., 2010; Park et al., 2017; Tee et al., 2016). Physical abuse against health care professionals has been connected to job exhaustion, customer demands, and decreasing patient-staff interactions (Phillips, 2016). About 80% of all significant violence in healthcare settings stems from encounters between nurses and patients, according to Occupational Safety and Health Administration (Wyatt et al., 2016).

When it came to psychological violence and bullying, Asia had the highest prevalence, with the US following just after. Meanwhile, Africa had the lowest percentage of non-physical violence, Europe had the second lowest percentage of physical violence, bullying, and sexual harassment, and Asia was placed worst for all three types of violence. Although it revealed that US nurses had the highest overall exposure to physical WPV, Asian nurses had the least exposure to that specific type of WPV. In Europe and Africa, the nurses were exposed to low levels of physical violence and high levels of nonphysical violence. Men reported the most experiences of physical WPV, accounting for the lower reported rate of physical WPV (Mobaraki et al., 2020).

A recent systematic review study carried out in the Saudi Arabia indicated that pediatric employees who work in a healthcare setting may face an increased risk of WPV. Parents or caregivers of the attending children can exhibit a range of forms of aggression against pediatric doctors and nurses due to the stress the child's condition can produce. Hospital personnel must be given explicit instructions on how to handle violent occurrences and how to report them (Albalwei et al., 2021). According to El-Hneiti et al studies, about 60% of participants reported being victims of violence or facing threats of violence in the workplace (El-Hneiti et al., 2020). There is a lack of accurate data on abuse in Saudi Arabia, thus we decided to explore this phenomenon in Saudi Arabia and its culture. In order to determine the prevalence of WPV among nurses working in public health facilities, this study will examine the relevant factors. The nurses' workplace also has mechanisms in place to cope with workplace violence.

MATERIAL AND METHODS

Settings: During the period of 5 September 2020 to 10 January 2021, this descriptive cross-sectional study was undertaken in two public hospitals in Saudi Arabia. As part of the study (n=550), all full-time licensed nurses with at least one prior hospital experience were included. A random sample of 275 nurses from each hospital was taken.

Study Instrument: The questionnaires (English version) developed by the International Labour Office (ILO), ICN, WHO, and Public Services International (PSI) in 2003 (ICN, 2003) served as the basis for the study instrument. The questionnaire was written in English at first, then translated to Arabic to suit local language requirements, and finally back to English to verify conceptual consistency. Non-Arabic speakers could fill out surveys in English. To minimize confusion, the definition of workplace violence

was given with both questionnaire versions (English and Arabic).

The questionnaire was divided into five portions, each with 39 questions. The first segment (7 questions) included questions about the participants' sociodemographic data, the second section (7 questions) included questions about workplace characteristics, and the third section covered physical workplace violence encountered by nurses in the previous 12 months (14 questions). This part evaluated the instance, location, time, and perpetrator type. Participants were asked how frequently they had encountered physical violence, how they responded to the occurrence, and how they dealt with the abuse. The fourth section (9 questions) had similar questions to the third section. The final portion includes two questions about the reasons for the instances of violence that happened with nurses, as well as the steps and policies that the employer implemented to deal with workplace violence.

Five experts evaluated the instrument's content validity. They were asked to review and rate the instrument based on its clarity, relevance, comprehensiveness, translation correctness, and cultural sensitivity. Following the suggestions of the experts, certain improvements were suggested. After that, the redesigned questionnaire was pilot tested with 25 participants, who were then eliminated from the study population. As a result, several changes were made. Cronbach alpha values ranged from 0.71-0.87 for all subscales.

IRB and consent: Ethical approval was obtained for this study from our institutional premises. The 550 participants involved in this study has signed the informed consent to participate in this study. Participation in the study was entirely optional, and individuals who were unwilling or desired to withdraw at any time were advised that they could do so without limitation. The hospital's central nursing department delivered a self-administered questionnaire along with a cover letter to 550 nurses. The nurses were given a two-week time frame to respond. Confidentiality was maintained throughout this study

Statistical analysis: The information gathered data was uploaded and analyzed using the Statistical Package for Social Sciences (SPSS Inc., software version 21 Chicago, IL, USA). Demographic and occupational violence factors were described in descriptive statistics. The Chi-square test was used to examine the relationship between socio-demographic data and kinds of violence. The logistic regression was used to predict the relationship between the participants' demographic data among the violation of nurses. The 95% confidence interval (CI) was used to examine the crude odds ratio. A P value of 0.05 was deemed statistically significant.

RESULTS AND DISCUSSION

This is a cross-sectional study conducted at Public Hospitals following the COVID-19 pandemic, and 550 nurses took part in it after signing an informed consent form. Table 1 lists the clinical characteristics of nurses. The nurses' ages

were classified into three groups, with 36.3% being under 30 years old, 48.1% between 31-45 years old, and 15.6% being above 45 years old. Male and female participation rates were discovered to be 19.6% and 80.4%, respectively. Almost 65% of nurses were married, while 31.6% were single. The proportion of nurses with a bachelor's degree was determined to be 72.1%, while 18.6% of subjects were documented with a diploma and 9.3% were post-graduated. Only 20.5% of the nurses had more than a decade of experience, 47.9% of the nurses had experience between the ages of 6-10, and 31.6% of the nurses had experience less than five years.

Table 1. Characteristics of participants (n=526)

Variables	Frequency	Percentage
The age		
≤ 30	191	36.3
31-45	253	48.1
>45	82	15.6
Gender		
Male	103	19.6
Female	423	80.4
Marital status		
Single	166	31.6
Married	344	65.4
Others	16	3.0
Educational status		
Diploma 2 years	98	18.6
Bachelor (BSC)	379	72.1
Graduate studies	49	9.3
Years of experience		
≤ 5	166	31.6
6-10	252	47.9
>10	108	20.5
Shift work		
Day	130	24.7
Evening	9	1.7
Night	12	2.3
Rotation	375	71.3
Clinical setting		
Medical/surgical	181	34.4
Emergency	68	12.9
Intensive care	69	13.1
Pediatric	56	10.6
Obstetric and Gynecology	51	9.7
Orthopedic	48	9.1
ENT/ Ophthalmology	18	3.4
Operating room/Recovery	22	4.2
Dialysis	13	2.6

The rotation shifting nurses accounted for 71.3% of the total, while the day shifting nurses accounted for 24.7%. The evening and night shift nurses were found to be 1.7% and 2.3%, respectively. One-third (34.4%) of the nurses worked in the medical and surgical departments, while 12.9%,

13.1%, and 10.6% worked in the emergency, intensive care, and pediatric departments, respectively. The percentages of nurses working in obstetrics/gynecology, orthopedics, ENT, operating rooms, and dialysis were 9.7%, 9.1%, 3.4%, 4.2%, and 2.6%, respectively.

Table 2. Violations among nurses during their WPV

Variable	Frequency	Percentage
Exposure to violent incidents in the last 12 months		
Yes	408	77.6
No	118	22.4
Types of violence		
Physical violence	39	9.6
Verbal Abuse	369	90.4
Both (physical and verbal)	51	12.7
Identity of perpetrator		
Patient	69	16.9
Relatives of patient	272	66.7
Staff nurse	37	9.1
Manager or supervisor	17	4.2
External colleague	9	2.2
Unknown	4	0.9
Time of violent incidence		
Morning shift (7 am - 2.30 pm)	151	36.9
Evening shift (2.30pm – 10.30pm)	186	45.6
Night shift (10.30pm-7am)	71	17.5
Violence place		
Patient room	191	46.8
Waiting room	70	17.2
Treatment room	26	6.4
Nursing station/reception	79	19.4
Corridor	42	10.2
Reasons of violence		
Intense workload	364	89.2
High level of stress related to patient illness	210	51.5
Pain	132	32.4
High patient expectation	173	42.4
Long waiting time	77	18.9
Lack of communication	22	5.4
Inadequate security		
Initial reaction to the incident		
Took no action	127	31.2
Tried to pretend it never happened	39	9.5
Told the person to stop	50	12.2
Told friends and family	35	8.5
Told a colleague	70	17.2
Reported to senior staff	87	21.4

Table 2 shows the WPV of the individuals in the study. In this research, 77.6% of nurses reported being violated on the job in the previous 12 months. The nurses have suffered the most verbal abuse (90.4%) and the least physical violence

(9.6%). However, 12.7% of nurses have had to deal with both of them. The highest percentage of violations was collected from a patient's relative (66.7%), followed by a patient (16.9%), a staff nurse (9.1%), a manager (4.2%), a colleague (2.2%), and 0.9% was unknown. The biggest violation was discovered to be 45.6% in the afternoon, followed by 36.9% in the morning, and the lowest violation was discovered to be 17.5% during the night shift. The patient room has seen the most violence, with 46.8%, followed by reception (19.4%), waiting room (17.2%), corridor (10.2%), and treatment room (6.4%).

Table 3. Details of nurses and their role in complaining about their violation during WPV

Reporting incident		
Yes	87	21.4
No	321	78.4
Reasons for not reporting the incident		
It was not important	102	31.7
Felt a shamed	42	13.1
Felt guilty	14	4.3
Afraid of negative consequences	40	12.4
Useless	123	38.5
Nurse's satisfaction with the manner In which the incident was handled		
Very dissatisfied	35	8.6
Dissatisfied	128	31.3
Moderately satisfy	224	54.8
Very satisfy	21	5.3
Opinion of nurses with the measures used to deal with workplace violence exist in their workplace (n=408)		
Security measures (e.g. guards, alarms, portable telephones, cameras)	221	54.2
Improve surroundings (e.g. lighting, noise, heat, access to food, cleanliness, privacy)	88	21.5
Restrict public access	202	49.6
Waiting room should be comfortable	128	31.4
Increased staff numbers	239	58.7
Changed shifts or rotas (i.e. working times)	79	19.4
Reduced periods of working alone	72	17.6
Training (e.g. workplace violence, coping strategies, communication skills, conflict resolution, self-defense)	162	39.7

The high degree of patient stress during treatment was discovered to be one of the main reasons for the violation, with 51.5% and 89.2% for the intensive workload. The patient expectation was documented to be 32.4%, the long waiting time was 42.4%, the lack of communication was 18.9%, and the inadequate security was 5.4%. In this study, 31.2% were found to be for no action, 9.5% for pretending not to happen, 12.2% for stopping the person, 8.5% for informing family members, 17.2% for informing a coworker, and 21.4% reported to senior staff.

Table 3 defines the nurses who report the occurrence of workplace violations. In this study, 21.4% of nurses reported the occurrence, while 78.6% did not disclose the occurrence of the violation. The majority of nurses, 38.5%, believed there was no use in complaining about a file, while 31.7% believed it was unimportant. 13.1% and 12.4% of nurses were embarrassed and worried of unfavorable consequences, respectively. Only 4.3% of the nurses felt guilty. The method in which the incident was handled was extremely satisfactory to 5.3% of the nurses. Nonetheless, 54.8% of nurses were moderately happy. Both 31.3% and 8.6% of the nurses were dissatisfied or very dissatisfied with their jobs.

Table 4. Multivariate adjusted odds ratios for exposure to violence among participants

Characteristic	Adjusted		P value
	OR	95%CI	
Gender			
Male	1.61	0.33-119	0.183
Female	1.0	Reference	
Age			
<30	3.41	2.91-16.46	0.001
≥30	1.0	Reference	
Marital status			
Single	1.39	0.79-3.85	0.176
Married	1.0	Reference	
Education			
<Bachelor's degree	4.21	4.21	0.010
≥ Bachelor's degree	1.0	1.0	
Years of experience			
< 10	7.63	7.63	0.001
≥ 10	1.0	1.0	
Shift work			
Fixed	0.68	0.68	0.582
Rotation	1.0	1.0	

Table 4 describes the multivariate logistic regression analysis among the baseline characteristics such as gender, age, marital status, education, work experience, and working shifts, and the study results confirmed that age ($p=0.001$), education ($p=0.01$), and work experience ($p=0.001$) were statistically associated. The aim of this study was to enroll WPV among the working nurses in the Saudi Arabia and the current study results indicated that 77.6% of nurses were violated at work. The majority of the violations (90.4%) were classified as verbal abuse. The relatives of the patients violated 66.7% of the nurses, and the occurrence occurred during the evening shifts of the working hours between 2.30-10.30pm. The most violations occurred in the patient room, accounting for 46.8% of all violations, with the severe workload accounting for 89.2% of all violations. However, among the violations, 21.4% of the nurses were reported to senior management. Nurses are exposed to more impolite behavior and more physical or emotional abuse under stressful situations, such as the death of a patient, the transfer of patients to another hospital, or

as they wait for a doctor or nurse to arrive at the scene of an accident or trauma (Shoghi et al., 2008).

Worldwide, the prevalence of WPV were documented and majority of the nurses were very well known with their past experiences. In our study, nurses were subjected to various forms of physical aggression while at work. During the job, nurses are exposed to violence and that exposure has an impact on them, making them more likely to get exhausted, to experience sleeping disorders, to be stressed, to have spasms, to lose self-confidence, to be disappointed, to want to drink, to smoke, and to attempt suicide. These difficulties experienced in the workplace may lead to absence from shifts, frequent absenteeism, inattention to patients, and a decrease in job satisfaction. This may result in resignation or even death. A study found that workplace violence against nurses may decrease the quality of care provided, cause greater levels of staff errors, compromise ethics, and end up costing treatment centers and the community substantially more, (Kobayashi et al., 2020).

Nursing workplace violence is likely a serious problem that has yet to be fully investigated and reported on (Jackson et al., 2002; Jones and Lyneham, 2001; Kaye, 1996). on the other hand, it has been observed that the incidence is rising (Taylor, 2000). O'Connell et al state that patients are the most frequent workplace violence perpetrators, with colleagues and managers in nursing next in line. Then, families follow (O'Connell et al., 2000). Patients were the most frequent source of violence in a previous research on mental health nurses and violence. Furthermore, in the study they observed that the risk of violent encounters with healthcare workers other than physicians or allied health professionals is greater than the risk of such encounters with medical practitioners or allied health workers (Nolan et al., 1999).

Concerning workplace violence and gender, there is contradicting information. For instance, in Queensland, it has been found that women are more likely to be harassed at work than men (Queensland Government, 2002). Rippon disagreed, saying that male nurses are more likely to face workplace violence than female nurses (Rippon, 2000). One study indicated that patient satisfaction is significantly greater in hospitals where there are fewer nurses that are disgruntled or burned out (McHugh et al., 2011).

The rate of WPV against nurses was shown to range from 10-50% (Hegney et al., 2003; Wells and Bowers, 2002), one study finding the higher incidence rate as 87% (Uzun, 2003). Our study identified a large proportion of workplace violence, particularly verbal aggressiveness, which has been documented in prior studies, (Koohestani et al., 2011; Magnavita and Heponiemi, 2011; Samadzadeh and Aghamohammadi, 2018; Tee et al., 2016, Budden et al., 2017; Cheung et al., 2019, Normohammadi et al., 2021). A couple of systemic reviews have validated and documented the WPV among nurses with a higher incidence (Konttila et al., 2018; Spector et al., 2014). One of the limitations of our study was we haven't mentioned the details of foreign countries and we have enrolled only 550 participants during the pandemic crisis. The study design was cross-sectional

and does not deliberate causality. The strength of our study was we have covered all types of violation and nurses came out with the WPV in our study. We haven't incorporated the details of the patients and their relatives who have abuses the nurses to avoid the conflicts.

CONCLUSION

This study has confirmed that the nurses who have participated in this were mentally strong in handling the WPV during their job. It is necessary to conduct further study, which may include studies looking at the factors that increase the chance of a repeat event and those that decrease it, as well as development of an intervention program for the emotional load that accompanies WPV. To prevent workplace violence in healthcare settings, it is equally vital to implement policies that supplement the existing call to action. To help prevent workplace violence, this study recommends to consider implementing anti-bullying programs in the nursing curriculum, hospital security, and legal policies, as well as designing violence reporting and surveillance systems.

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Biotechnological Communication

Biological Features and Molecular Genetic Structure of *Habrobracon hebetor* Populations

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ABSTRACT

One of the promising entomophages capable of controlling the abundance of the codling moth is *Habrobracon hebetor* Say. Natural populations of the gabrobragon can reduce the number of caterpillars of the corn moth to 22%, the garden moth to 35%, the cotton moth to 45%, and the gamma moth to 30%. This work aims to assess the parasitic activity of the gabrobragon as a regulator of the codling moth abundance in various geographic populations, to select a host insect for its mass breeding in laboratory conditions, and to assess the molecular genetic variability of the structure of *H. hebetor* populations. The capture of natural populations of the gabrobragon *H. hebetor* was carried out in apple orchards in Krasnodar Krai and Stavropol Krai of Russia using cassettes in which caterpillars of the codling moth were placed. As a result of the research, the natural starting population of the gabrobragon *H. hebetor* was captured, and a method for their maintenance and breeding was developed. The most effective host insect is the wax moth (*Galleria mellonella* L.), which resulted in 195 adults, compared to 98 of the mill moth (*Ephestia kuhniella* Zell.). The gabrobragon population introduced into the apple tree cenosis continued its reproduction in natural conditions and largely suppressed the number and harmfulness of the codling moth. The RAPD analysis of the Krasnodar and Stavropol populations of *Habrobracon hebetor* Say revealed a high level of DNA polymorphism and genetic diversity in the studied geographic populations of the gabrobragon. At the same time, intrapopulation variability was 87.1%, while interpopulation variability accounted for 12.9% of the total indicator. The limited gene flow ($N_m = 3.298$) results in relatively low identity ($GI = 0.906$) between populations and significant interpopulation variability. This indicates that the analyzed insect samples probably represent different geographic populations of the *H. hebetor* ectoparasite.

KEY WORDS: APPLE TREE, CODLING MOTH, DNA POLYMORPHISM, GABROBRAGON HABROBRACON HEBETOR SAY, RAPD ANALYSIS.

INTRODUCTION

Habrobracon hebetor Say is a hymenoptera parasite of many types of Lepidoptera pests in India, Pakistan, South Africa, Egypt, Canada, Western Europe, Central Asia, southern regions of Russia, Crimea, Transcaucasia, the Caucasus, Ukraine and Moldova (Dulgerova 1994; Agasyeva et al. 2019; Piekarska-Boniecka et al. 2019). It affects caterpillars of cotton moth, corn stalk moth, apple, plum, oriental moth, mill, acacia, boxwood moth and other (over 60 species) lepidoptera pests that actively colonize vegetable, fodder, fruit crops, cotton, soybeans and corn (Amarasekara et al. 2016; Chouinard et al. 2019). *H. hebetor* is a small insect

from the order Hymenoptera of the Braconidae family. A female finds a caterpillar, paralyzes it, and then lays her eggs on the caterpillar's body. The number of eggs laid per caterpillar can reach 45, while the gabrobragon larva, while developing, feeds on the hemolymph of the caterpillar, of which only the outer covers remain (Kovalenkov et al. 1995; Chouinard et al. 2019).

The duration of development of one generation of the gabrobragon is 9–14 days. Natural bragon is present in all stations; during the growing season it migrates from one to another (Jumaev et al. 2017; Chouinard et al. 2019). Natural populations of the gabrobragon are able to reduce the number of caterpillars of the corn moth to 22%, garden moth - up to 35%, cotton moth - up to 45%, and gamma moths - up to 30% (Kovalenkov et al. 1995). For many years, the entomophage has been the object of mass breeding and use against a number of harmful lepidoptera species. The

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Received 25/10/2021 Accepted after revision 22/12/2021

Published: 31st December 2021 Pp- 1642-1647

This is an open access article under Creative Commons License,

Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.41>

biological efficacy of a gabrobragon propagated in artificial conditions at low release rates (1-3 thousand individuals / ha) against a corn moth, cotton moth, acacia moth reaches 70-90% (Kovalenkov et al. 1995; Agasyeva et al. 2019).

It is used in the open field for biological protection of tomato, corn, soybeans, cotton, sunflower, apple against cotton moth (*Helicoverpa armigera* Hb.), corn stalk moth (*Ostrinia nubilalis* Hbn.), acacia moth (*Cyella zinckenella* Fr.), codling moth (*Cydia pomonella* L.) Average daily temperature in the range of 25-30°C, the lifespan of the imago of at least 15 days, and the relative humidity of 70-80% are optimal for the parasitic activity of the gabrobragon. However, a number of researchers noted that the trophic relationships of *H. hebetor* vary significantly, both in laboratory and in the field (Frolov 2014; Amarasekarea et al. 2016; Piekarska-Boniecka et al. 2019). Thus, it is artificially divided into pyralid, moth, leaf-roller and other races. In all likelihood, as a result of microevolutionary processes, many biological parameters of this species have changed, such as food specialization, stationary distribution, migration abilities, and morphogenetic structure of populations, which may complicate its application in biological plant protection programs. In this regard, the study of the biological characteristics of *H. hebetor* will make it possible to identify the reasons for the variability of the structure of gabrobragon populations and the prospects for its further use for biological control of a number of harmful Lepidoptera (Chouinard et al. 2019).

The aim of the present work is to assess the parasitic activity of the gabrobragon as a regulator of the codling moth abundance in various geographic populations, to select a host insect for its mass breeding in laboratory conditions, and to assess the molecular genetic variability of the structure of *H. hebetor* Say populations.

MATERIAL AND METHODS

The capture of natural populations of the gabrobragon *H. hebetor* was carried out in apple orchards in Krasnodar Krai and Stavropol Krai of Russia using cassettes in which caterpillars of the codling moth were placed. Mass breeding of gabrobragon for biological control of the codling moth was carried out using the wax moth (*Galleria mellonella* L.) as a host insect of middle-aged caterpillars. In clean glass jars with a capacity of 1 liter, we put 100 g of artificial nutrient medium (modification A) and 7-10 cocoons of galleria (before the butterflies leave them). The jars are covered with glass lids and placed in a dark thermostat with a temperature of 28-30 °C and an air humidity of 70-75%.

The emerged butterflies laid eggs directly on the medium, from which in 12-15 days caterpillars hatched, which immediately penetrated into the medium. In jars, caterpillars developed up to the 3rd age. Then the contents of the jars were divided into two portions, which were placed in new 1 liter jars, previously filled with a medium with a layer of 6-7 cm. This amount of medium was sufficient until the caterpillars were fully grown. Then galleria caterpillars of older ages, 100-400 ind. were placed in glass jars with a capacity of 1-3 liters with corrugated paper and infected

with gabrobragon. In each jar, 30-50 females of the parasite were introduced, which paralyzed the caterpillars and laid eggs on them. After 10-14 days adults flown out, which were packaged in plastic containers or glass jars and released into apple orchards during the appearance of middle-aged codling moth caterpillars at the rate of 1-2 thousand ind. per 1 hectare. Efficacy assessment of the bioagent was carried out using cassettes with caterpillars of the codling moth and trapping belts placed in the experimental garden and control plots of the garden.

Figure 1: Cassettes with codling moth caterpillars for catching gabrobragon in an apple orchard.



We determined the parasitic activity of natural gabrobragon populations and the dynamics of its numbers according to the damage degree of the caterpillars. When assessing the genetic structure of various geographic populations of *H. hebetor*, the object of the study was a sample of insects (n = 20) from the Krasnodar and Stavropol populations. Laboratory experiments were performed using the following equipment: iCycler amplifier (Bio Rad, USA), Sub Cell-GT electrophoresis devices (Bio Rad, USA), Power Pac-Basic electrophoresis power supply (Bio Rad, USA), transilluminator ECX-20-M (Vilber Lourmat, France), microcentrifuge "MiniSpin" (Eppendorf, Germany), thermostat for microtubes "Thermo 24" (Biokom, Russia).

DNA isolation was performed from adult insects (imago), amplification (RAPD-PCR) and electrophoresis in 1.8% agarose - as we described earlier (Kil et al., 2016b). In the PCR reaction, four primers highly specific for *H. hebetor* DNA were used: OPA05, OPA10, OPB04, UBC519 (Kil et al. 2016a; Kil et al. 2016b; Kil et al. 2018). The primers were synthesized by LCC Evrogen (Moscow); DNA polymerase, buffer, and other necessary components for PCR were supplied by Sibenzyme (Moscow). Genetic diversity, DNA polymorphism, and genetic similarity were assessed

using Nei and Shannon, from the POPGENE version 1.31 software package (Yeh et al. 1997; Kil 2019).

Statistical data processing was performed using the Statistica 13.0 software package with the Duncan's test. The studies were carried out on the basis of the laboratory of the State Collection of Entomocariphages and the primary assessment of biological plant protection products of the Federal Research Center of Biological Plant Protection (FRCBPP), Russia, Krasnodar.

RESULTS AND DISCUSSION

One of the promising entomophages capable of controlling the abundance of the codling moth is the gabrobragon *H. hebetor*. In (2019) and (2020), in the apple orchards of the FRCBPP (Krasnodar Krai) and in the SSPK Sady Stavropolya (Stavropol Krai), natural starting populations of the gabrobragon *H. hebetor* were caught on bait cassettes with older caterpillars of the codling moth. Local ecotypes were of practical value, which were captured in order to clarify their bioecological features and the possibility of practical application to protect the apple tree from the codling moth. Biological features and morphological signs of gabrobragon: ectoparasite wintering occurred in a state of diapause at the imago stage. The flown out of

the gabrobragon occurred not earlier than April when the temperature was about + 15°C. The entomophage feeds on nectar and pollen from flowering plants, for example, weeds (wild radish, dandelion, shepherd's purse, etc.), were accumulated in fruit gardens and vineyards (Agasyeva et al. 2019; Piekarska-Boniecka et al. 2019).

After feeding, mating and searching for target host species that may appeared on crops took place. On sunny, windless days at a temperature of + 25 ... + 30°C, the maximum searching and parasitic activity of the ectoparasite was noted. The males of the gabrobragon were polygamous, the females were monogamous. The search for an insect host for laying eggs was based on the orientation of females to odors produced by the host's fodder plant, as well as by the caterpillars themselves and their metabolic products. While attracting the parasite, the combination of various plants and phytophagous insects - objects of infection by the gabrobragon - played a huge role. Under the laboratory conditions, a host insect and optimal conditions for its mass reproduction, storage and maintenance were selected. Caterpillars of the wax moth (*G. mellonella*) and caterpillars of the mill moth (*E. kuhniella*) were tested as host insects. The effectiveness of each species was determined by the number of paralyzed caterpillars and the emergence of parasites of the filial generation (Table 1) (Chouinard et al. 2019).

Table 1. The number of gabrobragon emerged depending on the host insect

Host insect	Number of caterpillars, ind.	Number of gabrobragon used for infestation, ind.	Number of cocoons formed, pcs.	Number of the emerged gabrobragon, ind.
<i>Ephestia kuhniella</i> Zell.	100	25	106	98
<i>Galleria mellonella</i> L.	100	25	123	195

As a result of statistical processing by Student's t-test, the following values were obtained: temp. = -3.77003, t0.01 = 4.6041, t0.05 = 2.7764, which rejects the null hypothesis, therefore, the options are statistically significantly different.

Table 2. Parasitic activity of the ectoparasite *Habrobracon hebetor* Say against the codling moth (*Cydia pomonella* L.)

Codling moth, caterpillars	Number of caterpillars, ind.	Among them:		Number of cocoons formed, pcs.	Number of emerged parasites, %
		paralyzed, %	parasitized, %		
Krasnodar Krai					
Older age	42	100 ^d	76,19 ^d	32 ^d	78,13 ^c
Middle age	34	100 ^c	64,71 ^c	22 ^c	54,55 ^b
Young age	37	0 ^a	0 ^a	0 ^a	0 ^a
Stavropol Krai					
Older age	45	22,22 ^b	4,44 ^b	0 ^b	0 ^a
Middle age	39	5,13 ^a	0 ^a	0 ^a	0 ^a
Young age	35	0 ^a	0 ^a	0 ^a	0 ^a
*Note: there are no statistically significant differences according to Duncan's test at the 95% probability level between the options marked with the same letter indices, when compared within the columns.					

As the data presented in (Table 6) show, the most effective host insect was the wax moth (*G. mellonella*), in the variant with which the yield of adults was 195 individuals, in comparison with 98 on the mill moth (*E. kuhniella*). The timing of the release of entomophages and the developmental phases of the target insect were of paramount importance, especially for obtaining the maximum effect from the use of parasitic Hymenoptera. In this connection, in laboratory conditions, an assessment of the parasitic activity of the gabrobragon was carried out, depending on the age structure of the caterpillars of the codling moth (Table 2) (Chouinard et al. 2019).

As the data presented in (Table 2) show, gabrobragon effectively paralyzed the caterpillars of the codling moth of middle and older ages (caught in Krasnodar Krai), the emergence of parasites of which was 54.8 and 78.6%, respectively. The Stavropol population was not very effective against caterpillars of the codling moth of middle and young ages. Thus, it was found that the new moth population of the gabrobragon, propagated under laboratory conditions, met all the requirements for assessment as a bioagent for regulating the abundance of the codling moth (Chouinard et al. 2019).

Figure 2. 1: A trapping belt installed on an apple tree; 2 - gabrobragon cocoons, after the parasite leaves them.



In an organic apple orchard at the Shcherbakov farm, gabrobracons were released on the autumn ripening varieties Liberty and Florin. Studies on the use of *H. hebetor* were carried out over two years (2019) and (2020). Reproducing under natural conditions, the ectoparasite, upon reaching a sufficient population density, significantly regulated the number of the pest. As a result of the experiment, we observed the uniform dispersal of the gabrobragon over the entire garden area. The evidence was the infestation of caterpillars in cassettes installed in the apple orchard. The damage degree by gabrobracons of pest caterpillars was 50-65%, fruit damage was 3.5% (Chouinard et al. 2019; Agasieva et al. 2019).

During the growing season, cardboard trapping belts were installed on apple tree boles to record the dynamics of the pest abundance and the damage of caterpillars by the hymenopteran parasites. As a result of regular (weekly) observations, caterpillars of the codling moth infected with gabrobragon and empty cocoons of the parasite that flew out were revealed. The general parasitism of the pest caterpillars in the trapping belts reached 60-70% (Figure 2). Thus, the gabrobragon population introduced into the apple tree

cenosis continued its reproduction in natural conditions and largely suppressed the number and harmfulness of the codling moth (Chouinard et al. 2019). RAPD analysis of two geographic populations of the entomophage *H. hebetor* was carried out using four primers: OPA05, OPA10, OPB04, UBC519. The results of the RAPD analysis of the two studied insect populations using these primers are shown on electrophoregrams (Figure 3) (Agasieva et al. 2019).

Figure 3: Electrophoregrams of the products of DNA amplification of gabrobragon populations in 1.8% agarose with various RAPD primers. Tracks: 1-10 - Krasnodar; 11-20 - Stavropol; M - molecular weight markers (base pairs)

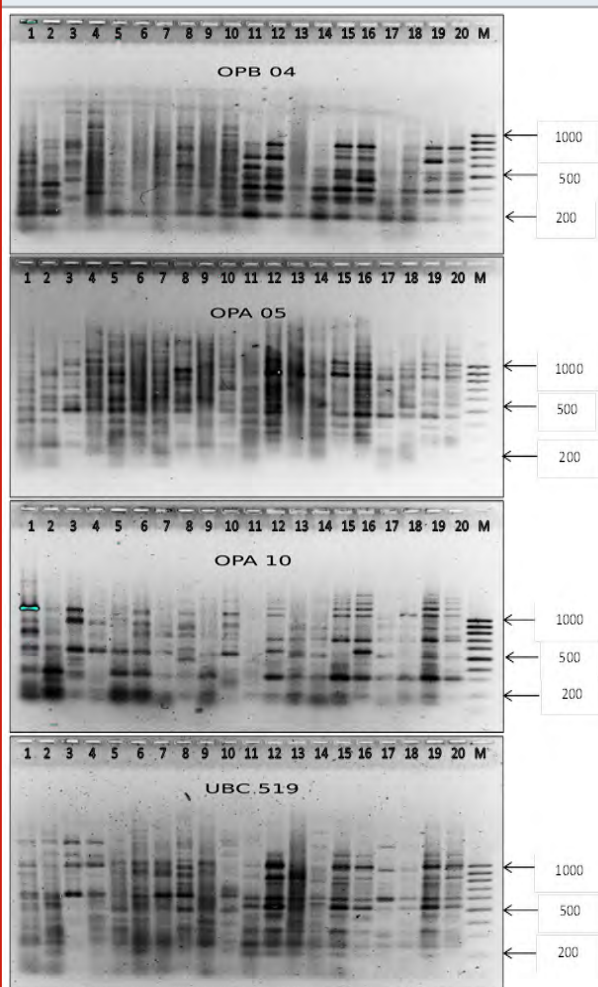


Table 3. Number of loci per primer and sizes of DNA fragments in RAPD analysis of gabrobragon populations

RAPD-primer	Base pairs 5'-3' subsequence	Number of loci	Sizes of DNA fragments (b.p.)
OPA 05	AGGGGTCTTG	13	≈ 1100-100
OPA 10	GTGATCGCAG	16	≈ 1500-100
OPB 04	GGACTGGAGT	13	≈ 1100-100
UBC 519	ACCGGACACT	20	≈ 1300-100

Table 4. DNA polymorphism and genetic diversity of gabrobragon populations by RAPD primers (62 loci)

Sample from the population	P (%)	H \pm SD*	I \pm SD*
Krasnodar	82,3	0,256 \pm 0,171	0,394 \pm 0,236
Stavropol	80,7	0,283 \pm 0,189	0,422 \pm 0,259

*t_{fact} \leq t₀₅ - differences are not significant;
P - % of polymorphic loci in the population;
H - genetic diversity according to Nei (1973);
I - Shannon informational index;
 \pm SD is the standard deviation.

As a result of the PCR analysis, 62 RAPD loci were obtained, ranging in size from 100 to 1500 base pairs (Table 3). Molecular genetic analysis of *H. hebetor* revealed a high level of DNA polymorphism (P = 80.7-82.3%) and genetic diversity (H = 0.256-0.283) in the studied geographic samples and the absence of significant differences between them in these indicators (table 4).

Genetic diversity (according to Nei) within geographic samples (H_s = 0.270) accounted for 87.1% of the total genetic diversity (H_s = 0.310) (Table 5). The revealed ratio of intra- and inter-population variability, estimated by the Shannon Index (I), showed similar data. The level of genetic flow between populations was Nm = 3.298, and the coefficient of genetic differentiation was G_{st} = 0.132.

Table 5. Total genetic variability of gabrobragon populations for all RAPD loci (n = 62)

Indicator	Pt (%)	Ht	Hs	Gst	Nm
Value (\pm SD)	100.0	0.310 \pm 0.018	0.270 \pm 0.015	0.132	3,298

Pt - % of polymorphic loci for all samples;
Ht - the general genetic variability in the population;
Hs - genetic variability within populations;
Gst - the coefficient of genetic differentiation;
Nm - an indicator of gene flow between populations.

The G_{st} value confirmed the fact that 12.9% of the total genetic variability falls on the share of variability between populations, which determines the differentiation between samples (Agasyeva et al. 2019). Analysis of genetic differences between the studied insect samples showed that their genetic similarity was relatively low (genetic identity (GI) = 0.906) (table 6).

Table 6. Genetic distances (GD) (under the diagonal) and genetic identity (GI) (over the diagonal) between gabrobragon populations (according to Nei, 1978)

Sample from the population	Krasnodar	Stavropol
Krasnodar	-	0.906
Stavropol	0.099	-

CONCLUSION

The findings of the present study indicate that the analyzed insect samples were likely to represent different geographic populations of the *H. hebetor* ectoparasite, which was confirmed by the data of biological studies. Thus, the RAPD analysis of the Krasnodar and Stavropol populations of *H. hebetor* revealed a high level of DNA polymorphism and genetic diversity in the studied geographic populations of the gabrobragon. At the same time, intrapopulation variability was 87.1%, while interpopulation variability

accounted for 12.9% of the total indicator. The limited gene flow (Nm = 3.298) resulted in relatively low identity (GI = 0.906) between populations and significant interpopulation variability.

ACKNOWLEDGEMENTS

This study was financially supported by the Kuban Science Foundation within the framework of the scientific project No. MFI-20.1 / 65.

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Biochemical Communication

Alterations in Various Biochemical Parameters Among Covid-19 Patients: An Observational Retrospective Analysis

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ABSTRACT

Novel coronavirus causing the pandemic infectious disease termed as COVID-19 is characterized by respiratory illness which may lead on to acute respiratory distress syndrome. Ferritin is a key mediator of immune dysregulation leading on to cytokine storm. Alterations in various biochemical parameters have been widely reported in COVID-19. Early identification of effective biomarkers to assess the severity of this disease is essential. Our study was aimed to evaluate the variations in the routinely analysed biochemical parameters and their association with ferritin levels among COVID patients. The study participants consisted of 270 members among which 149 were COVID positive and 121 were negative. Analysis of the routine biochemical parameters as well as ferritin level were carried out. Among the 149 positive cases, 84 (56.4%) were mild positive with ferritin levels $<500\text{ng/ml}$ and 65 (43.6%) were severe positive with ferritin levels $>500\text{ng/ml}$. We reported significant increase in serum ferritin levels in severe positive samples (1449.84 ± 249.47) compared to mild positive samples (230.04 ± 17.41). We observed increased levels of total bilirubin in 12.7%, direct bilirubin in 16.8%, indirect bilirubin in 8.7%, AST in 65.8%, ALT in 44.3%, ALP in 9.4%, GGT in 51.7%, urea in 18.4%, creatinine in 14.3%, BUN in 18.4% and decreased levels of total protein and albumin in 23.5% positive patients compared to negative patients. Ferritin and its associated biochemical parameters act as predictors of COVID severity. These biochemical alterations suggest the significance of early risk assessment and monitoring of COVID patients.

KEY WORDS: BIOCHEMICAL PARAMETERS, COVID-19, ELECTROLYTE ABNORMALITIES, FERRITIN, LIVER FUNCTION TESTS.

INTRODUCTION

COVID-19 which has been officially declared as a pandemic by the World Health Organization is characterised by respiratory illness which may progress on to severe pneumonia and acute respiratory distress syndrome (ARDS) (Park et al. 2020; Mbarka et al. 2020; Ashour et al. 2020). Since the outbreak of the coronavirus pandemic several abnormalities in various biochemical parameters have been reported but their clinical implications need to be investigated. Several meta-analyses have thrown light on the importance of serial assessment of biochemical parameters namely ferritin, lactate dehydrogenase (LDH), total bilirubin, total protein, albumin, aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), gamma

glutamyl transferase (GGT), urea, creatinine and electrolytes in evaluation of risk. Ferritin is a key mediator of immune dysregulation via direct immunosuppressive and pro-inflammatory effects contributing to cytokine storm (Ashour et al. 2020; Henry et al. 2020).

Ferritin may be considered as a strong discriminator for potential progression to critical illness of COVID-19 (Zhou et al. 2020). In view of the limited resources available, the early diagnosis of severe COVID-19 is of great importance to reduce morbidity and mortality. Assessing serum ferritin levels along with identification of derangements of other biochemical parameters during hospitalization can help to identify at risk individuals with COVID-19. The advantage of utilizing these parameters for effective triaging is the availability of highly standardized automated analyzers and reagent kits which offer rapid, reliable, reproducible results which are also economical (Henry et al. 2020). This study is proposed to identify potential biochemical laboratory markers which can be used to assess the progression of

Article Information:*Corresponding Author: vinodhiv1@srmist.edu.in
Received 25/10/2021 Accepted after revision 15/12/2021
Published: 31st December 2021 Pp- 1648-1659
This is an open access article under Creative Commons License,
Published by Society for Science & Nature, Bhopal India.
Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.42>

disease from mild to severe form and help in timely triaging of the patients.

MATERIAL AND METHODS

This retrospective study included patients of SRM Medical College Hospital and Research Centre, SRM Institute of Science and Technology, SRM Nagar, Kattankulathur, Kanchipuram, Chennai, Tamil Nadu, India. This study was carried out between the months of May and August 2020. Patients who tested positive for COVID-19 by RT-PCR testing were included as the study group and those who tested negative for COVID-19 by RT-PCR testing on admission were chosen as the control group. The study protocol was approved by the Scientific and Ethical committees of our Institution (IEC No: 1977/IEC/2020). All the samples were collected and processed as per the ICMR safety guidelines.

Table 1. Age Group of Participants

Age group	Positive (n-149)	Negative (121)
<20	0	6 (4.96 %)
20-29	13 (8.72 %)	21 (17.36%)
30-39	33 (22.15%)	39 (32.23%)
40-49	33 (22.15%)	22 (18.18 %)
50-59	25 (16.78 %)	23 (19.01 %)
≥60	45 (30.20 %)	10 (8.26 %)

Table 2. Chi Square Analysis Between Gender And Ferritin

Ferritin levels (ng/ml)	Positive (N-149)		Negative (N-121)	
	Male (N-111)	Female (N-38)	Male (N-75)	Female (N-46)
<500 ng/ml	52 (46.8%)	32 (84.2%)	63 (84.0%)	44 (95.7%)
>500 ng/ml	59 (53.2%)	6 (15.8%)	12 (16.0%)	2 (4.3%)

The parameters of renal function test, liver function test, serum electrolytes and lactate dehydrogenase were estimated using Beckman Coulter Auto analyser. Ferritin level was measured in Enhanced CLIA-Vitros ECI immunoanalyzer. After completion of estimations, the sample processing area and instruments were sterilized using hypochlorite solution and alcohol-based sanitizer. The biohazard materials used during the sample processing were disposed appropriately as per instructions in the safety guidelines. Based on the ferritin values obtained, the patients were categorized as having mild or severe coronavirus disease using a cut-off value of 500 ng/ml (Lin et al. 2020). The statistical analysis of study parameters was done using SPSS software (version 22).

Table 3. Analysis Of Positive And Negative Samples

Parameters	Positive		Negative		Significance P-value
	N	Mean ± SEM	N	Mean ± SEM	
Ferritin (ng/ml)	149	762.16 ± 119.62	121	264.99 ± 31.95	0.000***
LDH (U/L)	110	359.94 ± 22.76	105	272.66 ± 9.74	0.001**
Urea (mg/dl)	149	38.96 ± 3.56	121	24.37 ± 1.40	0.001**
Creatinine (mg/dl)	149	1.55 ± 0.21	121	0.76 ± 0.04	0.001**
BUN (mg/dl)	149	18.20 ± 1.66	121	11.38 ± 0.66	0.001**
Sodium (mmol/l)	146	134.84 ± 0.99	115	137.50 ± 0.28	0.021*
Potassium (mmol/l)	145	4.09 ± 0.05	115	3.94 ± 0.03	0.017*
Chloride (mmol/l)	145	100.67 ± 0.39	115	102.58 ± 0.33	0.000***
Bicarbonate (mmol/l)	145	24.19 ± 0.59	115	24.62 ± 0.28	0.544
Total bilirubin (mg/dl)	149	0.69 ± 0.03	120	0.59 ± 0.03	0.023*
Direct bilirubin (mg/dl)	149	0.20 ± 0.02	120	0.16 ± 0.02	0.049*
Indirect bilirubin (mg/dl)	149	0.49 ± 0.03	120	0.43 ± 0.02	0.092
Total protein (g/dl)	149	7.03 ± 0.08	120	7.34 ± 0.06	0.003**
Albumin (g/dl)	149	3.88 ± 0.06	120	4.08 ± 0.05	0.013*
Globulin (g/dl)	149	3.23 ± 0.04	120	3.25 ± 0.03	0.703
AG ratio	149	1.23 ± 0.02	121	1.26 ± 0.02	0.248
AST (IU/L)	149	59.16 ± 11.68	120	35.37 ± 2.02	0.071
ALT (IU/L)	148	50.01 ± 7.40	120	30.08 ± 1.80	0.018*
ALP (IU/L)	148	88.21 ± 6.83	120	84.21 ± 3.34	0.625
GGT (U/L)	149	51.68 ± 4.08	120	40.50 ± 3.63	0.047*

Significance: *P<0.05, **P<0.01, ***P<0.001

Table 4: Unpaired 'T Test Between Mild (Ferritin <500ng/ml) And Severe (Ferritin >500ng/ml) Positive Groups

Parameters	Mild positive		Severe positive		Significance P-value
	N	Mean \pm SEM	N	Mean \pm SEM	
Ferritin (ng/ml)	84	230.04 \pm 17.41	65	1449.84 \pm 249.47	0.000***
LDH (U/L)	69	285.97 \pm 13.25	41	484.41 \pm 51.65	0.000***
Urea (mg/dl)	84	27.70 \pm 2.23	65	53.51 \pm 7.27	0.000***
Creatinine (mg/dl)	84	1.18 \pm 0.22	65	2.02 \pm 0.39	0.048*
BUN (mg/dl)	84	12.93 \pm 1.04	65	24.99 \pm 3.40	0.000*
Sodium (mmol/l)	82	136.38 \pm 0.43	64	132.88 \pm 2.19	0.081
Potassium (mmol/l)	82	4.09 \pm 0.07	63	4.09 \pm 0.08	0.977
Chloride (mmol/l)	82	101.79 \pm 0.49	63	99.21 \pm 0.58	0.001**
Bicarbonate (mmol/l)	82	25.01 \pm 0.94	63	23.11 \pm 0.59	0.112
Total bilirubin (mg/dl)	84	0.60 \pm 0.04	65	0.80 \pm 0.05	0.004**
Direct bilirubin (mg/dl)	84	0.17 \pm 0.02	65	0.25 \pm 0.03	0.028*
Indirect bilirubin (mg/dl)	84	0.43 \pm 0.04	65	0.56 \pm 0.03	0.023*
Total protein (g/dl)	84	7.19 \pm 0.11	65	6.81 \pm 0.11	0.017*
Albumin (g/dl)	84	4.08 \pm 0.08	65	3.64 \pm 0.07	0.000***
Globulin (g/dl)	84	3.28 \pm 0.05	65	3.17 \pm 0.07	0.203
AG ratio	84	1.26 \pm 0.03	65	1.18 \pm 0.03	0.047*
AST (IU/L)	84	40.44 \pm 5.30	65	83.35 \pm 25.70	0.068
ALT (IU/L)	83	33.08 \pm 3.25	65	71.61 \pm 15.99	0.009**
ALP (IU/L)	83	79.23 \pm 3.81	65	99.68 \pm 14.72	0.138
GGT (U/L)	84	39.61 \pm 4.16	65	67.29 \pm 7.25	0.001**
Significance: *P<0.05, **P<0.01, ***P<0.001					

Student's test was used for comparison of parameters between the groups. Pearson's correlation was utilized to assess the association between the various parameters and multiple comparison analysis was done using ANOVA.

RESULTS AND DISCUSSION

The study participants included 270 symptomatic patients who underwent COVID testing on the day of admission. Among them 149 (55%) tested positive and 121 (45%) were negative. Among the 149 positive cases, 111 (74.5%) were males and 38 (25.5%) were females. Amidst the 121 negative cases, 75 (62.0%) were males and 46 (38.0%) were females. We observed that males were more prone to COVID infection as compared to females. The maximum numbers of positive cases were found in the age group of above 60 years (Table 1).

The lactate dehydrogenase levels in adults ranges from 140-280 U/L. We found 44 (40%) of Covid positive patients having LDH in the normal range whereas 64 (58.2%) had LDH levels greater than 280 U/L.

We observed significant increase in the levels of ferritin, lactate dehydrogenase, urea, creatinine, BUN, potassium, total bilirubin, direct bilirubin, alanine transaminase, gamma glutamyl transferase and significant decrease in the levels of sodium, chloride, total protein, albumin between positive and negative COVID samples (Table 3).

We observed significant increase in the levels of ferritin, lactate dehydrogenase, urea, creatinine, BUN, total bilirubin, direct bilirubin, indirect bilirubin, AG ratio, alanine transaminase and gamma glutamyl transferase and significant decrease in the levels of chloride, total protein, albumin in severe positive (ferritin levels >500ng/ml) compared to mild positive (ferritin levels <500ng/ml) [Table 4].

A significant positive correlation of ferritin was found with lactate dehydrogenase, urea, creatinine, BUN, potassium, total bilirubin, direct bilirubin, indirect bilirubin, aspartate transaminase, alanine transaminase and alkaline phosphatase. A significant negative correlation of ferritin was found with chloride and albumin in COVID positive patients [Table 5]. A significant positive correlation of ferritin was found with lactate dehydrogenase, bicarbonate and gamma glutamyl transferase in mild positive patients [Table 5]. A significant positive correlation of ferritin was found with lactate dehydrogenase, urea, creatinine, BUN, potassium, total bilirubin, direct bilirubin, aspartate transaminase, alanine transaminase and alkaline phosphatase. A significant negative correlation of ferritin was found with chloride in severe positive patients [Table 5].

We observed the frequency of participants in mild positive as 84 (31%), severe positive as 65 (24%) and negative as 121 (45%). We also observed a significant difference in

the levels of urea, creatinine, BUN, sodium, total bilirubin, indirect bilirubin, total protein, aspartate transaminase, alanine transaminase and alkaline phosphatase between

mild positive, severe positive and negative groups in ANOVA analysis [Tables 6, 7, 8].

Table 5. Pearson Correlation Of Ferritin With Study Parameters In Positive Group, Mild Positive And Severe Positive Groups

Ferritin association with study parameters	Positive group			Mild positive			Severe positive		
	N	R value	P-value	N	R value	P-value	N	R value	P-value
LDH (U/L)	110	0.562	0.000***	69	0.266	0.027*	41	0.495	0.001**
Urea (mg/dl)	147	0.590	0.000***	84	-0.034	0.762	63	0.577	0.000***
Creatinine(mg/dl)	149	0.575	0.000***	84	0.055	0.619	65	0.694	0.000***
BUN (mg/dl)	149	0.589	0.000***	84	0.760	-0.034	65	0.581	0.000***
Sodium (mmol/l)	146	-0.090	0.281	82	0.060	0.591	64	-0.035	0.782
Potassium (mmol/l)	145	0.167	0.045*	82	-0.105	0.350	63	0.275	0.029*
Chloride (mmol/l)	145	-0.305	0.000***	82	-0.116	0.300	63	-0.312	0.013*
Bicarbonate (mmol/l)	145	-0.105	0.208	82	0.233	0.035*	63	-0.170	0.182
Total bilirubin (mg/dl)	149	0.260	0.001**	84	0.193	0.079	65	0.256	0.039*
Direct bilirubin (mg/dl)	149	0.272	0.001**	84	0.167	0.128	65	0.270	0.030*
Indirect bilirubin (mg/dl)	149	0.163	0.047*	84	0.137	0.215	65	0.167	0.185
Total protein (g/dl)	149	-0.146	0.075	84	-0.043	0.697	65	-0.116	0.356
Albumin (g/dl)	149	-0.199	0.015*	84	0.031	0.782	65	-0.143	0.257
Globulin (g/dl)	149	-0.072	0.382	84	-0.118	0.286	65	-0.033	0.794
AG ratio	149	-0.131	0.112	84	0.136	0.217	65	-0.115	0.361
AST (IU/L)	149	0.440	0.000***	84	-0.077	0.489	65	0.438	0.000***
ALT (IU/L)	148	0.410	0.000***	83	0.064	0.565	65	0.374	0.002**
ALP (IU/L)	148	0.334	0.000***	83	0.054	0.630	65	0.330	0.007**
GGT (U/L)	149	0.112	0.174	84	0.385	0.000***	65	-0.030	0.811

Significance: *P<0.05, **P<0.01, ***P<0.001

We observed that the prevalence of COVID infection was more in males compared to females. Similar results were observed by Huang et al. (2020) and Chen et al. (2020). MERS-CoV and SARS-CoV have also been found to infect more males than females (Badawi et al. 2016). These findings could be linked to the protection associated with sex hormones which modulate both innate and adaptive immunity (Jaillon et al. 2019; Channappanavar et al. 2020). The mean age of COVID positive group was found to be 50.28 ± 15.19 years in our study. We observed that a greater number of COVID positive patients were in the age group of more than 60 years (30.2%) whereas an increased incidence of COVID infection in the age group of 50-59 years (30%) has been reported (Chen et al. 2020).

Ferritin is an iron-binding molecule which helps to store iron in its active form and protects from iron toxicity. Each apoferritin is made up of 24 subunits of two types namely the H and L subunits. The L-subunit rich ferritin predominantly occurs in liver, spleen whereas the H-subunit rich ferritin is present in heart and kidneys (Harrison et al. 1996; Knovich et al. 2009). Oxidative stress, growth factors, thyroid hormone, second messengers, hyperoxia and hypoxia-ischemia are some of the major factors regulating the expression of ferritin (Torti et al. 2002). The hepatocytes,

kupffer cells and macrophages secrete ferritin (Recalcati et al. 2008; Wang et al. 2010; Cohen et al. 2010). The serum ferritin (iron poor form) is mostly made up of L-subunits only. H-ferritin with its immunomodulatory effects causes diminished antibody production, allergic responses as well as phagocytosis (Broxmeyer et al. 1981; Hann et al. 1989; Morikawa et al. 1994; Recalcati et al. 2008). The L-subunit (predominant in serum) acts as a pro-inflammatory mediator (Ruddell et al. 2009; Wang et al. 2010; Channappanavar et al. 2020).

Ferritin expression is found to be induced by pro-inflammatory cytokines (IL-6) and in turn ferritin enhances the expression of the pro-inflammatory cytokines. Moreover, induction of the anti-inflammatory cytokines (IL-10) also by ferritin explains its immunosuppressive effects. Ferritin as well as the cytokines regulate both inflammation and immunosuppression. Hence ferritin can play a role as either an immunosuppressive or a pro-inflammatory agent through different receptors, pathways and effectors (L- versus H-ferritin). An already existing proinflammatory state, sepsis or genetic susceptibility may influence the pathogenic role of ferritin (Rosário et al. 2013). Several mechanisms have been suggested to explain the association

of hyperferritinemia with severity of disease in COVID-19 patients. The viral infection mediated production of the proinflammatory cytokines (IL-1 β , IL-6, TNF- α) as well as leakage of intracellular ferritin as a consequence of inflammation mediated cellular damage have been

implicated as the cause of hyperferritinemia commonly occurring in COVID-19 patients. Heightened reactive oxygen species driven iron release from ferritin may also promote a vicious cycle of inflammation (Kobune et al. 1994; Kell et al. 2014; Channappanavar et al. 2020).

Table 6. Multiple Comparison Of Study Parameters In Mild Positive, Severe Positive And Negative Groups By Anova

Study parameters		Sum of Squares	df	Mean Square	F	Sig.
LDH	Between Groups	11560407.152	249	46427.338	1.832	.056
	With in Groups	506958.167	20	25347.908		
	Total	12067365.319	269	1252.390	2.667	.007**
Urea	Between Groups	310592.609	248			
	With in Groups	8921.167	19	469.535		
	Total	319513.776	267			
Creatinine	Between Groups	1054.846	249	4.236	50.233	.000***
	With in Groups	1.687	20	.084		
	Total	1056.533	269			
BUN	Between Groups	68319.249	249	274.374	2.815	.004**
	With in Groups	1949.185	20	97.459		
	Total	70268.434	269			
Sodium	Between Groups	22278.939	242	92.062	7.498	.000***
	With in Groups	221.000	18	12.278		
	Total	22499.939	260			
Potassium	Between Groups	67.761	241	.281	1.338	.240
	With in Groups	3.782	18	.210		
	Total	71.542	259			
Chloride	Between Groups	4503.938	241	18.689	1.214	.328
	With in Groups	277.000	18	15.389		
	Total	4780.938	259			
Bicarbonate	Between Groups	5635.395	241	23.383	.155	1.000
	With in Groups	2717.667	18	150.981		
	Total	8353.062	259			

Significance: *P<0.05, **P<0.01, ***P<0.001

Table 7. Multiple Comparison Of Study Parameters In Mild Positive, Severe Positive And Negative Groups By Anova

Study parameters		Sum of Squares	df	Mean Square	F	Sig.
Total bilirubin	Between Groups	36.355	248	.147	2.253	.018*
	With in Groups	1.301	20	.065		
	Total	37.657	268			
Direct bilirubin	Between Groups	9.959	248	.040	1.653	.092
	With in Groups	.486	20	.024		
	Total	10.445	268			
Indirect bilirubin	Between Groups	19.426	248	.078	2.380	.013*
	With in Groups	.658	20	.033		
	Total	20.084	268			
Total protein	Between Groups	188.533	248	.760	2.553	.008**
	With in Groups	5.955	20	.298		
	Total	194.488	268			
Albumin	Between Groups	106.440	248	.429	1.536	.129
	With in Groups	5.590	20	.280		
	Total	112.030	268			
Globulin	Between Groups	49.981	248	.202	1.559	.121
	With in Groups	2.585	20	.129		
	Total	52.566	268			
AG ratio	Between Groups	16.718	249	.067	1.416	.181
	With in Groups	.948	20	.047		
	Total	17.667	269			
Significance: *P<0.05, **P<0.01, ***P<0.001						

Based on the ferritin values obtained, the patients were categorized into mild and severe groups using a cut-off value of 500 ng/ml. Among the 149 positive cases, 84 (56.4%) were found to have ferritin levels <500ng/ml and 65 (43.6%) had ferritin levels >500 ng/ml whereas of the 121 negative cases, 107 (88.4%) had ferritin level <500 ng/ml and 14 (11.6%) had ferritin levels >500 ng/ml. Serum ferritin levels are also found to be increased in a variety of diseases and other inflammatory states also especially diabetes mellitus which could explain this finding (Lin et al. 2020). Analysis of 147 confirmed cases of COVID-19 found 29.93 % of patients to have hyperferritinemia (>500 ng/ml) whereas in our study 43.6% of patients had hyperferritinemia (Lin et al. 2020).

We reported significant increase in serum ferritin levels in severe positive samples (1449.84 ± 249.47) compared to mild positive samples (230.04 ± 17.41). Zhou et al found that individuals with severe COVID infection exhibited greater elevations of serum ferritin levels (Zhou et al. 2020). Jenifer et al in their review of studies which documented serum ferritin levels among severe and non-severe COVID-19 patients only at the time of hospital admission observed ferritin concentrations to be within the normal range in non-severe disease and presence of hyperferritinemia in severe disease state (Gomez-Pastoraa et al. 2020). These findings are similar to the observations of our study. Bilateral pulmonary infiltration rate has been observed to be more in patients who had ferritin levels more than 500 ng/

Aravaanan et al.,ml (Lin et al.2020). On investing 201 COVID- 19 patients they found increased risk of development of ARDS to be associated with higher serum ferritin levels (Wu et al.2020). On the other hand, they

found ferritin levels to be elevated in both mild as well as in severe. Among the non survivors, ferritin levels remained elevated throughout the course of the disease (Mo et al. 2020; Zhou et al. 2020).

Table 8. Multiple Comparison Of Study Parameters In Mild Positive, Severe Positive And Negative Groups By Anova

Study parameters		Sum of Squares	df	Mean Square	F	Sig.
Aspartate Groups	Between transaminase	3103587.502	248	12514.466	90.450	.000***
	With in Groups	2767.167	20	138.358		
	Total	3106354.669	268			
Alanine Groups	Between transaminase	1256918.527	247	5088.739	16.166	.000***
	With in Groups	6295.667	20			
	Total	1263214.194	267	314.783		
Alkaline Groups	Between phosphatase	1131998.188	247	4582.989	2.108	.026*
	With in Groups	43489.167	20			
	Total	1175487.354	267	2174.458		
Gamma Groups transferase	Between glutamyl	518302.004	248	2089.927	.922	.634
	With in Groups	45353.000	20			
	Total	563655.004	268	2267.650		
Significance: *P<0.05, **P<0.01, ***P<0.001						

Thus, ferritin levels estimated at admission as well as during the progress of the disease may help to differentiate those with severe manifestations and help in planning effective treatment strategies. Circulating ferritin levels may not only indicate an acute phase response but may also play a key inflammatory role in pathogenesis of COVID-19. Hence the role of iron chelators as well as reduction of dietary iron can also be considered as treatment strategies in the setting of hyperferritinemia (Fleming et al. 2002; Mobarra et al. 2016). LDH, an intracellular enzyme which occurs in cells of most organs is composed of two major subunits and occurs as five major isozymes. Cytokine mediated tissue damage following severe infection causes LDH release into circulation (Martinez-Outschoorn et al. 2011; Ju et al. 2016; Henry et al. 2020).

The isoenzyme of LDH derived from the lungs (LDH-3) is found to be elevated as a consequence of interstitial pneumonia which may progress on to acute respiratory distress syndrome, a common feature of COVID (Kaplan et al. 2002; Patschan et al. 2006; Zhang et al. 2014). Early laboratory data analysis of COVID-19 patients had indicated significant variations in LDH levels among patients with severe disease manifestations (Henry et al. 2020). We observed that 58.2% of COVID positive patients

had LDH levels greater than 280 U/L. By January 2020, 73% of COVID infected patients with elevated LDH levels was reported (Huang et al.2020). LDH levels were also significantly elevated in the severe positive group (ferritin >500ng/ml). A meta-analysis of 9 published studies with 1532 COVID-19 patients to study the association between elevated LDH levels at the time of admission and disease outcome and found that elevated LDH levels represented a sixfold increase in the risk of developing severe disease and a sixteen-fold increase in mortality (Henry et al. 2020).

Greater increase in LDH levels was seen among patients admitted in the intensive care unit and those who progressed onto acute respiratory distress syndrome (ARDS) respectively (Huang et al 2020; Wu et al. 2020). Nearly 60 % of patients with SARS and also those infected with MERS-CoV have reported liver impairment (Chau et al. 2004; Alsaad et al. 2018). Direct viral infection of the hepatic cells has been implicated as the cause for liver damage in patients infected with coronavirus. Data from large scale case studies have indicated that 2-11% of COVID patients had pre-existing liver comorbidities, 14-53% had alterations in levels of AST and ALT as the disease progressed. Liver dysfunction is found to be more prevalent in severe than in mild cases of COVID (Zhang et al. 2020).

In our study elevations of total bilirubin occurred in 12.7%, AST in 65.8 %, ALT in 44.3 %, ALP in 9.4% and GGT in 51.7% of COVID positive patients. Hypoproteinemia and hypoalbuminaemia was observed in 23.5% of the patients.

An increase in ALT by 28%, AST by 35%, bilirubin by 18% and decrease in albumin by 98% and elevation of GGT by 54% was reported (Chen et al. 2020; Zhang et al. 2020).

Table 9. Incidence Of Liver Function Parameters

Parameters ranges	Positive participants	Negative participants
Total bilirubin (mg/dl)	Positive (n-149)	Negative (n-120)
<0.5	53 (35.6%)	55 (45.8%)
0.5-1.0	77 (51.7%)	55 (45.8%)
>1.0	19 (12.7%)	10 (8.4%)
Direct bilirubin (mg/dl)	Positive (n-149)	Negative (n-120)
<0.1	26 (17.4%)	44 (36.7%)
0.1-0.3	98 (65.8%)	67 (55.8%)
>0.3	25 (16.8%)	9 (7.5%)
Indirect bilirubin (mg/dl)	Positive (n-149)	Negative (n-120)
<0.2	11 (7.4%)	4 (3.4%)
0.2-0.8	125 (83.9%)	109 (90.8%)
>0.8	13 (8.7%)	7 (5.8%)
Total Protein (g/dL)	Positive (n-149)	Negative (n-120)
<6.6	35 (23.5%)	14 (11.7%)
6.6-8.3	110 (73.8%)	105 (87.5%)
>8.3	4 (2.7%)	1 (0.8%)
Albumin (g/dL)	Positive (n-149)	Negative (n-120)
<3.5	35 (23.5%)	18 (15%)
3.5-5.2	113 (75.8%)	102 (85%)
>5.2	1 (0.7%)	0 (0%)
Globulin (g/dL)	Positive (n-149)	Negative (n-120)
<2.5	7 (4.7%)	1 (0.8%)
2.5-3.0	45 (30.2%)	38 (31.7%)
>3.0	97 (65.1%)	81 (67.5%)
AG ratio	Positive (n-149)	Negative (n-120)
<1.4	120 (80.6%)	86 (71.7%)
1.4-1.7	23 (15.4%)	31 (25.8%)
>1.7	6 (4.0%)	3 (2.5%)
AST (IU/L)	Positive (n-149)	Negative (n-120)
<31	51 (34.2%)	62 (51.7%)
>31	98 (65.8%)	58 (48.3%)
ALT (IU/L)	Positive (n-149)	Negative (n-120)
<34	83 (55.7%)	83 (69.2%)
>34	66 (44.3%)	37 (30.8%)
ALP (IU/L)	Positive (n-149)	Negative (n-120)
<30	2 (1.3%)	0 (0%)
30-120	133 (89.3%)	111 (92.5%)
>120	14 (9.4%)	9 (7.5%)
GGT (U/L)	Positive (n-149)	Negative (n-120)
<38	72 (48.3%)	79 (65.8%)
>38	77 (51.7%)	41 (34.2%)

Inflammatory cytokines are known to play a role in inducing acute kidney injury and glomerulopathy. Endothelial injury and cardiovascular instability occurring in severely infected COVID patients may cause renal impairment resulting in cardio renal syndrome (González-Cuadrado et al. 1997;

Sanz et al. 2011; Lin et al. 2020; Fan et al. 2020). Previous studies have reported expression of angiotensin converting enzyme 2 (ACE-II) receptors in human kidneys thus indicating a potential pathway for COVID-19 infection (Lin et al. 2020; Fan et al. 2020). Acute kidney injury

is considered to be an independent predictor of covid-19 in-hospital mortality (Cheng et al. 2020; Carriazo et al. 2020). Analysis of urea and creatinine levels at the

onset of disease as well as later during the clinical course showed that impaired kidney function is seen in COVID-19 patients contributing to morbidity and mortality (Hassan Mohammed et al; Mahmoudi et al. 2020).

Table 10. Incidence Of Kidney Function Parameters

Parameters ranges	Positive participants	Negative participants
Urea (mg/dL)	Positive (n-147)	Negative (n-121)
<17	15 (10.2%)	31 (25.6%)
17-43	105 (71.4%)	84 (69.4%)
>43	27 (18.4%)	6 (5.0%)
Creatinine (mg/dL)	Positive (n-147)	Negative (n-121)
<0.5	2 (1.4%)	8 (6.6%)
0.5-1.2	124 (84.3%)	109 (90.1%)
>1.2	21 (14.3%)	4 (3.3%)
BUN (mg/dL)	Positive (n-147)	Negative (n-121)
<6	4 (2.7%)	8 (6.6%)
6-20	116 (78.9%)	107 (88.4%)
>20	27 (18.4%)	6 (5.0%)

Table 11. Incidence Of Electrolyte Imbalances

Parameters ranges	Positive participants	Negative participants
Sodium (mmol/L)	Positive (n-145)	Negative (n-115)
<130	11 (7.6%)	1 (0.9%)
130-145	132 (91%)	113 (98.2)
>145	2 (1.4%)	1 (0.9)
Potassium (mmol/L)	Positive (n-145)	Negative (n-115)
<3.5	14 (9.7%)	7(6.1%)
3.5-5.0	121 (83.4%)	107 (93%)
>5.0	10 (6.9%)	1 (0.9%)
Chloride (mmol/L)	Positive (n-145)	Negative (n-115)
<95	14 (9.7%)	1 (0.9%)
95-105	116 (80%)	96 (83.5%)
>105	15 (10.3%)	18 (15.6%)
Bicarbonate (mmol/L)	Positive (n-145)	Negative (n-115)
<21	28 (19.3%)	7(6.1%)
21-31	114 (78.6%)	107 (93%)
>31	3 (2.1%)	1 (0.9%)

In our study, elevations of urea levels occurred in 18.4%, creatinine in 14.3% and BUN in 18.4% was found in COVID positive patients. Creatinine elevations have been reported as 10 % and 3% in their COVID study group by Huang et al. (2020); Chen et al. (2020) respectively. An increase in BUN beyond the normal range was seen in 6% of the study group analysed by Chen et al. The meta-analysis on biochemical abnormalities also identified derangements in kidney function in patients with severe and fatal COVID-19 patients (Henry et al. 2020). Initial evidence from COVID studies have indicated the presence of electrolyte abnormalities (Guan et al. 2020; Huang et al. 2020).

Identification of such alterations help not only in effective patient management but also help to understand key pathophysiological mechanisms of the disease process. Among the COVID patients of our study, the incidence of hyponatremia and hypokalemia was found to be 7.6% and 9.7% respectively. Fall in serum chloride and bicarbonate levels below the reference range was noted in 9.7% and 19.3% respectively (Lippi et al. 2020). The abnormalities in serum electrolyte levels were found to be more prevalent among the positive patients compared to those who had tested negative. Results of pooled analysis identifies the most commonly encountered electrolyte abnormalities in COVID patients which indicates the COVID-19 severity to

be associated with lowered serum concentrations of sodium, potassium and calcium (Lippe et al. 2020).

Binding of the virus to its host receptor namely angiotensin converting enzyme-2 may cause increased renal loss of potassium leading onto hypokalemia. Electrolyte status among COVID patients also varies highly (Lippe et al. 2020; Huang et al. 2020). Studies with larger number of COVID positive samples analysed at different stages of progression of the disease will help to clearly establish the clinical significance and to initiate the appropriate interventions. The major limitation of our study is its retrospective cross-sectional nature and the non-availability of correlation with the clinical aspects of COVID patients. Analysis of a larger sample size sourced from multiple centres with clinical outcome can help in a better understanding of the clinical utility of these parameters.

CONCLUSION

The findings of the present study indicate that males were more commonly affected by COVID-19 than females. The maximum numbers of positive cases were found in the age group of more than 60 years. Among the 149 positive cases, 84 (56.4%) were found to have ferritin levels <500 ng/ml and 65 (43.6%) had ferritin levels >500 ng/ml. We reported significant increase in serum ferritin levels in severe positive samples (1449.84 ± 249.47) compared to mild positive samples (230.04 ± 17.41). We observed significant increase in the levels of lactate dehydrogenase, urea, creatinine, BUN, total bilirubin, direct bilirubin, indirect bilirubin, AG ratio, alanine transaminase and gamma glutamyl transferase and significant decrease in the levels of chloride, total protein, albumin in severe positive (ferritin levels >500 ng/ml) compared to mild positive (ferritin levels <500 ng/ml). The significant alterations in various biochemical parameters among the COVID-19 patients suggests the importance of initial assessment and monitoring of these laboratory parameters in risk assessment.

Conflict of interests: Authors declare no conflicts of interests to disclose.

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Biotechnological Communication

Molecular Detection of *epsA*-Mediated and Extracellular Polysaccharide-Mediated Biofilm Formation Among Multidrug Resistant Strains of *Acinetobacter baumannii*

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ABSTRACT

The *epsA* associated biofilm formation attributes to potent virulence in the drug resistant strains of *Acinetobacter baumannii*. This study is aimed to molecularly characterize the *epsA* gene among the multidrug resistant clinical isolates of *A. baumannii* and to assess the frequency of the same in different drug resistant groups. To detect the biofilm formation among the selected MDR strains of *A. baumannii*, semi-quantitative adherent bioassay was performed using crystal violet staining method. Further PCR amplification was done to screen the presence of *epsA* gene with further sequencing of the amplicons. Pearson's correlation analysis was done to check the correlation of the occurrence of *epsA* gene with drug resistant strains (p -value<0.05). 58.9%, 31.5% and 0.9% of the strains were recorded as high grade, low grade and negative biofilm formers under biofilm assay. The *epsA* gene was observed in 14 MDR strains (19.17%) of *A. baumannii* with an amplicon size of 451bp. Co-occurrence of *epsA* gene was 100% in β -lactam, cephem and folate resistant strains followed by 71.4% among aminoglycosides, 57.1% against carbapenems and 14.2% in fluoroquinolone and efflux pump mediated resistant strains. The findings of the study suggest the co-occurrence of *epsA* gene mediated biofilm formation among the multidrug resistant strains of *A. baumannii*. Further studies on the same helps in designing new vaccines and drugs for the prevention and treatment of *A. baumannii* infections.

KEY WORDS: A. BAUMANNII, BIOFILM, EPSA, MULTIDRUG RESISTANCE, VIRULENCE.

INTRODUCTION

Acinetobacter baumannii is a ubiquitous gram-negative and non-fermentative bacillus which causes a variety of diseases in humans. It exists mainly in health care settings such as hospitals and is recognized as one of the six dreadful nosocomial pathogens by the World Health Organization. Severe systemic complications linked with *A. baumannii* among hospitalized patients in intensive care units are associated with various infections like urinary tract infections, pneumonia, endocarditis, post-operative wound infections and septicemia have been recognized globally (Mayers et al. 2017; Matar 2018). The ability of *A. baumannii* to survive in the hospital setting is attributed with its biofilm formation. Initiation and formation of biofilms contributes to virulence in *A. baumannii*, as they were recognized as bacterial communities which enclosed

in a matrix of extracellular material like DNA, proteins and polysaccharides (Ketter 2015; Ramirez et al. 2021).

Additionally, the biofilm of *A. baumannii* promotes the survival rate in both biotic and abiotic surfaces through its cell adhesiveness (Biswas and Rather 2019; González et al. 2021). *A. baumannii* is grouped as one of the ESKAPE organisms that is *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *A. baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp., that pose a global threat to human health and a therapeutic challenge to researchers due to the constant emerging of increasing resistance to drugs (Kyriakidis et al. 2021).

A. baumannii infections account for >2% of all healthcare-associated infections in the Asia and the Middle East of which ~45% of all isolates are considered to be MDR, a rate up to four times increased when described in relation to other Gram-negative pathogenic organisms, such as *P. aeruginosa* and *K. pneumoniae* (Harding et al. 2018). Many phenotypes and genotypes vary in the expression of biofilm initiation, progression or development and distortion attributing to

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Received 15/09/2021 Accepted after revision 25/12/2021

Published: 31st December 2021 Pp- 1660-1665

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.43>

the virulence in *A. baumannii*. This is due to the different forms of the gene operons associated with the biofilm formation such as *ompA*, *csu*, *csg*, *ptk*, *bap*, *epsA*, *kpsmII* etc (Ahimou et al. 2007; Costerton et al. 2014). Amidst many genes, it is a known fact that *epsA* gene encodes for extracellular polysaccharide biofilms in *A. baumannii*. *epsA* based biofilms were also detected in many other bacteria resulting in the aggregation of the bacterial cells (Costerton et al. 2014; Chakravarty 2020).

These polysaccharide lines on the surface of *A. baumannii* organize into moderate to strong biofilms rendering effective protection for the bacterium to survive in harsh environments (Ghasemi et al. 2018). A recent study has documented its 95% occurrence among the drug resistant strains of *A. baumannii* (Zeighami et al. 2019). As an outcome of these statistics, the infections induced by carbapenem-resistant *A. baumannii* has been categorised in the critical group of all bacteria as a global threat to human health by WHO for prioritizing research and evolution of new antimicrobial treatments (Vázquez-López et al. 2020). With this background, assessment on the correlation of the occurrence of *epsA* gene among the multi-drug resistant clinical isolates of *A. baumannii* would be a timely investigation as it is not so vivid in many studies from South India. Not much studies are documented related to the *epsA* based biofilm formation in the strains of hospitalized patients. This study is thus aimed to molecularly characterize *epsA* gene among the clinical isolates of *A. baumannii* with further comparative genomic assessments of the sequenced amplicons of the *epsA* gene (Vranceanu et al. 2020).

MATERIAL AND METHODS

The formation of the biofilms by the drug-resistant strains was investigated by culturing the cells in 96-well flat-bottomed microtiter plates as described earlier (Kouidhi et al. 2010). The detection of biofilm formation by semi-quantitative adherence assay was carried out in triplicates for each strain, with 200 µl of the fresh broth culture in trypticase soy broth (HiMedia, Mumbai, India) with 0.25% glucose (w/v). The plate was incubated at 37°C /24 hrs with negative control (broth + 0.25% glucose) and positive control (known biofilm forming strain of *A. baumannii* earlier detected). After incubation the wells were washed thrice with phosphate buffered saline (PBS), to remove the free cells, and the adhered bacteria were fixed using 95% ethanol/5 min and the plates were dried. Finally, all the wells were stained with 100 µl of 1% w/v crystal violet solution (HiMedia), for 5 mins. Excess stains were removed by washing with distilled water and the wells were dried. Optical density was measured in the plate reader at 570 nm (OD₅₇₀) and the biofilm formation was graded as high (OD₅₇₀ ≥ 1), low (0.1 ≤ OD₅₇₀ < 1) or negative (OD₅₇₀ < 0.1) (Avila-Novoa et al. 2019).

73 different groups of drug-resistant strains of *A. baumannii* isolated in our earlier studies were maintained at -80 °C in 80% / 20% (v/v) glycerol in LB medium in our repertoire, and were retrieved by incubating at 37 °C for 24 hrs.

Chromosomal DNA was extracted using the Qiagen DNA extraction kit in accordance with the manufacturer's instructions (Inchai et al. 2015). The extracted genomic DNA was stored in -20 °C until further use. For the amplification of *epsA*, the PCR reaction mixture [15 µl] was prepared by adding 7.8 µl of 2x master mix [Taraka, Japan] in 5.6 µl of double distilled water with 0.31 µl of 100 pmol/ml concentration of the specific Primer and Primer [Eurofins Genomic India Pvt Ltd, Bangalore] of *epsA* genes. To the master mix 1 µl of the DNA was added and the amplification was carried out with PCR condition of 55°C as annealing temperature for 35 cycles in Eppendorf thermocycler, Germany.

Using Big-Dye terminator cycle sequencing kit and 3730XL Genetic Analyzer the amplicon product of *epsA* were bidirectional sequenced. The obtained sequences of F and R primers were aligned using Bioedit Sequence Alignment Editor v7.2.5 which were subjected to BLAST (Basic Local Alignment Search Tool) for nucleotide similarity search. With the help of ClustalW software version 1.83 for DNA multiple sequence alignment using default parameters the sequences were aligned. For analyzing *epsA* sequences of *A. baumannii* strain WCHAB005078 (CP027246.2), *A. baumannii* strain KC526901.2 and *A. baumannii* strain 11W359501 (CP041035.1) were used as templates.

RESULTS AND DISCUSSION

Correlation of *epsA* with multidrug resistance; Semi quantitative adherent bioassay for biofilm formation showed 58.9% (43/73) under high grade, 31.5% (23/73) under low grade and 0.9% (7/73) to be negative. Amidst the 43 strains of high-grade biofilm formers all were multidrug resistant (100%; 43/43) exhibiting resistance against more than 3 classes of the antibiotics tested followed by 91.3% (21/23) under low grade biofilm formers. Under the negative biofilm formers, only one strain was drug resistant. Pearson correlation analysis yielded positive value suggesting the correlation of the occurrence of *epsA* gene with drug resistant strains (p-value < 0.05).

From the screened 73 genomes of multi-drug resistant strains of *A. baumannii*, 19.17% (n=14) showed positive amplicons for the *epsA* gene associated with biofilm formation (Figure 1). Correlation of *epsA* occurrence was high (100%; 14/14) in the resistant groups of beta-lactam inhibitors (piperacillin and tazobactam), cephalosporins (ceftazidime, cefepime, ceftriaxone, ceftriaxime) and folate drugs (co-trimoxazole). It was followed by 71.4% (n=10/14) aminoglycosides (amikacin and kanamycin) and 57.1% (8/14) occurrence among the strains resistant to carbapenems (doripenem, meropenem and imipenem). The least occurrence was observed with 14.2% (2/14) among the strains resistant to fluoroquinolones (ciprofloxacin and levofloxacin) and efflux pumps (tetracycline, doxycycline and minocycline) drugs (Figure 2). None of the control susceptible strains have shown positive for *epsA* gene. Figure 3 and 4 show the sequence chromatogram and the multiple sequence alignment of the *epsA* amplicon.

In recent years, *A. baumannii* is considered as a potent nosocomial pathogen and in the past decade there has been

a high prevalence of multidrug resistant clinical isolates in hospitalized patients, ranging from 21–95% (Zhang et al. 2016).

Table 1. Primer sequence and PCR conditions to detect epsA gene in multi-drug resistant strains of *A. baumannii*

Gene of target	Primer details	Annealing temp	Amplicon size
epsA	AGCAAGTGGTTATCCAATCG ACCAGACTCACCCATTACAT	55	451 bp

Figure 1: Electropherogram of epsA gene product of size 451bp in lane 1 and 2 with marker ladder of size 100bp (M)

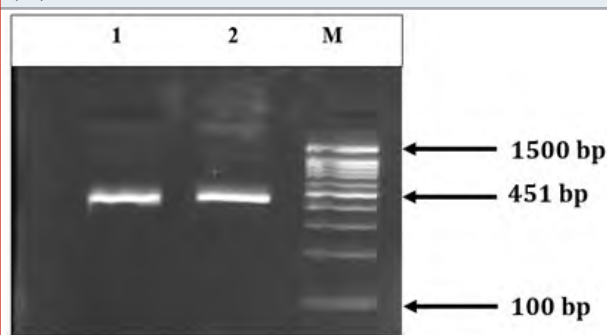


Figure 2: Frequency of epsA gene among different groups of antibiotic resistant strains of *A. baumannii*

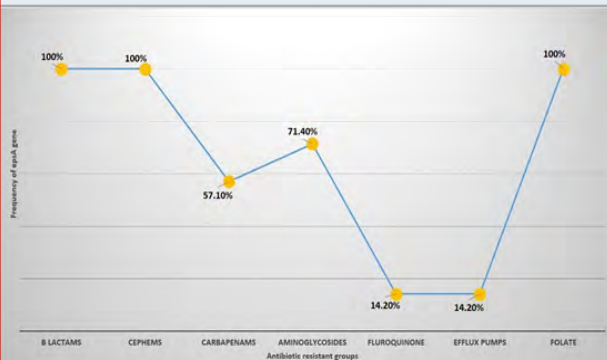


Figure 3: Partial sequence chromatogram of epsA gene from the amplicon of *A. baumannii*

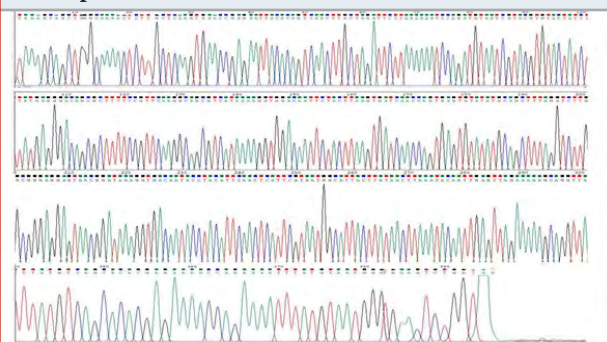


Figure 4: Partial sequence alignment of epsA gene from the present study (Ab) with reference sequences available in the database. The deleted regions are depicted as dashes (--), mismatch as gap () and conserved sequences as star (*).

KC526981.2:A.baumannii	-----TTACGAGCTCACCATTAAGATTCTTGTTTCT	39
Q228_247_3_PCR_EPSA_EPSAF	TAAGCAGCTGGCAAACTCTTGCCTAAGTGAACCGAATTACACA----	55
CP827246.2:A.baumannii	-TAAAGCAGCTGGCAAACTCTTGCCTAAGTGAACCGAATTACACA----	54
CP841835.1:A.baumannii	-TAAAGCAGCTGGCAAACTCTTGCCTAAGTGAACCGAATTACACA----	54
KC526981.2:A.baumannii	CGTGTGCTGACATAAATTTGTTCATTGGTTGCTACAAAAGTTTATGTAATGAATACCT	89
Q228_247_3_PCR_EPSA_EPSAF	TGCTAGATTCTTAAAAAATCCAGATGTAATCGTACGTGTACTA--TCTTATGAGGACAG	113
CP827246.2:A.baumannii	TGCTAGATTCTTAAAAAATCCAGATGTAATCGTACGTGTACTA--TCTTATGAGGACAG	112
CP841835.1:A.baumannii	TGCTAGATTCTTAAAAAATCCAGATGTAATCGTACGTGTACTA--TCTTATGAGGACAG	112
KC526981.2:A.baumannii	GCCTTTTC--TAACCTCAATTGTATTAAAGTTAAGTAATGCCATTACGAATGAGTTGA	156
Q228_247_3_PCR_EPSA_EPSAF	CGCTTTTCCTGTTCAAGGACGCTCATAAAGGTGGACAATTCTACTTAAGTGATCAACCA	173
CP827246.2:A.baumannii	CGCTTTTCCTGTTCAAGGACGCTCATAAAGGTGGACAATTCTACTTAAGTGATCAACCA	172
CP841835.1:A.baumannii	CGCTTTTCCTGTTCAAGGACGCTCATAAAGGTGGACAATTCTACTTAAGTGATCAACCA	172
KC526981.2:A.baumannii	ATGTAGTATTGTGACCTTTATCGGTTACTCTCCGCAAGACCTAAAGCTGACATATACA	216
Q228_247_3_PCR_EPSA_EPSAF	GTGAGTGTATATGACGCTTTAGGCTTTGCGGGAGGAGTAACGGATAAAGGTGACATATACC	233
CP827246.2:A.baumannii	GTGAGTGTATATGACGCTTTAGGCTTTGCGGGAGGAGTAACGGATAAAGGTGACATATACC	232
CP841835.1:A.baumannii	GTGAGTGTATATGACGCTTTAGGCTTTGCGGGAGGAGTAACGGATAAAGGTGACATATACC	232
KC526981.2:A.baumannii	CTCACTGTTGATCATTAAAGTAAATTTGTC-CACCTTTTATGACGCTGCTTGAACGGA	275
Q228_247_3_PCR_EPSA_EPSAF	TACATTCACTCATTGTAATGGCATTACTTATACTTAATACAAATGAGTTAGAAAAA	292
CP827246.2:A.baumannii	TACATTCACTCATTGTAATGGCATTACTTATACTTAATACAAATGAGTTAGAAAAA	291
CP841835.1:A.baumannii	TACATTCACTCATTGTAATGGCATTACTTATACTTAATACAAATGAGTTAGAAAAA	291
KC526981.2:A.baumannii	AAAGCCTGTGCTCTCAAGATAGTACACGTACGATTACATCTGGATTTTTAGAACTCT	335
Q228_247_3_PCR_EPSA_EPSAF	---AGCAGGTTATTCTAT-----TACATAAATTTTATG-----TAC	324
CP827246.2:A.baumannii	---AGCAGGTTATTCTAT-----TACATAAATTTTATG-----TAC	323
CP841835.1:A.baumannii	---AGCAGGTTATTCTAT-----TACATAAATTTTATG-----TAC	323
KC526981.2:A.baumannii	AGCAAGTTGACTGTGTAATTTCTGGTTCCTTGAAGAA-----GTTTGCACGCTGCTTT	392
Q228_247_3_PCR_EPSA_EPSAF	AACCAATGACACAATTATGTGACGACACGAGAAACCAAGAAATTTATGTAATGGGTT	384
CP827246.2:A.baumannii	AACCAATGACACAATTATGTGACGACACGAGAAACCAAGAAATTTATGTAATGGGTT	383
CP841835.1:A.baumannii	AACCAATGACACAATTATGTGACGACACGAGAAACCAAGAAATTTATGTAATGGGTT	383
KC526981.2:A.baumannii	A-----	393
Q228_247_3_PCR_EPSA_EPSAF	AAGTCTGTGTA	385
CP827246.2:A.baumannii	AGTCTGTGTA	383
CP841835.1:A.baumannii	AGTCTGTGTA	383

Assessment on its virulence properties in harsh environmental niches is highly attributed to its ability of biofilm formation. Biofilm associated bacteria is generally associated with two properties namely, the increased synthesis of exopolysaccharides and the development of antibiotic resistance. Increased production of these extracellular polysaccharides (EPS) in *A. baumannii* produces a protective environment causing difficulty in antibiotic penetration leading to the development of resistance (Lewis 2001; Hoiby et al. 2010). The spread of antibiotic resistance is by the ability of the bacterial pathogen to transfer genes horizontally and is commonly associated with biofilm formers (Ghasemi et al. 2018). Amidst

various biofilm associated genes. *epsA* is considered as a potent contributor for its correlation with drug resistance as the exopolysaccharide layers can influence the entry of antibiotics (Donlan and Costerton 2002; Leclercq et al. 2013; Tchuente et al. 2019; Bavelaar et al. 2021).

Thus, the present investigation is undertaken to molecularly assess the same and its correlation of its occurrence among the multi-drug resistant strains in hospital environments. A strong positive correlation of *epsA* and biofilm formation had already been documented, and in various other studies its contribution ranged from 30.2% respectively (Russo et al. 2010). The role of *epsA* in the biofilm formation on abiotic surfaces also reveal the existence of *A. baumannii* amidst various disinfectants and antimicrobial agents aiding in the transfer of resistance genes with the participating strains too (Sung 2018). Many studies reporting the confirmation of its association with the biofilm formation in nosocomial environments, the biofilm formation assay performed in the present study with the routine crystal violet staining method graded the strains as high- and low-grade biofilm formers. The high frequency of *epsA* observed in the present study hypothesizes that it would have aided *A. baumannii* to survive and persist in the hospital habitats, as the gene was detected by PCR from the clinical isolates of the same. This data is again a key fact that this sort of identification of the *A. baumannii* virulence factors periodically would offer more effective ways to eradicate them from the biotic and abiotic surfaces of the hospitals (Kongthai et al. 2021).

Our reports correlate with similar earlier studies associated with the presence of *epsA* genes in drug resistant clinical isolates of *A. baumannii*. Highest frequency of 95% *epsA* gene among MDR strains of *A. baumannii* was reported from Iran. In a similar study by Assaad et al. (2021) had documented 60% of the multi-drug resistant isolates of *A. baumannii* showing positivity for *epsA* (Zeighami et al. 2019; Asaad et al. 2021). In a study by Kongthai et al. (2021) 30.2% of the biofilm producers were *epsA* positive and were also multidrug resistant (Kongthai et al. 2021). In the same study, 86.2% *epsA* gene positive strains were multi drug resistant and the resistance ranged from 50% to 80% against different classes of antibiotics viz., piperacillin, cefixime, ciprofloxacin, levofloxacin, ceftazidime, gentamicin, ticarcillin and imipenem. In a study by Safari et al. (2015) 38% of the isolates encoded *epsA* gene with strong biofilms (Safari et al. 2015). In a similar study conducted by Sung et al 30% of the multi-drug resistant clinical isolates of *A. baumannii* showed the presence of *epsA* (Sung 2018). The present study also documents the frequency of *epsA* gene in different groups of antibiotics routinely employed against *A. baumannii* in hospital set-ups globally (Pompilio et al. 2021).

Results of the study showed high correlation of *epsA* with beta lactam, cepheims and folate resistant isolates, and may be related to the presence of *epsA* gene in all the resistant isolates. This is higher when compared to the study by Badave et al. (2015) where the *epsA* positive strains were 55.5% resistance to ampicillin and 42.6% resistance to piperacillin (beta lactams), 52.08% were resistant to

ceftazidime, and 50% were resistant to ciprofloxacin. In contrast, a study in South India, biofilm positive *Acinetobacter* showed resistance to both ceftazidime (95%) and cefepime (95%) (Badave 2015; Zeighami et al. 2019; Asaad et al. 2021).

The same study also had documented *epsA* positive biofilm formers showing 85% resistance to ciprofloxacin. Carbapenem resistance in *A. baumannii* is based on the production of carbapenemases or synergistic effects between carbapenemases, porin modifications or loss or modification of the penicillin-binding proteins (Towner et al. 1991; Poirel and Nordmann 2006). In our study, *epsA* mediated biofilm formers showed on 57.1% resistance to carbapenem group of drugs which is correlating with the study conducted by Zeighami et al. (2019), where 100% of isolates showed resistance to imipenem and this result is consistent with observations reported from various parts of the world which explain the high risk of failure of carbapenem treatment in *A. baumannii* infections but yet another study showed only 38.4% were resistant to imipenem (Rahbar et al. 2010; Peerayeh and Karmostaji 2015; Badave 2015; Saffari et al. 2017).

Overexpression of efflux pumps had been postulated to be a first step towards the development of a MDR phenotype in bacteria and most of them lead to the resistance to tetracyclines. A strong association of *epsA* genes was observed in the present study with 14.2% of the strains showing resistance against the cycline group of drugs. The present study observed the correlation of *epsA* genes with 100% resistant strains of drugs involved with folate pathway with the lowest occurrence of 14.2% among the strains resistant to fluoroquinolones. Similarly, correlation of *epsA* occurrence (71.4%) in aminoglycoside resistant strains of *A. baumannii*, correlates with the earlier study with 70% frequency and higher when compared to 53.4% amikacin resistant isolates in another study (Bambeke et al. 2000; Piddock 2006; Kongthai et al. 2021).

CONCLUSION

The findings of the present study investigated the presence of biofilm-forming *epsA* gene among the clinical isolates of *A. baumannii*. In spite of the effective infection management, it's thus alarming to note the augmented prevalence of biofilm-associated *epsA* gene among the multidrug-resistant clinical isolates of *A. baumannii*. Thus, it would be timely to suggest the periodical monitoring of the hospitals and laboratories for the presence of *epsA* mediated biofilm producing resistant groups of *A. baumannii*. This would also aid in designing new vaccines and drugs for the prevention and treatment of *A. baumannii* infections.

Conflict of interests: Authors declare no conflict of interest to disclose.

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Agricultural Communication

Investigations on the Source Material of Oat Seeds, *Avena sativa* through the Multidimensional Ranking Method in Natural Conditions of Yakutia, Siberia, Russia

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ABSTRACT

Eighty-three collection samples of oat seeds (*Avena sativa* L.) of various ecological, geographical, and breeding origins were studied in the conditions of Central Yakutia during 2017-2019 by the method of multidimensional ranking according to six economically valuable characteristics, namely, the duration of the growing season, grain yield, grain weight from the plant and panicles, the weight of 1.000 grains, and yielding tillering capacity. According to the results of the multidimensional ranking, the varieties were divided into three groups: the best, average, and worst. At that, from the data entered for 83 samples, the program determined priorities based on a combination of the duration of the growing season and yield. The group of best samples, based on a combination of economically valuable features, included 63% of samples from Europe, 30% from Russia, and 7% from Asia. The main share in the average group was made up of samples from Europe (63%), Russia (33%), and Asia (4%). The local zoned variety – Pokrovsky standard is included in the average group with a rank limit of 118.8. The worst group included the most samples from Europe (41%), Russia (26%), America (26%), Africa (3.7%), and Asia (3.7%). According to the precocity, 11 samples were identified that ripened earlier than the standard by 7-11 days. These are K-15062 (Omsk Region), K-15108 (USA), K-15111 (Colombia), K-15184 (Kemerovo Region), K-15191 (Slovakia), K-15357 (Norway), K-15375, K-15416, K-15418 (Germany), K-15392 (Sweden), and K-15408 (Belarus). Samples with high grain yield were included in the group of the best varieties. Among the selected varieties, cultivars K-15293 from Poland and K-15415 from Germany had the most stable yield over the years.

KEY WORDS: AGRONOMIC VALUABLE CHARACTERS, GRAIN YIELD, PRECOCITY, SEED OATS, SOURCE MATERIAL.

INTRODUCTION

Grain production for food and feed purposes remains one of the priority areas in agriculture and crop production. Oat crops in Yakutia occupy 57% of the total area (Sakha Yakutia Stat 2020). The demand and market attractiveness

of oats as a universal use crop has been growing in recent years. The Oats acreage occupies fifth place in the world after wheat, rice, corn, and barley. Grain is used for animal feed, in human nutrition, as well as a food raw material (Arendt and Zannini 2013; Loskutov and Polonsky 2017). Continuous improvement of grain varieties is possible only if there is a gene pool, which is represented by a wide range of samples from the collection of the Vavilov Institute of Plant Genetic Resources (VIR) (Bespalova 2015; Dzyubenko 2015; Petrova 2020).

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Received 17/10/2021 Accepted after revision 25/12/2021

Published: 31st December 2021 Pp- 1666-1672

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.44>

At that, direct use of the gene pool is difficult, as a rule, due to the low adaptability of the material and the predominance of recombinants with low yields in the offspring, which were rejected at the first stages of the selection process (Friedrich et al. 2014; Singh and Kumar 2016). To create new varieties with a complex of valuable characteristics, high yield, and high quality of products in various environmental conditions, the well-studied source material was required (Petrova 2018). The ideal genotype should work well for many years both in one place (certain environmental conditions) and in a wide range of environments formed by external conditions in different geographic areas (Romagosa and Fox 1993; Petrova 2020).

The identification of new genetic factors that determined the high degree of manifestation of agronomic valuable characters in the selected samples of the collection will contribute to the creation of varieties with the required parameters (Randhawa et al. 2013; Petrova 2020). To successfully overcome objectively emerging barriers, it is necessary to systematically conduct selection work to find new genotypes with a complex of agronomic valuable characters, which largely depends on the correct selection of the source material and its diversity (Lubnin 2006). Therefore, the introduction of species and varietal diversity of oats of various geographical and selection origins in a specific natural and climatic zone is of great scientific and practical importance. This issue is most relevant in the extreme conditions of Central Yakutia. The analysis of the natural and climatic conditions of Central Yakutia shows that agriculture in this zone develops in peculiar and more extreme conditions than in other regions not only of Russia but also worldwide. The climate of Central Yakutia is sharply continental: low temperatures in winter (the absolute minimum is 640C) with weak winds sharply contrast with high air temperatures in summer (the absolute maximum is 380C) (Toropov et al. 2020).

Only 210 mm of precipitation falls per year, including during the summer period (Gavrilova 1973). The ranking of collection samples according to the combination of agronomic valuable characters was carried out using the Snedecor computer program (Sorokin 2004). Ranking of collectible varieties of oats according to the set of characteristics using the algorithm developed by Yuzhakov and Sorokin (2000) allowed dividing them into three groups: the best (the sum of the ranks equal to 144.0-149.2) average (137.0-117.1), and worst (107.3-26.0). Ranking allowed to order the factors according to the degree of increasing or decreasing their influence on the social phenomenon of interest to the researcher (Korobov 2005). According to Montel (1998), there have never been two completely identical opinions or one grain was similar to another. For data processing, a ranking operation was used, which consisted in the fact that the observed values of a random variable were arranged in ascending order (Montel 1998; Yuzhakov and Sorokin 2000; Korobov 2005; Toropov et al. 2020).

The purpose of the research was to identify valuable collection samples of seed oats (*Avena sativa* L.) adapted

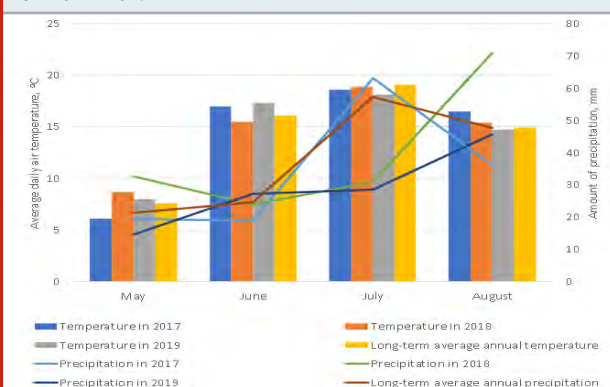
to the conditions of Yakutia, followed by the selection of promising (best) cultivars as parent forms for hybridization by the multidimensional ranking method.

MATERIAL AND METHODS

The research was carried out during 2017-2019 in the Pokrovsky Division of the Yakut Research Institute of Agriculture in the territory of the Khangalassky ulus of the Republic of Sakha (Yakutia), located in the middle taiga zone. At that, 114 collection samples were studied, among which later the most promising 83 samples were selected from the VIR and foreign selections. The main portion of the collection (50.6%) was made up of samples from Europe. Varieties from Russia occupied 35%. From the foreign assortment, varieties from America (9.6%), Asia (3.6%), as well as Africa (1.2%) prevailed. The most fully studied characteristics were precocity (duration of the growing season), grain yield, grain weight from a plant, grain weight from a panicle, weight of 1.000 grains, and yielding tillering capacity of plants. According to the data of the weather station in the Pokrovsk town of the Khangalassky ulus, Republic of Sakha (Yakutia), during the years of research (2017-2019), the growing season was characterized by insufficient moisture.

The sum of active air temperatures above +100C from sowing to the ripening of oats varied from 1688 to 1801, the amount of precipitation ranged from 116.6 to 144.0 mm. The lowest precipitation was in 2019 (HI (Hydrothermal Index) =0.65), more humid weather was in 2017 and 2018 (HI=0.77-0.81) (Fig. 1). In the first decade of May, (2017) average daily air temperatures were quite low equal to 3.3oC. The minimum temperature dropped to -2.8oC. The average monthly temperature in May was 6.1oC, which was lower than the average annual norm by 1.5oC. In general, 2017 was characterized by insufficiently humidified weather conditions. In June, when the vegetative mass of oats intensively increases (plants undergo the main phases, such as stem elongation, earing, formation of inflorescences), the moisture demand was greatest, however, the water supply of plants at elevated temperature conditions, lack of air humidity, and the dry hot wind was often insufficient.

Thus, 137 mm of precipitation fell from May to August, which was 0.9 times lower than the average annual norm (151 mm). The bulk of precipitation fell in July 63.1 mm, with an average annual norm of 57.3 mm. Current weather conditions led to an increase in the duration of the earing period – the wax ripeness of the samples. This resulted in a strong proliferation (sprouting), which greatly complicated the harvesting and reduced the quality of grain. June and July were warm – the average monthly air temperature was 17.8oC. The growing season of 2018, in general, can be characterized as quite favorable for oats growth and development. The average monthly temperature in May was 8.7oC, which was 1.1oC higher than the average annual norm. From May to August, precipitation amounted to 158 mm, which was 1.05 times higher than the average annual norm (151 mm).

Figure 1: Average daily air temperature and precipitation for 2017-2019

In May, precipitation for the month was 1.5 times higher than normal (33 mm versus 21 mm). Precipitation for July was 1.8 times below normal (31 mm with an average annual norm of 57 mm). In August, precipitation for the month was 1.5 times higher than normal (71 mm vs. 47 mm). The meteorological conditions of 2019 can be described as unfavorable for grain crops' growth and development. In May, precipitation for the month was 0.7 times lower than normal (21.3 mm versus 14.6 mm). The first and second decades of June were characterized by a severe drought, the amount of precipitation was 3.2 mm, which was 18.8 mm lower than normal. The third decade of June was rainy, the amount of precipitation exceeded the average annual norm by 3.1 mm. August was relatively cool. The monthly amount of precipitation was 45.7 mm, with a norm of 47.6 mm.

Table 1. Ranking of collectible varieties of oats by agronomic valuable characters (average for 2017-2019)

Group of the best objects		Group of the average objects		Group of the worst objects	
No. of the VIR catalog	Sum of ranks	No. of the VIR catalog	Sum of ranks	No. of the VIR catalog	Sum of ranks
15392	144.0	15287	137.0	15258	107.3
15357	143.7	15341	122.9	15390	119.8
15125	147.1	15348	148.7	15384	93.0
15336	168.7	15184	142.8	15376	112.1
15330	179.5	15278	130.9	15093	78.3
15342	137.6	15353	142.3	15249	106.4
15426	127.5	15335	149.4	15320	98.8
15380	159.1	15182	117.9	15428	92.0
15418	130.4	15420	129.2	15391	98.5
Nilola	160.7	15377	132.2	15064	105.2
15333	137.4	15279	129.2	15372	92.3
15180	146.8	15240	126.5	15111	86.5
15134	124.4	15425	121.3	15393	70.1
15186	118.7	15053	125.6	15394	60.2
15281	161.9	15412	95.2	15410	72.9
15293	153.2	15328	107.0	15267	88.5
15395	122.8	St Pokrovsky	118.8	15339	60.9
15291	143.0	15298	109.2	15264	80.1
15383	157.9	15417	64.3	15108	70.4
15415	143.8	15234	123.9	15006	64.2
15378	144.5	15069	94.9	15100	56.8
15421	144.2	15248	122.7	15062	43.7
15106	125.5	15416	110.4	15256	45.2
15294	134.3	15419	102.2	15121	38.9
15423	142.4	15301	105.4	15382	34.1
15275	150.0	15375	113.3	15408	34.9
15283	149.2	15338	117.1	15318	26.0

The agrochemical properties of the soil were determined using generally accepted methods in the laboratory of biochemistry and mass analysis of the Yakut Scientific Research Institute of Agriculture using the Infranid-61

infrared analyzer. The soil of the experimental plots was permafrost taiga-pale yellow, medium-loamy in mechanical composition with a humus content of 3-6% in the upper five-centimeter layer. With a depth, this indicator decreases

to 1-1.5%. The mobile phosphorus content according to Egner-Rome was 10.43 mg/100 g of soil, the exchange potassium according to Maslova was 27.4 mg/100 g of soil.

The reaction of the aqueous extract was alkaline throughout the profile, the area of 7.11-7.55, the hydrolytic acidity of the soil was 0.84-0.98 mg/eq. per 100 g of soil.

Table 2. Results of evaluation of collecting samples of oats by precocity

No. of VIR catalog	Variety	Vegetation period, days		
		average	±to standard	min-max
Standard	Pokrovsky	76		75-78
15111	Colombia	65	11	60-68
15062	Omsk region.	65	11	60-69
15418	Germany	67	9	63-74
15408	Belarus	67	9	60-73
15108	USA	68	8	65-71
15184	Kemerovo region	68	8	60-76
15357	Norway	68	8	65-71
15375	Germany	68	8	65-71
15191	Slovakia	69	7	66-74
15392	Sweden	69	7	67-73
15416	Germany	69	7	65-71

The type of salinity was sulfate-chloride (up to 49.1%). The composition of salts was dominated by sodium salts. Field experiments were based on one-factor experience, according to the generally accepted methods of field experience by I nthe previous studies (Dospekhov 1985).

Observations, estimates, and records of the harvest in collection nurseries were carried out according to the methodological guidelines for the study of the world collection of barley and oats (Methodological guidelines for the study of the world collection of barley and oats 1981) and the CMEA (Council for Mutual Economic Assistance) international classifier for *Avena sativa* L. genus. Mathematical data processing was carried out using an application software package developed by O.D. Sorokin (Sorokin 2004). The Pokrovsky variety was sown as a standard. In the experiment, manual sowing was used. The plot area of one square meter was placed on a complete fallow in a single repetition. The work was carried out taking into account the methodological guidelines of the VIR. After harvesting, the yield structure was analyzed.

RESULTS AND DISCUSSION

All six features were evaluated together, and the boundaries of the sum of ranks were determined, according to which the size of the group could be changed by the user towards decreasing from 1/3 of the total number of objects since three groups of ranks were determined, namely, the best, average, and worst (Sorokin). This was the peculiarity, novelty, and value of the statistical analysis of the multidimensional ranking of sown oats according to agronomic valuable characters. The cultivars that fall into the best group was used as parent forms for the selection of hybridization. The group of the best samples, based on the comprehensive agronomic valuable characters included 63% of samples from Europe, 30% from Russia, and 7% from Asia. The

main portion in this group was made up of samples from Europe (63%), Russia (33%), and Asia (4%). Local zoned grade –Pokrovsky standard was included in the average group with a rank boundary of 118.8 (Table 1). The worst group had the most samples from Europe (41%), Russia (26%), the USA (26%), Africa (3.7%), and Asia (3.7%) (Dzyubenko 2015; Toropov et al. 2020).

One of the most important features that determine the degree of adaptability of a crop to specific growing conditions was precocity. The duration of the growing season was the most important biological characteristic that reflects the interaction of the genotype of the variety and the environment. Considering the growing season as the main moment in plant selection, Vavilov (1935) pointed out that it was associated with many properties that determine the resistance against frost, drought, diseases, pests, and improvement of grain quality. In case of drought and a short growing season, the best type of plant was the one that may not have very high yield potential but was most resistant in all respects to adverse environmental factors. At the same time, the tougher the environmental factors, the more the selection possibilities were narrowed. In the context of a short northern summer, precocious varieties are needed, while against the background of drought – drought-resistant ones (Vasiliev 2000; Ivanov and Ivanova 2012; Toropov et al. 2020).

Precocious forms were characterized by a lower productivity potential compared to medium-ripened ones (Sinitsyn 1970). The duration of the growing season over the years of research varied on average from 60-79 days or more. Thus, 11 cultivars were identified, which turned out to be the most precocious. These were cultivars K-15111 from Colombia and K-15062 from the Omsk Region (11 days), K-15418 from Germany and K-15408 from Belarus (9 days), K-15108 from the USA, K-15184 from the Kemerovo

Region, K-15357 from Norway, K-15375 from Germany (8 days), K-15191 from Slovakia, K-15392 from Sweden, and K-15416 from Germany (7 days) (Petrova 2020).

The duration of the main interphase periods of oats (sprouting – sweeping, and sweeping – ripening) varied depending on the variety. In July, oats were forming and filling grain. At this time, with sufficient moistening, a moderate temperature regime was extremely necessary. The analysis of the duration of the germination-earing

period showed that the shortest period before earing in the collected samples was in 2019, on average, 27 and 33 days for various samples. The increased sum of temperatures (1801.2) in May, June, and July contributed to rapid ear formation. In (2017), the largest number of samples had an interphase period of ear formation – maturation lasting 48-53 days. The lowest indicator of the second period (45-47 days) is typical for precocious cultivars (Dzyubenko 2015; Toropov et al. 2020).

Table 3. Characteristics of varietal samples of spring oats, distinguished in collection Nurseries in terms of grain yield (average for 2017-2019)

No. of VIR catalog	Origin	Grain yield, g/m ²	Grain weight per plant, g	Grain weight per panicle, g	Weight of 1,000 grains, g	Yielding tillering capacity, itm.	Growing season, days
Pokrovsky	Yakutia	180	3.5	1.9	32.9	2.4	76
15392	Sweden	325	4.5	1.6	36.6	3.8	69
15357	Norway	322	5.0	1.4	36.1	3.8	68
15125	Ukraine	288	4.2	1.8	38.4	3.5	72
15336	Altai Territory	271	2.9	2.0	46.4	3.7	76
15330	Ulyanovsk region	263	5.0	2.0	39.2	4.3	74
15342	Buryatia	286	4.5	1.6	34.1	3.4	73
15426	Germany	299	3.3	1.5	40.2	2.2	72
15380	Germany	264	5.2	1.9	40.0	3.3	72
15418	Germany	283	3.8	1.6	39.8	3.1	67
Nikola	Kazakhstan	261	4.5	2.0	37.3	3.5	75
15333	Ulyanovsk region	273	4.1	2.0	38.3	2.8	71
15180	Ulyanovsk region	264	4.5	1.8	35.5	3.9	70
15134	Czech	274	4.0	1.7	37.6	2.6	70
15186	Kirov region.	279	3.9	1.6	35.1	3.0	72
15281	Moscow region.	250.3	4.6	1.9	42.1	3.3	74
15293	Poland	251	4.4	1.5	37.5	3.8	76
15395	Sweden	267	3.3	2.0	35.4	2.3	72
15291	Poland	260	4.3	2.1	36.8	3.2	73
15383	Ukraine	244	4.4	2.1	37.9	3.2	76
15415	Germany	247	4.4	1.6	39.7	3.0	74
15378	Germany	246	4.4	1.6	42.3	3.3	73
15421	Germany	241	4.3	2.0	43.2	2.5	72
15106	Portugal	246	3.9	1.5	38.5	3.6	71
15294	Poland	243	2.9	1.7	38.2	2.0	77
15423	Germany	237	4.0	2.4	43.2	2.7	71
15275	Kirov region	228	4.9	1.6	32.3	4.6	74
15283	Tyumen region	223	5.6	1.6	37.5	3.6	74

Due to heavy rains in July, abundant tillering began and the duration of the ear formation – maturation phase lengthened. The zoned Pokrovsky variety was subject to less fluctuation in the duration of the growing season, showing stability in all years (Table 2). The selected samples was used as sources of precocity in the selection process when creating varieties that reliably ripen in the natural conditions of Yakutia (Loskutov and Polonsky 2017; Petrova 2020).

One of the most important characteristics of any variety was its yield (Korobov, 2005). It was quite difficult to combine high productivity and precocity in one genotype. According to the evaluation results obtained by the method of multidimensional ranking, 27 varieties were distinguished in terms of the highest grain yield. These are samples from Germany, the Ulyanovsk Region, the Altai Territory, the Moscow Region, the Novosibirsk Region,

the Czech Republic, the Kirov Region, Poland, Ukraine, Portugal, the Tyumen Region, whose yield was 11-66% higher than the Pokrovsky variety standard. On average, over the years of research, the highest grain yield (299-325 g/m²) was shown by samples from Sweden (K-15392) and from Germany (K-15426), which is higher than the standard by 119 g/m². The lowest yield (26-37 g/m²) was noted in cultivars from the USA (K-15417 and K-15093), Ukraine (K-15382), and Poland (K-15248, K-15294, K-15248). Among the selected cultivars, K-15293 from Poland and K-15415 from Germany had the most stable yield over the years (Petrova 2020; Toropov et al. 2020).

The grain weight from the plant varied from 1.7 g in the sample from the USA (K-15256) to 5.2 g in the variety from Germany (K-15380). The largest weight of grain from a plant of the best group was shown by samples from Norway (K-15357), the Ulyanovsk Region (K-15330), Germany (K-15380), and the Tyumen Region (K-15283). The weight of 1,000 grains of early-maturing oat samples varied from 25.2 to 42.0 g. On average, over the years of research, the largest grain (40-46 g) was formed by samples K-15426, K-15380, K-15378, K-15421, and K-15423 from Germany, K-15281 from the Moscow Region, and K-15336 from the Altai Territory.

The highest yielding tillering capacity was observed in a sample from Germany K-15418 (3.6 g). On average, during the three years of research, five cultivars were the most productive in terms of yielding tillering capacity: K-15240 from the UK (4.7 g), K-15275 from the Kirov Region (4.6 g), K-15330 from the Ulyanovsk Region (4.3 g), K-15248 from Poland (4.4 g), and K-15420 from Germany (4.2 g). In general, in terms of the best yielding tillering capacity, the following varietal types can be distinguished by the method of multidimensional ranking: precocious samples K-15357 from Norway, K-15281 from the Moscow Region, K-15418, K-15375, and K-15416 from Germany, K-15408 from Belarus, K-15111 from Colombia, K-15062 from the Omsk Region, K-15108 from the USA, K-15184 from the Kemerovo Region, K-15191 from Slovakia, and K-15392 from Sweden; cultivars, combining precocity and yield: K-15418 from Germany, K-15357 from Norway, and K-15392 from Sweden; cultivars with a high yield of grain and its main elements from the best group: K-15392, K-15395 from Sweden, K-15357 from Norway, K-15125, K-15383 from Ukraine, K-15336 from the Altai Territory, K-15330, K-15333, K-15180 from the Ulyanovsk Region, K-15342 from Buryatia, K-15378, K-15426, K-15380, K-15415, K-15418, K-15421, K-15423 from Germany, Nikola from Kazakhstan, K-15134 from the Czech Republic, K-15186, K-15275 from the Kirov Region, K-15281 from the Moscow Region, K-15291, K-15293, K-15294 from Poland, K-15106 from Portugal, and K-15283 from the Tyumen Region (Loskutov and Polonsky 2017; Petrova 2018; Petrova 2020).

CONCLUSION

The findings of the present study confirmed that one of the main indicators of the density of a productive stem is considered to be yielding tillering capacity. The productive

stems formation is influenced by many factors. Employing the multidimensional ranking method in the analysis of grain yield and its structural elements allows identifying promising varieties of oats at the early stages of the selection process.

ACKNOWLEDGEMENTS

The study has been performed within the framework of a state assignment "Fundamentals of the creation of farming systems and agricultural technologies based on the preservation of the gene pool, selection of hybrids and varieties of a new generation of agricultural crops, their protection from harmful organisms, the preservation and reproduction of soil fertility in conditions of Cryolithozone of Yakutia" (registration number 0821-2019-0004).

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Biotechnological Communication

Antimicrobial Activity of Metabolites Extracted from Marine Actinomycetes

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ABSTRACT

Actinomycetes are free-living bacteria that are widely distributed and found in several habitats. These bacteria are essential organism in soil system, they contribute to agroindustry as the origin of active compounds. Their economical and biotechnological importance lies in the production of bioactive secondary metabolites including anticancer, insecticides, and antibiotic agents, such Actinomycetes-derived agents have been commonly used in both medical and industrial fields. Mainly, different Actinomycetes species isolated from coastal habitats are found to be novel sources of antibiotics. Thus, further investigating Actinomycetes will provide a better understanding of the physiological features and chemical composition of marine Actinomycetes. It also enables to use of large synthetic libraries of derived molecules (e.g., secondary metabolites) to develop biological drugs to combat advanced bacterial infections. Actinomycetes can produce more powerful biological compounds of medicinal and economic importance; moreover, it can provide insight into new antibiotics against different types of pathogens that cause infection to humans and support human health by overcoming complications caused by pathogenic bacteria and drug resistance. In particular, Actinomycetes of marine origin are a promising source of biomedical microbial products and natural products with an interesting microbial activity against many other pathogenic causing microorganisms. They are diverse in nature and have unique chemical compositions. During the past years, many new anti-microbial agents were discovered and deemed powerful therapeutic agents. The discovery of bioactive compounds continues to increase. However, the underlying potential of Actinomycetes has yet to be found. Therefore, this work conducts a review of the antimicrobial activity of metabolites extracted from marine Actinomycetes.

KEY WORDS: ACTINOMYCETES, ANTIBIOTIC, APPLIED MICROBIOLOGY, MARINE ENVIRONMENTS, METABOLITES.

INTRODUCTION

Marine environments contain enormous biological diversity. It is now thought to be a main source of a variety of pharmacologically and bioactive metabolites, among which actinomycetes are the most important source of natural bioactive compounds, which represent the storage pool for bioactive compounds with antibacterial, antiviral, anti-inflammatory, antimalarial, or antitumor activity. The rapid emergence of drug-resistant pathogens has led to the demand for new antimicrobial compounds. Drug-resistance

pathogens are considered a threat to public health globally, which poses an urgent need for developing new antibiotics to fight pathogens and help reduce emerging infectious diseases. Although developing new drugs through using combinatorial libraries of molecules, natural compounds and bioactive microbial metabolites are potent sources of bioactive scaffolds that are recognized as the basis for antibiotics development (Elmallah et al. 2020; Ghosh et al. 2020).

There are 30 phyla in the domain bacteria, the largest phylum is Actinobacteria with 6 classes, 16 orders, 14 suborders, 63 families, and more than 370 genera (Barka et al. 2016). It plays an essential role in biotechnology, producing secondary bioactive metabolites for pharmaceutical, medical and agricultural applications, they are being evaluated

Article Information:*Corresponding Author: najlabio@hotmail.com

Received 19/09/2021 Accepted after revision 25/12/2021

Published: 31st December 2021 Pp- 1673-1679

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.45>

for biofuel production and the compounds used in the development of plastics, detergents, and food preservatives/additives (Valliappan et al. 2014; Jagannathan et al. 2021). Actinomycetes are a group with filamentous morphology, they are Gram-positive bacteria and characterized by high C+ G genome. They are dominating marine ecosystems such as estuaries, seaweed meadows, and mangrove forests (Chaudhary et al. 2013; Shamikh et al. 2020; Jagannathan et al. 2021).

In recent decades, the antimicrobial activity of metabolites considered one of the hotspot areas in marine environments research. That's due to the diversity of marine ecosystems on microbial, metabolic, and genetic levels (Liu et al. 2010; Jose and Jha 2016). Many factors play a crucial role that affect the variety of living organisms in the marine environment, such as nutrients, temperature, salinity, and tidal movement. Water occupies the majority of the earth's surface (i.e., up to 97% of the) and holds 87% of undiscovered living organisms, making it distinguished by its enormous biodiversity, which attracts scientists and researchers' interest (Bérdy 2012; Duncan et al. 2015). The new discoveries of marine bacteria from ecosystems significantly support identifying new biologically active secondary metabolites, which increase biodiversity importance on how it can provide an extensive reservoir of potentially active compounds (Gozari et al. 2018; Suresh et al. 2020). Actinomycetes are rich sources of several bioactive compounds of biomedical prominence worldwide. They manifest a high physiological diversity and produce various bioactive independent reported compounds, antibiotics, antioxidants, and other critical pharmaceutical products (Mahapatra et al. 2019; Shamikh et al. 2020; Mani et al. 2021).

Furthermore, Actinomycetes are a valuable source of unique chemicals, secondary receptors, and new therapeutic compounds (Longnecker and Kujawinski 2020; Safaei et al. 2021). In a recent work, ninety-seven new species of Actinomycetes were found in the marine environment between (2013) and (2017). The marine Actinomycetes ecological systems are broadly classified, includes genera consisting of Streptomyces, Actinomyces, Arthrobacter, Corynebacterium, Frankia, Micrococcus, and various others. Importantly, almost 1% Actinomyces have been detected, classified, studied, and documented. From the 500,000 of biological source-derived-natural compounds that have been discovered worldwide, only 14% are derived from bacteria and fungi, and about 30% of them are Actinomycetes where 60% of utilized antibiotics are derived from Streptomyces (Rasool and Hemalatha 2017; Subramani and Sipkema 2019; Jagannathan et al. 2021).

The overspread of emerging infectious diseases and human pathogens resistant to many drugs have been an imminent threat to global health (Khan and Khan 2016). As a result, there has been a paramount need to discover biologically effective medicinal drugs and natural products relevant to meet these challenges. This review summarizes the diversity of Actinomycetes and bioactive compounds of marine Actinomycetes and their roles on medical and industrial fields (Elmallah et al. 2020; Safaei et al. 2021).

Characterization of marine metabolites: Marine organisms have received great popularity and attention among scientists for their enormous potential and ability to many bioactive compounds (Mahajan and Balachandran 2012). Actinomycetes have been reported as the leading and determinant producer of bioactive compounds for marine ecosystems. As such, peptides, terpenes, polyketides, and alkaloids (Dalisay et al. 2013; Pimentel-Elardo et al. 2010; Abdelfattah et al. 2019; Khalifa et al. 2019; Mayer et al. 2021).

They have various important bioactivities, such as antibacterial, antioxidants, anticancer, antifungal, and antialgae, antibiotics, anti-oncological agents, and many effective compounds are pharmaceutical with diverse biological activities. With new advances in sequencing technologies, marine organism-associated Actinobacterial 16S rRNA gene sequences are stored in NCBI database (Pimentel-Elardo et al. 2010; Valliappan et al. 2014; Abdelfattah et al. 2019; Khalifa et al. 2019; Elmallah et al. 2020; Guimarães et al. 2020; Mayer et al. 2021).

Bioactive Constituents of Marine Organisms:

Polyketides: Recently, novel aromatic polyketides were identified from the aquatic sponges are include strains of Saccharopolyspora and Streptomyces. One major source of aromatic polyketides is the sponge-associated Actinomycetes. Thence, from about 15 South China Sea sponges, a total of 77 Actinomycetes were collected. The isolated Actinomycetes phylogenetic was mainly based on gene sequencing (i.e., 16S rRNA), which facilitated their classification into 12 families and 20 genera. According to that, Marihabitans, Polymorphospora, and Streptomonospora were isolated for the first time from the marine sponges and considered 'rare genera'. Remarkably, the gene β -ketoacyl synthase (KS α) was utilized to assess the possibility of the Actinomycetes strains producing aromatic polyketides (Mani et al. 2021).

Alkaloids: Altemicidin is a monoterpene alkaloid, and it has an antibacterial activity that was isolated from Streptomyces species. Streptomyces and Micromonospora have the main antibacterial activity. Between these, there are five groups of Streptomyces, that are flavus, albosporus, roseosporus, viridis, and hygrosopicus. Almost 65% of all isolated antimicrobial, have an antimicrobial role in inhibition Gram-positive bacteria activity. And 47% were against Gram-negative bacteria, while 32% inhibit both bacteria (Mani et al. 2019; Mani et al. 2021).

Peptides: Bioactive peptides have presently been isolated and characterized from a variety of natural and processed foods. Within the biological process, peptides work as possible physiological modulators during intestinal absorption. Their release is sustained by their structure and their organic compound sequence. Overall, the bioactive peptides are found to own nutraceutical capabilities and promote human health. Properties have got a lot of publicity throughout the human health community, such as Antihypertensive and antimicrobial property. Distinct purified peptides that have been isolated from phyla like Porifera and Craniata have cytotoxic impacts on different

cell lines, such as pancreatic and breast cell lines cell line (Lazcano-Pérez et al. 2012; Pujiastuti et al. 2019). In addition, Apratoxin A, coibamide A, and lyngbyabellin B that have been isolated from *Leptolyngbya* sp and *Lyngbya majuscula*, respectively, are also showed cytotoxic impact against human HeLa cervical carcinoma cells (Khalifa et al. 2019; Suresh et al. 2020).

Recently, a total of 4 cyclic peptides- ogipeptins of secondary metabolites were isolated from *Pseudoalteromonas* with hydrophobic patterns and recognized as acylated cyclic peptides. Four ogipeptins showed potent antibacterial activity against *Escherichia coli* and slightly weaker activity against *Staphylococcus aureus*. It was also found that ogipeptins could block binding of cell surface receptor CD14 and lipopolysaccharide (LPS) and inhibit the discharge of tumor necrosis factor- α (TNF- α) caused by LPS (Wang et al. 2018; Suresh et al. 2020).

Terpenes: Terpenes are hydrocarbons that belong to a comprehensive broad family of natural compounds that include primary and secondary metabolites biosynthesized metaphorically from five-carbon isoprene units. Also, auxarthonoside, which consider a novel triterpene glycoside with a rare sugar moiety in nature, it has been discovered in the marine sponge-derived fungus *Auxarthron reticulatum* (Jiang et al. 2019; Núñez- Pons et al. 2020).

Terpenes are one of the most researched classes of molecules found in natural products. In addition to Terpenoids derivatives terpenes (MOU1). Depending on how many isoprene units they produce, they have sometimes named isoprenoids, also known as hemi-, mono-, etc. Antiviral, antibacterial, anticancer and anti-inflammatory activity are among the properties of marine diterpenoids, a diverse and promising class of terpenes (Ciaglia et al. 2017). Sesquiterpenoids have the molecular formula $C_{15}H_{24}$ and are made up of three isoprene units. They can be acyclic or contain rings, and they appear in several different configurations. Due to a longer chain and an extra binary link in sesquiterpene precursors, cyclic sesquiterpenes are more common than cyclic monoterpenes. Monoterpenes are made up of two isoprene units formed by a single condensation process (Avila et al. 2020). Red algae that live in the shallow waters of the polar regions produce polyhalogenated monoterpenes. These multi-faceted metabolites are frequently found in high concentrations and can be linear or cyclic in most common assumptions. They serve as a defense mechanism herbivory and providing resistance to fouling species. These compounds could also be used offensively, providing producers with allelopathic competitive advantages while harming potential competitors for space and resources (Núñez- Pons et al. 2020).

Antimicrobial activity of metabolites extracted from marine Actinomycetes: The increase in health problems worldwide has increased the demand for treatments that could rapidly solve these problems. Conventional treatments are an undesirable choice due to cytotoxicity, side effects, the long-term effect of use. There has been a new shift for researchers to focus on natural resources to discover biologically active molecules that deem effective

and safer for human use. Actinomycetes are a natural mine for many new secondary metabolites of new chemical compounds. They are found in many aquatic environments, including marine sediments and coral reefs (Xu et al. 2014; Avila et al. 2020).

Marine environments and swampy areas have become a crucial source in discovering novel bioactive natural products and biological variety. Consequently, as the use of therapeutic novel compounds increases, researchers started exploring oceans for bioactive compounds (Jadon et al. 2014). Currently, the antibiotic resistance is considered one of the largest public health issues to worldwide; therefore, the development and designing of unique antimicrobial is tremendously subject to the exploration of novel natural products (Jakubiec-Krzesniak et al. 2018; Avila et al. 2020).

Resistance to existing drugs is constantly being reported from all over the world. It creates a significant demand for the identification of new drug molecules that target these resistant pathogens. Actinomycetes have proven their ability to produce novel secondary metabolites with a pent anti-bacterial activity against these drug-resistant pathogens (Ravi and Kannabiran 2018). Despite that, Biosynthetic gene clusters (BGCs) play a crucial role in producing microbial natural products through utilizing metabolic pathways encoded by adjacent chromosomal genes. Furthermore, essential proteins and transporters for produce a metabolite are regulated by encoding the responsible enzymes by BGCs (Jakubiec-Krzesniak et al. 2018; Núñez- Pons et al. 2020).

Antitumor compounds: Chemotherapy is one of the most treatments to fight cancer. Natural products or their derivatives, primarily microorganism products, constitute a considerable number of antitumor compounds. Actinomycetes, mainly are derivatives of many natural products with various biological activities (Olano et al. 2009). Antitumor antibiotics, antibiotics include anthracycline and actinomycin, are among the foremost effective cancer chemotherapeutic agents. Also, Actinobacteria are consider rich of natural products bioactive that proved scientifically in Ontario Institute for Cancer Research (OICR) Drug Discovery as effective. Considerable amount of the marine antitumor compounds is derived from metabolites of the marine Actinobacteria (Degirmenci et al. 2020).

The signaling pathway with potent antineoplastic activity (RRME: a series of amino acids inside the cell that carries an indication assisted by the cell's surface receptor towards DNA within the nucleus of the cells). RRME pathway components were discovered in cancer cells (Liu et al. 1989). Drugs that reverse the 'on' or 'off' function are being tested as cancer therapies. Diazepinomicin binds and performs RAS kinase inhibition, thus preventing protein phosphorylation and activation downstream of the RAS signal transduction pathway (Abdelmohsen et al. 2012). This agent binds to a highly expressed receptor in the cells of some tumor cell types known as peripheral benzodiazepine receptor (PBR) triggering cell cycle arrest and apoptosis in

cells that express PBR. The barrier (BBB) may be crossed by Diazepinomicin (Degirmenci et al. 2020).

Diazepinomicin has also demonstrated antioxidant and antiparasitic activity against *Trypanosoma* with an IC_{50} of 70–90 mM (Hassan et al. 2017). Furan 2-ylacetate, a cytotoxic secondary metabolite formed by marine *Streptomyces* sp., and rhodomycin B, a cytotoxic secondary metabolite produced by *Streptomyces purpurascens*, has been documented by Suthindhiran and Kannabiran (F2A has demonstrated cytotoxic activity against different cancer cells). Anticancer activity against the HeLa cell, *Streptomyces* sp. KS190, KS190 was have anticancer effect on Hep2, HeLa, HL-60, and MCF7 (Ravi and Kannabiran 2018; Degirmenci et al. 2020).

Anti-inflammatory compounds: Actinomycetes secondary bioactive metabolites, including ozenoxacin is a powerful anti-microbial agent that is essential versus sensitive and Gram-positive-resistance strains (i.e., *Staphylococci* and *Streptococci*). Also, ozenoxacin exhibits efficacy against certain Gram-negative isolates (Morrissey et al. 2019; Tabara et al. 2020). Nevertheless, ozenoxacin's anti-inflammatory properties have not been fully investigated. Taking together that anti-inflammatory effects of ozenoxacin were studied both *In vitro* and *in vivo*. Inhibitory effects of ozenoxacin were proved to inhibit the releasing of interleukin (IL)-6 and IL-8 that induced by *Cutibacterium acnes*. That result on ozenoxacin recognition as a potential therapeutic agent on inflammatory acne. Actinomycetes are widely distributed in marine living organisms. They produce 80% of known antibiotics and exhibit numerous bioactivities in their other metabolites such as anti-inflammatory, and anticancer activities (Tabara et al. 2020).

Antiviral compounds: Viruses are organisms that are associated with disease in living organisms such as animals and plants. Recently, viruses frequently show their roles in the world's ocean (Bhatnagar et al. 2010). They comprise a huge portion of microorganisms inducing diverse infectious diseases. The presence of various viral diseases, such as human immunodeficiency virus (HIV) and viral hepatitis encourage the development of new immense efficacy therapeutic agents. Carboxyl has proven to inhibit virus replication in critical cells such as cells expressing CD4 receptor. Furthermore, carboxyl polyethers have pivotal role in fighting both acute and chronic infections (Kevin et al. 2009; Hassan et al. 2017; Tabara et al. 2020).

Antibacterial compounds: The major cause of turbidity and mortality around the world is the bacterial infections. To overcome such a challenge, the antibiotic-resistance bacterium has been generated very expeditiously, resulting in causing severe problems while both *Staphylococcus aureus* and other pathogens are mainly associated with infection (Hassan et al. 2017). According to World Health Organization (WHO) statistics, about 50,000 lives got lost every year in the USA and Europe due to antibiotic-resistance. The current developed antimicrobial agents cannot sufficiently address the Grams-negative bacteria's problem of large resistance (Tabara et al. 2020).

21 Actinomycetes are isolated from the sediment Caspian Sea sediment; 14 stains showed antibacterial activities against multidrug-resistance (MDR) indicator bacteria. According to the isolated Actinomycetes, they can produce bioactive compounds that can inhibit the growth of Methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant Enterococci (VRE), and the *P. Aeruginosa*, which have more than MDR bacteria (Norouzi et al. 2018). From the marine soil Actinomycetes, the first two bagremycins A and B, were extracted have showed antimicrobial activity against both *Arthrobacter aurescens* and *Streptomyces viridochomogenes* (Zhang et al. 2018; Tabara et al. 2020).

Another antimicrobial potential substance is the buanmycin which is isolated from the *Streptomyces* strain from a tidal mudflat in Busan, Korea. It is a new pentacyclic xanthone. However, buanmycin doesn't only show strong effect on inhibiting Gram-positive *S. aureus*, *B. subtilis*, *K. rhizophila*. And Gram-negative bacteria *Salmonella enterica*, and *P. hauseri*. It can also inhibit *S. aureus* sortase A enzyme; this enzyme can play an essential role in facilitating Gram-positive bacteria adhesion to the host (Jakubiec-Krzesniak et al. 2018). Moreover, *Streptomyces* sp. strain Al-Dabhi 90 from Saudi Arabian marine exhibited an important antimicrobial activity against the drug resistant pathogens such as *Klebsiella pneumonia*, *S. aureus*, *E. coli*, *Pseudomonas aeruginosa*, *Enterococcus faecium*, and *Proteus mirabilis*. From Andaman and Nicobar Islands soil, 12 Actinomycetes were isolated depending on different morphological appearances. All of the isolates are subjected to antibacterial activity, and among the 12 isolates *Streptomyces* sp. Strain VITAK1 showed an antibacterial activity (Mani et al. 2021).

Novel metabolites produced by marine Actinomycetes: During the last two decades, scientific studies of marine microbes and their ability to produce vital metabolites were tremendously increased. Many bioactive compounds have been derived from Actinomycetes, representing the largest diversity of populations of the identified species. 45% of the bioactive microbial metabolites are produced by Actinomycetes, and *Streptomyces* are the most important sources of secondary metabolites of higher clinical importance, producing 70% of the antibiotics (Khadayat et al. 2020). Tanking together that more than 20,000 bioactive compounds have been obtained from microorganisms (Subramani et al. 2013). Many essential drugs such as rapamycin belongs to immunosuppressive group, while mitomycin, and leomycin belongs to the anticancer drugs and antimicrobial drugs as medications of that are vancomycin and erythromycin. The increasing demand for antibiotics proves their effectiveness in fighting important bacterial pathogens (Tommasi et al. 2015). For this purpose, researchers have become oriented towards discovering new discovering and focus on natural products from underexplored habitats, especially in marine environments that contain rare genera of Actinomycetes (Subramani et al. 2013; Jackson et al. 2018; Safaei et al. 2021).

Actinomycetes have demonstrated their tremendous ability to produce new secondary or therapeutic metabolites,

including antibiotics, anticancer, growth factors, enzymes, and herbicides (Luzhetskyy et al. 2007). This confirms that Actinomycetes remain a promising source for drug discovery. A recent literature review survey done by Subramani and Sipkema (2019) showed that 167 new biological compounds were obtained from 58 different Actinomycetes species. Most of these compounds provided a diverse antimicrobial activity. *Nocardiopsis*, *Micromonospora*, *Salinispora* and *Pseudonocardia* are the largest genera from which compounds were extracted. The genus *Micromonospora* exhibited unique and chemically diverse bioactive compounds. Safaei et al. (2021) carried out a study on freshwater snail *Physa acuta* and discovered novel Streptomyces species (Subramani and Sipkema 2019; Safaei et al. 2021).

The antimicrobial activity was isolated, and then the extracts were tested against bacteria and fungi, where 7NS3 represented a new species of Streptomyces and was given the name Streptomyces sp. DSM 110735 at the Leibniz Institute-German Collection of Microorganisms and Cell Cultures (DSMZ). Al-Dhabi et al. (2019) studied Actinomycetes in the Persian Gulf regions and found marine Streptomyces sp. Al-Dhabi-90 had a good biological action (anti-bacterial) against clinical pathogens and a MDR (Al-Dhabi et al. 2019). In another field, Streptomyces isolates were tested against dermatophytes fungi, found that Streptomyces sp. ACT2 (GQ478247) achieved high efficiency anti-dermatophytid, and it is an essential source of bioactive compounds listed four novels bioactive obtained from marine Streptomyces sp. shell-016. They showed activity against different lines of cancer cells lines (Shamikh et al. 2020; Suresh et al. 2020; Han et al. 2020).

CONCLUSION

The findings of the present study has found that recent studies have moved from discovering bioactive molecules, specifically Actinomycetes in the soil, to new marine environments that sound like large extensive compounds awaiting discovery. Improving strategies of bioactivity-guided, cultivation variation centered and metabolome- and genome-based approaches for identifying and isolating bioactive molecules form the basis for the characterization of novel compounds from Actinobacteria. Likewise, approaches to genome sequencing, bioinformatics, and the partnership of molecular genetics that lead to an understanding of the principles of biogenesis should be developed to produce new secondary metabolites of practical value.

ACKNOWLEDGEMENTS

This study was financially supported by Mawakeb Alajer Association, through the Science Research and Innovation Unit at the Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia under research number (scigriu41/15).

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Biomedical Communication

Knowledge, Skill and Attitude of Nurses on the COVID-19 Pandemic Crisis in Jazan, Saudi Arabia: A Quantitative Cross-Sectional Study

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ABSTRACT

Coronavirus (COVID-19) was identified in the Chinese city of Wuhan in 2019. COVID-19 is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and it is now recognized that SARS COV-2 may infect and spread among humans. During the pandemic situation, nurses and health care workers (HCWs) played a vital role in handling the infected patients and this study was carried out in Jazan city of Saudi Arabia among the nurses. The purpose of this study was to document nurses' knowledge, skills, challenges, and information on the COVID-19. In this questionnaire-based study, we have enrolled 296 nurses based on inclusion and exclusion criteria. The 296 nurses involved in this study has shared their knowledge, experience, skills and challenges in handling the infected patients with COVID-19. Initially, 34.1% of nurses was expecting COVID-19 virus will be controlled by vaccine. The majority of nurses, 54.7%, were convinced that antibiotics could control the illness. In this study results, 97% of nurses were aware of COVID-19 symptoms such as fever, cough, sore throat, and shortness of breath. Around 96.3% of the nurses were aware of the risk of chronic diseases in the elderly population, which may be at risk of COVID-19 infection. The 78.7% nurses believed that mask can protect from the infecting from COVID-19 and ~94% of the nurses believed that avoiding of gatherings at public places is the best method to control the virus. The majority of the 97% of nurses believed that isolation is the best method to control the COVID-19 virus in an infected person. Nurses played a critical role in the treatment of COVID 19 infected patients in Saudi Arabia, as nurses and HCWs were at risk of becoming asymptomatic carriers due to their role in disease transmission. This study recommends the nurses, HCWs and all the residents of Saudi Arabia to take the vaccine to prevent the spread of COVID-19 as new strains are developing in the global population

KEY WORDS: COVID-19, EXPERIENCE, JAZAN CITY, KNOWLEDGE, NURSES.

INTRODUCTION

The story of coronavirus (COVID-19) has started in the last month of December (2019) from Wuhan market in China. This infectious disease spread through animal to human from China to the rest of the world. The World Health Organization (WHO) has declared COVID-19 a pandemic, as well as a novel coronavirus known as SARS-CoV-2, which is a virus with a similar pattern to the SARS-CoV virus that infected people in China in 2003 (Meng et al. 2020). COVID-19

spreads primarily over the snows or coughs through saliva droplets or nose discharge. Studies have shown that there are no effective treatments for this disease (Hafeez et al. 2020). SARS-CoV-2 is the third coronavirus to be transmitted worldwide over the past two decades, causing serious disease in humans. Looking under an electron microscope, coronaviruses range in diameter from 60 nm to 140 nm and have spiky projections on their surface, and are called crown-like viruses. The most common coronaviruses in clinical practice are 229E, OC43, NL63, and HKU1, which usually cause common cold symptoms in immunocompetent people (Singhal 2020; Wiersinga et al. 2020).

COVID-19 symptoms include a dry cough, headache, diarrhea, myalgia, and vomiting. People who have multiple

Article Information:*Corresponding Author: mfarasani@hotmail.com

Received 14/10/2021 Accepted after revision 25/12/2021

Published: 31st December 2021 Pp- 1680-1686

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.46>

congenital disorders are more vulnerable to severe infections (Farasani 2020). SARS-CoV-2 can be distributed by direct and indirect means. The cause of airborne infections can also be personal protective equipment. COVID-19 can occur when a person has a SARS-CoV-2 contaminated surface and the hands are then directly in contact with mucous membranes, including eyes, nose and mouth. Therefore, enough hand washing is recommended with soap and water or hand sanitizers (Lotfi et al. 2020). Countries have put in place unprecedented steps to avoid the spread of the SARS-CoV-2 virus, from school closure to large lockdowns. The greater uncertainty in epidemiological monitoring and disease development are making international comparisons more complex. Furthermore, though continued communication took place around mid-February in most of Europe, the fact that the virus was introduced at various locations at very different times made global comparisons difficult (Ragonnet-Cronin et al. 2021).

The European Medicines Agency and the Italian Medicines Agency had approved four vaccines as of March 13, 2021, including Pfizer, Moderna, AstraZeneca (Oxford), and J&J (Ad26.COV2.S). After 28 days of vaccination with a safety zone the efficacy rate has been reported as 95%, 94%, 81.3% and 86% respectively (Giordano et al. 2021). During the COVID-19 pandemic crisis, health care staff, especially nurses, played a pivotal role in dealing with COVID-19 infected patients. Basically, for the nurses, in 24-hours and seven consecutive days of week, the nurses had the highest levels of workplace stress and anxiety. Empirical evidence of COVID-19 and other outbreaks of infectious respiratory disease indicates high concern among nurses about their personal and family wellbeing in the face of close interaction and tension in balance with their moral responsibilities to continue providing healthcare a potentially fatal virus (Maben and Bridges 2020; Giordano et al. 2021).

Nurses working in primary health care (PHC) centers outside of hospitals play different roles around the world. PHC practitioners play an important role in community education through managing public response and psychological sequelae to COVID-19 (Halcomb et al. 2020). Al Tobaity et al. (2020) reveal that nurses have a pivotal role in fighting COVID-19 since they are always on the frontline, in addition to offering holistic care for different patients daily, as well as during disasters. The nurses had opted the more specific roles in managing this illness include triaging patients, noticing suspected COVID-19 cases and dealing with them cautiously, offering critical services during an emergency, de-contaminating surfaces, coordinating with colleagues and other health practitioners, contributing to the expansion of care services, in addition to handling patients' relatives. Considering these critical roles that nurses play during a pandemic, documented studies stated that nurses should be equipped with key skills and knowledge to manage the crisis (Tobaity and Alshammari 2020; Liu et al. 2020; Kasnakova and Ivanova 2021). Limited studies have been documented regarding the role of nurses during the COVID-19 crisis and the current study was carried out in Jazan region in the nurses about their knowledge, skills, challenges and information about the COVID-19.

MATERIAL AND METHODS

The study will use a quantitative cross-sectional design. The chosen design is the most common design used in health-based studies. According to Setia (2021), it can also be referred to as descriptive research since it is used to describe certain characteristics in a community. The design is appropriate for the proposed investigation because it does not require a long duration of follow-ups. Moreover, the design will allow the researcher to measure prevalence rates for each factor in the research (Bloomfield and Fisher 2019; Setia and Verma 2021).

The total number of nurses in the two hospitals was 1,100. It was impossible to collect data from all these nurses because it might require more resources and time. Thus, only a sample from the entire population was used in this study. Out of 1100 nurses, only 285 nurses were selected. Since the population size was 1,100, the margin error was 5%. Thus, the sample size was 285. A pilot sample was done to identify 10% of the total population, test the instruments, and ascertain validity and reliability. The study was done in Jazan, Saudi Arabia. This region in Saudi Arabia that stretches 30 km on the southern part of the red sea (Alanazi et al. 2017). Jazan region covers approximately 11,671km with a population of around 1,567,547 (Alhazmi et al. 2020). The region has several hospitals to cater to the growing needs of its population. However, the proposed study focused only on biggest two hospitals due to resources and constraints that do not allow large-scale research. One of the hospitals is known as King Fahad central hospital, and the other one is Prince Mohammed bin Nasser hospital.

The study population comprised of the registered nurses from King Fahad and Prince Mohammed bin Nasser hospitals. The nurses in Jazan have various needs, including training, resources, and motivation to manage and combat the Covid-19 pandemic. Only registered nurses working in the Jazan region were included in the proposed study. Moreover, only nurses who helped COVID-19 patients to manage the disease participated as they had vast experience. In this study, a non-probability sampling technique was used. Particularly, the investigation adopted a convenience sampling method. The technique was adopted because it was low cost (Etikan et al. 2016). Moreover, the subjects for the research were readily available for selection. In this study, only registered nurses were included to investigate the topic. Besides, nurses who directly dealt with COVID-19 patients were selected to participate. The study did not include other healthcare workers because of the differences in training, capacity, and regulatory mechanisms.

An online questionnaires and consent forms were sent to participants through their emails. Participants were allowed two weeks starting from 1-1-2021 to fill in and return the questionnaires. The descriptive statistic was used to analyse the data. Data management and analysis followed a systematic process that was presented in the following steps: Data coding, data entry, and descriptive analysis: in terms of frequency and percentage conversion of socio-demographic, knowledge, skills, and attitudes questions.

The data analysis was performed as per documented studies (Khan et al. 2019).

RESULTS AND DISCUSSION

In this study, based on inclusion and exclusion criteria, we opted for 296 participants. The mean age of the enrolled nurses was in the age range of 30.15 ± 5.22 with the involvement of 4.1% of males and 95.9% of females. The majority of enrolled nurses (80.7%) had a bachelor's degree, followed by 17.2% with a diploma and 2.1% with a master's degree. More than 52% of nurses are working in the King Fahd Central Hospital and remaining 47.6% of them were employed at Prince Mohammed Bin Naser Hospitals in the Jazan region of Saudi Arabia. The complete basic details were documented in Table-1.

Table 1. Basic details of the participants

Basic details	n=296 (%)
Age	30.15±5.22
Gender	
Male:	12 (4.1%)
Female:	284 (95.9%)
Levels of Education	
Diploma	51 (17.2%)
Bachelor	239 (80.7%)
Master	06 (2.1%)
Nurses (working Places)	
King Fahd Central Hospital	155 (52.4%)
Prince Mohammed Bin Naser Hospital	141 (47.6%)
Years of experience	
0-4 Years	142 (48%)
5-9 Years	82 (27.7%)
10-14 Years	50 (16.9%)
15-19 Years	17 (5.7%)
20-25 Years	05 (1.7%)

The years of experience were divided into five categories: 0-4 years, 5-9 years, 10-14 years, 15-19 years, and 20-25 years. There were 48% of nurses with 0-4 years of experience, and 27.7% with 5-9 years of experience. However, 16.9% of the nurses had 10-14 years of experience, while 5.7% had 15-19 years of experience. Only 1.7% of them had more than two decades of nursing experience.

Table 2 provides the nurses' perspectives on COVID-19 patients and infection. Almost all of the nurses believed that COVID-19 was a viral infection, with 34.1% expecting the virus to be controlled by the vaccine and the remaining 66% not convinced that the vaccine would control the virus. The majority of nurses, 54.7%, were convinced that antibiotics could control the illness, while the remaining nurses were not. Almost all of the nurses followed the MOH standards for COVID-19 infection prevention. In this study, more than 90% of nurses considered that health care workers were at

high risk when treating COVID-19-infected patients, and 76.7% of nurses considered COVID-19 may be fatal.

In this study, 97% of nurses were aware of COVID-19 symptoms such as fever, cough, sore throat, and shortness of breath, and 87.5% were aware of the lack of COVID-19 treatment availability. The limited infection signs such as sneezing, cold, stuffy and running nose were known by 63.9 % of the nurses, and 96.3% of the nurses were aware of the risk of chronic diseases in the elderly population, which may be at risk of COVID-19 infection. Approximately 80% of the nurses were not sure that COVID-19 patients could not spread the virus without the symptom of fever, and 93.6% were aware that COVID-19 is transferred by the droplets of an infected individual (Table-3).

The table-4 of this study describes the personal protection and isolation towards COVID-19 infection. The majority of the 78.7% nurses believed that mask can protect from the infecting from COVID-19. There was an assumption that 85.5% of the nurses were not in agreement that children and adolescents can avoid the measurements towards COVID-19. However, ~94% of the nurses believed that avoiding of gatherings at public places is the best method to control the virus. The majority of the 97% of nurses believed that isolation is the best method to control the COVID-19 virus in an infected person. 49% of the nurses confirmed that they have not visited any crowded place during the pandemic and 83.3% of them were confirmed that they use to wear the mask before leaving the home.

The nurses during the contacting any person admitted in the hospital is used to wear the personal protective equipment and 96% of them use to cover the mouth when they cough or sneeze. Almost 90% of nurses admit that they wash their hands when they touch their nose or cough/sneeze, 96% of them wash their hand after touching the contaminated objects. 93.3% of nurses has the habit of wearing mask without symptoms and 96.6% of the nurses' wear N95 mask when contacting the COVID-19 patients.

The general awareness in the nurses about COVID-19 disease were documented in Table-5. The 78.4% of the nurses believed that COVID-19 was successfully controlled by the Saudi Arabia and 88.2% of them believed that Saudi Arabia won against the battle towards COVID-19. Nearly, 92% of the nurses were self-isolated after treating the COVID-19 patients and 90% of the nurses believed that transmission of COVID-19 can be prevented by washing the hands. In this study, 85.5% of the nurses believe that COVID-19 can be controlled in the HCW with the prevention of hospital infection and control programme. Only 79.4% of the nurses were preferred to have the vaccine and 90.5% of the medical staff are ready to involve in the anti-epidemic community.

COVID-19 has become a life style disease in the global countries. Previously, it was declared as public health emergency of international concern in the last day of January (2020). The COVID-19 pandemic gives us many painful lessons, including the vulnerability and need for

preparedness, cooperation and monitoring of those living with chronic conditions (Davidson and Szanton 2020). Till now, it's a mystery about the existence of COVID-19/SARS-CoV-2 as how these original sources were transmitted. However, based on available genetic and epidemiological data indicate that SARS-CoV-2 is a zoonotic disease with the potential to spread directly from wildlife or through

intermediate animal hosts or their products. Sustained human-to-human transmission has been verified in China, where a large number of healthcare professionals have been infected in clinical settings, resulting in overt clinical disease and mortality. One of the countries affected by the virus was Saudi Arabia. The first confirmed Case COVID-19 in the country was announced by the Saudi Minister of Health on 2 March 2020 (Alboaneen et al. 2020).

Table 2. Opinions of Nurses regarding COVID-19

Nurses opinion	Yes	No	No Idea
Is COVID-19 is a viral infection?	295 (99.7%)	0 (0%)	01 (0.3%)
Does COVID-19 Vaccine will control the infection?	101 (34.1%)	159 (53.7%)	36 (12.2%)
Antibiotics is the treatment option?	162 (54.7%)	117 (39.5%)	17 (5.8%)
MOH guidelines for prevention of disease transmission	0.7%	0.3%	99%
Healthcare workers are at a higher risk of infection?	269 (90.9%)	18 (6.1%)	09 (3%)
Can COVID-19 is a fatal?	227 (76.7%)	24 (8.1%)	45 (15.2%)

Table 3. Knowledge of COVID-19 symptoms

	Yes	No	Unknown
Clinical symptoms (Fever, Cough, Sore throat and shortness of breath)	287 (97%)	04 (1.3%)	05 (1.7%)
Currently no Treatment towards COVID-19	259 (87.5%)	19 (6.4%)	18 (6.1%)
Cold, sneezing, stuffy and running nose are the less infection for COVID-19	189 (63.9%)	75 (25.3%)	32 (10.8%)
Elder patients with chronic diseases are high risk of infecting COVID-19	285 (96.3%)	03 (1%)	08 (2.7%)
Person diagnosed with COVID-19 cannot infect without the symptom of fever	38 (12.8%)	237 (80.1%)	21 (7.1%)
COVID-19 is transmitted by respiratory droplet of the infected person	277 (93.6%)	12 (4.1%)	07 (2.3%)

Table 4. Personal protection and Isolation towards COVID-19 infection

	Yes (%)	No (%)	Sometimes (%)
Can normal person wear the mask to avoid COVID-19 virus?	233 (78.7%)	41 (13.9%)	22 (7.4%)
Can children and adolescents can avoid the measures for COVID-19 virus?	34 (11.5%)	253 (85.5%)	09 (3%)
Should we avoid the gatherings and public places to prevent the COVID-19 virus?	278 (93.9%)	07 (2.4%)	11 (3.7%)
Is isolation and treatment for COVID-19 is the best way to reduce COVID-19 virus?	287 (97%)	05 (1.7%)	04 (1.3%)
Is isolation being mandatory for the person who is communicated with infected person	283 (95.6%)	06 (2%)	07 (2.4%)
Did you visit any crowded places?	48 (16.2%)	145 (49%)	103 (34.8%)
Do you use to wear a mask before leaving the home?	248 (83.8%)	21 (7.1%)	27 (9.1%)
Do you use to remove the protective equipment carefully?	283 (95.6%)	05 (1.7%)	08 (2.7%)
Do you use to cover the mouth when you cough and sneeze?	284 (96%)	02 (0.6%)	10 (3.4%)
Do you wash your hands immediately after coughing, sneezing or rubbing the nose?	264 (89.2%)	04 (1.3%)	28 (9.5%)
Do you have a habit of wearing a mask with or without symptoms?	276 (93.3%)	08 (2.7%)	12 (4%)
Do you wash your hands after touching the contaminated objects?	284 (96%)	00 (0%)	12 (4%)
Do you use to wear the N95 mask during contacting with COVID-19 patients?	286 (96.6%)	02 (0.7%)	08 (2.7%)

As of May 20, 2021, the Kingdom of Saudi Arabia had documented 437,569 infected cases with COVID-19, of which 421,726 were recovered. Unfortunately, there were approximately 7214 cases of fatality, and the remaining 8629 cases were active. Based on worldometer data, Saudi Arabia ranked at 44th position among 222 countries were infected with COVID-19. Presently, USA and India are in the top row with the infected cases followed by Brazil and France. The recovery rate in the kingdom was documented to be 98% and mortality rate is less than 2%. The controls measures performed an important influence in limiting SARS-CoV-2 transmission with reduced deaths. In Saudi

Arabia mortality is extremely low compared to other countries since the Kingdom of Saudi Arabia offers the best health services at all. The present study was designed as a questionnaire study carried out in Jazan city of Saudi Arabia. In this study, 296 nurses were included based on inclusion and exclusion criteria. The overall study concludes on an average of about 90-99% of the nurses have good knowledge COVID-19 and in treating the patients as per the MOH guidelines. All the nurses from both the hospitals followed precautionary measures by wearing masks, N95 masks, gloves, protective equipment, washing hands and sanitizing (Alshammari et al. 2020).

Table 5. General awareness about COVID-19 among the nurses

General Awareness	Agree (%)	Disagree (%)	Neutral (%)
Do you agree that COVID-19 was successfully controlled in the Saudi Arabia	232 (78.4%)	15 (5.1%)	49 (16.5%)
Does Saudi Arabia win against the battle towards COVID-19	261 (88.2%)	13 (4.4%)	22 (7.4%)
Does COVID-19 infected nurses will be self-isolating?	271 (91.6%)	17 (5.7%)	08 (2.7%)
Transmission of COVID-19 can be prevented by washing hands	267 (90.2%)	14 (4.7%)	15 (5.1%)
Can COVID-19 be controlled in the HCWs with the prevention of hospital infection and control program	253 (85.5%)	14 (4.7%)	29 (9.8%)
Will you prefer to have the vaccine?	235 (79.4%)	22 (7.4%)	39 (17.2%)
Does medical staff is ready to participate in the anti-epidemic community	268 (90.5%)	08 (2.7%)	20 (6.8%)

However, on limited information such as vaccine, antibiotic treatment, asymptomatic transmission of infection from person to person, and prevention of COVID-19 transmission by washing hands were not known during the initial period of the pandemic for some of the staff. After the approval of MOH guidelines, all nurses were obliged to take strict precautions, measurements, and knowledge when dealing with infected patients in hospitals throughout the pandemic. In this questionnaire-based study, we have recorded the basic details, nurses' opinion, knowledge and general awareness about COVID-19 and additionally we have recorded about the PPE kit and quarantine after handling the infected persons. Limited studies were performed in the HCWs in the Saudi Arabia and among them nurses were also enrolled (Huynh et al. 2020; Asaad et al. 2020; Saqlain et al. 2020; Nepal et al. 2020; Qattan et al. 2021; Rabbani and Saigul 2021; Aleanizy and Alqahtani 2021).

The WHO has lot of impact in updating the information and requesting for the precautions. During the pandemic crisis, massive amounts of up-to-date information regarding COVID-19 were disseminated around the world. The WHO communicates with global research and development scientists and global health experts, and set new standards to prevent and assist in the spread of the coronavirus pandemic. The Ministry of Health in Saudi Arabia has played a major role in controlling the pandemic virus within the Kingdom. A special care was taken for the infected Saudi and non-Saudi person with free treatment, comfortable quarantine and strict lock-down was implemented. Based on WHO approval guidelines MOH prepared the COVID-19 protocols and were implemented for an effected and confirmed COVID-19 patients. Additionally, COVID-19 infected persons and

previously diagnosed with sickle cell disease, infectious diseases, chronic diseases, pregnancy women and neonates were taken the special care in treating them (Rabbani and Saigul 2021; Aleanizy and Alqahtani 2021).

MOH has taken many precautions in the Saudi Arabia to combat this pandemic and virus. Initially, there was no vaccine was available but presently, Pfizer-BioNTech and Oxford-AstraZeneca was available in the kingdom. In this study, almost 80% of nurses were shown interest towards vaccine. Both these vaccines were approved by the US Food and Drug Administrations (Tanne 2020). A recent study from Saudi Arabia in the HCW was carried out in acceptability of the COVID-19 vaccine and study results confirmed about 50.5% are willing towards having the vaccine. However, in this study 80% of HCWs are interested towards taking the vaccine. Specially nurses have a significant role in the effectiveness of the vaccine programme; research has shown that vaccine knowledge and attitudes impact the intentions and recommendation for the vaccine (Ellingson et al. 2010; Nzaji et al. 2020; Qattan et al. 2021).

Personal protective equipment (PPE) has become critical during the COVID-19 pandemic due to the virus's extraordinarily contagious nature and aggression, as well as the virus's limited treatment options. PPEs are used in a variety of settings, including industrial, pollution control, sports, and healthcare (Setia and Verma 2021). The biggest issue at the start of the pandemic was a lack of PPE for HCWs, as well as a lack of training in its use. Global studies document the importance of PPE kits during the pandemic in HCWs and in this study 95.6% of the nurses/HCWs were

known to use and remove the PPE carefully. Additionally, hospital infection and control program played a major role in controlling of COVID-19 (Hossain et al. 2021).

CONCLUSION

The findings of the present study recommends the nurses, HCWs and all the residents of Saudi Arabia to take the vaccine and prevent the spread of COVID-19 as new strains are developing in the global population. Future studies to implement in the large sample size from the Saudi population. Nurses played a critical role in the treatment of COVID 19 infected patients in Saudi Arabia, as nurses and HCWs were at risk of becoming asymptomatic carriers due to their role in disease transmission.

Conflict of Interests: Authors declare no conflict of interests to disclose.

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Ecological Communication

A Comparative Diversity Analysis of Soil Nematodes of Rajouri, Jammu and Kashmir, India

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ABSTRACT

Diversity of Nematode communities in Pir Panjal range of Jammu and Kashmir along with nematodes' driven indices were studied. Himalayan mountainous areas of district Rajouri were selected. Community composition and trophic structure (feeding types) were assessed and were analyzed at various altitudes and across varied habitats, which differ significantly on moving from low elevation to high elevation areas. A total of 47 genera were recorded across mountain clines. In terms of taxonomic groups, in higher elevations, the order Dorylaimids represent 55.18%, followed by Tylenchida 28.85%, Mononchida 2.38%, Rhabditida 2.18%, and Aphelenchida 2.05%, whereas in the lower reaches, the order Rhabditida represent 30.18%, followed by Dorylaimids 28.75%, Tylenchida 15.85%, Mononchida 10.05% and Aphelenchida 1.05%. In terms of trophic groups, in the upper reaches, omnivores (56.6%) predominate, representing highest number, followed by plant parasitic (33.4%), bacterivore (4.2%), predatory (3.2%) and fungivore (2.60%). In the lower reaches, Bacterivores (38.08%) predominates, followed by omnivores (29.85%), plant parasitic (18.5%), predatory (12.5%) and fungivores (1.07%). The total nematode abundance and diversity were found increasing with elevation. This pattern applied to most genera and feeding types. Across the regions, nematode diversity and community composition increases positively with elevations and richer habitats as given by Simpson index and Shannon-Weaver index. We conclude that nematode assemblages are potentially good bioindicators of climate change. They reacted sensitively and predictably to the changing environment. Thus, nematodes have suitability for long-term monitoring of biodiversity and community changes. Sampling techniques are well standardized and inexpensive. Furthermore, feeding types of nematodes can be determined with minimal taxonomic skills.

KEY WORDS: BIODIVERSITY, COMMUNITY COMPOSITION, ECOSYSTEM FUNCTIONING, SOIL NEMATODES.

INTRODUCTION

It has been established that 25% of the biodiversity of world sustains in the soils of terrestrial ecosystems. Nematodes are one of the most successful organisms and placed in a lower position in the taxonomic hierarchy. They are quite abundant and probably the most diverse organisms on the earth. Based on trophic grouping, nematodes may be bacterivores, predators, fungivores, omnivores and plant parasites, (Yeates et al. 1993; Bach et al. 2020).

Abundance, diversity and effects on soil processes of nematodes make their way to the usefulness of indices of nematodes assemblage as potential bio-indicators of ecosystem functioning. Most nematodes are beneficial in terms of maintaining earth's nutrients' cycle and enhancing

the diversity of natural ecosystem (Freckman 1982; Freckman 1988; Beare et al. 1992). Nematodes belonging to various species inhabiting in a pristine or a disturbed environment constitutes the nematode community of that particular area and are very much sensitive to changes in different soil parameters like pH, salinity, soil moisture, conductivity, food supply etc. (Yeates 1987).

Nowadays, nematodes are considered as bio-indicators owing to their suitability for long-term monitoring of biodiversity and community changes. Soil inhabiting communities particularly nematodes inhabiting in high elevations are largely unknown, they are good indicators of the changing ecosystem diversity because they themselves are very diverse in soils (Overgaard, 1949; Procter 1984; Yeates and Bongers 1999; Yeates 2003). Biodiversity in the highest elevations especially of nematodes in terms of species richness, abundance and biomass is greater as compared to the low latitudes, as hypothesized in previous studies (Procter 1990). Any disturbance in the niche of nematodes affects their population dynamics. The change

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Received 25/09/2021 Accepted after revision 15/11/2021

Published: 31st December 2021 Pp- 1687-1700

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.47>

in the factors like soil quality, soil characteristics, habitat stability, climate change and environment pollution is strongly reflected by nematodes and therefore they constitute an important part of the soil biota (Bongers 1990; Crawford, Neilson and Boag 1991; Goede and Bongers 1994; Ruess 1995; Ruess, Michelsen and Jonasson 1999; Sohlenius and Bostrom 1999; Bach et al. 2020).

Soil nematodes contribute and inform on the changes in ecosystem functioning across contrasted landscapes. Nematodes amassed around and in food sources and composed of various taxa of soil food web of various trophic levels (Yeates 2010). High altitude habitats represent a unique bio-region primarily because of its varied topography and habitat heterogeneity along a wide elevational range. In soil ecosystem, various functional trophic guilds can be assigned to nematodes and thus can be seen having wide range of ecological adaptations, from colonizer (r-strategists) to persister (K-strategists) along a cp (colonizer-persister) scale (Bongers 1990). Studying the species abundance and distribution of the high-altitude summits, their causes and consequences gives an insight into the community assembly and ecosystem functioning (Gaston 2000; Doherty, Callaway and Zedler 2011; Oliver et al. 2015; Wilschut et al. 2019). Of the total number of nematode species that exist on earth, only about 27,000 are known so far (Hugot, Baujard and Morand, 2001). The soil conditions differ with the changing habitat and elevation and are indeed important determinants of the composition of soil nematode communities. The vegetation of Pir Panjal Range represents appreciably diverse types, mainly because of the diversity of habitat. Forest cover of the Pir Panjal in the lower reaches comprise usually of conifers, like Blue Pine (*Pinus wallichiana*), Silver Fir (*Abies pindrow*), Himalayan Deodar (*Cedrus deodara*), The Himalayan Yew (*Taxus wallichiana*) and spruce (*Picea smithiana*) along with some associated shrubs and broad-leaved trees (Wu et al. 2011; Nielsen et al. 2014; Song et al. 2017; Li et al. 2020).

In the mid reaches, the Silver Fir assumes dominance, whereas natural stands of Birch (*Betula utilis*) occur in the upper forest reaches, forming the timberline in Himalaya. Beyond the tree line, alpine scrub vegetation, comprising mainly the species of *Salix*, *Lonicera*, *Juniperus*, *Cotoneaster* and *Rhododendron*, is quite common. The mountains at higher altitudes are dotted with the lush green meadows ('Dhoks') with characteristic herbaceous elements, such as species of *Gentiana*, *Ranunculus*, *Aconitum*, *Aquilegia*, *Iris* and *Potentilla*. Nematodes are also associated with these plant varieties as herbivores or plant-parasitic nematodes. Studies on the diversity of soil nematode communities of Pir Panjal Range of district Rajouri are very meagre. The aim of this study is to unravel nematode fauna for a better understanding of their population structure. Standard techniques were used to isolate nematodes. Moreover, identification and counting on generic level was done in the laboratory. Various Ecological indices were used to assess the diversity of different nematodes like Shannon-Weaver Index (H'), Simpson index (D) and Inverse Simpson index (Dar and Khuroo 2013; Li et al. 2020).

MATERIAL AND METHODS

The current study was conducted in Rajouri district of Jammu and Kashmir, India which varies in topography, geological formations, climatic conditions and the altitudinal range. Nematodes were sampled between May 2018 to June 2020. Soil samples from various locations were collected in air tight bags labelled with information like sample type, habitat, associated plant, collection date, elevation, collection site, etc. The nematodes were extracted by Cobb's sieving and decantation and modified Baermann's funnel techniques. Alongside, the extracted nematodes were killed and fixed using hot fixative in the ratio 4:1, followed by dehydration in glycerin-alcohol (5 parts glycerin + 95 parts 30% alcohol) and then mounted in anhydrous glycerin on glass slides using wax ring technique and then identified under Olympus BX51 DIC microscope using literature of (Seinhorst 1959; Andr  ssy 1983; Jairajpuri and Ahmad 1992).

Nematodes were counted to genus level under the Olympus Stereo-zoom SZX16 microscope and assigned a functional guild based on their life histories and trophic groups (Yeates et al. 1993). The following parameters were calculated to determine the diversity of soil-inhabiting nematodes (Tomar, Baniyammuddin and Ahmad 2006).

1. Frequency (N) is frequency of nematode genus (the number of samples in which the genus was present).
2. Absolute Frequency (AF %) is frequency of genus X 100/ total number of samples counted.
3. Mean Density (MD) is the number of nematode specimens of the genus counted in all samples / total number of the samples collected.
4. Relative density (RD %) is the mean density of the genus X 100/ sum of mean density of all nematode's genera. (Tomar, Baniyammuddin and Ahmad, 2006).

• The following indices were calculated.

1. Shannon-Weaver Index (H') = $-\sum P_i \ln P_i$ (Shannon and Weaver, 1949).
2. Simpson index (D) = $1/\sum P_i^2$
3. Inverse Simpson index (Hill's reciprocal) $N_2 = 1/D$.

In these indices, P_i = proportion of individual of taxon i in the total population

Statistical analyses were performed using R software, R version 4.0.3 (2020-10-10). Copyright (C) 2020 The R Foundation for Statistical Computing. In order to describe nematode communities across various locations, Correlation analysis was performed.

RESULTS AND DISCUSSION

There are 47 nematode genera found in Eight Major Areas of district Rajouri of Pir Panjal.

Nematode community analyses in Manjakote Area: A total of 34 genera were recorded from the Manjakote area, with *Dorylaimida* (33.3%), representing the highest

percentage followed by *Rhabditida* (27%), *Tylenchida* (12.12%), *Araeolaimida* (9.09%), *Mononchida* and *Aphelenchida* (6.06%), *Monhysterida* (3%) and *Enoplida* (3%), (Figure 1). On assigning the 34 genera to trophic groupings, the bacterivores represented the highest percentage (48%), followed by omnivores (18.18%) and

fungivores (15.5%) and then plant parasites (9.09%) and predators (9.09%), (Table I). *Mesorhabditis* and *Acrobeles* were the most dominant genera with the highest frequency of occurrence (100%) and (83.3%), respectively; whereas the genera *Teratocephalus* and *Heterocephalobus* were the least common, with frequency of occurrence (16.6%).

Table 1. Population structure of soil dwelling nematodes of Manjakote Area of district Rajouri

Feeding Habits	Genera	Manjakote Area			
		N	AF%	MD	RD%
Bacterivores	Anaplectus	3	50.00	5.00	1.860
	Mesorhabditis	6	100.00	35.67	13.267
	Pelodera	4	66.67	25.67	9.548
	Acrobeles	5	83.33	22.33	8.308
	Cuticularia	2	33.33	5.33	1.984
	Wilsonema	3	50.00	11.67	4.340
	Alaimus	1	16.67	2.00	0.744
	Diploscapter	3	50.00	16.83	6.262
	Teratocephalus	1	16.67	1.83	0.682
	Rhabditis	4	66.67	12.67	4.712
	Plectus	2	33.33	22.67	8.432
	Heterocephalobus	1	16.67	2.00	0.744
	Monhystera	2	33.33	7.83	2.914
	Ceratoplectus	1	16.67	5.83	2.170
	Prismatolaimus	1	16.67	2.33	0.868
Fungivores	Eucephalobus	1	16.67	3.67	1.364
	Tylencholaimus	1	16.67	1.17	0.434
	Aphelenchoides	1	16.67	0.83	0.310
	Leptonchus	1	16.67	1.00	0.372
	Aphelenchus	2	33.33	2.67	0.992
Herbivores	Diphtherophora	1	16.67	0.50	0.186
	Paratylenchus	2	33.33	7.83	2.914
	Helicotylenchus	2	33.33	11.83	4.402
Predators	Pratylenchus	1	16.67	3.33	1.240
	Mylonchulus	3	50.00	7.33	2.728
	Discolaimus	1	16.67	2.00	0.744
Omnivores	Mononchus	2	33.33	2.50	0.930
	Eudorylaimus	4	66.67	16.17	6.014
	Epidorylaimus	2	33.33	5.83	2.170
	Prodorylaimus	2	33.33	6.67	2.480
	Mesodorylaimus	3	50.00	7.50	2.790
	Dorylaimus	2	33.33	3.83	1.426
	Pungentus	2	33.33	4.50	1.674

Frequency: Among predators, the genus *Mylonchulus* was the most prevalent, with absolute frequency (AF) of 50%, whereas the least frequent genera were *Discolaimus* and *Mononchus*, with AF of 16.7% and 33.3%, respectively. *Mesorhabditis* and *Acrobeles* were the most frequent genera among the bacterivores with AF of 100% and 83.3%, respectively; whereas the least frequent genus was *Teratocephalus* with AF of 16.6%. Among Fungivores,

Aphelenchus was the most dominant genus with AF of 33.3% and *Diphtherophora* was the least dominant with AF 16%. Among omnivores, *Eudorylaimus* was the most frequent genus with AF of 66%, and the least frequent genus was *Dorylaimus* with AF of 33.3%. Among plant parasitic, genus *Helicotylenchus* was the most frequent with AF of 33%, whereas *Pratylenchus* was the least frequent genus with AF of 16%.

Mean density: *Mylonchulus* was the most dominant genus with MD= 7.3 among predators, with relative density (RD) of 2.7%. The least dominant genus was *Discolaimus* with MD=2 and RD=0.7%. Among the bacterivores, *Mesorhabditis* was the most dominant genus with MD=35.6% and RD=13.2% and least dominant genus was *Heterocephalobus* MD= 2% with RD=0.7%. Among Fungivores, dominant genera were *Aphelenchus* with

MD=2.67 and RD=0.9%, and *Diptherophora* having least MD=0.5 and RD=0.1%. The genus *Eudorylaimus* MD=16 was the most dominant genus among the omnivores with relative density (RD) of 6%. The least dominant genus was *Dorylaimus*, MD=3.8 with relative density of 1.4%. Among the plant parasites, *Helicotylenchus* was the most dominant genus MD=11.8 with RD=4.4%; whereas the least dominant genus was *Pratylenchus*, with MD=3.3 and RD=1.2% each.

Table 2. Population structure of soil dwelling nematodes of Darhal Area of district Rajouri:

Feeding Habits	Genera	Darhal Area			
		N	AF%	MD	RD%
Bacterivores	<i>Mesorhabditis</i>	2	50	4.75	0.583
	<i>Pelodera</i>	1	25	3.5	0.429
	<i>Curviditis</i>	1	25	5	0.613
	<i>Wilsonema</i>	1	25	11	1.349
	<i>Alaimus</i>	3	75	33.25	4.079
	<i>Diploscapter</i>	1	25	5	0.613
	<i>Teratocephalus</i>	4	100	30.25	3.711
	<i>Rhabditis</i>	1	25	5.5	0.675
	<i>Plectus</i>	2	50	35	4.293
	<i>Monhystera</i>	4	100	26.5	3.251
	<i>Ceratoplectus</i>	3	75	25.25	3.097
	<i>Prismatolaimus</i>	2	50	10	1.227
	<i>Panagrolaimus</i>	3	75	21.25	2.607
	<i>Eucephalobus</i>	1	25	3	0.368
Fungivores	<i>Tylencholaimus</i>	3	75	12.25	1.503
	<i>Aphelenchoides</i>	1	25	3.5	0.429
	<i>Aphelenchus</i>	1	25	3.25	0.399
	<i>Diphtherophora</i>	1	25	1	0.123
	<i>Tylencholaimellus</i>	2	50	6.25	0.767
Herbivores	<i>Paratylenchus</i>	4	100	96.5	11.837
	<i>Helicotylenchus</i>	4	100	67.5	8.280
Predators	<i>Nagelus</i>	2	50	6.5	0.797
	<i>Pratylenchus</i>	4	100	41.25	5.060
	<i>Rotylenchus</i>	4	100	47	5.765
Omnivores	<i>Mylonchulus</i>	2	50	12	1.472
	<i>Mononchus</i>	2	50	8.5	1.043
	<i>Tripyla</i>	2	50	7.25	0.889
	<i>Clarkus</i>	1	25	1.5	0.184
	<i>Paravulvulus</i>	1	25	3.25	0.399
	<i>Eudorylaimus</i>	4	100	98.75	12.113
	<i>Epidorylaimus</i>	4	100	57.75	7.084
	<i>Prodorylaimus</i>	4	100	73.5	9.016
	<i>Mesodorylaimus</i>	4	100	48.5	5.949

Nematode community analyses in Darhal Area: A total of 33 genera were recorded from the Darhal area, with *Dorylaimida* (27.2%), representing the highest percentage followed by *Rhabditida* (24.2%), *Tylenchida* (15.1%), *Araeolaimida* (9.09%), *Mononchida* (9.09%), *Aphelenchida*

(6.06%), *Monhysterida* (3%) and *Enoplida* (6%), (Figure 1). On assigning the 33 genera to trophic groupings, the bacterivores represented the highest percentage (42%), followed by predators, fungivores and plant parasites each (15%) and omnivores (12%), (Table II) *Teratocephalus* and

Monhystera were the most dominant genera with the highest frequency of occurrence (100%); whereas the genera *Eucephalobus* and *Wilsonema* were the least common, with frequency of occurrence (25%).

Frequency: Among predators, the genus *Mylonchulus* was the most prevalent, with absolute frequency (AF) of 50%, whereas the least frequent genera were *Clarkus* and *Paravulvulus*, with AF of 25% each. *Teratocephalus* and *Monhystera* were the most frequent genera among the bacterivores with AF of 100%; whereas the least

frequent genus was *Eucephalobus* with AF of 25%. Among Fungivores, *Tylencholaimus* was the most dominant genus with AF of 75% and *Diptherophora*, *Aphelenchus* and *Aphelenchoides* were the least dominant genera with AF 25%. Among omnivores, *Eudorylaimus*, *Mesodorylaimus*, *Prodorylaimus* and *Epidorylaimus* were the most frequent genera with AF of 100%. Among plant parasitic, *Helicotylenchus*, *Paratylenchus*, *Pratylenchus* and *Rotylenchus* were the most frequent genera with AF of 100%, whereas *Nagelus* was the least frequent with AF of 50%.

Table 3. Population structure of soil dwelling nematodes of Kotranka Area:

Feeding Habits	Genera	Kotranka Area			
		N	AF%	MD	RD%
Bacterivores	Genera	N	AF%	MD	RD%
	Anaplectus	3	50.00	10.83	5.000
	Mesorhabditis	4	66.67	18.67	8.615
	Pelodera	5	83.33	18.83	8.692
	Acrobeles	5	83.33	25.17	11.615
	Cuticularia	1	16.67	4.17	1.923
	Wilsonema	6	100.00	28.00	12.923
	Alaimus	1	16.67	9.17	4.231
	Diploscapter	5	83.33	27.00	12.461
	Rhabditis	3	50.00	16.00	7.385
	Plectus	2	33.33	7.17	3.308
	Heterocephalobus	1	16.67	1.83	0.846
	Monhystera	2	33.33	6.33	2.923
	Eucephalobus	1	16.67	1.00	0.462
Fungivores	Tylencholaimus	1	16.67	1.00	0.462
	Leptonchus	1	16.67	2.50	1.154
	Aphelenchus	2	33.33	5.00	2.308
	Diptherophora	2	33.33	2.00	0.923
	Helicotylenchus	1	16.67	1.33	0.615
Herbivores	Nagelus	1	16.67	1.67	0.769
	Pratylenchus	1	16.67	4.33	2.000
	Longidorella	1	16.67	4.17	1.923
	Tylenchorhynchus	1	16.67	1.33	0.615
Predators	Discolaimus	1	16.67	2.83	1.308
	Mylonchulus	2	33.33	3.00	1.385
	Eudorylaimus	1	16.67	1.67	0.769
Omnivores	Heterodorus	1	16.67	1.33	0.615
	Allodorylaimus	1	16.67	2.00	0.923
	Prodorylaimus	1	16.67	1.67	0.769
	Dorylaimus	1	16.67	6.67	3.077

Mean density: *Mylonchulus* was the most dominant genus with MD= 12 among predators, with relative density (RD) of 1.4%. The least dominant genus was *Clarkus* with MD=1.5 and RD=0.1%. Among the bacterivores, *Alaimus* was the most dominant genus with MD=33% and RD=4.0% and least dominant genus was *Eucephalobus* MD= 3% with RD=0.3%. Among Fungivores, dominant

genus was *Tylencholaimus* with MD=12.2 and RD=1.5%. and *Diptherophora* having least MD=1 and RD=0.1%. The genus *Eudorylaimus* MD=98.7 was the most dominant genus among the omnivores with relative density (RD) of 12.1%. The least dominant genus was *Mesodorylaimus*, MD=48.5 with relative density of 5.9%. Among the plant parasites, *Paratylenchus* was the most dominant genus

MD=96.5 with RD=11.8%; whereas the least dominant genus was Nagelus, with MD=6.5 and RD=0.7%.

Nematode community analyses in Kotranka Area:

A total of 29 genera was recorded from the Kotranka area, with Dorylaimida (34.3%), representing the highest

percentage followed by *Rhabditida* (34%), *Tylenchida* (20%), *Araeolaimida* (6.8%), *Mononchida*, *Aphelenchida* and *Monhysterida* (3%) each, (Figure 1). On assigning the 29 genera to trophic groupings, the bacterivores represented the highest percentage (44%), followed by omnivores and plant parasites (17.2%) each, fungivores (13.7%) and predators (6.8%), (Table III).

Table 4. Population structure of soil dwelling nematodes of Bakori Area:

Feeding Habits	Genera	Bakori Area			
		N	AF%	MD	RD%
Bacterivores	Mesorhabditis	2	50	4.5	0.897
	Acrobeles	1	25	5.25	1.047
	Alaimus	3	75	15.75	3.141
	Diploscapter	1	25	2	0.399
	Teratocephalus	4	100	24.25	4.835
	Plectus	2	50	14.25	2.841
	Monhystera	3	75	10.5	2.094
	Prismatolaimus	4	100	14	2.792
	Panagrolaimus	3	75	11	2.193
	Eucephalobus	1	25	6.25	1.246
Fungivores	Tylencholaimus	4	100	13.75	2.742
	Aphelenchoides	3	75	7	1.396
	Deladenus	2	50	10.75	2.144
	Aphelenchus	1	25	3.5	0.698
	Tylencholaimellus	3	75	6	1.196
	Paratylenchus	4	100	24	4.786
	Helicotylenchus	4	100	28	5.583
Herbivores	Nagelus	1	25	2.75	0.548
	Pratylenchus	4	100	43	8.574
	Longidorella	1	25	7.5	1.496
	Tylenchorhynchus	2	50	9	1.795
	Dorylaimellus	3	75	7.75	1.545
Predators	Mylonchulus	1	25	1.75	0.349
	Tripyla	1	25	4	0.798
	Clarkus	1	25	2	0.399
	Paravulvulus	1	25	2.5	0.499
Omnivores	Eudorylaimus	2	50	8.25	1.645
	Heterodorus	4	100	52.75	10.518
	Allodorylaimus	4	100	64.5	12.861
	Prodorylaimus	3	75	25.25	5.035
	Dorylaimus	4	100	69.75	13.908

Frequency: Among predators, the genus *Mylonchulus* was the most prevalent, with absolute frequency (AF) of 33%, whereas the least frequent genus was *Discolaimus* with AF of 16.7%. *Wilsonema* and *Pelodera* were the most frequent genera among the bacterivores with AF of 100% and 83.3%, respectively; whereas the least frequent genus was *Eucephalobus* with AF of 16.6%. Among Fungivores, *Aphelenchus* and *Diptherophora* were the most dominant

genera with AF of 33.3% each and *Tylencholaimus* and *Leptonchus* were the least dominant genera with AF 16% each. Among omnivores, *Eudorylaimus*, *Heterodorus*, *Allodorylaimus*, *Prodorylaimus* and *Dorylaimus* were the most frequent genera with AF of 16% each. Among plant parasitic, genus *Helicotylenchus*, *Nagelus*, *Pratylenchus*, *Longidorella* and *Tylenchorhynchus* were the most frequent with AF of 16.7% each.

Mean density: Mylonchulus was the most dominant genus with MD= 3 among predators, with relative density (RD) of 1.3%. The least dominant genus was Discolaimus with MD=2.8 and RD=1.3%. Among the bacterivores, Wilsonema was the most dominant genus with MD=28 and RD=12% and least dominant genus was Eucephalobus MD= 1% with RD=0.4%. Among Fungivores, dominant genus was Aphelenchus with MD=5 and RD=2.3%. and

Tylencholaimus having least MD=1 and RD=0.4%. The genus Dorylaimus MD=6.6 was the most dominant genus among the omnivores with relative density (RD) of 3.0%. The least dominant genus was Heterodorus, MD=1.3 with relative density of 0.6%. Among the plant parasites, Pratylenchus was the most dominant genus MD=4.3 with RD=2%; whereas the least dominant genera were Helicotylenchus and Tylenchorhynchus with MD=1.3 and RD=0.6% each.

Table 5. Population structure of soil dwelling nematodes of Rajouri Area:

Feeding Habits	Genera	Rajouri Area			
		N	AF%	MD	RD%
Bacterivores	Anaplectus	3	50	7	3.328
	Mesorhabditis	5	83	16	7.904
	Pelodera	4	67	50	25.042
	Acrobeles	3	50	13	6.489
	Cuticularia	2	33	5	2.329
	Curviditis	1	17	4	2.163
	Wilsonema	3	50	6	3.161
	Alaimus	1	17	3	1.248
	Diploscapter	3	50	12	6.157
	Teratocephalus	1	17	1	0.582
	Rhabditis	3	50	8	4.077
	Plectus	3	50	17	8.569
	Heterocephalobus	4	67	6	2.995
	Monhystera	2	33	6	3.161
	Ceratoplectus	1	17	6	2.829
Fungivores	Leptonchus	1	17	2	0.749
	Diphtherophora	3	50	8	3.744
Herbivores	Tylenchorhynchus	1	17	2	0.832
Predators	Discolaimus	1	17	2	0.749
Omnivores	Eudorylaimus	3	50	20	10.150
	Prodorylaimus	1	17	4	2.163
	Mesodorylaimus	1	17	3	1.581

Nematode community analyses in Bakori Area: A total of 31 genera were recorded from the Bakori area, with Dorylaimida (29%), representing the highest percentage followed by Tylenchida (25.8%), Rhabditida (19%), Mononchida, Enoplida and Aphelenchida (6.06%) each, Araeolaimida and Monhysterida (3.2%) each, (Figure 1). On assigning the 31 genera to trophic groupings, the bacterivores represented the highest percentage (32%), followed by plant parasitic (22%), omnivores and fungivores (16%) and then predators (12%), (Table IV).

Frequency: Among predators, the genera Mylonchulus, Tripyla, Clarkus and Paravulvus were the most prevalent, with absolute frequency (AF) of 25%. Teratocephalus and Pristomatolaimus were the most frequent genera among the bacterivores with AF of 100% each; whereas the least frequent genera were Acrobeles, Diploscapter and Eucephalobus with AF of 25%. Among Fungivores,

Tylencholaimus was the most dominant genus with AF of 100% and Aphelenchus was the least dominant with AF 25%. Among omnivores, Dorylaimus was the most frequent genus with AF of 100%, and the least frequent genus was Eudorylaimus with AF of 50%. Among plant parasitic, genus Helicotylenchus was the most frequent with AF of 100%, whereas Nagelus and Longidorellawere the least frequent genera with AF of 25%.

Mean density: Tripyla was the most dominant genus with MD= 4 among predators, with relative density (RD) of 0.7%. The least dominant genus was Clarkus with MD=2 and RD=0.3%. Among the bacterivores, Teratocephalus was the most dominant genus with MD=24.25 and RD=4.8% and least dominant genus was Diploscapter MD= 2% with RD=0.3%. Among Fungivores, dominant genus was Tylencholaimus with MD=13.7 and RD=2.7% and Aphelenchus having least MD=3.5 and RD=0.6%. The

genus *Dorylaimus* MD=69.7 was the most dominant genus among the omnivores with relative density (RD) of 13.9%. The least dominant genus was *Eudorylaimus*, MD=8.2 with relative density of 1.6%. Among the plant parasitic nematodes, *Helicotylenchus* was the most dominant genus MD=28 with RD=5.5%; whereas the least dominant genus was *Nagelus*, with MD=2.7 and RD=0.5% each.

Nematode community analyses in Rajouri Area: A total of 22 genera were recorded from the Rajouri area, with Rhabditida (45.4%), representing the highest percentage followed by Dorylaimida (27.2%), Araeolaimida (13.6%), Tylenchida (9%), and Monhysterida (4.5%), (Figure 1). On assigning the 22 genera to trophic groupings, the bacterivores represented the highest percentage (68%),

followed by omnivores (13.6%) and fungivores (9%) and then plant parasites and predators (4.5%) each, (Table V).

Frequency: Among predators, the genus *Discolaimus* was the most prevalent, with absolute frequency (AF) of 17%. *Mesorhabditis* and *Pelodera* were the most frequent genera among the bacterivores with AF of 83% and 67%, respectively; whereas the least frequent genus was *Teratocephalus* with AF of 17%. Among Fungivores, *Diptherophora* was the most dominant genus with AF of 50% and *Leptonchus* was the least dominant with AF 17%. Among omnivores, *Eudorylaimus* was the most frequent genus with AF of 50%, and the least frequent genus was *Mesodorylaimus* with AF of 16.9%. Among plant parasitic nematodes, genus *Tylenchorhynchus* was the most frequent with AF of 17%.

Table 6. Population structure of soil dwelling nematodes of Thanamandi Area:

Feeding Habits	Genera	Thanamandi Area			
		N	AF%	MD	RD%
Bacterivores	<i>Mesorhabditis</i>	2	50.0	3.8	0.803
	<i>Pelodera</i>	1	25.0	0.8	0.161
	<i>Acrobeles</i>	1	25.0	20.0	4.283
	<i>Alaimus</i>	3	75.0	15.3	3.266
	<i>Diploscapter</i>	2	50.0	12.5	2.677
	<i>Teratocephalus</i>	4	100.0	16.3	3.480
	<i>Rhabditis</i>	2	50.0	8.0	1.713
	<i>Plectus</i>	2	50.0	28.5	6.103
	<i>Monhystera</i>	1	25.0	4.0	0.857
	<i>Ceratoplectus</i>	3	75.0	11.8	2.516
	<i>Prismatolaimus</i>	2	50.0	7.5	1.606
	<i>Panagrolaimus</i>	2	50.0	9.5	2.034
	<i>Eucephalobus</i>	2	50.0	5.5	1.178
Fungivores	<i>Tylencholaimus</i>	3	75.0	4.0	0.857
	<i>Aphelenchus</i>	3	75.0	8.5	1.820
	<i>Tylencholaimellus</i>	3	75.0	11.0	2.355
Herbivores	<i>Paratylenchus</i>	3	75.0	11.0	2.355
	<i>Helicotylenchus</i>	3	75.0	11.5	2.463
	<i>Nagelus</i>	4	100.0	6.8	1.445
	<i>Pratylenchus</i>	2	50.0	5.0	1.071
	<i>Tylenchorhynchus</i>	3	75.0	7.5	1.606
	<i>Dorylaimellus</i>	2	50.0	2.8	0.589
Predators	<i>Mylonchulus</i>	1	25.0	7.5	1.606
	<i>Mononchus</i>	1	25.0	4.5	0.964
	<i>Paravulvulus</i>	1	25.0	1.5	0.321
Omnivores	<i>Eudorylaimus</i>	4	100.0	121.3	25.964
	<i>Prodorylaimus</i>	4	100.0	112.5	24.090
	<i>Mesodorylaimus</i>	2	50.0	8.5	1.820
	<i>Dorylaimus</i>	4	100	69.75	13.908

Mean density: *Discolaimus* was the most dominant genus with MD= 2 among predators, with relative density (RD) of 0.7%. Among the bacterivores, *Pelodera* was the most dominant genus with MD=50% and RD=25%

and least dominant genus was *Teratocephalus* MD= 1% with RD=0.5%. Among Fungivores, dominant genus was *Diptherophora* with MD=8 and RD=3.7%. and *Leptonchus* having least MD=2 and RD=0.7%. The genus *Eudorylaimus*

MD=20 was the most dominant genus among the omnivores with relative density (RD) of 10.1%. The least dominant genus was Mesodorylaimus, MD=3 with relative

density of 1.5%. Among the plant parasitic nematodes, Tylenchorhynchus was the most dominant genus with MD=2 with RD=0.8%.

Table 7. Population structure of soil dwelling nematodes of Khawas Area:

Feeding Habits	Genera	Khawas Area			
		N	AF%	MD	RD%
Bacterivores	Anaplectus	2	33.33	3.33	1.596
	Mesorhabditis	3	50.00	7.33	3.512
	Pelodera	4	66.67	10.50	5.028
	Acrobeles	3	50.00	14.83	7.103
	Wilsonema	6	100.00	30.50	14.605
	Alaimus	1	16.67	5.83	2.793
	Diploscapter	4	66.67	14.33	6.864
	Teratocephalus	1	16.67	0.67	0.319
	Rhabditis	3	50.00	11.50	5.507
	Plectus	2	33.33	10.17	4.868
	Heterocephalobus	2	33.33	5.83	2.793
	Monhystera	1	16.67	1.33	0.638
Fungivores	Tylencholaimus	1	16.67	1.83	0.878
	Aphelenchoides	2	33.33	3.17	1.516
	Leptonchus	1	16.67	2.00	0.958
	Aphelenchus	3	50.00	3.83	1.836
	Diphtherophora	3	50.00	5.50	2.634
Herbivores	Helicotylenchus	1	16.67	3.50	1.676
	Rotylenchus	3	50.00	9.17	4.390
Predators	Mylonchulus	1	16.67	2.00	0.958
	Enchodelus	1	16.67	1.67	0.798
	Clarkus	1	16.67	2.50	1.197
Omnivores	Eudorylaimus	1	16.67	3.83	1.836
	Epidorylaimus	1	16.67	14.33	6.864
	Heterodorus	1	16.67	2.50	1.197
	Allodorylaimus	2	33.33	8.33	3.990
	Mesodorylaimus	2	33.33	3.67	1.756
	Dorylaimus	2	33.33	9.17	4.390
	Pungentus	3	50.00	15.67	7.502

Nematode community analyses in Thanamandi Area: A total of 28 genera were recorded from the Thanamandi area, with Rhabditida (28%), representing the highest percentage followed by Dorylaimida (25%), Tylenchida (21%), Araeolaimida and Mononchida (7.1%) each, Aphelenchida, Monhysterida and Enoplida (3.5%) each, (Figure 1). On assigning the recorded 28 genera to trophic groupings, the bacterivores represented the highest percentage (46.4%), followed by plant parasites (21.4%), and then omnivores, fungivores and predators (10.7%) each, (Table VI).

Frequency: Among predators, the genera Mylonchulus, Mononchus and Paravulvulus were the most prevalent, with absolute frequency (AF) of 25% each. Teratocephalus and Ceratoplectus were the most frequent genera among the bacterivores with AF of 100% and 75%, respectively;

whereas the least frequent genera were Pelodera and Monhystera with AF of 25% each. Among Fungivores, Aphelenchus, Tylencholaimus and Tylencholaimellus were the most dominant genera with AF of 75% each. Among omnivores, Eudorylaimus was the most frequent genus with AF of 100%, and the least frequent genus was Mesodorylaimus with AF of 50%. Among plant parasitic nematodes, genus Nagelus was the most frequent genus with AF of 100%, whereas Pratylenchus was the least frequent genus with AF of 50%.

Mean density: Mylonchulus was the most dominant genus with MD= 7.5 among predators, with relative density (RD) of 1.6%. The least dominant genus was Paravulvulus with MD=1.5 and RD=0.3%. Among the bacterivores, Plectus was the most dominant genus with MD=28.5% and

RD=6.1% and least dominant genus was *Pelodera* MD=0.8% with RD=0.1%. Among Fungivores, dominant genera were *Tylencholaimellus* with MD=11 and RD=2.3%. and *Tylencholaimus* having least MD=4 and RD=0.8%. The genus *Eudorylaimus* MD=121 was the most dominant genus

among the omnivores with relative density (RD) of 25.9%. The least dominant genus was *Mesodorylaimus*, MD=8.5 with relative density of 1.8%. Among the plant parasites, *Helicotylenchus* was the most dominant genus MD=11.5 with RD=2.4%; whereas the least dominant genus was *Dorylaimellus*, with MD=2.8 and RD=0.5%.

Table 8. Population structure of soil dwelling nematodes of Budhal Area:

Feeding Habits	Genera	Budhal Area			
		N	AF%	MD	RD%
Bacterivores	<i>Mesorhabditis</i>	3	75.0	3.25	0.502
	<i>Pelodera</i>	1	25.0	2.25	0.348
	<i>Acrobeles</i>	2	50.0	28.75	4.444
	<i>Wilsonema</i>	2	50.0	2	0.309
	<i>Alaimus</i>	2	50.0	25.5	3.941
	<i>Diploscapter</i>	2	50.0	8	1.236
	<i>Teratocephalus</i>	3	75.0	6.5	1.005
	<i>Rhabditis</i>	2	50.0	3.75	0.580
	<i>Plectus</i>	1	25.0	11.5	1.777
	<i>Heterocephalobus</i>	1	25.0	1	0.155
	<i>Monhystera</i>	2	50.0	6	0.927
	<i>Ceratoplectus</i>	2	50.0	9.5	1.468
	<i>Prismatolaimus</i>	2	50.0	11.25	1.739
	<i>Panagrolaimus</i>	3	75.0	15	2.318
	<i>Eucephalobus</i>	3	75.0	12.25	1.893
Fungivores	<i>Aphelenchoides</i>	1	25.0	4.25	0.657
	<i>Leptonchus</i>	2	50.0	12.25	1.893
	<i>Aphelenchus</i>	1	25.0	3.5	0.541
	<i>Tylencholaimellus</i>	4	100.0	7	1.082
Herbivores	<i>Paratylenchus</i>	4	100.0	18.5	2.859
	<i>Helicotylenchus</i>	3	75.0	34.75	5.371
	<i>Pratylenchus</i>	3	75.0	7.25	1.121
	<i>Rotylenchus</i>	1	25.0	1.25	0.193
	<i>Longidorella</i>	2	50.0	12	1.855
	<i>Tylenchorhynchus</i>	4	100.0	18.5	2.859
Predators	<i>Mylonchulus</i>	1	25.0	11	1.700
	<i>Mononchus</i>	1	25.0	7	1.082
	<i>Enchodelus</i>	3	75.0	18.5	2.859
	<i>Paravulvulus</i>	1	25.0	6.25	0.966
Omnivores	<i>Eudorylaimus</i>	4	100.0	34.25	5.294
	<i>Epidorylaimus</i>	4	100.0	84.25	13.022
	<i>Heterodorus</i>	4	100.0	43.75	6.762
	<i>Allodorylaimus</i>	4	100.0	101.25	15.649
	<i>Prodorylaimus</i>	2	50.0	7	1.082
	<i>Mesodorylaimus</i>	3	75.0	26.75	4.134
	<i>Pungentus</i>	4	100.0	41.25	6.376

N= Frequency; c) AF= absolute frequency; d) MD = mean density;
e) RD= relative density

Nematode community analyses in Khawas Area: A total of 29 genera were recorded from the Khawas area, with Dorylaimida (37.9%), representing the highest percentage followed by Rhabditida (27.5%), Tylenchida (10%), Araeolaimida, Mononchida and Aphelenchida (6.8%) each

and Monhysterida (3.4%), (Figure 1). On assigning the 29 genera to trophic groupings, the bacterivores represented the highest percentage (41.3%), followed by omnivores (24.1%) and fungivores (17.2%), predators (10%) and then plant parasites (6.8%), (Table VII).

Table 9. Summary of nematode diversity indices of Major areas of district Rajouri:

Ecological indices	Manjakote	Kotranka	Rajouri	Khawas
Simpson index (D)	0.64	0.61	0.49	0.71
Shannon-Weaver Index (H')	2.8	2.8	2.5	3.04
Inverse Simpson index (Hill's Reciprocal)	1.5	1.63	2.04	1.40

Table 10

Ecological indices	Darhal	Bakori	Thana mandi	Budhal
Simpson index (D)	0.78	0.75	0.51	0.84
Shannon-Weaver Index (H')	3.07	2.9	2.6	4.07
Inverse Simpson index (Hill's Reciprocal)	1.27	1.3	1.96	1.19

Frequency: Among predators, the genera Mylonchulus, Enchodelus and Clarkus were the most prevalent, with absolute frequency (AF) of 16.7% each. Wilsonema and Pelodera were the most frequent genera among the bacterivores with AF of 100% and 66.7%, respectively; whereas the least frequent genus was Teratocephalus with AF of 16.6%. Among Fungivores, Aphelenchus and Diptherophora were the most dominant genera with AF of 50% each and Leptonchus and Tylencholaimus were the least dominant genera with AF 16% each. Among omnivores, Pungentus was the most frequent genus with AF of 50%, and the least frequent genus was Heterodorus with AF of 16%. Among plant parasitic nematodes, genus Rotylenchus was the most frequent with AF of 50%, whereas Helicotylenchus was the least frequent with AF of 16%.

Mean density: Clarkus was the most dominant genus with MD= 2.5 among predators, with relative density (RD) of 1.1%. The least dominant genus was Enchodelus with MD=1.6 and RD=0.7. Among the bacterivores, Wilsonema was the most dominant genus with MD=30% and RD=14.6% and least dominant genus was Teratocephalus MD= 0.6% with RD=0.3%. Among Fungivores, dominant genera were Diptherophora with MD=5.5 and RD=2.6%. and Leptonchus having least MD=2 and RD=0.9%. The genus Pungentus with MD=15.6 was the most dominant genus among the omnivores with relative density (RD) of 7.5%. The least dominant genus was Heterodorus, MD=2.5 with relative density of 1.1%. Among the plant parasitic nematodes, Rotylenchus was the most dominant genus MD=9.1 with RD=4.3%; whereas the least dominant genus was Helicotylenchus, with MD=3.5 and RD=1.6%.

Nematode community analyses in Budhal Area: A total of 36 genera were recorded from the Budhal area, with

Dorylaimida (30.5%), representing the highest percentage followed by Rhabditida (25%), Tylenchida (19.4%), Araeolaimida (8.3%), Mononchida and Aphelenchida (5.5%), Monhysterida (2.7%) and Enoplida (2.7%), (Figure 1). On assigning the 36 genera to trophic groupings, the bacterivores represented the highest percentage (41.6%), followed by omnivores (19.4%), then plant parasites (16.6%) and fungivores (11%) and predators (11.1%), (Table VIII).

Frequency: Among predators, the genus Enchodelus was the most prevalent, with absolute frequency (AF) of 75%, whereas the least frequent genus was Mononchus, with AF of 25%. Mesorhabditis and Teratocephalus were the most frequent genera among the bacterivores with AF of 75% each; whereas the least frequent genus was Heterocephalus with AF of 25%. Among Fungivores, Tylencholaimus was the most dominant genus with AF of 100% and Aphelenchus was the least dominant genus with AF 25%. Among omnivores, Eudorylaimus, Epidorylaimus, Allodorylaimus and Heterodorus were the most frequent genera with AF of 100%, and the least frequent genus was Prodorylaimus with AF of 50%. Among plant parasitic, genera Tylenchorhynchus and Paratylenchus were the most frequent with AF of 100%, whereas Rotylenchus was the least frequent with AF of 25%.

Mean density: Enchodelus was the most dominant genus with MD= 18.5 among predators, with relative density (RD) of 2.8%. The least dominant genus was Paravulvulus with MD=6.2 and RD=0.9%. Among the bacterivores, Acrobeles was the most dominant genus with MD=28.7% and RD=4.4% and least dominant genus was Heterocephalobus MD= 1% with RD=0.1%. Among Fungivores, dominant genus was Leptonchus with MD=12.2 and RD=1.8%. and genus Aphelenchus having least MD=3.5 and RD=0.5%.

The genus *Allodorylaimus* MD=101.2 was the most dominant genus among the omnivores with relative density (RD) of 15.6%. The least dominant genus was *Prodorylaimus*, MD=7 with relative density of 1.0%. Among the plant parasitic nematodes, *Helicotylenchus* was the most dominant genus MD=34.7 with RD=5.3%; whereas the least dominant genus was *Rotylenchus*, with MD=1.2 and RD=0.1%.

Figure 1: Taxonomic diversity of soil-inhabiting nematodes of various areas of district Rajouri

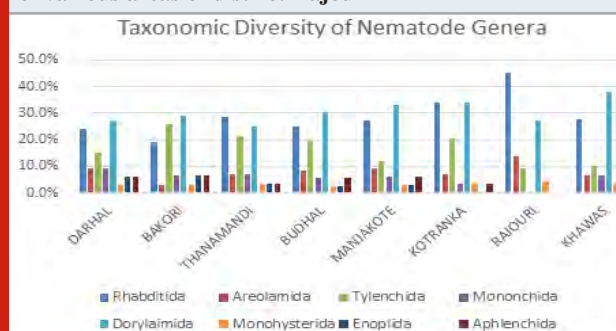
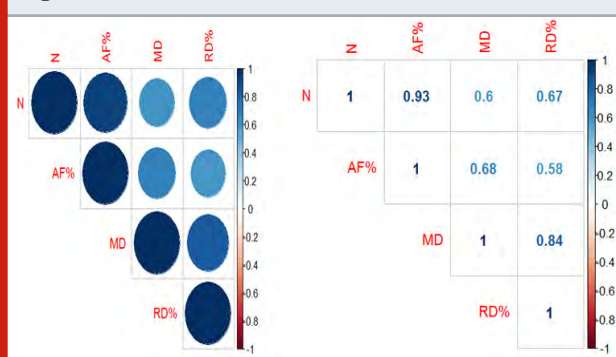


Figure 2: Correlation Plots:



Nematodes form a large and diverse group in the animal kingdom and are important ecologically as well economically. Their varied life cycles and the ways of life presence in almost all habitats are features unmatched by other groups of animals. Nematodes play an extremely important role in the soil ecology and biology. They occupy all levels of the food chain from bacterivores secondary degradation to predators (primary consumers). Because of the varied mode of feeding and their sensitivity to ecological and toxicological factors nematodes have become extremely important as ecological and biological indicators. Numerous soil nematode species have significant economic implications as well. As parasites of plants (both ecto- and endoparasites) they cause substantial damage. As parasites of insects, they are capable of destroying many insect pests and parasites and thereby play an important role in biocontrol program. As bacterial and fungal feeders they indicate the primary decomposition pathways of the soil, whether fungal dependent or bacterial dependent. An analysis of the community structures of these and other groups of nematodes would provide good indices on the state and fertility of soil as well (Hoogen et al. 2019; Hoogen et al. 2020).

The belowground group of nematodes are key components of the soil ecosystem. The nematode communities become more abundant and richer going from lower areas to higher reaches. The total number of nematodes were presented as number of individuals per 100g of dry soil sample and the diversity is calculated as Simpson index(D). Moreover, both nematode abundance and nematode diversity increases at higher altitudes. Increase in the Simpson index, Shannon Weiner index indicates increase in the diversity of soil dwelling nematodes at respective sites. Inverse Simpson index shows the same (Table X). Nematodes dwell in every ecosystem, they have the ability to colonize the harsh environments such as extreme temperatures in the alpine zones. (Yeates, 2010). The increase in the diversity of high elevation zones was also given by (Hoschitz and Kaufmann, 2004) in which high densities of nematodes and high diversity within the communities of nematodes were recorded (Hoogen et al. 2019; Hoogen et al. 2020).

However, study of taxonomic diversity can inform on biodiversity changes along various ecological gradients, the functional characterization of nematodes being major players in the community are necessary to link biodiversity to ecosystem functioning (Wall and Lynch 2000; Tilman 2001). Studies on taxonomic diversity along with functional diversity specially in the previous decade, are mostly focused on analyzing the basic mechanisms of ecosystem functioning (Thebault and Loreau 2006; Reiss et al. 2009; Montoya, Yallop and Memmott 2015). Moreover, these newly described trophic functional guilds gives a more vivid picture of nematode assemblage and thus better understanding of ecosystem functioning (Hoogen et al. 2019; Hoogen et al. 2020).

The dominant nematode communities are mostly formed by taxa like: *Eudorylaimus*, *Pratylenchus*, *Paratylenchus* and *Teratocephalus*. Among them *Eudorylaimus* and *Teratocephalus* have a worldwide distribution and known to colonize arctic or other extreme temperature habitats (Loof 1971; Ruess, Michelsen and Jonasson 1999; Hoschitz and Kaufmann 2004). *Pratylenchus* is also known to have wide host range that can be the reason of its high abundance in the upper regions (Jones and Fosu-Nyarko 2014). However, in lower reaches, nematode dominant communities are mostly bacterivores like *Acrobeles*, *Pelodera*, *Plectus* and *Cephalobus*, probably because of the required temperature for carrying out bacterial processes like decomposition which is otherwise little bit difficult on the upper zones. Thus, we can say, areas with high elevations, mostly have taxa which are more sensitive to environmental disturbances are found with high cp values showing a persistent mode of life history, adopting k-strategists, having longer life cycles and sensitive to soil perturbation marking their way as good bioindicators. They have all the attributes to be recognized as efficient bioindicators (McGeoch 1998; Ritz and Trudgill 1999; Hilty and Merenlender 2000; Hoogen et al. 2020).

CONCLUSION

The present work was planned to study the nematodes to ascertain their relationship with the environment. Very few disturbances, indicates the progression of sensitive

genera of nematodes towards the hilly areas, whereas in the lower areas, the genera found in abundance are less sensitive to disturbances. Thus, crucial for bio-monitoring of the environment. The diversity indices representing the diversity pattern of nematodes of various areas selected. Since nematodes play a well-defined role as bio-indicators, a thorough study of the dominance and distribution of nematodes will be highly significant in the bio-monitoring of the ecological changes in the areas.

ACKNOWLEDGEMENTS

This work was financially supported by the Council of Scientific and Industrial Research (CSIR, India, File number 380 09/1172(0002)/EMR-2018), which granted a research fellowship to YK.

Conflict of Interests: Authors declare no conflict of interest to disclose.

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Biotechnological Communication

Influence of Microwave Radiation on Whiskey Distillate Quality Indicators

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ABSTRACT

The paper aims to evaluate the quality indicators of the whiskey distillate during its maturation under the influence of microwave radiation based on the method of multiple regression analysis to determine the optimal technological parameters for its production. In the course of solving the problem of product import substitution, we tested the patented technology for producing strong alcoholic beverages at the stage of their aging, using oak chips with various degrees of heat treatment. One of the promising areas for intensifying the process of whiskey distillate maturation is applying “green technologies”, with microwave heating being one of them. Positive results were obtained for the effect of microwave radiation on the process of target component extraction from oak chips with a water-alcohol solution. The duration of this extraction process was reduced by more than 2 times, which confirmed its intensity in relation to the classical method. The obtained regression models proved adequate, since the mean relative error varied within 10%. The chromatographic analysis of the whiskey produced at the optimal technological parameters showed that all physical and chemical properties and product safety meet the requirements of the applicable regulations and standards.

KEY WORDS: EXTRACTION, MALT, OAK CHIPS, PHYSICAL AND CHEMICAL PROPERTIES, RATIONAL TECHNOLOGICAL MODES.

INTRODUCTION

The main raw materials for the production of whiskey and whiskey-based beverages are malted grain (barley, wheat, rye, corn); the other raw materials that affect the taste and aroma of whiskey – water and oak barrels where the aging process traditionally takes place – are equally important. In the classical production technology, the technological stage of whiskey distillates aging in oak barrels requires a certain amount of time. In addition, the barrels themselves require specific preparation, which makes both the barrels and the resulting product – whiskey – more expensive.

The analysis of Russian and foreign scientific literature published in the past ten years has shown that one of the promising areas for intensifying the process of whiskey distillate maturation is applying “green technologies”, which imply more eco-friendly production processes (concerning both the environment and the product itself) compared to the traditional technologies. The “green technologies” are implemented in the technological and innovative spheres to solve the issues of adapting alternative production processes

to improve the technological chain as a whole (Dumitriu et al. 2019).

“Green technologies” include microwave heating (Camarano et al. 2019). Studies of the extraction of polyphenol compounds using microwave heating and conventional solvent extraction showed that selective microwave heating can increase the yield and quality of the extracted polyphenols. It was found that at the same volume heating rate, there was no difference in the processing time and, consequently, in the theoretical energy requirements for the process (Galan et al. 2017; More and Gogate 2018; González-Centeno et al. 2019; Drevelegka and Goula 2020). The purpose of this paper is to evaluate the quality indicators of the whiskey distillate during its maturation under the influence of microwave radiation based on the method of multiple regression analysis to determine the optimal technological parameters for its production.

MATERIAL AND METHODS

The paper seeks to solve the following research tasks:

1. A multiple regression analysis of the effect of any two independent variables (the time of whiskey distillate exposure to microwave radiation t ; the power of microwave radiation N ; alcohol concentration in the whiskey distillate C) on the dependent variables – the

Article Information:*Corresponding Author: prosinmv@yandex.ru

Received 30/09/2021 Accepted after revision 25/12/2021

Published: 31st December 2021 Pp- 1701-1713

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.48>

- quality indicators of the obtained whiskey distillates (optical density (D), polyphenol content (PPh) and solid compound content (SC)) in the process of maturation under microwave radiation;
2. A multiple regression analysis of the influence of all the independent variables (t), (N) and (C) on the dependent variables: (optical density (D), polyphenol content (PPh) and solid compound content (SC)) in the whiskey distillate during its maturation under microwave radiation;
 3. A chromatographic analysis on the whiskey obtained at the optimum process parameters;
 4. An organoleptic assessment of the quality of whiskey.

The research team studied the whiskey distillates samples meeting the standards of modern industrial and state standards. Heat-treated French oak chips of strong charring were selected as the solid phase. The chips had the same size of about 5×5×5 mm and did not contain bark particles. Alcohol concentration in the studied whiskey distillate samples was 45%, 60%, and 75 %. During the research, a number of measuring methods were used to evaluate the extract samples. Spectrophotometry was used to assess the optical density of the distillates, the Erumanis method was used to measure the content of polyphenol, and the content

of solid compounds was measured by drying (Dyshlyuk et al. 2017; Korotkova et al. 2018; Borodulin et al. 2019a).

The optical density of the samples was analyzed to assess the change in their colour. The analysis was performed using the photoelectric colorimeter KFK-2, in sample pans with a layer thickness of 10 mm and a wave length of 540 nm against pure whiskey distillate. The work of the photoelectric colorimeter KFK-2 consists in allowing a stream of light pass through the studied solution and measuring the optical transmission coefficient. The arithmetic mean of three replicates (to three decimal places) was used as a final optical density value (Borodulin et al. 2019b). Specification of the photoelectric colorimeter KFK-2:

The Erumanis method was used to measure the content of polyphenols. This method was based on the reaction of polyphenols with trivalent iron salts in an alkaline medium. The polyphenol content (PPh) in the samples was calculated by the formula: $PPh = A - B + C \times 820$, where A was the optical density of the solution in the main experiment; B was the optical density of the solution in the control sample 1; C was the optical density of the solution in the control sample 2; and 820 was the conversion factor for polyphenols (Borodulin et al. 2020; Page 2019; Phetxumhou et al. 2020).

• Spectral wavelength:	315 ~ 980 nm
• Transmission coefficient measurement range:	100 ~ 5%
• Absolute error:	not exceeding $\pm 1\%$
• Light source:	small halogen tube KGM 6.3-15
• Cuvette working length (cuvette set No. 2):	50, 30, 20, 10, 5 mm
• Input power:	not exceeding 75 V*A
• AC power supply:	(220 \pm 22) W, (50/60 \pm 0.5) Hz
• Overall dimensions:	not exceeding 435 x 335 x 330 mm
• Weight:	14 kg

• Scale capacity, g	71
• Weighing sensitivity, g	0,0001
• Moisture content error, %	0,01
• Drying temperature, °C	30-200
• Humidity accuracy, %, (sample size: >1 g)	0,5
• Humidity accuracy, %, (sample size: >5 g)	0,01
• Weights external calibration	20g or 50g
• In-memory measuring programmes, ea	20
• Memory size, measurements	100
• Response rate, s	< 1
• Platform diameter, mm	85
• Overall dimensions, mm	215x320x173
• Power supply	220 W
• Operating temperature range, °C	+5 ~ +40
• Operating humidity, %	not exceeding 85
• Weight, kg	6

The content of solid compounds was measured by drying. The studies were performed using a thermogravimetric humidity analyzer MS-70. Devices of this type were based

on the principle of thermogravimetric measurements, where samples with a known initial mass were dried, residues were weighed and the relative change in mass was determined with accuracy to four decimal places. After setting the accuracy and drying temperature, the sample weight and completion temperature were determined automatically. When the drying speed reaches the set value, the measurement stops automatically. The value of the solid content in the sample was calculated automatically by the formula: $SC = (MW / MD) \times 100\%$, where MW was the mass of the wet sample, MD was the mass of the dried sample (Pradal et al. 2018; Phetxumhou et al. 2020). Specification of the humidity analyzer MS-70:

The chromatographic and organoleptic analysis of the studied whiskey samples was performed by the Federal Budgetary Institution "State Regional Standardization, Metrology and Testing Centre of Kemerovo Region". Certain properties were assessed using the gas chromatograph "Crystallux-4000M" according to the modern industrial standards. To analyze the effect of microwave radiation on the quality of whiskey distillate samples, a full-factorial experiment was performed, during which the following parameters obtained within previous studies were varied:

- the time of whisky distillate exposure to microwave radiation ($t = 1, 2, 3$ min.);
- the power of microwave radiation ($N = 450$ W – LOW mode; 600 W – MEDIUM mode; 750 W – HIGH mode);
- alcohol concentration in the whiskey distillate ($C = 45\%$, 60%, 75%).

Young whiskey distillates meeting the modern industrial standards were stored glass tanks. Similar amounts of French oak chips of strong charring were added to the distillates (1 g per 500 ml of the solution). After that the samples were exposed to microwave radiation of different power N (400 W, 600 W, and 750 W) for a different time t (1, 2, and 3 minutes). These radiation power values were selected since at a lower radiation power no visible differences are observed in the process of whiskey distillates maturation compared to the classical method (i.e., microwave radiation has no effect): the resulting quality indicators (optical density, polyphenol content and solid compound content) do not change.

Qualitative changes in the numerical values of the indicators under consideration were observed when whiskey distillates mature with exposure to microwave radiation with a power of 450 W. Therefore, processing whiskey distillates with microwave radiation at a lower power was not advisable. When the microwave radiation power exceeds 750 W, the whiskey distillate started to boil, which led to the loss of solution during evaporation. In this regard, to intensify the process of whiskey distillate maturation, it was exposed to the influence of microwave radiation at the power ranging within 450-750 W. The distillates exposed to microwave radiation matured for 9 months, after which the studied quality indicators were measured. The obtained values were put in the table. The multiple regression analysis of the obtained numeric values was performed using Statistica 10 software ("General regression models" module).

obtained numeric values are provided in Table 2. Possible measurement errors are due to the standard error of the measuring devices.

The study setup for microwave radiation influence on the quality indicators of the whiskey distillate is shown in Figure 1. The levels and variation intervals of the full-factorial design are shown in Table 1.

Table 1. Levels and variation intervals of the full-factorial design

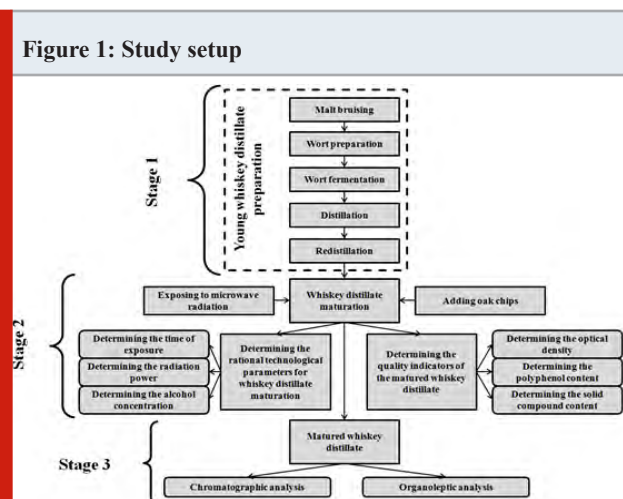
Factor and notation	Upper level	Lower level	Center point	Interval
Time of exposure to micro-wave radiation, min., X1	3	1	2	1
Power of microwave radiation, W, X2	750	450	600	150
Alcohol concentration in the whiskey distillate, %, X3	75	60	45	15

Research task 1 was solved by determining regression dependences of the quality indicators of the obtained whiskey distillates (optical density (D), polyphenol content (PPh) and solid compound content (SC)) in the process of maturation under microwave radiation on any two independent variables (the time of whiskey distillate exposure to microwave radiation t ; the power of microwave radiation N ; alcohol concentration in the whiskey distillate C) using Statistica software. Further analysis of the obtained data contained three stages, with optimal technological parameters influencing the quality indicators of the maturing whiskey distillates exposed to microwave radiation determined for each of the stages (Rudoy 2016; Wu et al. 2018; Gnetko et al. 2019; Yang et al. 2020).

Stage 1 of solving Research task 1 involved multiple regression analysis of the influence of any two independent variables (the time of whiskey distillate exposure to microwave radiation t ; the power of microwave radiation N ; alcohol concentration in the whiskey distillate C) on the dependent variable: the optical density (D) of the maturing whiskey distillates exposed to microwave radiation (Table 2). Data array processing allowed obtaining the regression models for the dependence of (D) in the whiskey distillates on the variables (t, N, C) (Wu et al. 2018; Habschied et al. 2019; Wanikawa 2020). The relationship between the optical density of whiskey distillates and the time of exposure to and the power of microwave radiation is determined by the equation:

$$D = 0.140481 - 0.007833t + 0.000278t^2 - 0.000229N \quad (1)$$

The dependence of the optical density of whiskey distillates on the time of exposure to microwave radiation and alcohol concentration in the distillate is determined by the equation:



RESULTS AND DISCUSSION

The optical density (D), polyphenol content (PPh) and solid compound content (SC) of the studied samples were measured for different values of the variables: (t), (N), and (C). The data of the full-factorial experiment and the

$$D = 0.108037 - 0.006500t + 0.000278t^2 - 0.001933C + 0.000021C^2 + 0.000122t \times C \quad (2)$$

The dependence of the optical density of whiskey distillates on the power of microwave radiation and alcohol concentration in the distillate is determined by the equation:

$$D = 0.167815 - 0.000233N - 0.002022C + 0.000021C^2 \quad (3)$$

The regression equation (1) shows that the time of exposure to microwave radiation t and its square (t^2) have the highest

influence on the dependent parameter (the optical density (D) of the whiskey distillate samples) related to the power of microwave radiation. The regression equation (2) shows that the time of exposure to microwave radiation t and its square (t^2) have a significant influence on the optical density (D) of the whiskey distillates related to the alcohol concentration value (C), just like in the equation (1). The regression equation (3) shows that the power of microwave radiation (N) has the highest influence on the optical density (D) of the whiskey distillate samples related to the value alcohol concentration (C) in them (Pradal et al. 2018; Phetxumthou et al. 2020).

Table 2. Changes in qualities indicators depending on technological parameters

Sample No.	Values of technological parameters						Optical density D	Polyphenol content, mg/dm ³ PPh	Solid compound content, g SC
	X1	X2	X3	t, min	N, W	C, %			
1	-1	-1	-1	1	450	45	0.065±1%	186.9±1%	0.0009±0.01%
2	0	-1	-1	2	450	45	0.066±1%	200.6±1%	0.00091±0.01%
3	1	-1	-1	3	450	45	0.061±1%	213.3±1%	0.001±0.01%
4	-1	0	-1	1	600	45	0.064±1%	245.8±1%	0.0013±0.01%
5	0	0	-1	2	600	45	0.053±1%	272.1±1%	0.00133±0.01%
6	1	0	-1	3	600	45	0.056±1%	300.5±1%	0.0014±0.01%
7	-1	1	-1	1	750	45	0.068±1%	312.8±1%	0.00145±0.01%
8	0	1	-1	2	750	45	0.063±1%	334.2±1%	0.00162±0.01%
9	1	1	-1	3	750	45	0.074±1%	361.3±1%	0.0017±0.01%
10	-1	-1	0	1	450	60	0.064±1%	194.5±1%	0.001±0.01%
11	0	-1	0	2	450	60	0.072±1%	214.4±1%	0.0012±0.01%
12	1	-1	0	3	450	60	0.077±1%	249±1%	0.0015±0.01%
13	-1	0	0	1	600	60	0.071±1%	278.7±1%	0.0016±0.01%
14	0	0	0	2	600	60	0.074±1%	322.2±1%	0.0016±0.01%
15	1	0	0	3	600	60	0.061±1%	347.9±1%	0.00174±0.01%
16	-1	1	0	1	750	60	0.066±1%	366.0±1%	0.00181±0.01%
17	0	1	0	2	750	60	0.07±1%	374.5±1%	0.0019±0.01%
18	1	1	0	3	750	60	0.088±1%	391.1±1%	0.0021±0.01%
19	-1	-1	1	1	450	75	0.086±1%	223.6±1%	0.0012±0.01%
20	0	-1	1	2	450	75	0.085±1%	260.1±1%	0.00135±0.01%
21	1	-1	1	3	450	75	0.082±1%	274.4±1%	0.0015±0.01%
22	-1	0	1	1	600	75	0.079±1%	299.3±1%	0.00171±0.01%
23	0	0	1	2	600	75	0.093±1%	310.7±1%	0.00184±0.01%
24	1	0	1	3	600	75	0.096±1%	330.4±1%	0.0022±0.01%
25	-1	1	1	1	750	75	0.092±1%	374.7±1%	0.0024±0.01%
26	0	1	1	2	750	75	0.094±1%	389.5±1%	0.0025±0.01%
27	1	1	1	3	750	75	0.095±1%	419.2±1%	0.0028±0.01%

The performance metrics for the regression models (1-3) are provided in Table 3.

Table 3 shows that the multiple correlation coefficient R and multiple determination coefficient R^2 for the regression models (1-3) lie in the range of 0.7308÷0.8934. Thus, the spread of values of the independent variables in the regression model in relation to their mean values is 73% or more. The F-criterion and the p-criterion are used to prove

the regression significance. The p-criterion coefficients tend to zero, which indicates the statistical significance of the regression models (Wanikawa 2020). The regression equation (1) was used to build the response surface showing the dependence of optical density (D) on the time of whiskey distillates exposure to and the power of microwave radiation (Figure 2). Figure 2 shows that the maximum values of (D) lie in the red and dark-red ranges with the highest values of the variables (t) and (N). Thus, the optimal parameters

of exposing whiskey distillates to microwave radiation are $t = 3$ min., $N = 750$ W, with the optical density value (D) equaling 0.085 (Wanikawa 2020).

The regression equation (2) was used to build the response surface showing the dependence of optical density (D)

on the time of whiskey distillates exposure to microwave radiation and alcohol concentration in the distillates (Figure 3). Figure 3 shows that the maximum values of (D) lie in the red and dark-red ranges with the optimal parameters: time of exposure to microwave radiation $t = 3$ min, and alcohol concentration in the whiskey distillate $C = 75\%$, with the optical density value (D) being > 0.09 .

Table 3. Performance metrics the regression models (1-3)

Indicator	Regression models		
	(1)	(2)	(3)
Multiple correlation coefficient R	0.8437	0.8719	0.8934
Multiple determination coefficient R^2	0.7308	0.7602	0.7982
Adjusted correlation coefficient R^2	0.6851	0.7032	0.7502
F-criterion	11.5254	13.32147	16.61727
p-criterion	0.000007	0.000006	0.000001

Figure 2: Response surface showing the dependence of optical density on the time of whiskey distillates exposure to and the power of microwave radiation

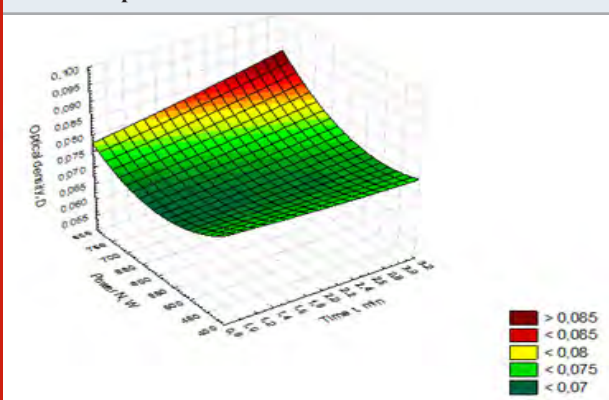
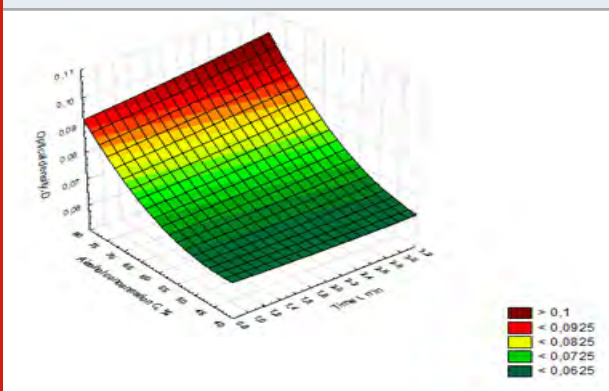


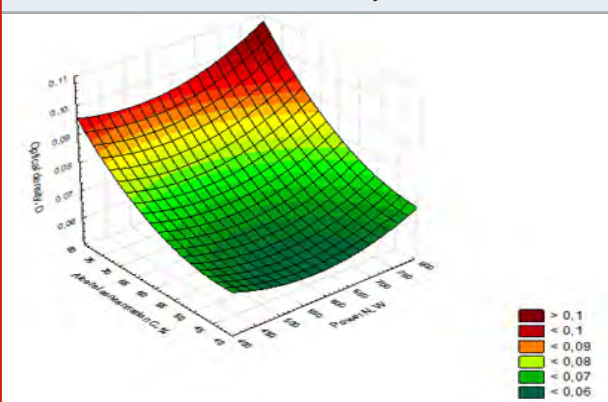
Figure 3. Response surface showing the dependence of optical density on the time of whiskey distillates exposure to microwave radiation and alcohol concentration in the distillate



Further, the regression equation (3) was used to build the response surface showing the dependence of optical density on the power of microwave radiation and alcohol

concentration in the whiskey distillates (Figure 4). Figure 4 shows that the best values for (D) were found for the whiskey distillates with alcohol concentration $C = 75\%$ exposed to microwaves radiation with $N = 750$ W. Summing up the results of the three response surfaces in Figures 2-4, we can conclude that the maximum value of optical density (D) is obtained at the following technological parameters of whiskey distillate maturation: $t = 3$ min., $N = 750$ W, $C = 75\%$. Finally, the obtained experimental values of optical density of the whiskey distillates (D) were compared to the ones modeled (predicted) in regression equations (1-3) (Phetxumphou et al. 2020). Relative error was calculated for each of the experimental and the modeled (predicted).

Figure 4: Response surface showing the dependence of optical density on the power of microwave radiation and alcohol concentration in the whiskey distillate

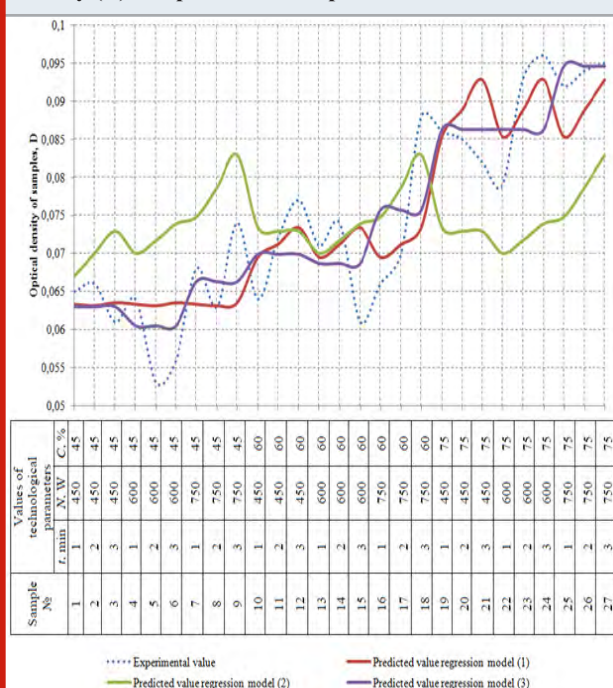


$$\delta = \left| \frac{D_{exp} - D_{pr}}{D_{exp}} \right| \times 100 \% \quad (4)$$

where δ is the relative error, %;
 D_{exp} is the experimental value for optical density;
 D_{pr} is the predicted value for optical density.

The obtained values are shown in the scatter graph (Figure 5).

Figure 5: Scatter graph for the predicted values of optical density (D) compared to the experimental values



The mean relative error between the obtained experimental data on the optical density of whiskey distillates and values obtained with regression models varies within 6.5 % – 13.98%. Thus, the obtained models may be considered adequate and may be applied with a sufficient degree of accuracy for determining the optical density (D) of whiskey distillates. Stage 2 of solving Research task 1 involved multiple regression analysis of the influence of any two independent variables (the time of whiskey distillate exposure to microwave radiation t ; the power of microwave radiation N ; alcohol concentration in the whiskey distillate C) on the dependent variable: the polyphenol content (PPh) in the maturing whiskey distillates exposed to microwave radiation (Table 2) (Phetxumphou et al. 2020). Data array processing allowed obtaining the regression models for the polyphenol content (PPh) in the whiskey distillates on the variables (t , N , C) (Phetxumphou et al. 2020). The dependence of the polyphenol content in whiskey distillates on the time of exposure to and the power of microwave radiation is determined by the equation:

$$\text{PPh} = -107.919 + 24.178t + 0.711t^2 + 0.722N - 0.008t \times N \quad (5)$$

The dependence of the polyphenol content in whiskey distillates on the time of exposure to microwave radiation and alcohol concentration in the distillate is determined by the equation:

$$\text{PPh} = 9.91481 + 20.71111t + 0.71111t^2 + 6.67259C - 0.04128C^2 \quad (6)$$

Table 4. Performance metrics for the regression models (5-7)

Indicator	Regression models		
	(5)	(6)	(7)
Multiple correlation coefficient R	0.937	0.422	0.95
Multiple determination coefficient R^2	0.879	0.1782	0.903
Adjusted correlation coefficient R^2	0.8507	-0.0173	0.88
F-criterion	30.634	0.911	39.49
p-criterion	0.000000	0.492	0.000000

The dependence of the polyphenol content in whiskey distillates on the power of microwave radiation and alcohol concentration in the distillate is determined by the equation:

$$\text{PPh} = -275.919 + 0.667N + 6.241C - 0.041C^2 + 0.001N \times C \quad (7)$$

The regression equation (5) shows that the time of exposure to microwave radiation t has the highest influence on the dependent parameter: the polyphenol content in the whiskey distillate samples, related to the power of microwave radiation (N). The regression equation (6) shows that the time of exposure to microwave radiation t has the highest influence on the polyphenol content in the whiskey distillate samples related to the power of microwave radiation, yet the latter factor should not be neglected as it shows a significant numerical characteristic. The regression equation

(7) proves the significance of alcohol concentration in whiskey distillates (C) related to the power of microwave radiation (N) for determining the polyphenol content (PPh) (Phetxumphou et al. 2020).

Table 4 shows that the regression model (5) has the multiple correlation coefficients $R = 0.937780$ and multiple determination coefficients $R^2 = 0.879432$, which means that the spread of values of the independent variables in relation to their mean values is 88%. The F-criterion and the p-criterion are used to prove the regression significance. As for the regression model (5), $F = 30.63497$, and $p = 0.000000$; thus, the model is statistically significant. The multiple correlation coefficient and multiple determination coefficient for the regression model (6) have rather low values: $R = 0.422$ and $R^2 = 0.1782$. Thus, the obtained regression model shows a 17% spread of values of the

independent variables in relation to their mean values. The values of the F-criterion and the p-criterion are low as well; thus, the model has little statistical significance.

Figure 6: Response surface showing the dependence of polyphenol content on the time of whiskey distillates exposure to and the power of microwave radiation

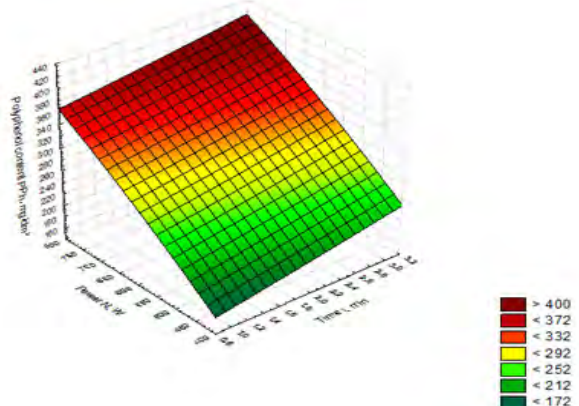


Figure 7: Response surface showing the dependence of polyphenol content on the time of whiskey distillates exposure to microwave radiation and alcohol concentration in the distillate

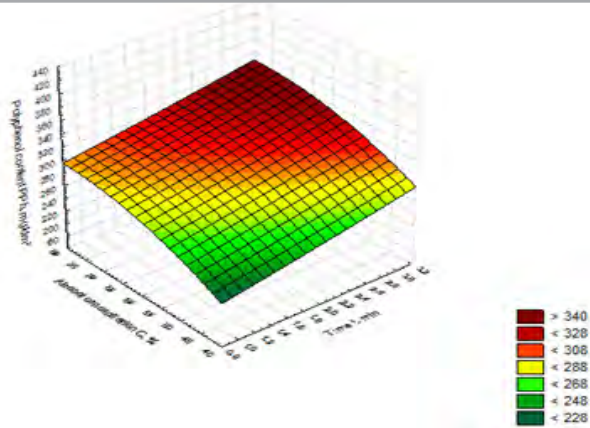
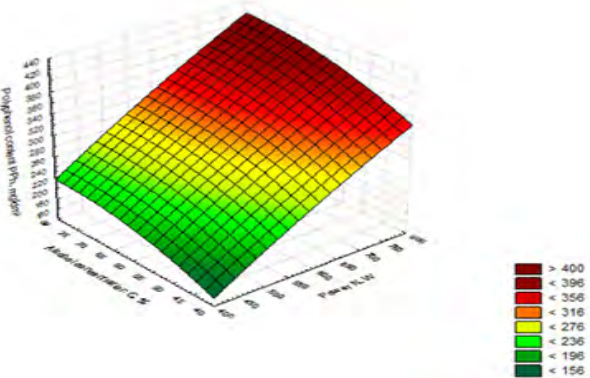


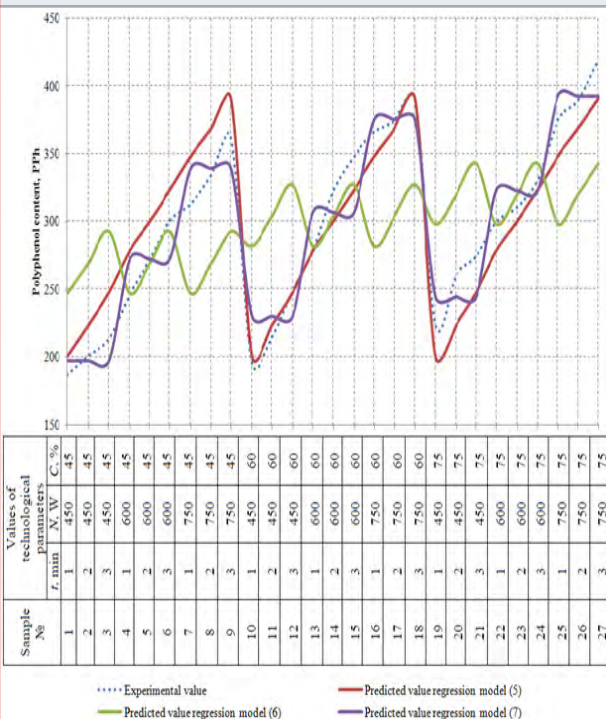
Figure 8: Response surface showing the dependence of polyphenol content on the power of microwave radiation and alcohol concentration in the whiskey distillate



The coefficients $R = 0.95$ and $R^2 = 0.903$ of the obtained regression model (7) show the maximum values in Table 4: the regression model shows a spread of values of the independent variables in relation to their mean values 90% or more. Since $F = 39.49$, and $p = 0.000000$, the statistical significance of the obtained model is high. The regression equation (5) was used to build the response surface showing the dependence of polyphenol content on the time of whiskey distillates exposure to and the power of microwave radiation (Figure 6). Figure 6 shows that the maximum values of (PPh) lie in the red and dark-red ranges with the highest values of the variables (t) and (N). Thus, the optimal parameters of exposing whiskey distillates to microwave radiation are $t = 3$ min., $N = 750$ W, with polyphenol content reaching the value of 400 mg/dm^3 . The performance metrics for the regression models (5-7) are provided in Table 4.

The regression equation (6) was used to build the response surface showing the dependence of polyphenol content on the time of whiskey distillates exposure to microwave radiation and alcohol concentration in the distillate (Figure 7). Figure 7 shows that the maximum values of (PPh) in the distillates were obtained at the following optimal parameters of exposing whiskey distillates to microwave radiation: $t = 3$ min., $N = 750$ W, with polyphenol content reaching the value of 340 mg/dm^3 .

Figure 9: Scatter graph for the predicted values of polyphenol content (PPh) compared to the experimental values



Further the regression equation (7) was used to build the response surface showing the dependence of polyphenol content on the power of microwave radiation and alcohol concentration in the whiskey distillate (Figure 8). Figure 8 shows that the maximum values of polyphenol content

(PPh) in the distillates were obtained for the samples with alcohol concentration $C = 75\%$ exposed to microwave radiation of $N = 750$ W. Summing up the results of the three response surfaces in Figures 5-7, we can conclude that the maximum value of polyphenol content (PPh) is obtained at the following technological parameters of whiskey distillate maturation: $t = 3$ min., $N = 750$ W, $C = 75\%$.

At the end of this stage, the obtained experimental values of polyphenol content in the whiskey distillates (PPh) were compared to the ones modeled (predicted) in regression equations (5-7). Relative error was calculated for each of the experimental and the modeled (predicted) values using the formula (4) (with optical density values replaced by polyphenol content values). The obtained values are shown in the scatter graph (Figure 9).

The mean relative error between the obtained experimental data on the polyphenol content in whiskey distillates and the values obtained with regression models (5) and (7) varies within $6.3\% - 7.003\%$. Thus, the obtained models may be considered adequate and may be applied with a sufficient degree of accuracy for determining the polyphenol content (PPh) in whiskey distillates. The mean relative error in the regression equation (6) equals 19% , which exceeds the limit for engineering calculations. Thus, the model (6) can be considered adequate and be applied for determining the polyphenol content (PPh) in whiskey distillates only as a first approximation (Habschied et al. 2019).

Stage 3 of solving Research task 1 involved multiple regression analysis of the influence of any two independent variables (the time of whiskey distillate exposure to microwave radiation t ; the power of microwave radiation N ; alcohol concentration in the whiskey distillate C) on the dependent variable: the solid compound content (SC) in the maturing whiskey distillates exposed to microwave

radiation (Table 2). Data array processing allowed obtaining the regression models for the solid compound content (SC) in the whiskey distillates on the variables (t , N , C). The dependence of the solid compound content in whiskey distillates on the time of exposure to and the power of microwave radiation is determined by the equation:

$$SC = -0.000749 - 0.000037t + 0.000045t^2 + 0.000005N \quad (8)$$

The dependence of the solid compound content in whiskey distillates on the time of exposure to microwave radiation and alcohol concentration in the distillate is determined by the equation:

$$SC = 0.000841 - 0.000284t + 0.000045t^2 + 0.000007C + 0.000004t \times C \quad (9)$$

The dependence of the solid compound content in whiskey distillates on the power of microwave radiation and alcohol concentration in the distillate is determined by the equation:

$$SC = 0.00051 + 0.000001N - 0.000022C \quad (10)$$

The regression equation (8) shows that the time of exposure to microwave radiation t and t^2 has the highest influence on the dependent parameter: the solid compound content in the whiskey distillate samples (SC), related to the power of microwave radiation (N). The regression equation (9) shows that the time of exposure to microwave radiation t has the highest influence on the solid compound content in the whiskey distillates related to alcohol concentration (C), just like in the equation (8). The regression equation (9) proves the significance of alcohol concentration in whiskey distillates (C) related to the power of microwave radiation (N) for determining the solid compound content (SC) (Wanikawa 2020). The performance metrics for the regression models (8-10) are provided in Table 5.

Table 5. Performance metrics for the regression models (8-10)

Indicator	Regression models		
	(8)	(9)	(10)
Multiple correlation coefficient R	0.782	0.623	0.952
Multiple determination coefficient R ²	0.611	0.389	0.907
Adjusted correlation coefficient R ²	0.541	0.243	0.885
F-criterion	8.66	2.677	41.19
p-criterion	0.000234	0.0504	0.0000

Table 5 shows that the multiple correlation coefficients R and multiple determination coefficients R² for the regression models (8-9) lie in the range of $0.389 - 0.782$. Thus, the spread of values of the independent variables in the regression model in relation to their mean values is 38% or more. The p-criterion coefficients tend to zero, which indicates the sufficient statistical significance of the regression models. The coefficients $R = 0.952$ and $R^2 = 0.907$ of the obtained regression model (10) show the maximum values in Table 5: the regression model shows

a spread of values of the independent variables in relation to their mean values 90% or more. Since $F = 41.19$, and $p = 0.000000$, the statistical significance of the obtained model is high. The regression model (8) was used to build the response surface showing the dependence of solid compound content on the time of whiskey distillates exposure to and the power of microwave radiation (Figure 10). Figure 10 shows that the maximum values of solid compound content (SC) in the distillates lie in the red and dark-red ranges with the highest values of the (t) and

(N) parameters. Thus, the optimal parameters for whiskey distillate exposure are: $t = 3$ min and $N = 750$ W, with the solid compound content reaching up to 0.0022 g (Habschied et al. 2019; Wanikawa 2020).

Figure 10: Response surface showing the dependence of solid compound content on the time of whiskey distillates exposure to and the power of microwave radiation

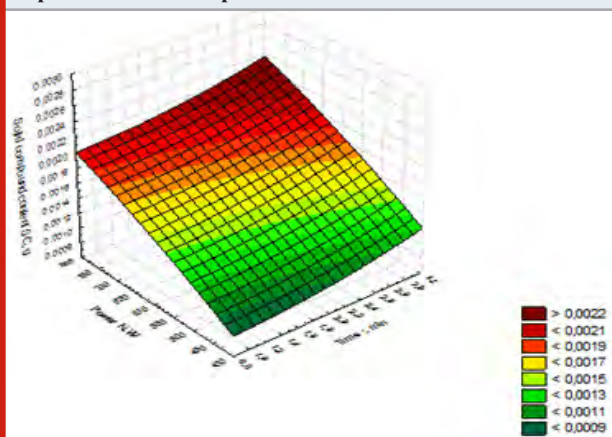
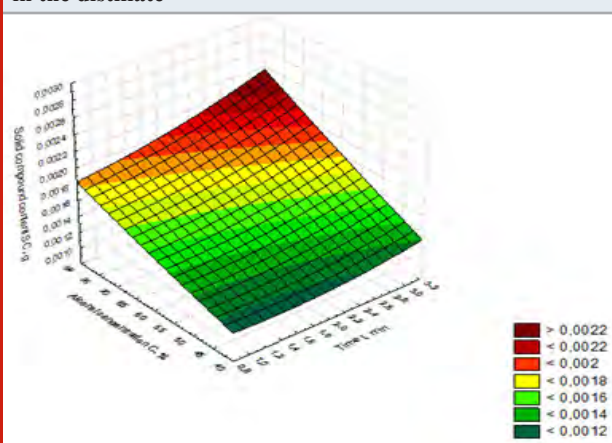


Figure 11: Response surface showing the dependence of solid compound content on the time of whiskey distillates exposure to microwave radiation and alcohol concentration in the distillate



The regression model (9) was used to build the response surface showing the dependence of solid compound content on the time of whiskey distillates exposure to microwave radiation and alcohol concentration in the distillate (Figure 11). Figure 11 shows that the maximum values of solid compound content (SC) are obtained at the following exposure parameters: $t = 3$ min. and alcohol concentration in the whiskey distillate $C = 75\%$, with the solid compound content reaching up 0.0024 g. Further the regression model (12) was used to build the response surface showing the dependence of solid compound content on the power of microwave radiation and alcohol concentration in the whiskey distillate (Figure 12).

Figure 10 shows that the maximum values of solid compound content (SC) were obtained in the whiskey

distillate samples with alcohol concentration $C = 75\%$, exposed to microwave radiation at $N = 750$ W. The value of the solid compound content in the sample reached 0.0028 g. Summing up the results of the three response surfaces in Figures 10-12, we can conclude that the maximum value of solid compound content ($SC = 0.0028$ g.) is obtained at the following technological parameters of whiskey distillate maturation: $t = 3$ min., $N = 750$ W, $C = 75\%$. At the end of this stage, the obtained experimental values of solid compound content in the whiskey distillates (SC) were compared to the ones modeled (predicted) in regression equations (8-10). Relative errors were calculated for the experimental value and each of the modeled values calculated using the formula (4) (values of solid compound content in the whiskey distillate were used instead of optical density values). Relative error was calculated for each of the experimental and the modeled (predicted) values using the formula (4) (with optical density values replaced by solid compound content values). The obtained values are shown in the scatter graph (Figure 13) (Wu et al. 2018; Habschied et al. 2019; Wanikawa 2020).

Figure 12: Response surface showing the dependence of solid compound content on the power of microwave radiation and alcohol concentration in the whiskey distillate

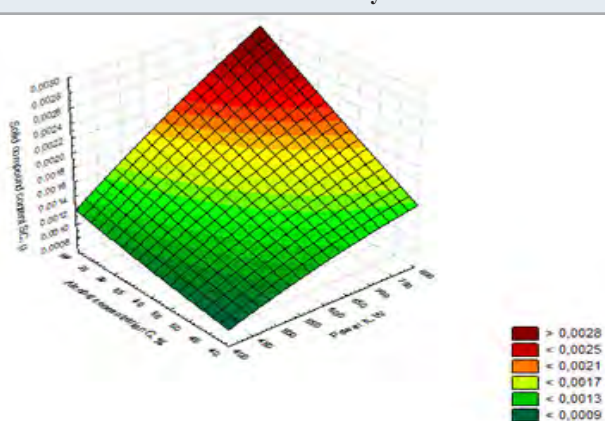
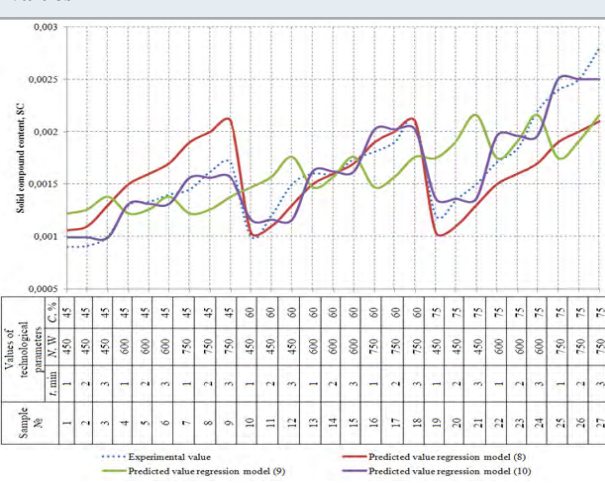


Figure 13: Scatter graph for the predicted values of solid compound content (SC) compared to the experimental values



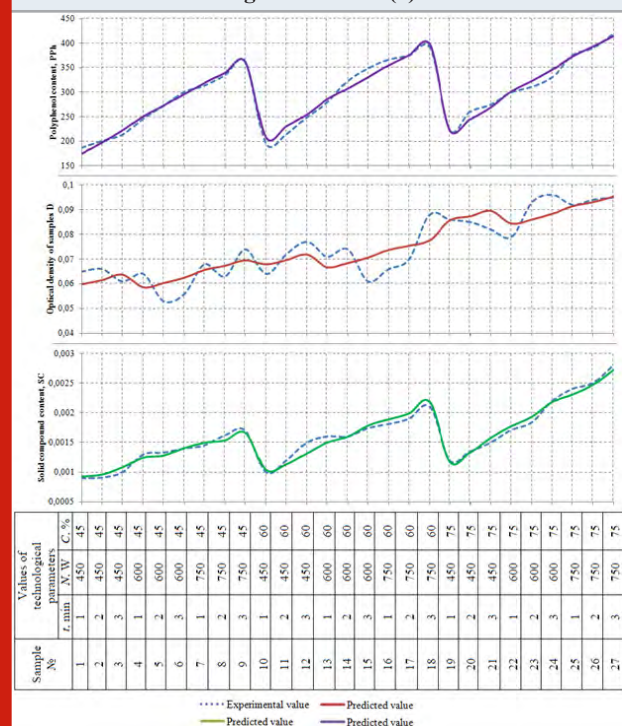
The mean relative error between the obtained experimental data on the solid compound content in whiskey distillates and the values obtained with regression models (8) and (9) varies within 12.5 % – 15.28%. Thus, they can be applied for determining the solid compound content in whiskey

distillates only as a first approximation. Yet, the mean relative error for the regression equation (10) equals 7.2%; thus, it is considered adequate and may be applied with a sufficient degree of accuracy for determining the solid compound content (SC) in whiskey distillates.

Table 6. Performance metrics for the regression models (11-13)

Indicator	Regression models		
	(11)	(12)	(13)
Multiple correlation coefficient R	0.899	0.99	0.988
Multiple determination coefficient R ²	0.809	0.98	0.977
Adjusted correlation coefficient R ²	0.752	0.97	0.966
F-criterion	74.204	97.446	83.109
p-criterion	0.000003	0.000000	0.000000

Figure 14: Relative errors of the experimental and modeled values calculated using the formula (4)



$$\text{PPh} = -329.726 + 25.244t + 0.711t^2 + 0.682N + 6.277C - 0.041C^2 - 0.008t \times N - 0.018 \times C + 0.001 N \times C \quad (12)$$

The dependence of the solid compound content in whiskey distillates on the variables (t, N, C) under exposure to microwave radiation:

$$\text{PPh} = -275.919 + 0.667N + 6.241C - 0.041C^2 + 0.001 N \times C \quad (13)$$

The regression equation (11) shows that alcohol concentration in whiskey distillates (C) and the time of their exposure to microwave radiation t have the highest influence on the depended parameter of optical density (D), since their numeric characteristics have maximum values. Yet, the numeric value of the power of microwave radiation (N) is rather significant; thus, it should not be excluded from further analysis. The regression equation (12) shows that the time of exposure to microwave radiation (t) has the highest influence on the polyphenol content in whiskey distillates, just like in the equation (11). The influence of alcohol concentration in whiskey distillates (C) is slightly lower. The regression equation (13) proves the significant influence of the time of exposure to microwave radiation t influence of alcohol concentration in whiskey distillates (C) on the polyphenol content (PPh) in whiskey distillates (Wu et al. 2018; Habschied et al. 2019; Wanikawa 2020). The performance metrics for the regression models (11-13) are provided in Table 6.

Research task 2 was solved as follows.

Data array processing allowed obtaining the following regression models. The dependence of optical density (D) in the whiskey distillates on the variables (t, N, C) under exposure to microwave radiation:

$$D = 0.144852 + 0.000833t + 0.000278t^2 - 0.0002N - 0.001689C + 0.000021C^2 \quad (11)$$

The dependence of the polyphenol content in whiskey distillates on the variables (t, N, C) under exposure to microwave radiation:

Table 6 shows that the multiple correlation coefficients R and multiple determination coefficients R² for all the regression models lie in the range of 0.80÷0.99. Thus, the spread of values of the independent variables in the regression model in relation to their mean values is 80% or more. The p-criterion coefficients tend to zero, which indicates the sufficient statistical significance of the regression models.

The F-criterion for the regression model (12) reaches 97.47 with p = 0.000000, which profess the high statistical significance of the obtained model. Finally, the obtained experimental values for optical density (D), polyphenols

content (PPh) and solid compound content (SC) in whiskey distillates were compared to the ones modeled (predicted) in regression equations (11-13). The relative errors were calculated for each of the experimental and modeled values using the formula (4). The results are presented in Figure 14 (Habschied et al. 2019).

Figure 14 show that the mean relative error varies within 2.7 % – 6.7%, which proves the full adequacy of the obtained regression models (11-13) and allows using them for predicting the quality indicators of whiskey distillates with a high degree of accuracy.

Research task 3 was solved by performing the analysis of the whiskey produced with the rational technological parameters. The physical and chemical properties of the whiskey and its safety were analyzed according to the requirement of the applicable regulations. At the initial stage, chromatographic analysis was performed to determine the following quality indicators: the strength of the drink and mass concentrations of furfural, aldehydes, fusel oil, and esters. The presence of toxic elements (cadmium, lead, and mercury) was also determined. The obtained data are shown in the chromatogram (Figure 15) (Sidorenko and Surikova 2015).

Figure 15: Chromatogram of the whiskey

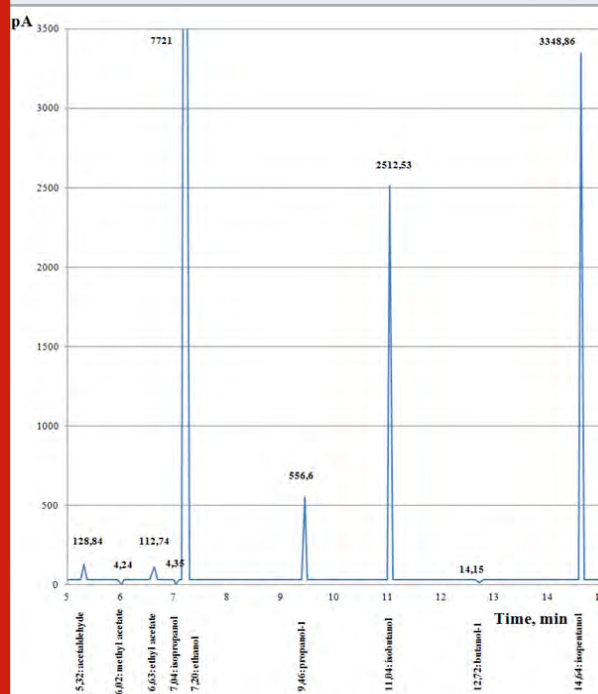


Table 7. Physical and chemical characteristics of the quality of the whiskey

Parameter	Industry standard	Obtained results
Alcohol by volume, %.	42	40.6+ 0,1
Mass concentration of furfural in 1 dm ³ of ethyl alcohol, mg	30	< 8.0
Mass concentration of aldehydes in 1 dm ³ of ethyl alcohol, mg	10-350	135.92+ 0.1
Mass concentration of fusel oil in 1 dm ³ of ethyl alcohol, mg	500-6000	5812.15+ 93.18
Mass concentration of esters in 1 dm ³ of ethyl alcohol, mg	50-1500	117.30 + 17.60
Volume ratio of methyl alcohol on ethyl alcohol basis, %,	≤0.05	0.0097+ 0.0015

The chromatogram shows the concentration peaks for the elements that make up the aged whiskey distillate. The highest peak at 7.20 min indicates the presence of ethanol in the drink, which is the active ingredient in alcoholic beverages. At 11 min and 15 min, the highest peaks indicate the concentrations of isobutanol and isopentanol; these components are classified as fusel oils. Mercury, cadmium, arsenic and other toxic elements were not found in the drink: after 15 min, the chromatogram shows no unwanted elements in the whiskey distillate. The obtained results of the chromatographic analysis were grouped and compared to the permissible values as determined by industry standards (Table 7).

Table 7 shows that the strength of the whiskey obtained with the selected optimal parameters is 42%, which meets the requirements of regulatory documents. Furfural is a toxic substance that negatively affects the human nervous system and causes irritation of the skin and mucous membranes. In this regard, its content is strictly regulated. In the resulting whiskey, the furfural content is less than 8 mg per 1 dm³ of ethyl alcohol, which indicates good quality. The increased content of fusel oils and methyl alcohol in the distillate leads to a deterioration in its quality and indirectly reflects the shortcomings of purification in the production technology. Summarizing the obtained data, we can highlight that the obtained whiskey sample meets the requirements as it has

a low content of these substances. Esters give the drink a fruity smell and affect the saturation of the aroma. In the resulting whiskey, the concentration of esters is within the normalized limits.

The number of toxic elements in the resulting whiskey does not exceed the permissible values and is < 0.001 mg/kg for cadmium and lead, < 0.002 mg/kg for arsenic, and < 0.0001 mg/kg for mercury (the normal values being 0.03, 0.3, 0.2,

and 0.005 respectively). Summarizing the obtained data of physical and chemical tests, we can state that the whiskey meets the quality and safety requirements (Wu et al. 2018; Habschied et al. 2019; Wanikawa 2020). Research task 4 required performing an organoleptic assessment of the obtained whiskey. The visual appearance, colour, aroma, and taste of the whiskey were assessed to obtain objective quality assessment results. The obtained data are provided in Table 8.

Table 8. Organoleptic characteristics of the quality of the whiskey

Parameter	Characteristics	
	Industry standard	In the obtained sample
Visual appearance	Transparent, free of foreign or residual matter	Transparent, free of foreign or residual matter
Colour	Ranging from light yellow to dark brown	Light brown
Aroma	Complex, with tones of sherry, almond, caramel, honey, hazelnut, wormwood, dried fruits or other tones, without foreign aroma	Complex whiskey smell with oak tones, without foreign aromas
Taste	Harmonious, well-balanced, sweet-dry, with tones of nuts, caramel, or coffee, with shades of bitterness and apples or other tones, without foreign taste	Harmonious, sweet-dry, with oak tones, without foreign taste

CONCLUSION

The findings of the present study noted that the developed new technology for whiskey distillate maturation, with the selected rational technological parameters of microwave radiation influence on the distillate, makes it possible to increase the efficiency of the process of whiskey aging and reduce its maturation time by 32%, compared to the drink prepared in the classical way. Additionally, it allows reducing the production cost (without purchasing expensive oak barrels) and the space for distillate maturation (without long-term storage of barrels). All this can find a worthy industrial application for private or newly opened whiskey distilleries, or in general for newcomers in this business who want to shorten the longest process of whiskey preparation – its maturation, and do not fear introducing technological innovations.

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Medical Communication

C850T Polymorphism in Tumor Necrosis Factor Alpha gene in Women with Polycystic Ovarian Syndrome: A Physiological Genetic Validation

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ABSTRACT

The most common endocrinopathy in women is polycystic ovarian syndrome (PCOS). Obesity is linked to PCOS and tumor necrosis factor- α (TNF- α), which is followed by hyperandrogenism and enhanced insulin resistance. Previous case-control and meta-analysis studies on the TNF- α gene and PCOS women relied heavily on the -C850T polymorphism. The aim of this study was to investigate the Elisa levels and -C850T (rs1799724) polymorphism in TNF- α gene with PCOS women in the Saudi women. In this case-control study, 50 PCOS patients and 50 healthy controls were recruited, and plasma levels of TNF- α were evaluated using an enzyme linked immunosorbent assay (Elisa), and extracted DNA was utilized to explore the -C850T polymorphism using Polymerase Chain Reaction (PCR). The limited and digested PCR products were run on an Agarose gel to test for the -C850T polymorphism. Elevated Elisa levels were found in CT genotype and gene polymorphism studies showed 12% of CT genotypes was documented in PCOS women and 14% in control women. None of the genotypes or allele frequencies were associated with a positive relationship between PCOS women and controls. The CT genotype had higher TNF- α levels than the CC genotype, and the C850T polymorphism was not related with PCOS in women, according to the findings of this study.

KEY WORDS: PCOS, C850T, TNF-A, ELISA AND PCR.

INTRODUCTION

PCOS (Polycystic Ovarian Syndrome) is one of the most common metabolic spectrum diseases in the human population, as well as one of the most commonly identified endocrine abnormalities (Ma et al., 2021). According to Rotterdam criteria, PCOS women were impacted, with a prevalence of 15-20% among reproductive-age women (Aboeldalyl et al., 2021). Ovarian hyperandrogenism and micropolycystic morphology in PCOS patients are clinical and biochemical manifestations (Aversa et al., 2020).

Currently, the etiology of PCOS is unknown, and there is no cure; therefore, management is based on symptoms, treatment, and risk factor reduction (Tatkare, 2021). The genetics contribution of PCOS is well established to be a complex condition in which heritable genetics is suggested due to familial clustering symptoms (Combs et al., 2021).

PCOS is associated with numerous of clinical symptoms, Obesity, metabolic syndrome, decreased glucose tolerance, and type 2 diabetes mellitus (T2DM), cardiovascular disease, and dyslipidemia, and prior research has shown a correlation between gestational diabetes mellitus (GDM) and PCOS (Pan et al 2015, Lentscher et al., 2021).

Signaling proteins produced by various types of immune cells have a significant effect on other cells and organisms. Leukocytes, oocytes, and follicular cells produce it in the ovary. Many genes have been proposed as contributing factors to PCOS, however none have yet been accepted as a main cause of this clinical disorder. TNF- α (tumor necrosis factor alpha) levels in muscle and adipose tissue have been associated to an increased risk of insulin resistance in humans. To comprehend the pathophysiology of PCOS, it is necessary to comprehend the involvement of TNF- α . TNF- α is a proinflammatory cytokine that has been proven to be elevated in at least a portion of PCOS women (Thathapudi et al., 2014, Guo et al., 2015, Gnanadass et al., 2021).

Several TNF- α promoter polymorphisms have been found and connected to TNF- α transcription regulation. The

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Received 13/10/2021 Accepted after revision 20/12/2021

Published: 31st December 2021 Pp- 1714-1718

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.49>

polymorphisms (SNPs) in the promoter region of the TNF- α gene have been thoroughly investigated (Guzmán-Flores et al., 2013). Kato group discovered the -C850T polymorphism in the Promoter region of TNF- α gene, which is proven to be multifunctional and cytokine is produced mostly by macrophages and lymphocytes. TNF- α gene is located on the 6p21.3 chromosome and spans around 3kb. It has four exons (Khan et al., 2015). Elisa and C-850T polymorphism studies were carried out in PCOS women from the Saudi population in this study. There have been no previous reports with Elisa, although one Saudi study (Alkhuriji et al.) revealed in PCOS women with cytokine markers, including TNF- α gene polymorphism. The purpose of this study is to look into TNF- α levels in PCOS women using Elisa tests and C-850T (rs1799724) polymorphism analyses in the TNF- α gene.

MATERIAL AND METHODS

Selection of PCOS and control women: In this study, we have recruited 50 patients diagnosed with polycystic ovarian syndrome from Saudi Arabia. Ethical grant was approved from Institutional Review Board and all the women participated in this study has signed the informed consent form. The women who denied in signing the consent form was excluded from this study. Based on Rotterdam criteria, PCOS women was diagnosed and included in

this study. The women who doesn't fit into the Rotterdam criteria were excluded from this study. The women without any history of infertility is considered as normal controls were involved in this study. The women with any history of infertility or PCOS will be excluded in this study as normal controls. Based on inclusion and exclusion criteria, 60 control subjects were recruited in this study. Based on Mohamadin et al studies, the cases and controls were selected (Mohamadin et al., 2010).

Sample collection: One milliliter of ethylenediaminetetraacetic acid blood was collected from each patient and used for DNA isolation. Another one milliliter of blood was used for measuring Elisa levels with the extraction of plasma from blood.

Elisa studies: Plasma samples were separated from 50 PCOS women and using TNF- α kit (Cat# BMS223HS, Invitrogen, USA) as per the prescribed protocol from Invitrogen company. The enzyme reaction was ended by adding 100ul of stop solution and read the absorbance on a spectrophotometer using 450nm and 620nm as the primary and reference wave lengths. All the samples were performed in a duplicate. Finally, the concentration of TNF- α of each sample was calculated from the obtained standard curve. The reagents were stored between 2-8°C and lyophilized controls were stored at -20°C based on the recommended protocol.

Table 1. Demographic and biochemical data obtained with PCOS cases and control women

	PCOS (n=50)	Controls (n=50)	P Value
Age	32.1 \pm 5.74	34.64 \pm 4.74	0.39
Weight, kg	72.5 \pm 11.89	80.71 \pm 14.94	0.02*
Height, cm	158.44 \pm 5.40	159.71 \pm 9.29	0.47
Body Mass Index (BMI).	28.69 \pm 5.01	31.94 \pm 7.54	0.04*
Fasting Blood Glucose (FBG), mmol/L	4.98 \pm 0.79	4.91 \pm 0.85	0.75
Follicle Stimulating Hormone (FSH), IU/L.	6.81 \pm 2.63	6.48 \pm 2.87	0.65
Luteinizing Hormone (LH), IU/L.	7.79 \pm 4.72	5.61 \pm 2.41	0.06
Tumor Necrosis Factor alpha (TNF- α), pg/mL	11.57 \pm 0.57	NA	NA

Arithmetic means \pm standard error, * indicates significant difference between PCOS and control (P<0.05). NA, not applicable.

Table 2. Genotype and allele frequencies between PCOS women and control subjects

C-850T	PCOS (n=50)	Controls (n=50)	Statistical analysis
CC	44 (88%)	43 (86%)	-
CT	06 (12%)	07 (14%)	OR-0.84 [95%CI:0.26-2.69] p=0.72
TT	00 (0%)	00 (0%)	OR-0.97 [95%CI:0.01-50.36] p=0.99
C	94 (0.94)	93 (0.93)	-
T	06 (0.06)	07 (0.07)	OR-0.84 [95%CI:0.27-2.61] p=0.77
CC vs TT+CT	06 (12%)	07 (14%)	OR-0.83 [95%CI:0.26-2.69] p=0.76

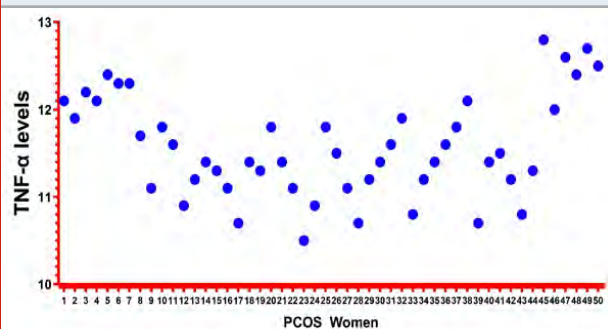
DNA analysis: Genomic DNA was extracted from collected 1ml of the blood using Qiagen kit and the technique was implemented as per the recommended protocol. The separated DNA was measured with NanoDrop spectrophotometer and stored in the freezer at -80°C . Genotyping was performed with polymerase chain reaction and followed with RFLP analysis. PCR reaction was carried out with a total volume of $50\mu\text{l}$ for rs1799724 polymorphism, which consists of 10pmols of primers (+/-), 20ng of genomic DNA, $30\mu\text{l}$ of Qiagen master mix and $15\mu\text{l}$ of purified water. The cycling conditions was carried out in thermal cycles based on initial denaturation at 94°C for

10 mins, 94°C for 30seconds of denaturation, 66°C for 30s as annealing temperature, 72°C for 1 min as extension and 72°C for 10 mins as final extension which was followed for 40 cycles and holds at 4°C (Al-Otaiby et al., 2021, Alshammery and Khan, 2021). The amplified obtained PCR product was 131bp and after digesting with HindII restriction enzyme at 18 hours for room temperature the digested PCR product produces 105 and 26bp at C allele and 131bp at T allele. All PCR products were separated using a 3% agarose gel electrophoresis and stained with ethidium bromide. The complete RFLP analysis was performed based on the Khan et al studies (Khan et al., 2015).

Table 3. Anova analysis involved with C850T genotypes in PCOS women

	CC (n=44)	CT (n=06)	TT (n=00)	P values
Tumor Necrosis Factor alpha levels (TNF- α), pg/mL	11.4 \pm 0.48	12.5 \pm 0.28	0.0 \pm 0.0	0.001**
Body Mass Index (BMI).	28.5 \pm 5.27	30.4 \pm 1.78	0.0 \pm 0.0	0.01*
Fasting Blood Glucose (FBG), mmol/L	4.94 \pm 0.80	5.29 \pm 0.69	0.0 \pm 0.0	0.68
Follicle Stimulating Hormone (FSH), IU/L.	6.85 \pm 2.79	6.55 \pm 0.85	0.0 \pm 0.0	0.79
luteinizing Hormone (LH), IU/L.	7.53 \pm 4.67	9.71 \pm 5.10	0.0 \pm 0.0	0.29

Figure 1: Representation of Elisa levels using GraphPad



Statistical analysis: The distribution for normality of continuous variables was examined with Kolmogorov-Smirnov test between cases and controls. The mean \pm SD, was used to present the normal distribution and HWE equilibrium was performed with -850C/T controls was assessed using a software defined (Khan et al., 2015). Genotype and allele frequencies were obtained from PCOS cases and controls using OR, 95%CI and specific p values. The ANOVA was performed with C-850T polymorphism with three genotypes in PCOS women (Khan et al., 2019). Elisa studies on TNF- α levels showed different levels in PCOS cases, which was represented using Graphpad software. The Elisa levels were measured in duplicates for 90% of PCOS cases.

RESULTS AND DISCUSSION

Demographic and Biochemical Details: Both the demographic and biochemical values of both PCOS and controls were shown in Table-1. The mean age of the PCOS cases and controls were found to be 32.1 ± 5.74 and 34.64 ± 4.74 . The weight was found to be high among controls rather than cases. The mean height was found to

be 158.4 in PCOS and 159.7 in controls. The PCOS cases were found to be overweight and controls were confirmed as obesity. The mean FBG was found to be 4.9 in both Cases and controls. The FSH levels were found to be high in PCOS cases than controls and elevated LH levels were found in PCOS cases than controls.

Elisa details: The Elisa levels were measured for 50 PCOS cases using Elisa Invitrogen kit. The mean levels were found to be 11.57 . The minimum and maximum values for TNF-a levels were found to be 10.5 and 12.8 . The values were recorded in pg/ml. The details were shown in Table-1. Simultaneously, figure-1 represents the Elisa levels in PCOS cases.

Genotyping details: The genotyping for -850C-T polymorphism was performed between PCOS cases and controls was shown in Table-2. CC and CT genotypes for PCOS cases was documented to be 88% and 12% and 86% and 14% was found in controls. None of the TT genotypes was documented in none of the PCOS cases and women. Genotype (CT vs CC-OR-0.84 [95%CI:0.26-2.69] $p=0.72$ and TT vs CC- OR-0.97 [95%CI:0.01-50.36] $p=0.99$) and dominant model (CC vs TT+CT- OR-0.83 [95%CI:0.26-2.69] $p=0.76$) don't show any statistical association when compared between PCOS cases versus control subjects. The minor allele frequency of T allele showed 6% in PCOS and 7% in controls, whereas in C allele, PCOS women was confirmed to be 94% and 93% in controls (T vs C- OR-0.84 [95%CI:0.27-2.61] $p=0.77$).

In this case-control study, Anova analysis was performed between CC and CT genotypes in PCOS cases and compared with TNF- α levels, BMI, FBG, FSH and LH levels which is tabulated in Table-3. All the parameters involved in this study such as TNF- α levels, BMI, FBG, FSH and LH levels showed higher values in CT genotype when compared

with CC genotypes. Both TNF- α levels ($p=0.001$) and BMI values ($p=0.01$) showed significant association when compared with CC, CT and TT genotypes. As usual, there was no values obtained in TT genotypes.

In the present study, the genotype and Elisa levels were measured for TNF- α gene. Genotyping was performed with PCOS cases and controls and the study results showed negative association either with genotypes (CT vs CC-OR-0.84 [95%CI:0.26-2.69] $p=0.72$ and TT vs CC- OR-0.97 [95%CI:0.01-50.36] $p=0.99$) and dominant model (CC vs TT+CT- OR-0.83 [95%CI:0.26-2.69] $p=0.76$) and allele frequencies (T vs C- OR-0.84 [95%CI:0.27-2.61] $p=0.77$). However, Elisa levels were measured only PCOS cases and duplicates was measure in above 90% of plasma samples. The minimum level was obtained is 0.5 μ l and maximum value was 2.8 μ l. However, in CC genotypes, the lower and upper levels of TNF- α values were found to be 0.5-2.4pg/ml, whereas in CT genotypes, 2-2.8pg/ml levels were measured. The current study results confirmed as there was negative association with -850C/T polymorphism in PCOS cases versus controls. Additionally, there was no strong association when compared with genotyping and biochemical levels such as FBG, FSH and LH ($p>0.05$) and positive association was documented with TNF- α levels and BMI ($p<0.05$), when performed Anova analysis.

PCOS is defined as the presence of multiple ovaries and prolonged oligo-anovulation in a proinflammatory state, which causes ovarian dysfunction and metabolic disorders. C-reactive protein levels were commonly elevated in PCOS women, and this low-grade inflammatory marker may lead to a serious of cardiac problems, as adipose tissue is associated with inflammation. TNF- α is one of the documented inflammatory markers in which their biological role of function varies with complexed mechanism. TNF- α plays enormous therapeutic roles in the body with immunostimulation. TNF- α and PCOS were associated in an in vitro study on rats, which found that TNF- α can alter the reproductive axis. It can also induce theca cell growth, stereogenesis, and has an apoptotic impact (Deligeoroglou et al., 2012, Ebejer and Calleja-Agius, 2013).

This study has performed based on limited sample size. From each group 50 cases and 50 controls were selected. The study results confirmed the negative association and this study is in agreement with the previous studies confirmed in PCOS women in global studies (Deepika et al., 2013, Escobar-Morreale et al., 2001, Guo et al., 2015, Korhonen et al., 2002, Milner et al., 1999, Vural et al., 2010, Yun et al., 2011) and only single study from India has confirmed the positive association (Thathapudi et al., 2014). Additionally, a previous meta-analysis carried out in PCOS and cytokine inflammatory markers which include -C850T polymorphism showed the negative association (Wu et al., 2015).

Global studies have performed the Elisa studies with TNF- α gene in PCOS women (Escobar-Morreale et al., 2001, Ihsan et al., 2018, Korhonen et al., 2002, Milner et al., 1999, Thathapudi et al., 2014) and our levels were found to be low when compared with other study results and global

studies have also compared their serum/plasma levels with in controls. In this study, only PCOS cases were studies and controls were excluded. This study has certain limitations as only 50 PCOS cases and 50 controls were studies and Elisa levels were measured only in PCOS women and excluded the controls. The other limitation of this study was only C-850T polymorphism was studies and documents as negative association. The strength of this study was carried out with 50 PCOS women and 50 controls selected from Saudi women. RFLP analysis was performed in this study to detect the polymorphism frequencies.

CONCLUSION

In conclusion, this study confirms the negative association with -850C/T polymorphism in TNF- α gene and elevated Elisa levels were observed in CT genotypes. The -850C/T polymorphism which was previously documented from Saudi population, global and meta-analysis studies.

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Biomedical Communication

Sero-Prevalence of IgG Rubella Antibodies in Indian Adolescent Women from Tertiary Care Hospitals, Kanchipuram, Tamil Nadu, India

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ABSTRACT

Rubella causes a mild self-limiting illness in children with fever and rash, however in pregnant women, rubella infection causes miscarriage, fetal death, or an infant born with congenital birth defects known as congenital rubella syndrome. The present study attempts to investigate the seroprevalence of IgG rubella antibodies in Indian adolescent girls. The study being a cross-sectional hospital based, was carried at the department of Obstetrics and Gynecology OPD and ward at Meenakshi Medical College Hospital and Research Institute (MMCHRI), Enathur, Kanchipuram, Tamil Nadu, India. In all the cases the association was statistically not significant (P value > 0.05) when compared with joint family. The odds of Rubella IgG positive was 0.656 times in nuclear family and the association was statistically not significant (P value > 0.05) when compared with several children in three. The odds of Rubella IgG positive were 1.711 times in only 1 child, the odds of Rubella IgG positive in 2 children were 1.371 times. In the present study we conclude that the statistically no significant association between the socioeconomic status, parents' age, father's education level, father's employment status, mother's education level, mother's employment status, and Rubella IgG status.

KEY WORDS: ADOLESCENT GIRLS, CHILDREN, IGG ANTIBODY, PREGNANT WOMEN.

INTRODUCTION

In India, Congenital Rubella Syndrome (CRS) is the most common cause of non-traumatic childhood cataracts after the hereditary cataract. Cataract due to CRS accounts for about 10% of pediatric cataracts in India. India has set a goal to eliminate measles and rubella/ CRS by 2023 (Herini et al. 2021). World Health Organization (WHO) has recognized CRS which results in vision and hearing loss among babies can be prevented. Children inflicted with CRS have special needs throughout their life causing a lot of disease burden (Qin et al. 2021). Hence live Rubella vaccines (RCV) are vigorously promoted by WHO in many countries (Herini et al. 2021; Rasool et al. 2021).

Before the introduction of the Rubella vaccine in 1969, the global incidence of CRS was 0.8-4/1000 live births during epidemics and the endemic periods from 0.1-0.2/1000 live births during endemics (Gubio et al. 2019). Since 2010 the "trivalent Measles-Mumps-Rubella vaccine" is available in India but it was not included in the regular immunization schedule of a newborn. The same was the case with the Rubella vaccine, but it was included in the National Immunization Schedule (India) recently (Shanmugasundaram et al. 2021). Immunization studies reported that less than 50% of the children are covered with MMR vaccine. This forced the Indian government to take stringent measures thereby it was decided in 2017 to include the Rubella vaccine in the National Immunization Program. Each country adopted its strategy as per their requirements and implementation feasibility. Covering all adolescent girls and susceptible women of reproductive age is one such strategy (Gupta et al. 2019; Shanmugasundaram et al. 2021).

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Received 11/10/2021 Accepted after revision 12/12/2021

Published: 31st December 2021 Pp- 1719-1723

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.50>

Once infected with Rubella and developing antibodies, these antibodies persist throughout a person's life providing immunity (Chotta et al. 2017). In India at the age of 5 years, 50% of them develop Rubella antibodies due to the previous infection and almost 80–90% become immune to Rubella even without vaccination, naturally by the time they reach 15 years (Gupta et al. 2019). This childhood exposure and development of antibodies provide immunity to women, but periodic epidemics affect children and susceptible adult women, leading to epidemics of CRS (Shanmugasundaram et al. 2021).

The lack of standard assay techniques and different methods adopted by different laboratories poses a challenge in comparing data from different places (Shahapur and Kandi, 2020). To determine an effective strategy for prevention and control of Rubella and thereby CRS, it is essential to have an understanding of the specific epidemiology of Rubella in a country. India has witnessed multiple Rubella and Mumps outbreaks among children, while they are milder among this vulnerable population Administration of two doses of the Measles-Mumps-Rubella (MMR) (Rasheed et al. 2019). In this study serologic surveys were planned to monitor the rubella seroprevalence among the adult women during 2018 to 2020. The serosurvey conducted based on the seroprevalence data from the two phases, we estimated the incidence of CRS and the total number of CRS cases in India (Albrecht et al. 2021).

MATERIAL AND METHODS

The present study was carried at the department of Obstetrics and Gynaecology OPD and ward at Meenakshi Medical College Hospital and Research Institute (MMCHRI), Enathur, Kanchipuram, Tamil Nadu, India. All adolescents' girls aged 10 years to 19 years residing in and around Kanchipuram. This is a hospital-based cross-sectional study, 240 adolescent girls were included in this study. The duration period of the study is from July 2018 to July 2020 for 2 years. The institutional human ethics committee was approved (IEC-25/Jan-2019) this study. All the Subjects who signed Informed written consent before the commencement of the study were allowed to participate. The risks and benefits involved in the study and the voluntary nature of participation were explained to the participants before obtaining consent. Confidentiality of the subjects was maintained.

Amongst the patients presenting to the Obstetrics and Gynaecology outpatient department, adolescent girls (according to WHO criteria) were chosen irrespective of their immunization status. The following information was obtained using a predesigned proforma from each patient, which includes name, date of birth, gender, residence, occupation/educational status, father and mother's educational status, family income, number of children in the family, type of family, vaccination status. Consent was taken from the patient. For pediatric cases, parent/guardian and patient consent were obtained. A blood sample (2 ml) was collected by venipuncture and tested for Rubella IgG antibody using a commercially available ELISA kit (Gupta et al. 2019). The test was performed and results were

interpreted by plotting graphs as per the manufacturer's instructions. According to international guidelines Samples that show IgG antibody titer > 20 IU were positive, <15 IU as negative, and 15-20 IU as equivocal (Viswanathan et al. 2019).

Descriptive analysis was carried out for quantitative variables as mean and standard deviation and categorical variables as frequency and proportion. Data represented using appropriate diagrams, bar diagrams, pie diagrams, and box plots. Univariate binary logistic regression analysis was applied to check the association between the explanatory variables and outcome variables. An unadjusted Odds ratio along with 95% CI. P-value < 0.05 was considered statistically significant. Statistical analysis was carried using IBM SPSS version 22.

RESULTS AND DISCUSSION

Table.1. showed that the descriptive analysis of age in years in the study population, A total of 240 subjects were included in the final analysis. The mean child age was 15.29 ± 1.66 in the study population, ranged between 10 years to 19 years. The mean father's age was 44.98 ± 2.18 in the study population, ranged between 40 years to 51 years. The mean mother's age was 36.74 ± 2.82 in the study population, ranged between 31 years to 45 years.

Table 1. Descriptive analysis of age in years in study population (N=240)

Parameter	Mean \pm SD	Minimum	Maximum
Child Age (in years)	15.29 ± 1.66	10.00	19.00
Father Age (in years)	44.98 ± 2.18	40.00	51.00
Mother Age (in years)	36.74 ± 2.82	31.00	45.00

Table 2. Descriptive analysis of vaccination status in the study population (N=240)

Vaccination Status of a study group	Frequency	Percentages
Yes	122	50.83%
No	118	49.17%

Descriptive Analysis of Vaccination status in the study population: Table.2. Indicated that the descriptive analysis of vaccination status in the study population Among the study population 122 (50.83%) children were vaccinated remaining 118 (49.17%) were non vaccinated, children.

Compilation of Factors affecting Rubella IgG Status in study population (bivariate analysis): Table.3 indicated that the Compilation of Factors affecting Rubella IgG Status in the study population. The mean age of the people with Rubella IgG positive was 15.28 ± 1.68 and it was 15.33 ± 1.59 in people with Rubella IgG negative (P=0.858). The mean age of the father with Rubella IgG positive was

44.99 ± 2.2 and it was 44.93 ± 2.11 in people with Rubella IgG negative (P=0.868). The mean age of the mother with Rubella IgG positive was 36.68 ± 2.71 and it was 37.02 ± 3.3 in people with Rubella IgG negative (P=0.468). Among the people with previous exanthematous fever, all of them 26 (100%) participants had Rubella IgG positive (P=0.998). Out of the 122 children vaccinated, all of them 122 (100%) children had Rubella IgG positive (P=0.995) (Albrecht et al. 2021).

Among the people with the primary education of the father, 103 (85.53%) participants had Rubella IgG positive (P=1.000). Among the people with secondary education of father, all of them 24 (100%) participants had Rubella IgG positive (P=0.999). Among the people with bachelor's education of the father, 71 (74.74%) participants had Rubella IgG positive (P=1.000). Among the people with the occupation of the father as a farmer, 149 (81.42%) participants had Rubella IgG positive (P=1.000). Among the people with the occupation of the father as business, 45 (88.24%) participants had Rubella IgG positive (P=1.000). Among the people with the occupation of the father as engineer, 1 (50%) participant had Rubella IgG positive (P=1.000). Among the people with the occupation of the father as the landlord, all of them 1 (100%) participant had

Rubella IgG positive (P=0.999). Among the people with the occupation of the father as the driver, all of them 2 (100%) participants had Rubella IgG positive (0.999). Among the people with primary education of the mother, 69 (76.67%) participants had Rubella IgG positive (P=0.077) (Albrecht et al. 2021).

Among the people with secondary education of the mother, 60 (84.51%) participants had Rubella IgG positive. Among the people with bachelor's education of the mother, 69 (87.34%) participants had Rubella IgG positive. Among the people with housewife occupation of the mother, 149 (83.24%) participants had Rubella IgG positive (P=0.672). Among the people with duty work occupation of the mother, 35 (92.11%) participants had Rubella IgG positive (P=0.230). Among the people with tailor's occupation of mother, 7 (50%) participants had Rubella IgG positive (P=0.194). Among the people with the weaver occupation of the father, 7 (77.78%) participants had Rubella IgG positive. Among the people of the upper economic class, 47 (81.03%) participants had Rubella IgG positive (P=0.995). Among the people of the lower economic class, 59 (83.1%) participants had Rubella IgG positive (P=0.794). Among the people of the upper-middle economic class, 32 (86.49%) participants had Rubella IgG positive (P=0.530) (Murhekar et al. 2020).

Table 3. Compilation of Factors affecting Rubella IgG Status in study population (bivariate analysis)

Parameters	Rubella IgG status (Mean± SD)		Odds ratio (95% CI)	P value
	Yes	No		
Age in years	15.28 ± 1.68	15.33 ± 1.59	0.982 (0.804-1.200)	0.858
Any previous exanthematous fever (Baseline=No)				
Yes (N=26)	26 (100%)	0 (0%)	39447641.5 (0.001-0.001)	0.998
No (N=214)	172 (80.37%)	42 (19.63%)		
Vaccination status (Baseline=No)				
Yes (N=122)	122 (100%)	0 (0%)	89276241.2 (0.001-0.001)	0.995
No (N=118)	76 (64.41%)	42 (35.59%)		
Upper (N=58)	47 (81.03%)	11 (18.97%)	0.997 (0.348-2.856)	0.995
Lower (N=71)	59 (83.1%)	12 (16.9%)	1.147 (0.409-3.215)	0.794
Upper Middle (N=37)	32 (86.49%)	5 (13.51%)	1.493 (0.427-5.218)	0.530
Lower Middle (N=37)	30 (81.08%)	7 (18.92%)	1.000 (0.312-3.201)	1.000
Upper Lower (N=37)	30 (81.08%)	7 (18.92%)		
Type of family (Baseline=Joint)				
Nuclear (N=153)	123 (80.39%)	30 (19.61%)	0.656 (0.317-1.359)	0.257
Joint (N=87)	75 (86.21%)	12 (13.79%)		
Number of children in the family (Baseline=3)				
1 (N=64)	55 (85.94%)	9 (14.06%)	1.711 (0.681-4.297)	0.253
2 (N=112)	93 (83.04%)	19 (16.96%)	1.371 (0.634-2.963)	0.423
3 (N=64)	50 (78.13%)	14 (21.88%)		

Among the people of the lower middle economic class, 30 (81.08%) participants had Rubella IgG positive (P=1.000). Among the people of the upper-lower economic class, 30 (81.08%) participants had Rubella IgG positive. Among the people of the nuclear family, 123 (80.39%) participants had Rubella IgG positive (P=0.257). Among the people of

the joint family, 75 (86.21%) participants had Rubella IgG positive. Among the family with one child, 55 (85.94%) participants had Rubella IgG positive (P=0.253). Among the family with two children, 93 (83.04%) participants had Rubella IgG positive (P=0.423). Among the family with three children, 50 (78.13%) participants had Rubella

IgG positive. The univariate logistic regression analysis had shown statistically no significant association with

Rubella IgG status with all explanatory factors as presented (Murhekar et al. 2020).

Table 4. Compilation of Factors affecting Rubella IgG Status in Male and female population (bivariate analysis)

Parameters	Rubella IgG status (Mean± SD)		Odds ratio (95% CI)	P value
	Yes	No		
Age in years	15.28 ± 1.68	15.33 ± 1.59	0.982 (0.804-1.200)	0.858
Father Age in years	44.99 ± 2.2	44.93 ± 2.11	1.013 (0.870-1.180)	0.868
Mother Age in years	36.68 ± 2.71	37.02 ± 3.3	0.958 (0.852-1.076)	0.468
Father education (Baseline=Masters)				
Primary (N=120)	103 (85.83%)	17 (14.17%)	97883286.0 (0.001-0.001)	1.000
Secondary (N=24)	24 (100%)	0 (0%)	2.610 (0.001-0.001)	0.999
Bachelors (N=95)	71 (74.74%)	24 (25.26%)	47793336.0(0.001-0.001)	1.000
Masters (N=1)	0 (0%)	1 (100%)		
Father occupation (Baseline=Tailor)				
Farmer (N=183)	149 (81.42%)	34 (18.58%)	7079703965 (0.001-	
0.001)	1.000			
Business (N=51)	45 (88.24%)	6 (11.76%)	1.212	
(0.001-0.001)	1.000			
Engineer (N=2)	1 (50%)	1 (50%)	1615502918(0.001-	
0.001)	1.000			
Land Lord (N=1)	1 (100%)	0 (0%)	2.610 (0.001-0.001)	0.999
Driver (N=2)	2 (100%)	0 (0%)	2.610 (0.001-0.001)	0.999
Tailor (N=1)	0 (0%)	1 (100%)		
Mother education (Baseline=Bachelors)				
Primary (N=90)	69 (76.67%)	21 (23.33%)	0.476 (0.209-1.085)	0.077
Secondary (N=71)	60 (84.51%)	11 (15.49%)	0.791 (0.314-1.991)	0.618
Bachelors (N=79)	69 (87.34%)	10 (12.66%)		
Mother occupation (Baseline=Weaver)				
House Wife (N=179)	149 (83.24%)	30 (16.76%)	1.419 (0.281-7.168)	0.672
Duty Work (N=38)	35 (92.11%)	3 (7.89%)	3.333 (0.467-23.77)	0.230
Tailors (N=14)	7 (50%)	7 (50%)	0.286 (0.043-1.889)	0.194
Weaver (N=9)	7 (77.78%)	2 (22.22%)		

The odds of Rubella IgG positive were 0.982 times in age and the association was statistically not significant (P-value 0.858). The odds of Rubella IgG positive were 1.013 times in father's age and the association was statistically not significant (P-value 0.868) (Table 4). The odds of Rubella IgG positive were 1.013 times in mothers and the association was statistically not significant (P-value 0.468). Compare to bachelors, the odds of Rubella IgG positive were 0.476 times in primary, the odds of Rubella IgG positive in secondary were 0.791 times. In all the cases the association was statistically not significant (P value>0.05). Compared to the weaver, the odds of Rubella IgG positive were 1.419 times in housewives, the odds of Rubella IgG positive in duty work were 3.333 times, the odds of Rubella IgG positive in tailors were 0.286 times. In all the cases the association was statistically not significant (P value>0.05). Compare to upper lower, the odds of Rubella IgG positive were 0.997 times in upper, the odds of Rubella IgG positive in lower was 1.147 times, the odds of Rubella IgG positive in upper-middle were 1.493 times the odds of Rubella

IgG positive in lower-middle were 1.000 times. In all the cases the association was statistically not significant (P value>0.05) (Murhekar et al. 2020).

Compare to joint family, the odds of Rubella IgG positive were 0.656 times in nuclear family and the association was statistically not significant (P value>0.05). Compare to the number of children in three, the odds of Rubella IgG positive were 1.711 times in only 1 child, the odds of Rubella IgG positive in 2 children were 1.371 times. In all the cases the association was statistically not significant (P value>0.05) (Table.3 and 4).

In 2017 the Indian government had included the Rubella vaccine in the National Immunization Program. For making policy decisions on implementing the Rubella control program, it is necessary to collect background data on the serological status of reproductive-age women (Murhekar et al. 2020). IgG positive and those belonging to families with three children, 78.13% were Rubella IgG

positive. Thus in the current study, there was no statistically significant association between the socio-economic factors, parents' age, father's education, father's employment status, mother's education, mother's employment status, number of children in the family (one child p-value 0.253; two children p-value 0.423), socio-economic class (lower (p-value 0.794), upper-lower, lower-middle (p-value 1.000), upper-middle (p-value 0.530), upper (p-value 0.995)), type of family (nuclear/joint p-value 0.257) and Rubella IgG status (Murhekar et al. 2020).

The study by Clark et al. (2016) showed no difference in IgG status in both parents meaning that Rubella is being transmitted equally among all income groups (Clark et al. 2016). In Poethko-Müller et al. (2012) study a high maternal educational level was associated with seronegative to Rubella (Poethko-Müller and Mankertz 2012). In (2018), Indian study reported that there was no significant difference in Rubella susceptibility among different socioeconomic classes, ages, and gravidity (Bavdekar et al. 2018; Murhekar et al. 2020).

Conclusion: The findings of the present study found that Rubella IgG positive was 0.656 times in nuclear family when compared with joint family the correlation was statistically not significant (P value > 0.05) when compared with several children in three. The odds of Rubella IgG positive were 1.711 times in only 1 child, the odds of Rubella IgG positive in 2 children were 1.371 times we conclude that there is a statistically no significant association between the socioeconomic status, parents' social economic status of father's, mother's education level, mother's employment status, and Rubella IgG status.

ACKNOWLEDGEMENTS

The infrastructure support for this study was provided by Meenakshi Medical College Hospital and Research Institute, Kanchipuram.

Conflict of Interests: Authors have no conflict of interests to disclose.

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Agricultural Communication

Expanded Reproduction of Chernozem Fertility in Biological Agriculture

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ABSTRACT

The current stage of development of zonal farming systems in the Central Black Earth region involves the creation of balanced, highly productive, and sustainable agricultural landscapes, maximally adapted to the natural conditions of the region and ensuring the preservation and improvement of soil fertility. The solution to the problem of reproduction of soil fertility in traditional agriculture is associated with the use of a large number of energy-intensive resources and, first of all, irreplaceable ones. However, the level and direction of soil/biological processes are not sufficiently taken into account, whereas they to a certain extent ensure the reproduction of soil fertility. In this regard, the problem of the formation of the scientific foundations of the reproduction of soil fertility through the integrated use of methods of biologization of agriculture in the Central Black Earth Region and the activation on this basis of the soil/biological factor in the long-used chernozems acquires special importance. The purpose of the study is to review the changes in soil fertility indicators in conditions of biological agriculture. The paper presents the results of a study to determine changes in soil fertility indicators in typical chernozem of the Belgorod region (Russia) in a field experiment with various technologies of crop cultivation. The study demonstrates the regularities of the transformation of the fertility of chernozem. In terms of its fertilizing efficiency, compost in the applied norm turned out to be equivalent to a half dose of mineral fertilizers since both variants under consideration provided equivalent increases in the corn yield. The full dose of mineral fertilizers on the background of organic matter turned out to be excessive since its introduction did not contribute to a reliable increase in corn productivity.

KEY WORDS: COMPOST, GRAIN PRODUCTIVITY, MINERAL NUTRITION, THREE-FIELD CROP ROTATIONS.

INTRODUCTION

During the transition of agricultural production to an ecological basis, the main objective seems to be the expanded reproduction of the fertility of chernozems, improving their productivity in specific conditions and at the same time, obtaining agricultural raw materials that meet the most demanding quality requirements, optimizing material, labor and other types of costs (Azarov 2004; Kloster and Azarov 2015). The main soils of the region, chernozems, due to long-term agricultural use, suffer from degradation: their reserves

of organic matter, biogenic elements are reduced, and their physical and other characteristics deteriorate (Linkov et al. 2012; Kuznetsova and Akinchin 2014; Linkov et al. 2016). In the Belgorod region, over the 30 years preceding the study, about 280 thousand hectares of unsuitable land were withdrawn from agricultural use. Their irrational use leads to a violation of the stability of the entire ecosystem of the region (Kuznetsova and Akinchin 2014; Linkov et al. 2016; Gorodov et al. 2017; Prodana et al. 2021; Lee et al. 2021).

In these circumstances, the optimization of arable land productivity, reproduction of the fertility of chernozems dictates the need to develop and implement innovative energy-saving elements of agricultural technologies, with the introduction of which, to obtain profitable, stable yields, and rational use of soil resources would occur, which would

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Received 13/10/2021 Accepted after revision 26/12/2021

Published: 31st December 2021 Pp- 1724-1729

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.51>

resolve the issues of preserving soil fertility indicators, replenishing stocks of nutrients and humus (Linkov et al. 2012; 2016). In the Belgorod region, the closest attention is paid to solving this problem. The solution can be the introduction of biologization elements into the technology of cultivation of the main grain crops of the region, such as soybeans and corn, namely the introduction of organic fertilizers, the use of minimal soil preparation, and sowing of after-harvest and post-cut crops (Turyansky et al. 2014; Orazava et al. 2017; Ippolito et al. 2021). The main objective of our study is to review the trend of the influence of the components of biologization in the technologies of cultivation of grain crops on the ecological state of arable land, the nature of the rationality of their use as an irreplaceable natural resource, the environmental friendliness of the proposed agricultural technologies.

MATERIAL AND METHODS

The experiment was carried out during the full rotation of experimental three-field crop rotations, corresponding to the general specialization of the farm, recognized to provide full-fledged high-quality feed to the main production of the company group (poultry). The experiment was stationary and two-factor, developed in time and space. Variants of the experiment are as :

1. Control without fertilizers (variant 11)
2. Mineral fertilizers at a $\frac{1}{2}$ dose calculated for the planned yield (wheat: 5 t/ha, corn: 6 t/ha of grain) (variant 12)

3. Mineral fertilizers at a full dose calculated for the planned yield (soybeans: 2.5 t/ha, wheat: 5 t/ha, corn: 6 t/ha of grain) (variant 13)
4. Organic fertilizers (compost based on poultry manure) at a dose of 14 t/ha (variant 21)
5. Organic fertilizers (compost based on poultry manure) at a dose of 14 t/ha + Mineral fertilizers at a $\frac{1}{2}$ dose calculated for the planned yield (variant 22)
6. Organic fertilizers (compost based on poultry manure) at a dose of 14 t/ha + Mineral fertilizers at a full dose calculated for the planned yield (variant 23)

The fundamental indicators of soil fertility, which were not subject to significant fluctuations during the period of the experiment, such as the humus content, hydrolytic acidity, nitrification ability of the soil, were determined at the beginning and the end of the crop rotation to establish the nature of the dynamics of these indicators depending on the factors studied.

RESULTS AND DISCUSSION

As the results of the study showed, the humus content in the arable (0-20 cm) and sub-arable (20-40 cm) soil horizons averaged 5.13%, and in the sub-arable horizon, it equaled 4.35%, which corresponds to the gradation of the average organic matter content in the soil. Down the soil profile, humus reserves were reduced due to the lack of an influx of fresh organic matter due to the accumulation of root plant residues.

Table 1. Humus content in the soil in the experimental field, %

Organic fertilizers, t/ha	Nitrogen, phosphorus, and potassium (NPK)	Soil layer, cm	humus content, %		
			2016	2019	changes
0	0	0-20	4.92	4.56	-0.36
		20-40	4.09	3.62	-0.47
	0.5 dose	0-20	5.09	4.58	-0.49
		20-40	4.45	4.13	-0.32
	1 dose	0-20	5.01	4.78	-0.23
		20-40	4.16	3.99	-0.17
1 dose	0	0-20	5.08	5.09	+0.01
		20-40	4.57	4.68	+0.13
	0.5 dose	0-20	5.22	5.32	+0.10
		20-40	4.39	4.91	+0.52
	1 dose	0-20	5.53	5.58	+0.07
		20-40	4.48	4.88	+0.51

Upon completion of the phases of the three-field experimental crop rotation on control variants or variants with NPK, there was a decrease in humus content by an amount from 0.23 to 0.49% in the upper soil layer and by 0.17-0.47% in the soil layer up to 40 cm. The explanation for this fact should be sought in the intensive mineralization of soil organic matter during the cultivation of grain crops without fresh organic matter entering the soil (Table 1). In the variants of the experiment, where an organic fertilizer complex was

used (compost based on poultry manure), the trend was radically changing: the disposition to some stabilization and increased in humus content has been noted in all biological variants.

In this case, it should be noted that there was a pattern of a significant increase in humus reserves in the lower soil layer in the variants with an organic mineral fertilizer system by about 0.5%. On these plots, when sealing organic matter

with heavy disk machines, most of it fall into the underlying layers and, when assimilated by plants, it was easily turned into humus and increases the fund of organic matter of the

soil. Table 2 presents the experimental data on the value of the hydrolytic acidity of the soil in the experimental field (Gutorova et al. 2021).

Table 2. Hydrolytic acidity of chernozem (mmol/100g) in the experimental field

Organic fertilizers, t/ha	NPK	Soil layer, cm	Hydrolytic acidity (mmol/100g)		
			2016	2019	+/-
0	0	0-20	2.64	2.68	+0.03
		20-40	2.78	2.86	+0.06
	0.5 dose	0-20	3.18	3.12	-0.08
		20-40	1.97	2.05	+0.06
	1 dose	0-20	2.55	2.56	-0.01
		20-40	2.62	2.64	0.00
1 dose	0	0-20	2.38	1.98	-0.40
		20-40	1.90	1.87	-0.03
	0.5 dose	0-20	2.55	2.42	-0.15
		20-40	1.81	1.83	+0.02
	1 dose	0-20	2.18	2.15	-0.05
		20-40	2.04	2.12	+0.06

Table 3. Soil nitrification capacity (mg/kg) in the experimental field

Organic fertilizers, t/ha	NPK	Soil layer, cm	Nitrification capacity, mg/kg		
			2016	2019	+/-
0	0	0-20	7.28	8.48	+1.20
		20-40	5.23	6.56	+1.33
	0.5 dose	0-20	5.69	6.59	+0.90
		20-40	4.54	5.24	+0.70
	full dose	0-20	6.51	7.12	+0.61
		20-40	3.38	4.85	+1.47
full dose	0	0-20	7.91	8.75	+0.84
		20-40	7.22	7.98	+0.78
	0.5 dose	0-20	7.59	8.12	+0.53
		20-40	5.15	7.33	+2.18
	full dose	0-20	6.24	7.88	+1.64
		20-40	5.19	6.98	+1.71

The indicator of the value of hydrolytic acidity indicates the degree of saturation of the soil absorbing complex with hydrogen cations. The higher it was, the smaller the capacity of cation exchange and the weaker the metabolic processes were; in this case, the agrochemical and agrophysical indicators of soil fertility were significantly reduced (Pepo 2021; Gutorova et al. 2021). As the analysis of the materials in Table 2 shows, there was a pronounced heterogeneity of the initial state of soil fertility of the experimental field concerning acid-base characteristics. After three years of intensive cultivation of crops, this soil characteristic did not show significant differentiation.

We have recorded a trend of a slight decrease in the indicator under consideration with the mixed use of organic and mineral fertilizers in the upper layer of the soil and

a moderately negative trend (up to 0.06 points) under similar conditions on the mineral fertilizer system. The nitrification ability of the soil belongs to the biological categories of soil fertility, as it characterizes the potential ability of the studied soil to transform available nitrogen compounds from its total reserves, which are part of the nutrient complex of the soil, by the activity of soil biota. The higher the nitrification capacity of the soil, the higher its potential fertility. Naturally, this indicator will grow with an increase in the dose of organic fertilizers (Futa et al. 2021; Peng et al. 2021).

Table 3 shows data on the nitrification capacity of the soil in the experimental field of experimental crop rotations for the period of the study. The change in nitrification capacity indicators by soil layers and experimental variants varies

from 3.37 to 7.90 mg/kg of mineral nitrogen (N-NO₃). The explanation of this fact should be sought in the variety of quantitative and qualitative composition of the microflora of soil biota, which the reference soils of Russia and the whole world possess, namely, typical chernozems (Gorban et al. 2020). In this regard, we analyzed soil samples from each experimental plot to record the dynamics of changes in the nitrification capacity of the soil in time and space.

As the data in Table 3 show, there is an increase in the nitrification capacity of chernozem in all variants of the experiment. This indicator showed an increase of 0.53-2.18 mg/kg of soil, depending on the filling of the variant with a tendency to increase with the biological fertilization

system of grain crops. Optimal agrophysical properties of the soil create prerequisites for ensuring favorable conditions for water, air, and temperature regimes for crop growth and development. The most significant indicator of the agrophysical properties of the soil is the density and structural composition of the root layer of the soil. Data on the agrophysical properties of the soil in the experimental field after the completion of the full cycle of vegetation of crops of the experimental crop rotation are presented in Table 4. As the results of our studies of the agrophysical properties of the soil have shown, the obtained parameters of the density and structural coefficient of the arable layer of chernozem are in optimal values with minor deviations depending on the factors studied (Gutorova et al. 2021).

Table 4. Agrophysical properties of the 0-20 cm soil layer Depending on the applied fertilizers

Indicators	Content of experiment variants					
	without fertilizers	0.5 NPK	1NPK	Compost (background)	Background + 0.5 NPK	Background + 1NPK
Soybean						
Density, g/cm ³	1.20	1.21	1.20	1.1	1.15	1.17
Pedality coefficient	2.5	2.6	2.6	3.0	3.2	3.2
Winter wheat						
Density, g/cm ³	1.22	1.20	1.20	1.12	1.18	1.19
Pedality coefficient	2.6	3.3	3.2	3.5	3.6	3.7
Grain corn						
Density, g/cm ³	1.20	1.18	1.18	1.12	1.14	1.14
Pedality coefficient	3.2	3.2	3.3	3.5	3.6	3.5

Thus, the density of the soil on the variant without fertilizers and their use in the field, where the cultivated soybean was in the same values (of 1.21 g/cm³), and the application of organic fertilizer (compost) contributed to the loosening of the soil (1.1 g/cm³), the introduction of mineral fertilizers at a dose of 1 kg and 2 kg/ha in physical weight was accompanied by soil compaction to the value of 1.15-1.17 g/cm³. It should be noted that the optimal indicators of the density of the arable horizon of typical chernozem range from 1.05 to 1.25 g/cm³, which was generally favorable for crop cultivation.

Under winter wheat and grain corn, the density on all fertilization backgrounds maintained the same trends in its values as under the cover of soybeans with a slight increase in friability against the background of organic fertilizers. The soil pedality coefficient by definition was the ratio of agronomically valuable soil aggregated with a diameter of 0.25 to 10 mm to the rest of the dry soil sample taken for analysis from each experimental plot. The higher the pedality coefficient, the better properties the soil has in terms of optimizing air, temperature, water, and nutrient regimes (Gutorova et al. 2021). The data in Table 4 also indicate that the soil structure coefficient increases in the soy-corn-winter wheat series and increases significantly against the background of composting based on poultry manure. The use of this local fertilizer stimulated the formation of agronomically valuable soil aggregates due to the connective effect of the substances that make up its composition.

The productivity of soybeans, as a crop, which we entered into the experiment, was formed mainly under the influence of fertilizers applied. In the variants without the use of fertilizers, its value was about 13 c/ha, which is a rather low value for the Central Black Earth region. This fact was explained by the use of intensive-type varieties in the experiment, as well as by the level of soil fertility of the experimental site belonging to old arable lands with an insufficient value of available forms of macronutrients. It should be noted that under the condition of fertilization, this value increased in proportion to the number of fertilizers and on plots with the use of 11 tons/ha of compost based on poultry manure and a full dose of mineral fertilizers, the yield of soybean grain was already 22.9 c/ha. It should be noted that in the experimental field as a whole, the agriculture standards were kept at a fairly high level. Regarding the structure of the soybean crop, we can note a tendency to increase almost all values as the level of fertilization increases, which is quite understandable, since in this case a larger grain is formed (Pepo 2021; Gutorova et al. 2021).

Under the scheme of the field experiment, we used winter wheat for two backgrounds of application of organic fertilizers (compost based on poultry manure) and three levels of mineral nutrition 0; 40 NPK (half dose) and 80 NPK (full dose). The experiment was two-factor because three levels of providing plants with nutrients from synthetic fertilizers were superimposed with two organic backgrounds, amounting to 0 and 11 t/ha of compost. Observations and

records on the experimental field under winter wheat were conducted according to the main indicators of wheat quality productivity: the coefficient of productive tillering capacity; the number of productive stems; the number of grains in the ear; the mass of grains in the ear; the mass of 1,000 grains; the nature of grain; the grain: straw ratio; the plant height. Our study has established that the coefficient of the productive tillering capacity of wheat increases with an increase in the amount of plant nutrition from 2.1 in the control variant without the use of fertilizers, to 2.6 at the half and 3.1 with a full dose of mineral fertilizers. The use of organic matter in a full dose contributed to an improvement in the tillering capacity of wheat by 0.3 points with a Least Significant Difference (LSD) index of 0.2 (Pepo 2021; Gutorova et al. 2021).

The organic mineral fertilizer system contributed to the formation of the extremely high tillering capacity of plants up to 4.1 stems per plant. The number of productive stems per unit area increases with an increase in the level of mineral nutrition of the crop from 272 units on the variant without fertilizers to 371 against the background of organic matter and a full dose of mineral fertilizers. At the same time, it should be noted that the high excess background of plant nutrition in a favorable climatic year contributed to the fact that wheat formed a large vegetative mass, as a result of which, at the end of the earing phase during a heavy downpour with gusty wind, plants were lodging with an estimate of 4-5 points on variants with the combined use of mineral and organic fertilizers in full doses. The negative effect of the lodging of wheat plants was noted in the reduction of the grain mass in the ear, the mass of 1,000 grains, and an increase in the proportion of straw to a ratio of 1:1.5 concerning the grain weight during the sheaf analysis of samples from experimental plots of the organic mineral fertilizer system (Pepo 2021).

The yield of winter wheat grain at the control without fertilizers amounted to 43.4 c/ha, which was explained both by the high fertility of the experimental site as a whole and by the use of highly productive varieties of local breeding in the experiment. However, the cultivation of wheat with the absence or lack of mineral nutrition greatly impoverishes the soil, since, for the formation of this crop, the removal of only assimilated nitrogen reaches over 150 kg/ha. The use of a half dose of NPK increased wheat productivity by 7.9 c/ha compared to the absolute control variant with an LSD in the (LSD 05) factor of 3.5 c/ha. The increase in the dose of mineral fertilizers to N 80 P 80 K 80 was not reflected in the further increase in the yield of winter wheat grain: the increase was 1.5 c/ha and is unreliable, i.e. it cannot be mathematically proven. The use of poultry manure-based compost in full norm contributed to an increase in the yield of winter wheat grain by 5.5 c/ha, which undoubtedly proves the high efficiency of local organic fertilizer. The introduction of an incomplete dose of NPK against the background of organics turned out to be optimal. The yield of winter wheat grain on these plots is noted at its maximum values, namely 56.7 c/ha (Gutorova et al. 2021).

The results of the study on winter wheat give the right to assert that with high effective soil fertility and optimal

implementation of all agrotechnical measures (sowing, soil preparation, protection from pests and diseases, selection of varieties), the full dose of mineral fertilizers was excessive, especially against the background of organic fertilizers. To increase the productivity of winter wheat at fertilization levels that meet the biological needs of plants, it was necessary to sow short-stem varieties or use special preparations based on trace elements and biologically active substances that help strengthen internodes and increase grain productivity of winter wheat. Cameral processing of the experimental material obtained experimentally according to the indicators of grain productivity of corn showed that the yield of grain according to the experimental variants increased with an increase in the level of fertilization from 67% on the variant without fertilizers to 80% with the combined use of organic and mineral fertilizers. The mathematically proven reliability of excess grain yield is observed only with significant changes in the nutritional regime of plants. Of considerable scientific and practical interest is the data on the number of ears on a plant, depending on the level of fertilization (Orazaeva et al. 2017; Ippolito et al. 2021).

According to the calculations made, the number of cobs in the control variant was on average 0.85/plant, and with a half dose of NPK it was already 1.2, with a full dose of NPK it equaled 1.3, and under similar conditions, but with organic matter, the number of cobs per plant increased in value to 1.5 units. Studies have also revealed that the number of rows and grains in the cob, the weight of the grain from the cob depend on the level of fertilization of corn. Against the background of organic fertilizers, when applying a full dose of mineral fertilizers, a slight decrease in the mass of grains in the cob and the weight of 1,000 grains was recorded, which indicates the frailty of grain with excessive fertilization of the crop. The optimal ratio of grain and aboveground mass of corn was observed by us on plots with a maximum saturation of fertilizers equaling 1:3, while the lowest ratio was observed in the control variant without their use and equaled 1:2.1. Grain productivity of corn in the control variant was fixed at the level of 50.4 c/ha, which confirms our assumption that the typical chernozem of the experimental field has a high level of natural fertility. Grain corn responded well to the additional introduction of nutrients (Orazaeva et al. 2017; Ippolito et al. 2021).

When using a half dose of NPK, the grain yield increased by 11.3 c/ha compared to the control variant, from the use of a full dose, it increased by another 7.7 c/ha compared to the previous version with an LSD05 of 4.5 c/ha. The use of compost based on chicken manure as an organic fertilizer at a full rate of 14 t/ha increased the yield of corn grain by 11.7 c/ha against the variant without the use of fertilizers. It should be noted that in terms of its fertilizing efficiency, compost in the applied norm turned out to be equivalent to a half dose of mineral fertilizers since both variants under consideration provided equivalent increases in the corn yield. The introduction of a half dose of NPK against the background of the use of organic local fertilizers increased grain yield by 11 c/ha. The full dose of mineral fertilizers on the background of organic matter turned out to be somewhat excessive since its introduction did not contribute to a

reliable increase in corn productivity (Orazaeva et al. 2017; Ippolito et al. 2021).

CONCLUSION

The findings of the present study confirmed that grain corn has huge potential in successfully solving the problem of providing intensive animal husbandry with high-quality feed. At the current level of development of breeding science, promising corn hybrids have been developed and tested, capable of forming grain productivity over 100 c/ha on the optimal background of mineral nutrition. As an example, we can present the advanced farms of the Belgorod region (such as the Krasnoyarskaya Zernovaya Kompaniya LLC or the BESRK-Belgrankorm company group) that received 120 kg/ha of corn on significant acreage in their fields.

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Biotechnological Communication

Synthesis, Characterisation and Antimicrobial Potential of Novel N-Alkylated Pyrrole Derivatives of Chitosan

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ABSTRACT

Chitosan, a cationic biopolymer is a major derivative of chitin. It is biocompatible, non-toxic and environ-friendly material and has broad spectrum antimicrobial activity. However, it is less effective in neutral or basic conditions due to its solubility only in acidic medium. Therefore, chemical modification with suitable groups is necessary to enhance the potency of chitosan. The present study was mainly conducted to explore the effect of structural modifications on antimicrobial potential of chitosan. N-Methyl, N-Ethyl and N-Propyl pyrrole were reacted with N-chloroacyl-6-O-triphenylmethylchitosan prepared by stepwise modification of chitosan to form N-Methyl, N-Ethyl and N-Propyl pyrrole derivatives of chitosan. Structural characterization of these pyrrole derivatives was done by IR, NMR, XRD, DSC and Elemental Analysis. The gram-negative bacterium *Escherichia coli*, gram-positive bacterium *Staphylococcus aureus* were selected for antibacterial activity and the fungus *C. albicans* was selected for antifungal activity by agar diffusion method and MIC method. Antimicrobial activity of the N-Methyl, N-Ethyl and N-Propyl pyrrole derivatives on *E. coli*, *S. aureus* and *C. albicans* showed an inhibitory effect on all the organisms. The potency of inhibition was found to be varied with the substitutions. The maximum activity was shown by N-pyrrolylpropylchitosan against *E. coli* (zone of inhibition 1.2 ± 0.05 cm, MIC 0.15 ± 0.03 mg/ml), *S. aureus* (zone of inhibition 1.4 ± 0.03 cm, MIC 0.15 ± 0.01 mg/ml), *C. albicans* (zone of inhibition 0.8 ± 0.03 cm, MIC 0.2 ± 0.03 mg/ml). The study also confirmed that all the three derivatives exhibited higher inhibition than that of chitosan against *E. coli* (zone of inhibition 0.7 ± 0.03 cm, MIC 0.09 ± 0.02 mg/ml), *S. aureus* (zone of inhibition 0.8 ± 0.03 cm, MIC 0.09 ± 0.02 mg/ml), *C. albicans* (zone of inhibition 0.6 ± 0.03 cm, MIC 0.09 ± 0.03 mg/ml). Results demonstrated that these three N-alkylpyrrole chitosan derivatives exhibited improved potency and hence can have the more applicability as antimicrobials..

KEY WORDS: ANTIMICROBIAL ACTIVITY, CHITOSAN, CHARACTERIZATION, N-CHLOROACYL-6-O-TRIPHENYLMETHYLCHITOSAN, STRUCTURAL MODIFICATION.

INTRODUCTION

Chitosan, a linear heteropolymer is now considered as a functional biopolymer. It is widely exploited for its applications on account of its biodegradability, biocompatibility, versatility and also is found plentiful in nature (Mati-Bauche et al. 2014). Chitosan is the major derivative of the chitin. It is readily soluble in diluted acetic acid (Kumari et al. 2017). Chitosan gained curiosity of researchers not only because of its various properties but also due to its unique biological applications such as antimicrobial, hypocholesterolemic, antitumor, anti-inflammatory, antioxidant, angiotensin-I-converting enzyme (ACE) inhibition, excluding toxins from the intestines, reducing heavy-metal poisoning in humans, mucoadhesive

haemostatic, analgesic, radio-protective properties, preventing tooth decay and tooth diseases and immunity enhancing activities (Chien et al. 2016; Pragłowska et al. 2019). It is also widely used in biomedical industries for enzyme immobilization and purification, in chemical plants for wastewater treatment and in food industries for food formulations as binding, gelling, thickening and stabilizing agent (Hosseinnejad et al. 2016; Nadia et al. 2019; Adnan et al. 2020).

Chemical modification of chitosan has thus provided a new way for developing more and more new derivatives having promising biological activities and physicochemical properties. Chitosan has a primary amino group (C2), and a primary and secondary free hydroxyl group (C3, C6) and this strong functionality of chitosan (per-repeat unit) gives it a considerable chance of chemical modification (Aranaz et al. 2010; Adnan et al. 2020). Pyrrole containing heterocyclic derivatives has been reported for exhibiting wide biological

Article Information:*Corresponding Author: rahatekalpana@gmail.com

Received 25/10/2021 Accepted after revision 26/12/2021

Published: 31st December 2021 Pp- 1730-1736

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.52>

applications, like antibacterial, antioxidant, cytotoxic, insecticidal, anti-inflammatory, anticoagulant, antiallergic, antiarrhythmic, hypotensive and anticonvulsant, due to which they are preferred in a variety of medicinal agents (Parmar et al. 2012; Masci et al. 2019; Kundu and Pramanik 2020).

Due to limited solubility chitosan has not been an effective antimicrobial agent. Thus, in order to enhance the solubility and to broaden antimicrobial activity of chitosan, present study was taken up to synthesize N-alkylated pyrrole derivatives of chitosan and to compare their antibacterial and antifungal property with chitosan.

MATERIAL AND METHODS

Chitosan (CS) was purchased from Nitte gelatin India Limited (Kochi, Kerala, degree of deacetylation 76%). All the other chemicals were purchased from Loba Chemie, Sisco research laboratories, Spectrum Reagents and Chemicals, Alfa Aesar, Nice Chemicals. All the chemicals were used without any further purification. Synthesis of N-Chloroacetyl-6-O-triphenylmethyl chitosan intermediate from chitosan (Fig. 1) was done as per the procedure reported earlier. N-Chloroacetyl-6-O-triphenylmethyl chitosan intermediate was synthesized stepwise from Chitosan. It (10g) was dispersed overnight in DMF (200 mL), treated with Phthalic anhydride (27.6 g, 186 mmol) at 120°C for 8 h to obtain N-Phthaloylchitosan.

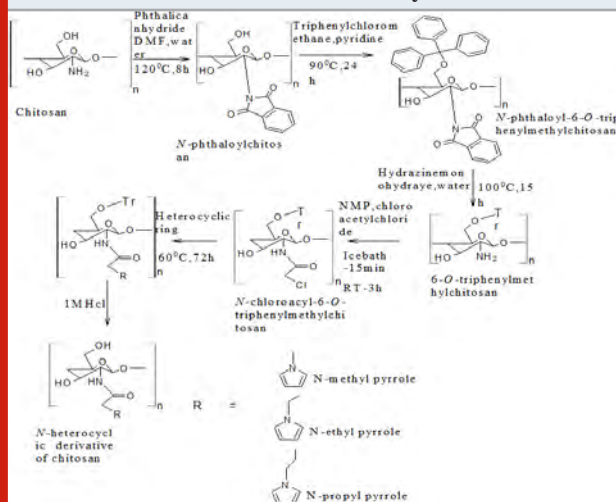
Triphenyl chloromethane (43.8 g, 157.2 mmol) was then added to a solution N-phthaloylchitosan (14.31 g, 52.45 mmol) in pyridine (215 mL) and stirred at 90°C for 24 h to form N-Phthaloyl-6-O-triphenylmethylchitosan. Upon reaction with hydrazine monohydrate (148 mL, 3.04 mol) at 100°C for 15 h, N-Phthaloyl-6-O-triphenylmethylchitosan (26.37 g, 45.06 mmol) was then converted into 6-O-Triphenylmethylchitosan, which was then acetylated by dropwise addition of chloroacetyl chloride to form N-Chloroacetyl-6-O-triphenylmethylchitosan intermediate (Holappa et al. 2005).

The N-alkylated pyrrole derivatives of chitosan (NPCS) were prepared by treating N-chloroacetyl-6-O-triphenylmethyl chitosan intermediate with the corresponding N-alkylated heterocyclic derivatives. N-Chloroacetyl-6-O-triphenylmethyl chitosan was stirred in N-methyl, N-ethyl, N-propyl pyrrole (25mg/mL) separately at 60°C for 72 h under argon. Results are mentioned in figure 1. The corresponding alkylated derivatives were evaporated from the reaction mixtures and washed with methanol and diethyl ether and dried in hot air oven at 80°C for 5 h and melting point was determined by using capillary melting point apparatus (Ma et al. 2008). Percent yield was computed by the following equation: Percent yield = Degree of substitution x (Molecular weight of starting material – Molecular weight of product / Molecular weight of starting material) x 100 aDegree of substitution can be obtained from elemental analysis.

For the study of physicochemical properties, the intrinsic viscosity of CS and NPCS was measured by Ostwald viscometer. Mixture of 0.1 M acetic acid and of 0.2 M

sodium chloride solution (1:2) was used as solvent. The solutions were filtered through a filter paper to remove insoluble impurities and then were passed through the viscometer. Intrinsic viscosity ($[\eta]$) was calculated. The molecular weight of chitosan and NPCS was determined by Staudinger equation $\log [\eta] = \log K + a \log M_v$,

Figure 1: Synthetic route for novel N-alkylated pyrrole derivatives of chitosan via N-chloroacetylation



where, K and a are constant values: 1.8×10^{-3} and 0.93 respectively (Lallana et al. 2017).

For the determination of swelling index, swelling capacity of the CS and NPCS was studied in different media. Previously weighed derivative samples (0.5 g for each test) were individually immersed in 30 mL 0.1 M acetic acid, Phosphate buffer saline of pH 7.4, distilled water and 0.1 M NaOH. The samples were then recovered from the media when they reached equilibrium swelling (24h), wiped with filter paper and weighed. The swelling ratio of each sample was determined according to the following equation: Swelling ratio (%) = $(W_t - W_o) / W_i \times 100$.

For characterisation, Fourier transform infrared (FT-IR) spectra of NPCS were measured in the 4000–400 cm^{-1} regions using a Thermo Nicolet Avatar 370 FT-IR spectrometer in KBr discs. Solid state ^{13}C and ^1H NMR spectra were recorded from a Bruker spectropin-400MHz spectrometer. The percentage elemental analysis (C, H, N and S) was performed on Elemental Vario EL III analyser. XRD measurement of the powder samples were performed with a D8 advance diffractometer (Bruker) with $\text{CuK}\alpha$ radiation ($\lambda = 0.154 \text{ nm}$, 40 kV, 100 mA, scanning rate 40/min). DSC temperature scan was performed on Mettler Toledo DSC 822e (temperature rate $10^\circ/\text{min}$). The degree of substitution was calculated by using the values of C/N ratio obtained from the elemental analysis (Shang et al. 2011; Jiao et al. 2011; Wang et al. 2021).

The antimicrobial activity of chitosan (CS) and NPCS was examined against Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacterial and fungal strain (*Candida albicans*) by Agar diffusion

method and Minimum Inhibitory Concentration (MIC) method. Ciprofloxacin and Ketoconazole (0.25 mg) were used as standards respectively. Preliminary screening tests were performed at concentrations ranging from 0.1 to 3.0 mg/ml. Nutrient agar was used as culture media for antibacterial studies and Sabouraud dextrose agar was used for antifungal study (Hamid et al. 2011; Gharieb et

al. 2015). MIC was recorded in each case as the minimum concentration of compound which inhibited the growth of tested microorganism after incubation at 37°C for 24 h. From the MIC observed, the intermediate concentrations between MIC values were prepared by suitable dilution of stock solution to determine accurate MIC values. Each concentration was tested in triplicate (Li and Zhuang 2020; Lo et al. 2020).

Table 1. Percent Yield (%), M.P. (°C), viscosity [η], and swelling index (%) of chitosan and N-alkylated pyrrole derivatives of chitosan

Sample	% Yield	M. P. (°C)	Intrinsic Viscosity [η]	Swelling ratio %		
				0.1M NaOH	Phosphate buffer	0.1M CH ₃ COOH
CS	-	-	1154	60	54	58
S1	57	233-236	2868	64	78	80
S2	38	245-251	8578	66	70	78
S3	31	285-288	9855	70	84	94

Table 2. Elemental composition and degree of substitution of chitosan and N-alkylated pyrrole derivatives of chitosan

Sample	Theoretical values			Experimental values			C/N Ratio	Degree of substitution (DS)
	C	N	H	C	N	H		
CS	43.52	7.25	7.83	40.34	7.14	7.25	5.6	-
S1	57.68	4.97	5.74	55.73	4.33	5.30	12.8	1.02
S2	58.88	6.58	8.03	57.18	4.93	7.97	11.6	0.75
S3	62.98	6.23	8.29	61.18	5.16	8.02	11.8	0.69

RESULTS AND DISCUSSION

Yield and Physicochemical properties: The yield of N-methylpyrrole derivative of chitosan was found to be highest as compared with N-ethylpyrrole and N-propylpyrrole derivatives. It was found that after the chemical modification there was increase in intrinsic viscosity [η] of the resultant derivatives. There has been significant increase in the swelling index of NPCS compared to that of CS. The results are mentioned in table 1. The improved swelling index due to increase in rate of hydration indicated for better absorption of the drug.

Elemental analysis: After elemental analysis of CS and N-methyl, N-ethyl and N-propylpyrrole derivatives (S1, S2, S3) the values of percent of carbon, hydrogen and nitrogen were found experimentally close to the values obtained theoretically (Jauris et al. 2017). The C/N ratios were also calculated and from that the degree of substitution (DS) was determined. The values of DS suggested full N substitution in all the NPCS (Barbosa et al. 2019). The results are shown in table 2.

X-Ray Diffraction (XRD) analysis: XRD diffractogram of CS showed the characteristic peaks at 10.3° and 20.1°, which suggested the formation of inter and intra-molecular

hydrogen bonds in the presence of free amino groups. Absence of these two peaks in the spectra of all three NPCS confirmed that CS was substituted by the corresponding substituents added. In the samples S1 peak at 4.5° was attributed to the alkyl chain and the other two peaks at 26.6° and 15.5° were formed for pyrrole moiety. S2 showed two peaks at 4.30 and 4.5° for alkyl chain and two peaks at 25.7° and 15.80 for the pyrrole moiety and in S3 the peaks at 4.3° and 5.5° attributed to the alkyl chain and the peaks at 25.6° and 14.6° for the pyrrole nucleus chitosan (Mobarak et al. 2010; Wang et al. 2014; Pati et al. 2020). The crystalline index of CS as well as PCS was determined by following formula:

$$\text{Crystalline index (\%)} = \frac{(I_{110} - I_{am})}{I_{110}} \times 100$$

Where, I_{110} is the maximum intensity approximate to 200 and I_{am} is the intensity approximate to 160 (Cheng et al. 2020).

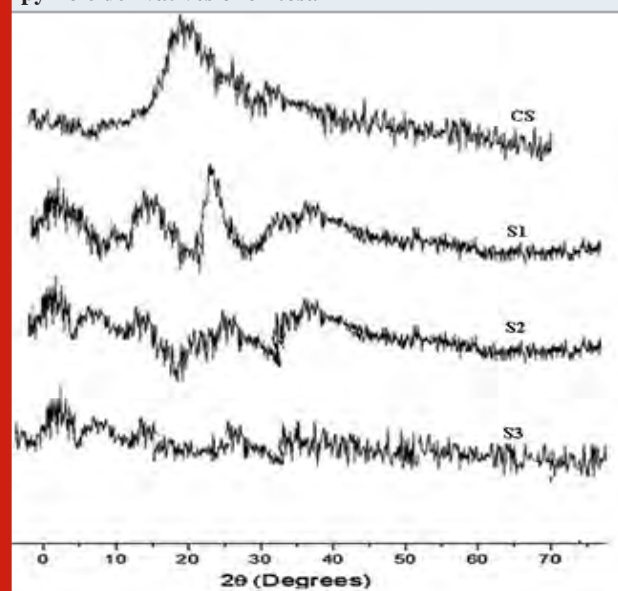
From the XRD results it was observed that the crystallinity of NPCS changed reasonably from that of CS. It is observed that S1, S2 and S3 showed crystalline index comparable to that of CS. It is also seen that S3 showed more crystalline

index than S1 and S2. The crystallinity index of CS and NPCS were shown in table 5. The diffractograms of CS and PCS were shown in figure 2.

Table 3. Crystalline index of chitosan and N-alkylated pyrrole derivatives of chitosan

Samples	Crystalline index (%)
CS	48
S1	41
S2	38
S3	42

Figure 2: XRD diffractogram of chitosan and N-alkylated pyrrole derivatives of chitosan



DSC analysis: Thermogram of CS showed typical polysaccharide behaviour with two distinct degradation stages. The first wide peak started at 30°C and continued up to 150°C, which corresponding to a dehydration process. The second stage started at 245°C and an elevation in the baseline, corresponding to combustion of the sample, was observed. No exothermic melting peak was displayed due to the amorphous state of the CS. Main melting point of chitosan was attributed to the peak which started from 245°C. This coincided with the melting point found out from the capillary melting point apparatus. Thermogram of CS was presented in figure 3a. The thermogram of S1 showed three distinct peaks of which two peaks corresponded to the degradation endothermic peak and the third one for the exothermic peak. The results were exhibited in figure 3b (Alipour et al. 2019; Ferreira et al. 2020).

From the DSC values it could be concluded that the peak at 95.59°C corresponded to dehydration of the sample. The second peak at 163.52°C corresponded to the melting of sample. The third stage showed a positive peak which was exothermic in nature which was due to the curing of the sample. The peak at 163.52°C clearly coincided with

the melting point found out from the capillary melting point apparatus. Thermogram of S2 showed two distinct endothermic peaks at 118.98°C and 218.98°C which showed the degradation as well as the melting of sample. The melting point of S2 obtained from DSC agreed with the value obtained by capillary melting point apparatus. S3 showed two endothermic peaks at 76.55°C and 112.75°C which corresponded to the loss of water and the melting of the sample. The sample showed similar melting points for both DSC as well as capillary tube method. The DSC result coincided with the results of XRD in such a way that the chitosan as well as the derivatives were weakly crystalline (Alipour et al. 2019; Ferreira et al. 2020).

Figure 3a: Thermogram of chitosan

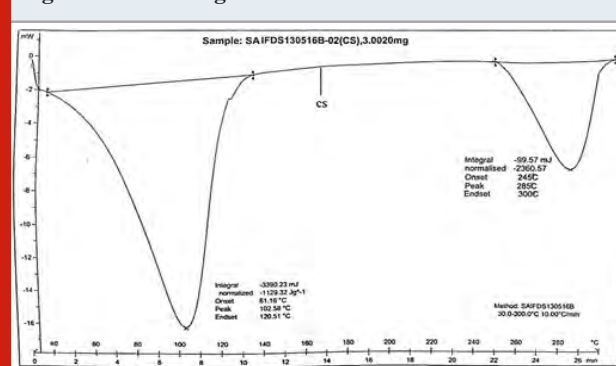
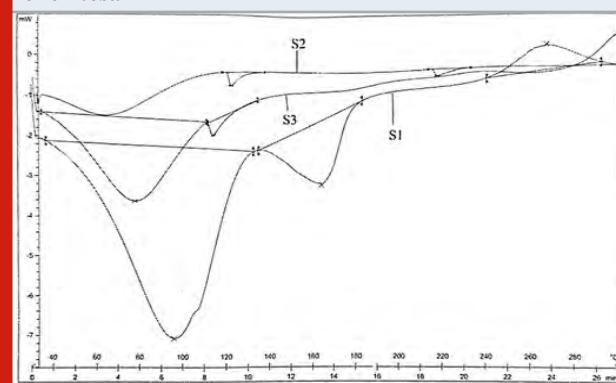


Figure 3b: Thermograms of N-alkylated pyrrole derivatives of chitosan

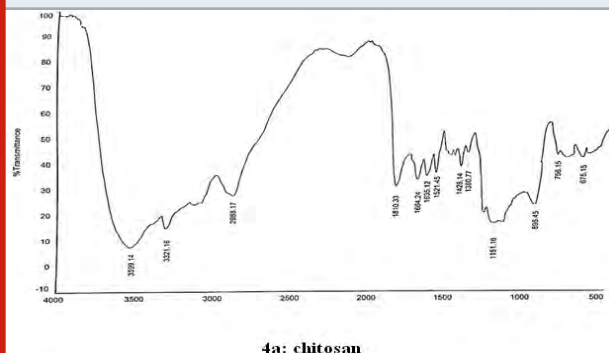


Spectral data of chitosan (CS): FT-IR: 3587 (O-H stretching), 3498 (N-H stretching), 2924 (N-H bond in CH₂), 2881 (C-H bond in CH₃), 1680 (N-H deformations of amide I), 1589 (Amide II), 1378 (CH₃ symmetrical deformation), 1080 (C-O-C bonds), 1421 (Methyl), 1153 (Asymmetric vibration of C-O in oxygen bridge), 896 (Wagging of saccharide structure of chitosan), 645 (C-H bend in aromatics). ¹³CNMR: δ (98.32, 56.95, 70.92, 78.07, 75.74, 61.43). ¹H NMR: 2.09–2.12 (s, NHCOCH₃), 3.15–3.30 (m, H-2 of GlcN residue), 3.57–4.10 (m, H-3,4,5,6 of GlcN unit and H- 2,3,4,5,6 of GlcNAc unit) and 4.88–5.00 (m, H-1 of GlcN and GlcNAc units). Spectra of chitosan coincided with that reported earlier (Verlee et al. 2017; Haj et al. 2020; Lal et al. 2020).

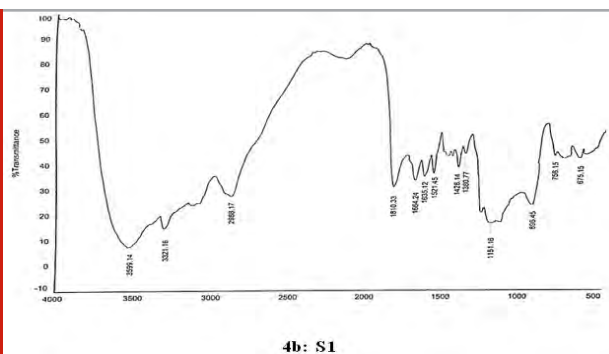
Spectral data of N-pyrrolylmethyl chitosan (S1): FT-IR: 3599 (O-H stretching), 3320 (N-H stretching), 2988 (C-H

stretching in CH₂), 1810 (C=O stretching), 1665 (Amide I), 1630 (C=C stretching in ring), 1519 (Amide II), 1429 (C=N stretching in ring), 1380 (C-N stretching), 1150 (Assymmetric vibration of C-O in oxygen bridge), 896 (Wagging of saccharide structure of chitosan), 747 (C-H bend mono in aromatics), 675 (C-H bend mono in aromatics). ¹³C NMR δ (98.33, 56.98, 70.91, 78.08, 75.45, 62.44, 178.09, 23.12, 109.22, 121.58, 35.82). ¹H NMR: 1.824 (s, 1H), 3.338 (s, 1H), 3.57–4.10 (m, H-3,4,5,6 of GlcN unit and H-2,3,4,5,6 of GlcNAc unit), 3.626 (s, 3H), 4.519 (s, 2H), 4.896 (d, J=2.4, 1H), 6.128 (s, 2H), 6.638 (s, 2H).

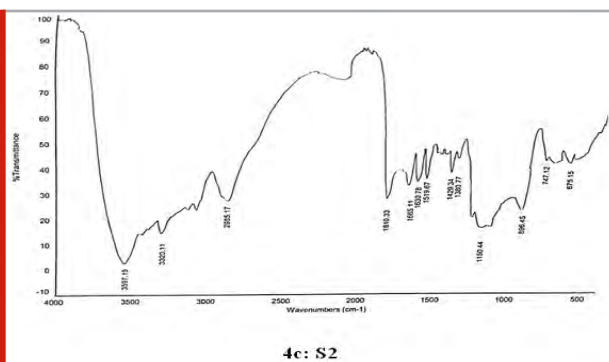
Figure 4: Infrared spectra of chitosan and N-alkylated pyrrole derivatives (S1,S2,S3) of chitosan



4a: chitosan

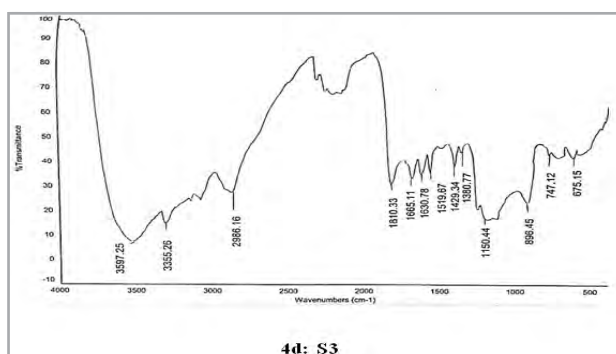


4b: S1



4c: S2

Spectral data of N-pyrrolylethyl chitosan (S2): FT-IR: 3597 (O-H stretching), 3335 (N-H stretching), 2986 (C-H stretching in CH₂), 1810 (C=O stretching), 1665 (Amide I), 1630 (C=C stretching in ring), 1519 (Amide II), 1429 (C=N stretching in ring), 1380 (C-N stretching), 1150 (Assymmetric vibration of C-O in oxygen bridge), 896 (Wagging of saccharide structure of chitosan), 747 (C-H bend mono in aromatics), 675 (C-H bend mono in aromatics). ¹³C NMR:



4d: S3

δ (99.53, 57.98, 71.99, 78.21, 75.77, 62.45, 179.81, 22.33, 109.05, 121.19, 44.68, 16.89). ¹H NMR: 1.391 (s, 2H), 1.823 (s, 1H), 3.331 (s, 1H), 3.57–4.10 (m, H-3,4,5,6 of GlcN unit and H-2,3,4,5,6 of GlcNAc unit), 3.670 (s, 3H), 4.356 (s, 2H), 4.519 (s, 2H), 4.897 (d, J=1.6, 1H), 6.221 (s, 2H), 6.964 (s, 2H).

Spectral data of N-pyrrolylpropylchitosan (S3): FT-IR: 3589 (O-H stretching), 3355 (N-H stretching), 2986 (C-H stretching in CH₂), 1810 (C=O stretching), 1665 (Amide I), 1630 (C=C stretching in ring), 1519 (Amide II), 1429 (C=N stretching in ring), 1380 (C-N stretching), 1150 (Assymmetric vibration of C-O in oxygen bridge), 896 (Wagging of saccharide structure of chitosan), 747 (C-H bend mono in aromatics), 675 (C-H bend mono in aromatics). ¹³C NMR: δ (98.32, 55.98, 70.91, 78.08, 75.76, 62.43, 178.58, 22.12, 108.10, 122.18, 45.66, 16.88, 10.67). ¹H NMR: 1.362 (s, 2H), 1.824 (s, 1H), 1.893 (s, 2H), 2.101 (s, 2H), 3.332 (s, 1H), 3.57–4.10 (m, H-3,4,5,6 of GlcN unit and H-2,3,4,5,6 of GlcNAc unit), 3.678 (s, 3H), 4.515 (s, 2H), 4.895 (d, J=1.6, 1H), 6.216 (s, 2H), 6.362 (s, 2H), 6.965 (s, 2H).

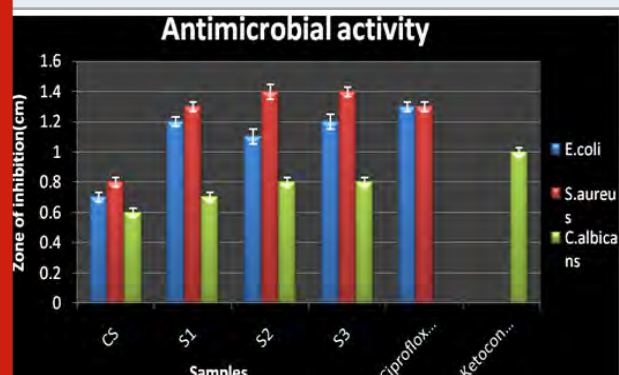
Antimicrobial activity: Chitosan and the three N-alkylated pyrrole derivatives of chitosan S1, S2, and S3 were screened for antimicrobial activity at different concentrations 2 μ l, 5 μ l, 10 μ l, 15 μ l, 20 μ l, and 25 μ l against *E. coli*, *S. aureus* and *C. albicans* by Agar diffusion assay method. The antimicrobial results of the chitosan derivatives against the bacteria and fungi were shown in table 6.

In general, NPCS showed significant antibacterial as well as antifungal activity against the bacteria *E. coli* and *S. aureus* and fungi *C. albicans* than native CS against *E. coli* (zone of inhibition 0.7 ± 0.03 cm, MIC 0.09 mg/ml), *S. aureus* (zone of inhibition 0.8 ± 0.03 cm, MIC 0.09 mg/mL) and *C. albicans* (zone of inhibition 0.6 ± 0.03 cm, MIC 0.09 mg/mL). The maximum activity was shown by N-pyrrolylpropyl chitosan against *E. coli* (zone of inhibition 1.2 ± 0.05 cm, MIC 0.15 ± 0.03 mg/mL), *S. aureus* (zone of inhibition 1.4 ± 0.03 cm, MIC 0.15 ± 0.01 mg/mL), *C. albicans* (zone of inhibition 0.8 ± 0.03 cm, MIC 0.2 ± 0.03 mg/mL). From the results it was also a relevant fact that as the number of alkyl group increased the antimicrobial activity was also seen to be increasing as reported earlier (Panzariu et al. 2016; Mahira et al. 2019). Thus, it was concluded that antimicrobial activity of chitosan was enhanced obviously via chemical modification. The introduction of the substituent group at C-2 position increased the antimicrobial activity of CS.

Table 4. Antimicrobial activity of chitosan and N-alkylated pyrrole derivatives of chitosan by agar diffusion and MIC method

Sample	zone of inhibition (cm)			*Minimum Inhibitory Concn (mg/ml)*		
	<i>E.coli</i>	<i>S. aureus</i>	<i>C. albicans</i>	* <i>E. coli</i>	* <i>S. aureus</i>	* <i>C. albicans</i>
CS	0.7±0.03	0.8±0.03	0.6±0.03	0.09±0.02	0.09±0.02	0.09±0.03
S1	1.2±0.03	1.3±0.03	0.7±0.03	0.15±0.03	0.15±0.02	0.2±0.02
S2	1.1±0.05	1.4±0.05	0.8±0.03	0.15±0.02	0.15±0.02	0.2±0.03
S3	1.2±0.05	1.4±0.03	0.8±0.03	0.15±0.03	0.15±0.01	0.2±0.03
Ciprofloxacin	1.3	1.3	-	-	-	-
Keroconazole	-	-	1	-	-	-

* Values mean±SD mentioned for n=3.

Figure 5: Antimicrobial activity of chitosan and N-alkylated pyrrole derivatives of chitosan

CONCLUSION

The findings of the present study determine the antimicrobial activity that showed an improved antibacterial and antifungal activity as compared to chitosan. The activity of the N-propylpyrrole chitosan was maximum and comparable to that of the standards suggested that with increase in alkyl chain length the solubility and hence antimicrobial activity of the synthesized compounds also increased. The results may play a significant role in designing more potent chitosan derivatives. For this study, three novel N-alkylpyrrole derivatives of chitosan were synthesized by substitution on the reactive amino group of chitosan and characterized by FTIR and NMR spectral study. The compounds had low crystallinity and thermal stability comparable to chitosan.

ACKNOWLEDGEMENTS

The laboratory facilities for this study were provided by the Amrita School of Pharmacy, Amrita Vishwavidyapeetham, Kerala, India.

Conflict of Interests: Authors declare no conflict of interests to disclose.

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Ecological Communication

Morphometric and Meristic Analysis of *Sillaginopsis panijus* Along with Seasonal Variation from Rupnarayan River, West Bengal, India

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ABSTRACT

Present study is a first-time report of flathead Sillago, *Sillaginopsis panijus* (Hamilton, 1822), from Rupnarayan River of West Bengal. Seasonal sampling performed from January 2019- February 2020 by collection of water sample and fish sample in the morning time 5.00 A.M. – 8.00 A.M. A total of 116 specimens of *Sillaginopsis panijus* (Hamilton, 1822) were collected from four different sampling stations of Rupnarayan river (22.23°N 88.03°E to 22.40°N 87.36°E), West Bengal, India. Present work is a morphometric and meristic data analysis has been provided in detail. Total 23 morphometric characters and 13 meristic characters were analyzed. Morphological characteristics of the species were present to confirm the occurrence and distribution of *Sillaginopsis panijus* (Hamilton, 1822) along the riverine water of Rupnarayan. The physico-chemical parameters of water have been measured such as temperature of water, dissolved oxygen, pH and salinity. The statistical analysis of multivariate test with post-Hoc analysis and correlation were established with the abundance of *S. panijus* (Hamilton, 1822) in relation to water parameters. The result shows the dissolved oxygen, temperature, pH and salinity played a most important role in the distribution of *S. panijus* (Hamilton, 1822). The result shows a statistically significant difference in distribution of fish species, $F(12, 8)=18.86, p<0.0005$; Wilk's $\Lambda=0.001$, partial $\eta^2=0.966$. Present study certainly provides the baseline information of *Sillaginopsis panijus* (Hamilton, 1822) from the Rupnarayan river of West Bengal, India. This record of *Sillaginopsis panijus* (Hamilton, 1822) may assist the fishery scientist, researchers, policy planners and conservationists to develop sustainable fishery management. Therefore, this study was considered as a first step on morphometric characters for its development and documenting the extension of the distribution and ecological changes in its natural habitat which helps to conserve this species abundance in this area and prevent overexploitation.

KEY WORDS: BIOMETRY, FIRST REPORT, PHYSICO-CHEMICAL PARAMETERS, RUPNARAYAN RIVER, *SILLAGINOPSIS PANIJUS*.

INTRODUCTION

The *Sillaginopsis panijus* which is a flathead sillago of the family Sillaginidae and order Perciformes is a migratory amphidromous fish found in the areas of Gangetic delta (Type locality), Pondicherry (Coromondal coast), Bangladesh (Siddik et al. 2015), Burma-Malaysia and rarely to the Indonesia (Hamilton 1822; Hamilton- Buchanon 1822; Rayappa et al. 1962; Roper et al. 1984; Talwar and Jhingran 1991; Rahman 2005; Azim et al. 2012). This family is found widespread in the Indian ocean and western Pacific Ocean also. The sillaginids are easily identified

with their uniformity of body shapes. The morphological identifications are considered the most common cost-effective tool in the characterisation of fish species (Cadrian and Silva 2005; Chaklader et al. 2015; Sidik et al. 2021). *S. panijus* is an estuarine and inshore marine fish but adapted in the muddy substrate in shallow water and it migrates to the upper reaches of the tidal river for extending their habitat for breeding and in search of food (Hamilton 1822; Talwar and Jhingran 1991; Alam et al. 2007). The spawning of *S. panijus* (Hamilton, 1822) occurs twice in a year (probably August-September and November- February) and the juveniles migrate toward the upper region of the tidal river during the month of December and March-April (Talwar and Jhingran 1991; Liu et al. 2021).

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Received 11/09/2021 Accepted after revision 25/11/2021

Published: 31st December 2021 Pp- 1737-1745

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Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.53>

It comes with a predatory habit and consumes small fish, planktonic crustaceans and algae. The morphometric and meristic study such as measuring of length, counting of fins and fin-rays, counting of scales and other parameters are important tools used for the proper identification of the species (Cavalcanti et al. 1999). Fish populations are highly dependent upon the physico-chemical parameters of the riverine water body, which supports the abundance of fish population and to perform their biological functions (Ali 1999). Among all the physico-chemical factors salinity, pH, temperature and dissolved oxygen (DO) are the determinants and by their regular or irregular fluctuations a fish population is determined (Thirumala. et al. 2011). Literature survey reveals that a very little work has been done on the fish faunal diversity till date in the river Rupnaryan except the work done by Mishra et al. in (2003) and Ghorai et al. in (2015) and they have listed seventeen and thirty-eight number of species respectively. The present investigation reveals strong evidence that the existence of *S. panijus*, will certainly enrich the biodiversity data of the river Rupnarayan (Hamilton 1822; Chakraborty et al. 2021).

MATERIAL AND METHODS

During present study a total of 116 specimens were collected seasonally by the help of the local fishermen, which captured in the early morning using trawl nets and gill nets from four different study sites of the Rupnarayan River namely Bandarghat (S1), Baksi (S2), Kolaghat (S3), and Gadiara (S4). After collection, photographs were taken for the fresh specimens and were preserved in a wide mouth jar having 4% formalin solution and brought to the laboratory of Raja N. L. Khan Women's College (Autonomous) for further studies. A total of 27 individuals of various size ranges of *S. panijus* were studied morphometrically and meristically. Twenty-three morphometric characters were measured by using a digital slide calipers scale with 0.1 cm accuracy (Hamilton 1822; Bagra and Das 2016).

Morphometric characteristics were studied by the help of existing literature like Talwer and Jhingran (1991); Jayaram (1999), Turan (1999), and the standard method followed after Hubbs and Lagler (1958) and Hubbs and Lagler (2016). Statistical analysis has done by using correlation matrix, multivariate tests, the test of between-subjects effects, multiple comparisons with Tukey HSD test method were performed to established the significant variation among the water parameters (pH, DO, Temperature, Salinity) with occurrence of fish species population seasonally in different four sampling stations (Gonzalez 2013; Nanda et al. 2021).

RESULTS AND DISCUSSION

The fresh fish have a shiny reddish silver colour in the anterior part and whitish silver colour in the abdomen (Fig.1). Fins are pale brownish with black dusting spots. The body shape is elongated and sub cylindrical with a greatly depressed head with scale. Mouth is small and terminal with villiform feeble teeth. Eyes are laterally present, slightly upwards with fleshy orbits. Body covered with small ctenoid

scales, lateral line with 91 to 93 scales. Presence of two well separated dorsal fins with ten fin rays in first dorsal fin in where second dorsal spine of first dorsal fin is very elongated and extended to the caudal fin and second dorsal fin with 26-27 fin rays. The paired pectoral fin with 19 to 22 fin rays and the anal fin with two spine and 25 to 27 soft finrays. The caudal fin with 18 to 20 fin rays. Opercle with a well-developed spine.

Fin formula:

D1 X, D2 I+26-27, P 19-22, P I+V, A II+26-27, C18-20 (Present Study)

D1 X, I+24-28, P 17-22, P I+5, A II+25-27 (Pradhan et al. 2020)

D IX, I+27-28, P 23-24, P I+5, A II+24-28 (Islam et al. 2012)

D IX, I+26-27, P 23-24, P I+5, A II+25-26 (Rahman 1989,2005)

D X, I+26-27, P 24, V I+5, A II+24-26 (Talwar and Jhingran,1991).

Synonyms: *Cheilodipterus panijus* (Hamilton-Buchanan 1822) (Fishes of Ganges:221,381), *Sillago panijus* (Hamilton 1822) (i-vii + 1-405, Pls. 1-39), *Sillago domina* (Cuvier 1829) (Histoire naturelle des poissons. v. 3: i-xxviii + 2 pp. + 1-500, Pls. 41-71.), *Sillaginopsis domina* (Cuvier 1829) (Histoire naturelle des poissons. v. 3: i-xxviii + 2 pp. + 1-500, Pls. 41-71).

Type locality: Ganges estuaries (Hamilton-Buchanan 1822) (Fishes of Ganges:221,381).

Conservation status: According to IUCN Red List 2017 – 2020 Report, not evaluated in Bay of Bengal, India (Image and Bat 2020). Not evaluated globally (Pramanik et al. 2017).

Figure 1: Lateral View of *Sillaginopsis panijus* (Hamilton,1822) Showing Different Body Parts Measurements (Morphometry). aq-Total Length, ap- Fork Length, ar-Standard Length, ae- Head Length, ab- Snout Length, cd- Eye Diameter, ac- Pre-Orbital Length, ad- Post-Orbital Length, af- Pre-Pectoral Length, fv- Pectoral Fin Length, ag- 1st Pre-Dorsal Length, ai- 2nd Pre-Dorsal Length, gj- Longest Fin Ray, ik- 2nd Dorsal Fin Length, ay-Pre-Pelvic Length, yx- Pelvic Fin Length, au- Pre-Anal Fin Length, ut- Anal Fin Length, nq- Caudal Fin Length, hw- Body Depth, ms- Caudal Peduncle, az- Jaw Length.



Table 1. Meristic count of examined *Sillaginopsis panijus* (Hamilton, 1822) from Rupnarayan River

Sl. No.	Meristic characters	Number
1	First dorsal fin rays	10
2	Second dorsal fin rays	26-28
3	Pectoral fin rays	19-22
4	Pelvic fin rays	6
5	Anal fin rays	26-27
6	Caudal fin rays	18-20
7	Scales on lateral line	91-93
8	Scales above lateral line	6
9	Scales below lateral line	14-15
10	First gill raker (Upper)	3-4
11	First gill raker (Lower)	7-8
12	Pre dorsal scale	37-39
13	Circumpeduncular scale	9-10

The descriptive data of 23 morphometric characters of 27 identified samples of *S. panijus* comprised the range of minimum and maximum value, mean value, standard deviation and standard error of each of the characteristics presented in Table 2 and thirteen meristic counts of identified specimens are enlisted in table 1. The morpho-meristic characters differ in the same species due to environmental conditions of different geographical areas (Hamilton 1822; Franičević et al. 2005). The collected specimen is agreed with some diagnosis done by Talwar and Jhingran (1991) except pectoral fin with nineteen to twenty-two fin rays, anal fin with twenty-six to twenty-seven fin rays and caudal fin with eighteen to twenty finrays. Such differences in count of second dorsal fin rays, pectoral fin rays and caudal fin rays are observed in the previous studies (Pradhan et al. 2020).

Some earlier authors also established the meristic counts like Talwar and Kacker (1967); Robins (1986); Rahman (1989); McKay (1992); Rahman (2005), Kaga and Ho (2012); Islam et al. (2012). Species distribution is influenced

Table 2. Morphometric measurement of examined *Sillaginopsis panijus* (Hamilton, 1822) from Rupnarayan River

Sl. No.	Morphometric characters	Maximum (cm)	Minimum (cm)	Mean	SD	SE
1	Total length	25.5	8.6	18.1	6.8964	3.0842
2	Fork length	24.29	8	17.3	6.6421	2.9705
3	Standard length	22.5	7.8	15.8	6.2043	2.7747
4	Head length	6.38	1.66	4.35	1.9221	0.8596
5	Head depth	2.01	0.7	1.5	0.544	0.2433
6	Eye diameter	0.87	0.3	0.65	0.2316	0.1036
7	Snout length	2.55	0.6	1.74	0.8	0.3578
8	First pre dorsal length	7.34	2.57	5.06	1.9635	0.8781
9	Second pre dorsal length	9.92	3.49	6.98	2.7281	1.2201
10	Pre pectoral length	6.67	2.3	4.58	1.7551	0.7849
11	Pre pelvic length	6.93	2.4	4.83	1.8964	0.8481
12	Pre anal length	10.9	4	7.76	2.8248	1.2633
13	Length of longest fin-ray	13.65	3	8.58	4.3226	1.9331
14	Pectoral fin length	3.57	1.42	2.73	0.9924	0.4438
15	Pelvic fin length	2.64	0.9	1.94	0.7387	0.3304
16	Anal fin length	7.64	2.73	5.63	2.1222	0.9491
17	Caudal fin length	3.31	1.27	2.41	0.8194	0.3665
18	Body depth	3.27	1.1	2.13	0.824	0.3685
19	Pre orbital length	2.61	0.94	1.82	0.7283	0.3257
20	Post orbital length	3.41	1.22	2.21	0.8148	0.3644
21	Lower jaw length	1.04	0.33	0.72	0.3015	0.1348
22	Upper jaw length	1.44	0.49	0.97	0.3871	0.1731
23	Length of Caudal peduncle	1.61	0.47	1.16	0.4248	0.19

by a large number of physico-chemical factors such as surface water temperature, pH, salinity, dissolved oxygen was recorded during the study period. The multivariate tests (Table 3) represent the statistically significant difference

in distribution of fish species, $F(12, 8) = 18.86$, $p < 0.0005$; Wilk's $\Lambda = 0.001$, partial $\eta^2 = 0.966$. The correlation matrix (Table 4) showed significance at the level 0.05 in the single star marking values and correlation is significant at the 0.01

level marked with double star marking values. The positive correlation value shows salinity-spot (0.665*), pH- season

(0.603*) at 0.05 level and species no.- spot (0.861**) at 0.01 significance level (Mallya 2007; Ross and Behringer 2019; Velasco et al. 2019).

Table 3. The Multivariate Tests analysis of *S. panijus* (Hamilton,1822) from Rupnarayan river, West Bengal.

	Effect	Value	F	Hypothesis df	Error df	Sig.	Partial Eta Squared
Intercept	Pillai's Trace	1	3972.3	6	4	0	1
	Wilks' Lambda	0	3972.3	6	4	0	1
	Hotelling's Trace	5958.48	3972.3	6	4	0	1
	Roy's Largest Root	5958.48	3972.3	6	4	0	1
Seasons	Pillai's Trace	1.694	4.612	12	10	0.011	0.847
	Wilks' Lambda	0.001	18.855	12	8	5E-04	0.966
	Hotelling's Trace	260.418	65.104	12	6	5E-04	0.992
	Roy's Largest Root	258.109	215.09	6	5	5E-04	0.996

Table 4. Correlation Matrix established with water parameter and number of species of *S. panijus* from Rupnarayan river, West Bengal (Hamilton 1822).

		SPOTS	SEASONS	pH	TEMP	D.O.	SALINITY	SPECIES NO
SPOTS	Pearson Correlation	1	0	0.242	0.018	0.17	0.665*	0.861**
	Sig. (2-tailed)		1	0.449	0.957	0.6	0.018	0
	N	12	12	12	12	12	12	12
SEASONS	Pearson Correlation	0	1	0.603*	-0.927**	0.13	0.637*	0.163
	Sig. (2-tailed)	1		0.038	0	0.69	0.026	0.613
	N	12	12	12	12	12	12	12
pH	Pearson Correlation	0.242	0.603*	1	-0.751*	0.32	0.216	0.068
	Sig. (2-tailed)	0.449	0.038		0.005	0.31	0.501	0.833
	N	12	12	12	12	12	12	12
TEMP.	Pearson Correlation	0.018	-0.927**	-0.751*	1	-0.02	0.572	0.067
	Sig. (2-tailed)	0.957	0	0.005		0.52	0.052	0.836
	N	12	12	12	12	12	12	12
D.O.	Pearson Correlation	0.169	0.128	0.319	-0.204	1	0.075	-0.109
	Sig. (2-tailed)	0.599	0.692	0.313	0.524		0.817	0.736
	N	12	12	12	12	12	12	12
SALINITY	Pearson Correlation	0.665*	-0.637*	-0.216	0.572	-0.07	1	0.463
	Sig. (2-tailed)	0.018	0.026	0.501	0.052	0.82		0.13

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Table 5. Tests of Between-Subjects Effects analyses with water parameter and seasonal occurrences of *S. panijus* from Rupnarayan river, West Bengal (Hamilton 1822).

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	PH	1.696a	2	0.848	10.606	0.004	0.702
	TEMP	480.667b	2	240.333	262.182	0	0.983
	DO	0.095c	2	0.047	0.089	0.915	0.019
	SPECIES NO	61.167d	2	30.583	0.375	0.697	0.077
	SPOTS	0.000e	2	0	0	1	0
	SALINITY	6.562f	2	3.281	3.285	0.085	0.422
Intercept	PH	631.475	1	631.475	7900.3	0	0.999
	TEMP	8164.08	1	8164.08	8906.27	0	0.999
	DO	162.068	1	162.068	304.67	0	0.971
	SPECIES NO	1121.33	1	1121.33	13.759	0.005	0.605
	SPOTS	75	1	75	45	0	0.833
	SALINITY	19.001	1	19.001	19.027	0.002	0.679
Seasons	PH	1.696	2	0.848	10.606	0.004	0.702
	TEMP	480.667	2	240.333	262.182	0.001	0.983
	DO	0.095	2	0.047	0.089	0.915	0.019
	SPECIES NO	61.167	2	30.583	0.375	0.697	0.077
	SPOTS	0	2	0	0.001	1	0.001
	SALINITY	6.562	2	3.281	3.285	0.085	0.422
Error	PH	0.719	9	0.08			
	TEMP	8.25	9	0.917			
	DO	4.787	9	0.532			
	SPECIES NO	733.5	9	81.5			
	SPOTS	15	9	1.667			
	SALINITY	8.988	9	0.999			
Total	PH	633.89	12				
	TEMP	8653	12				
	DO	166.95	12				
	SPECIES NO	1916	12				
	SPOTS	90	12				
	SALINITY	34.55	12				
Corrected Total	PH	2.415	11				
	TEMP	488.917	11				
	DO	4.882	11				
	SPECIES NO	794.667	11				
	SPOTS	15	11				
	SALINITY	15.549	11				

a. R Squared = .702 (Adjusted R Squared = .636); b. R Squared = .983 (Adjusted R Squared = .979); c. R Squared = .019 (Adjusted R Squared = -.198); d. R Squared = .077 (Adjusted R Squared = -.128); e. R Squared = .000 (Adjusted R Squared = -.222); f. R Squared = .422 (Adjusted R Squared = .294)

The negative correlation value shows salinity-season (0-.637*) at 0.05 level and season-temp (-0.927**), pH-temp (-0.751**) at 0.01 level. The test of between-subject effects (Table 5) shows the significant value of the pH= F (2, 9) = 10.60; $p < 0.004$; partial $\eta^2 = 0.702$; Temperature= F (2, 9) = 262.19; $p < 0.001$; partial $\eta^2 = 0.983$ and the not significant values are DO = F (2, 9) = 0.09; $p > 0.915$; partial $\eta^2 = 0.019$; Species No. = F (2, 9) = 0.38; $p > 0.697$; partial $\eta^2 = 0.077$; Spots= F (2, 9) = 0.001; $p > 1$;

partial $\eta^2 = 0.001$; Salinity= F (2, 9) = 3.29; $p > 0.085$; partial $\eta^2 = 0.422$. Post Hoc Tukey HSD test for multiple comparisons (Table 6) the significant values are in pH of the pre -monsoon -post monsoon (0.022), monsoon – post monsoon (0.004); temperature of pre-monsoon- monsoon (0.012), pre-monsoon- post monsoon (0.001) at the level $p < 0.05$. The values of hydrogen ion concentration of water varied from (6.55-7.93) (Velasco et al. 2019).

Table 6. Multiple Comparisons

Tukey HSD

Dependent Variable	(I) SEASONS	(J) SEASONS	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
pH	1	2	0.2225	0.19991	0.53	-0.3357	0.7807
		3	-0.6625*	0.19991	0.022	-1.2207	-0.1043
	2	1	-0.2225	0.19991	0.53	-0.7807	0.3357
		3	-0.8850*	0.19991	0.004	-1.4432	-0.3268
	3	1	0.6625*	0.19991	0.022	0.1043	1.2207
		2	0.8850*	0.19991	0.004	0.3268	1.4432
TEMP	1	2	2.5000*	0.677	0.012	0.6098	4.3902
		3	14.5000*	0.677	0.001	12.6098	16.3902
	2	1	-2.5000*	0.677	0.012	-4.3902	-0.6098
		3	12.0000*	0.677	0.001	10.1098	13.8902
	3	1	-14.5000*	0.677	0.001	-16.3902	-12.6098
		2	-12.0000*	0.677	0.001	-13.8902	-10.1098
DO	1	2	-0.025	0.51572	0.999	-1.4649	1.4149
		3	-0.2	0.51572	0.921	-1.6399	1.2399
	2	1	0.025	0.51572	0.999	-1.4149	1.4649
		3	-0.175	0.51572	0.939	-1.6149	1.2649
	3	1	0.2	0.51572	0.921	-1.2399	1.6399
		2	0.175	0.51572	0.939	-1.2649	1.6149
SPECIES NO	1	2	-5.5	6.38357	0.676	-23.323	12.323
		3	-3.25	6.38357	0.869	-21.073	14.573
	2	1	5.5	6.38357	0.676	-12.323	23.323
		3	2.25	6.38357	0.934	-15.573	20.073
	3	1	3.25	6.38357	0.869	-14.573	21.073
		2	-2.25	6.38357	0.934	-20.073	15.573
SPOTS	1	2	0	0.91287	1	-2.5487	2.5487
		3	0	0.91287	1	-2.5487	2.5487
	2	1	0	0.91287	1	-2.5487	2.5487
		3	0	0.91287	1	-2.5487	2.5487
	3	1	0	0.91287	1	-2.5487	2.5487
		2	0	0.91287	1	-2.5487	2.5487
SALINITY	1	2	1.2	0.70662	0.258	-0.7729	3.1729
		3	1.775	0.70662	0.077	-0.1979	3.7479
	2	1	-1.2	0.70662	0.258	-3.1729	0.7729
		3	0.575	0.70662	0.704	-1.3979	2.5479
	3	1	-1.775	0.70662	0.077	-3.7479	0.1979
		2	-0.575	0.70662	0.704	-2.5479	1.3979

Based on observed means. The error term is Mean Square (Error) = .999.

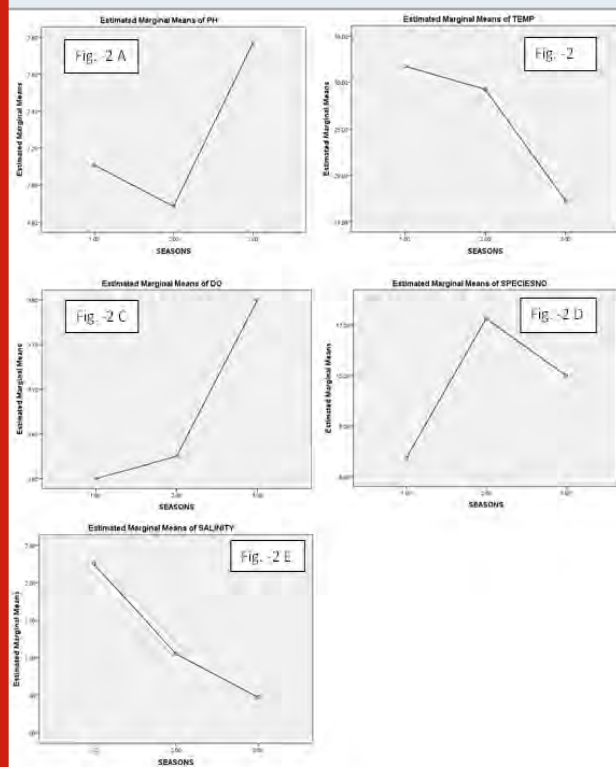
* The mean difference is significant at the .05 level.

The marginal means of pH value (Fig.2 A) in the pre-monsoon season ranged between 7.00-7.20 and in the monsoon was low (below 7.00) and in post-monsoon was high. It was minimum in monsoon at S1 and maximum in post monsoon time in S3. River water with a pH 5.5 and

below is particularly at risk (Sulochanan and Muniyandi 2005). The graphical representation of temperature value (Fig.2 B) showed a lower temperature in post monsoon season than the monsoon season and the surface water temperature high in pre-monsoon season. The minimum

temperature (16°C) was recorded in post monsoon period (December, 2019) in station S3 and the maximum temperature (33°C) in summer season (May, 2019) in S4. The surface water temperature depends on the intensity of solar radiation, evaporation, tidal flow etc. similar findings reported in the previous studies (Thangaraj 1984; Raju et al. 2016; Cheung et al. 2018; Roy and Shamim 2020a; Rahman 2021).

Figure 2 (A-E): Shows the graphical representation of the relation with pH, temperature, DO, specimen no and salinity in respect to the season of *Sillaginopsis panijus* (Hamilton, 1822) from Rupnarayan river, West Bengal.



The estimated marginal means of dissolved oxygen (DO) represented in (Fig.2 C) low level in pre-monsoon and in post-monsoon it was higher than the monsoon period. Dissolve oxygen (DO) is one of the most important parameters which reflects the physical and biological processes of water (Kibria 2017). The average concentration of DO in the water body varied 3.0- 5.6 during the study time. The minimum DO was recorded in the post monsoon season in S1 and maximum in S3 station. The individuals of *S. panijus* were established in a larger number in monsoon season than post-monsoon and a very few in pre-monsoon season (Fig.2 D). Salinity was observed throughout the study period, minimum salinity (0.1ppm) recorded in post-monsoon season in S1 & S2 and the maximum salinity (4.0ppm) in pre-monsoon season in S4.

The salinity ranged lower in the post-monsoon season than the monsoon and salinity level was high in the pre-monsoon season (Fig.2 E). The present result agrees with the result of Mahapatro et al. (2017), that fish always seek better environmental conditions and they extended their habitat

and geographical location due to environmental changes which depends on variable environmental parameters, as a result the species distributed in new areas far from their natural habitat (Hamilton 1822; Hanif et al. 2017; Cheung et al. 2018). The different parameters in an optimum level control the water quality which helps the proper growth of aquatic life (Roy et al. 2021). Several studies on different rivers in India were conducted and portrayed the deterioration of the water body and depletion of valuable aquatic life in its natural habitat (Roy and Shamim 2020a; Rahman 2021).

CONCLUSION

The findings of the present study ensure the presence of *Sillaginopsis panijus* as a first-time record ever in the upstream and downstream of the Rupnarayan river of West Bengal, India. Present morphometric study describes thorough and vivid comparison among individuals in a species qualitatively. It provides the basic information for fishery management and research. The *S. panijus* population size is found larger in monsoon than post-monsoon and smaller in pre-monsoon season. This seasonal and morphometric study could be used as primary information in the near future in fish research and management.

ACKNOWLEDGEMENTS

This study was not financially supported by any other funding agency. Authors are also thankful to Principal, Raja Narendra Lal Khan Women's College (Autonomous) and Head of the Department of Zoology, for providing laboratory facilities and support during the study periods. Authors are grateful to anonymous reviewers and editors for their valuable suggestions and feedback.

Conflict of Interests: Authors declare no conflict of interests to disclose.

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Biotechnological Communication

Control of Fusarium Wilt Using *Streptomyces griseus* with Plant Growth-Promoting Effect on Tomato

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ABSTRACT

The efficacy of antagonistic *Streptomyces griseus* was evaluated against tomato wilt disease incited by *Fusarium oxysporum*. Among the different formulations, *Streptomyces griseus* with chitin amended formulation showed effective increase in seed germination and seedling vigour. Further, talc-based formulations of *S. griseus* mixed with or without chitin was developed and tested under greenhouse conditions. Lowest disease severity of 19.1% was observed in plants treated with self fusant (SFSg 5) *S. griseus* suspension (root dipping - 9×10^8 cfu / mL) followed by 19.5% in treatment of chitin amended *S. griseus* (root dipping - 9×10^8 cfu/mL) was recorded over control. Plant growth of the treated traits were analyzed and compared with control. The shoot length, root length, leaf area was increased significantly over the controls by the treatment of self fusant (SFSg 5) *S. griseus* suspension followed by nearby values were reached in chitin amended *S. griseus* was recorded. The chemical treatments had less effect compared with these formulations. Histochemical studies showed that cambium layers, xylem vessels per bundle, and the vessel diameter decreased in the plants inoculated with *F. oxysporum* over control and changes in variables were observed in infected plants treated with *S. griseus*. In conclusion, *S. griseus* can be a potential biocontrol agent against *F. oxysporum* for better crop production practices.

KEY WORDS: DISEASE SEVERITY, *S. GRISEUS*, SEED/ROOT INOCULATION, TOMATO GROWTH PROMOTION, WILT.

INTRODUCTION

Plants in their environment face potential deleterious organisms such as fungi, bacteria, viruses, nematodes, etc., in soil and air are responsible for losses in crop yield worldwide. Soil-borne pathogens are saprophytic in nature. They reside in the soil for brief or extended periods; enter via roots and cause disease of the roots or stem, disrupting the uptake and translocation of water and nutrients from the soil (McGovern 2015). Plant disease incidence and prevalence varies from season to season depending on the nature of the pathogen, environmental factors, and cultivars and varieties. Acquired resistance of pests & pathogens towards the chemical fertilizers, which have detrimental effects on the environment is the major concern (Abbasi et al. 2019). The use of microorganisms to control plant pathogens is now in

practice (Al-Ani and Adhab 2012, Al-Ani et al. 2012; Al-Ani and Adhab 2013; Passari et al. 2019).

The potential use of plant associated bacteria as agents for stimulating plant growth and managing soil and plant health has been well described (Compant et al. 2005). The growth-promoting bacteria colonizing the root surfaces and closely adhering soil interface (rhizosphere) and enters root interior and established as endophytic populations. The general mechanisms involved in biocontrol are to reduce the population of pathogens at their entry level and to provide strong growth promoting activity during its symbiosis (Chen et al. 2000; Naik et al. 2008). Many plants growth promoting rhizobacteria, endophytic and actinobacteria specifically belonged to the genus *Streptomyces* sp., were used to improve crop fertility by enabling the plant to get nutrients from the soil (Nejad and Johnson 2000; Araujo et al. 2000; Bloembergen et al. 2001; Kanini et al. 2013; Kamal and Sharma 2014; Abbasi et al. 2019; Passari et al. 2019).

Microbial endophytic communities are currently the focal point to explain their function as plant growth promoters

Article Information:*Corresponding Author: anithavarshini22@gmail.com

Received 14/10/2021 Accepted after revision 21/12/2021

Published: 31st December 2021 Pp- 1746-1753

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.54>

and their involvement in plant protection. In consideration of the above, the study was aimed to evaluate growth characteristics of tomato with respect to antagonistic *Streptomyces griseus* against wilt disease induced by *Fusarium oxysporum* under greenhouse conditions.

MATERIAL AND METHODS

For the plant material, Tomato variety Co-4 susceptible to wilt disease was employed during the investigation. For the preparation of antagonist suspension inoculum, the antagonists *S. griseus* was grown on MS broth (with/without chitin) with constant shaking for 7 days at 30°C and at 125 shakes/min. (Remi – C 24). The bacterial cells were harvested by centrifugation at 8000 rpm for 15 min and resuspended in phosphate buffer (0.01M, pH 7.0) and used as inoculum (Bharathi et al. 2004). To determine the efficacy of antagonists on seed germination and seedlings vigour, the mycelial suspension of the *S. griseus* prepared was tested for their plant growth promoting activity, which was carried out by the standard roll towel method ISTA (1996). The seeds were soaked in 10 mL of the bacterial suspension (10^8 cfu/mL) for 2 h and blot dried, placed in wet blotters and incubated at room temperature for 10 days. The seeds soaked in sterile water served as control. The germination percentage of seeds was recorded and the vigour index was calculated using the following formula: Vigour index = per cent Germination x seedling length (shoot length + root length).

To determine the efficacy of *S. griseus* against fusarium wilt under greenhouse conditions, the tomato variety Co-4 was used for *in vitro* and the greenhouse experiments in the entire period of investigation. All seeds were surface-disinfected with 1% sodium hypochlorite for 5 min and rinsed three times in sterile distilled water prior to sowing. Tomato seeds for glasshouse experiments were sown in trays (27 x 42 x 7 cm³) containing an autoclaved mixture of 1:1 (v:v) ratio of vermiculite and sand. Trays were maintained on benches in a glasshouse without artificial light for 4–5 weeks before transplanting. After some days, when the seedlings had 2–4 leaves, they were carefully transplanted into the pots.

To assess the effect of *S. griseus* on disease severity, various formulations were assessed for their efficiency in controlling *F. oxysporum* f. sp. *lycopersici* induced wilt incidence in greenhouse conditions. A pot culture study was undertaken with the following treatments by using completely randomized design (CRD) with three replications. The treatments were imposed as seed treatment and seedlings dip (Kamal and Sharma 2014). Seeds treated with formulation was sowed in the trays and 60 days old seedlings were transferred to the pot and marked as T₁, T₂. Tomato seeds for glasshouse experiments were sown in trays (27 x 42 x 7 cm³) containing an autoclaved mixture of 1:1(v: v) ratio of vermiculite and sand. After 60 days, the seedlings were pulled out from the trays and roots were immersed in various formulation. Various methods of treatment details include, were T₁ - the plants raised from seeds treated with talk formulation of *S. griseus* (10 g/kg

of seeds), T₂ - the plants raised from seeds treated with chitin amended talk formulation of *S. griseus* (10 g/kg of seeds), T₃ - dipped in water containing talc formulation (20 g/L) for 2 h.

The roots alone were immersed in the chitin amended suspension of *S. griseus* (9×10^8 cfu/mL) formulation, T₄ - dipped in water containing talc formulation (20 g/L) for 2 h. The roots alone were immersed in the suspension of self fusant (SFSg 5) *S. griseus* (9×10^8 cfu/mL) formulation, T₅ - dipped in crude chitinase enzyme prepared from *S. griseus* with enzyme activity of 113.3 IU/mL, T₆ - dipped in partially purified endo chitinase enzyme of *S. griseus* with enzyme activity of 1000 IU/mL, T₇ - the plants raised from carbendazim-treated seeds (2 g/ kg of seeds), served as chemical check and T₈ - Healthy control. After treatment, all the treated seeding was transplanted in to the pathogen infested (substrate at 5 percent (w/w) was mixed with sterilized soil) pots at the rate single seedlings per pot. Plants in the greenhouse were watered daily.

Beginning a week after inoculation, external symptoms of Fusarium wilt (wilting and yellowing leaves) were assessed. And the disease severity was assessed as DS = (Proportion of leaves with symptoms / Total number of leaves) X 100. To assess the effect of *S. griseus* on growth attributes, the efficacy of the formulations on various growth parameters viz., root length, shoot length, fresh & dry root weight, fresh & dry shoot weight were recorded in all the treatments at monthly intervals. All the experiments were analyzed independently. The treatment means were compared by Duncan's Multiple Range Test (DMRT) (Gomez and Gomez 1994).

For the microscopic sections and histological studies of tomato tissues, samples from greenhouse were used for histological studies. In all of the experiments, hypocotyls were cut into 1-cm-long segments and stored in FAA (Formalin-Acetic-Alcohol) at least a week before continuing the processing for embedding. Plant tissue was transferred to aluminum weighing dishes and filled with molten paraffin and placed in a warm oven (60°C) overnight. Segments were transversally cut into 25- to 50-µm-thick sections with a freezing microtome. The sections were affixed in the slides and stained with toluidine blue for 5 min, rinsed with sterile water, and mounted with 50% glycerin. Then viewed with microscope and photomicrographs were obtained from the microscope (Cal et al. 2000).

RESULTS AND DISCUSSION

Efficacy of antagonist on seed germination: The use of chemical fertilizers has become common which has had a harmful impact on the environment (Fravel et al., 2005). Hence, it is essential to identify endophytic microorganisms that can be used as a method for increasing plant host resistance to disease and the improvement of soil health (Barnawal et al. 2017). In this regard, a number of actinobacteria and endophytic *Streptomyces* sp. have been reported to stimulate plant growth, function as biocontrol agents against diverse pathogenic fungi; and in general,

increase abiotic and biotic stress tolerance in plants (Kanini et al. 2013; Kamal and Sharma 2014; Passari et al. 2017; Abbasi et al. 2019; Passari et al. 2019).

In the present study, the antagonistic strain *S. griseus* revealed the seedlings vigour (1679) and germination

percentage (92.00). The seeds, which were not treated with any PGPR strain, showed poor performance and from this screening the strain *S. griseus* was selected for developing formulation. The *S. griseus* with chitin showed 96.10% germination and the highest seedling vigour of 3954 and it was improved over control (Table 1).

Table 1. Efficacy of antagonists on seed germination and vigour index of seedling

Treatments	Germination %	Shoot length (cm)	Root length (cm)	Vigor index
<i>S. griseus</i>	92.00 ^c	7.65 ^d	10.40 ^a	1679
<i>S. griseus</i> with chitin	96.10 ^d	6.25 ^{bc}	14.06 ^d	3954
Carbendazim	85.00 ^{ab}	5.25 ^{ab}	12.00 ^c	1331
Control	80.59 ^a	3.95 ^a	9.00 ^a	1044

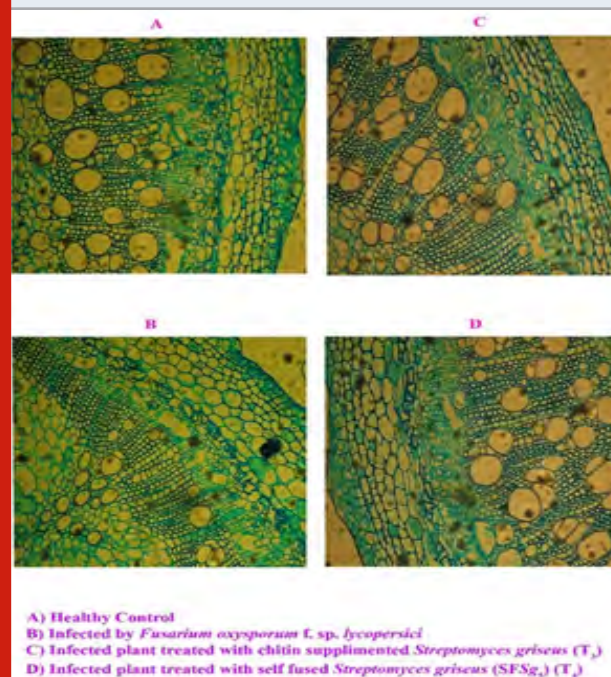
Values are the mean of three replications. Means followed by a common letter are not significantly different at 5% level by DMRT. (% - percentage; Cm – Centimeter)

With an increased number of colonies, improved performance on seed germination and seedling vigour was observed. The studies on growth promotion indicated that antagonistic strain *S. griseus* promote plant growth directly by the production of plant growth regulators or by stimulating nutrient uptake by producing siderophores or antibiotics to protect plants from soil borne pathogens. Further significant increase in seedling vigour would have occurred by better synthesis of hormones like auxins, cytokinins, etc. This finding is consistent with the result obtained by Passari et al., (2019), were demonstrated the four bacterial isolates (BPSAC77, BPSAC101, BPSAC121, and BPSAC147) mediated enhancement in the germination of tomato seeds. Inoculation of BPSAC147 increased the germination percentage to 100% compared to 90% in the untreated, control seeds. Similarly, Lasudee et al. (2018) who stated that *S. thermocarboxydus* S3 increased the germination percentage of mung bean seeds (95–98%) which was statistically higher than the control. Kanini et al. (2013) reported the emergence of the tomato plants, the in vivo experiments were the tomato seeds treated with *S. rochei* ACTA1551 germinated at a higher rate into *F. oxysporum* infected soil compared to untreated seeds (Kanini et al. 2013; Lasudee et al. 2018; Passari et al. 2019).

Green house experiments on tomato plants: Efficacy of formulations on fusarium wilt disease of tomato: *Fusarium oxysporum* is a soil-borne pathogen and can spread through root system and it would be better to protect the infection sites rather than alter the entire soil microbial community. Biocontrol agents can protect the infection site by producing specific nutrients and other compounds against pathogen (Passari et al. 2019). Therefore, biocontrol agent must be introduced to root systems well in advance of *Fusarium oxysporum* f. sp. *lycopersici* infestation (Lu et al., 2004; Kanini et al. 2013). Additionally, this procedure is much easier to implement and more applicable for large scale cultivation, compared to the classic one which

includes enrichment of the soil with the biocontrol agents which is time consuming one and difficult in real farming conditions (Whipps 2001; Nerey et al. 2010). Actinobacteria are identified to be existing in the rhizosphere of plants, and *Streptomyces* sp. are well known as endophytic in root tissues, where they can be able to function as probable biocontrol agents and furthermore modulate plant growth (Rey and Dumas 2017; Jones et al. 2019).

Figure 1: Cross sections of tomato stems (×100).



In the present study, lowest disease severity of 19.1% in treatment of self fusant (SFSg 5) *S. griseus* suspension (root dipping - 9×10^8 cfu / mL) followed by 19.5% in treatment

of chitin amended *S. griseus* (root dipping - 9×10^8 cfu/mL) was recorded (Table 2).

Isolate *S. griseus* exhibited biocontrol activity against *F. oxysporum* in tomato plants which may be due secondary metabolites production are in agreement with previews studies on the potential of *Streptomyces* sp., isolates to be used as biocontrol agents was documented in the previous studies (Passari et al. 2017). The finding is correlated with the earlier findings, were tomato seedlings treated with application of biocontrol agents mainly belongs to *S. enissocaesilis* IC10 and *S. rochei* Y28; *S. flavofuscus* CPP-53; *S. griseorubens* E44G from Saudi Arabia; *S. rochei*

ACTA1551; *S. miharaensis* KPE62302H; *S. psammotomicus* KP1404 (Kim et al. 2011) from Korea and *S. plicatus* from Egypt against *Fusarium* wilt disease of tomato incited *F. oxysporum* f. sp. *lycopersici* showed efficient reduction in wilt disease severity and were reported as successful biocontrol agents to control tomato *Fusarium* wilt (Abd-Allah 2001; Kim 2012; Kanini et al. 2013; Rashad et al. 2017; Abbasi et al. 2019). In the same line, application of non-pathogenic *Fusarium oxysporum* and *Trichoderma harzianum* also showed efficient reduction in wilt incidence (Silva and Bettio 2005; Shishido et al. 2005; Yigit and Dikilitas 2007; Verma et al. 2017; Kamal and Sharma 2019).

Table 2. Efficacy of biocontrol agents on disease severity and yield of tomato under greenhouse conditions

Treatments	Disease severity (%)	Yield (g/plant)
T ₁ – <i>S. griseus</i> (Seed treatment – 10 g / kg)	26.0 ^a	485.0
T ₂ – Chitin amended <i>S. griseus</i> (Seed treatment – 10 g/ kg)	23.1 ^a	455.0
T ₃ – Chitin amended <i>S. griseus</i> suspension (Root dipping – 4×10^8 cfu/mL)	19.5 ^a	520.0
T ₄ – self fusant (SFSg 5) <i>S. griseus</i> suspension (Root dipping - 4×10^8 cfu/mL)	19.1 ^a	550.0
T ₅ – foliar spray using crude chitinase enzyme (1L) of <i>S. griseus</i> with Apsa 80 (113.3 IU/mL) after planting	28.9 ^{ab}	416.0
T ₆ – carbendazim treated seeds (2 g/kg of seeds)	31.5 ^{ab}	425.0
T ₇ – Healthy Control	49.3 ^{de}	425.0
T ₈ – Inoculated Control	61.1 ^h	120.0

Values are the mean of three replications. In a column, means followed by common letters are not significantly different at 5% level by DMRT.

Efficacy of *S. griseus* on growth attributes of tomato:

Better growth may be responsible for better synthesis of auxin and cytokinin which would have helped in better partitioning efficiency which later resulted in increased economic yield. PGPR synthesize phytohormones that promote plant growth at various stages (Passari et al. 2017). In the present study, compared with control, highest shoot length of 70.60 cm, fresh weight of 23.60 g, dry weight of 6.65 g and root length of 55 cm, fresh 15.55 g, dry weight dry weight 3.08 g was observed in the treatment of self fusant (SFSg 5) *S. griseus* suspension followed by nearby values were reached in treatment of chitin amended *S. griseus* (root dipping) was recorded (shoot and root length - 67.50 cm, 52.65 cm; Fresh & dry weight of shoot - 24.7 g; 15.75 g; Fresh & dry weight of root - 6.8 g, 2.85 g). Correspondingly, the chemical treatments had less effect compared with these formulations nevertheless showed moderate increase over control (Table 3). The possible reason might be associated with the initial increase in root growth by the application of PGRP strains, which would have helped in promoting better absorption of essential nutrients that are responsible for highly active photosynthesis as well as protein synthesis (Passari et al. 2017; Jones et al. 2019).

Our study, however, is the first report on the ability of endophytic *S. griseus* to exhibit PGP and biocontrol

activity in tomato plants. Similar results were reported in the previous studies who evidenced that endophytic *S. thermocarboxydus* BPSAC147; *Streptomyces* sp. isolate PM4 and PM5; *Streptomyces* sp. isolate SNL2 and *S. caeruleatus* isolate ZL2 significantly increased the growth against *F. oxysporum* in tomato plants, relatively non-inoculated control plants (Zamoum et al. 2015; Goudjal et al., 2016; Dias et al. 2017; Passari et al. 2019). Furthermore, Goudjal et al. (2014) and Kanini et al. (2013) also reported that *Streptomyces* sp. CA-2 and AA-2 and *Streptomyces rochei* ACTA1551 significantly increased the shoot and root length of tomato plants, relative to control plants. In earlier studies, inoculation of *Trichoderma* sp., *G. intraradices* showed significant increase in growth and yield of tomato plant (Ilham et al. 2003; Akkopru and Demir 2006; Martínez-Medina et al. 2014; Ghazalibiglar et al. 2016; Lee et al. 2016; Jones et al. 2019).

Microscopic sections and histological studies: After assessing the disease severity plant tissues of specific treatment and the corresponding controls also were studied histochemically. Control plant showed normal cambium layers, xylem vessels per bundle (Plate. 1c). The cambium layers, xylem vessels per bundle, and the vessel diameter decreased in plants infected with *F. oxysporum* and not treated with *S. griseus* (Plate. 1a). Cambium is in charge of

secondary growth and regression or its total differentiation has a negative effect on vascular development with inefficient water transport in the plant and subsequent

stunting (Cal et al. 2000). Several authors have described the reduction in the number of xylem vessels as an effect of vascular pathogens including *F. oxysporum* f. sp. *lycopersici* (Cal 1997; Cal 2000; Abbasi et al. 2019).

Table 3. Efficacy of formulations on growth attributes of tomato under greenhouse condition.

Treatments	Shoot			Root		
	Length (cm)	Fresh weight (g)	Dry weight (g)	Length (cm)	Fresh weight (g)	Dry weight (g)
T ₁ – <i>S. griseus</i> (Seed treatment – 10 g / kg)	63.98 ^{cf}	22.75 ^e	6.30 ^{fg}	51.55 ^{gh}	14.5 ^f	2.48 ^d
T ₂ – Chitin amended <i>S. griseus</i> (Seed treatment – 10 g/ kg)	65.68 ^{fg}	25.8 ^h	6.5 ^{gh}	52.05 ^{gh}	14.75 ^g	2.55 ^{de}
T ₃ – <i>S. griseus</i> suspension (root dipping - 9 x 10 ⁸ cfu/mL)	67.50 ^g	24.7 ^g	6.8 ^{gh}	52.65 ^{gh}	15.75 ^h	2.85 ^{fg}
T ₄ – self fusant (SFSg 5) <i>S. griseus</i> suspension (root dipping - 9 x 10 ⁸ cfu / mL)	70.60 ^h	23.60 ^f	6.65 ^h	53.0 ^h	15.55 ^b	3.08 ^{gh}
T ₅ – Crude chitinase enzyme of <i>S. griseus</i> suspension (root dipping - 113.3 IU / mL)	58.35 ^d	21.75 ^{de}	5.8 ^{de}	50.6 ^{gh}	13.85 ^{cf}	2.35 ^{cd}
T ₆ – Partially purified chitinase enzyme of <i>S. griseus</i> suspension (root dipping – 1000 IU / mL)	60.35 ^{de}	20.8 ^{cf}	5.05 ^b	49.85 ^g	13.85 ^e	2.015 ^{ab}
T ₇ – carbendazim (seed treatment - 2 g/ kg)	54.7 ^{bc}	19.85 ^e	4.5 ^a	22.65 ^a	13.35 ^b	1.91 ^a
T ₈ – Inoculated control	46.85 ^a	15.85 ^a	5.08 ^b	39.35 ^{de}	9.8 ^a	1.75 ^a

Values are the mean of three replications. In a column, means followed by a common letter are not significantly different at 5% level by DMRT.

(g / kg – gram/kilo gram; cfu – colony forming units; IU / mL – International Units/Milli Liter; Cm – Centimeter; g – Gram)

The mode of application of *S. griseus* showed marked difference in the suppression of morphological effects caused by *F. oxysporum* f. sp. *lycopersici* in tomato plants. Changes in ciambium layers, xylem vessels per bundle and the vessel diameter under microscopy were observed (Plate. 1b) and these changes were most apparent in plants of treated with *S. griseus* (treatment 3). Morphological changes observed in plants treated with *S. griseus* in seeding before transplanting might have a role in resistance to *Fusarium* tomato wilt, and they remain systemic in the tomato plants (Passari et al. 2019).

The changes could be related to the growth regulators production causing cell growth, division and differentiation also the phytohormones, the major factor which controlling the plant vascular differentiation. This result was supported by the previous studies that showed tomato (*Lycopersicon esculentum*) plants of 'Lorena' were induced with a conidial suspension (107conidia/mL) of *Penicillium oxalicum* before inoculation with *F. oxysporum* f. sp. *lycopersici*, the wilt pathogen. In non-induced plants, the pathogen produced almost a complete loss of cambium (75 to 100% reduction) whereas plants induced with *P. oxalicum* showed less

disease, did not lose the cambium, had a lower number of bundles, and had less vascular colonization by *F. oxysporum* f. sp. *lycopersici* (35 to 99%). Further the suppression of disease caused by *F. oxysporum* f. sp. *lycopersici* due to application of *P. oxalicum* was demonstrated previously to be related to mechanisms of induced resistance in tomato plants (Cal et al. 1995; Cal et al. 1997; Cal et al. 2000; Passari et al. 2019; Abbasi et al. 2019).

Different patterns of root colonization between cultivars and *F. oxysporum* f. sp. pathosystems have been reported previously, such as *F. oxysporum* f. sp. *lentis*; purple passionfruit – *F. oxysporum* and *F. solani*; chickpea - *F. oxysporum* f. sp. *ciceris*; melon - *Fusarium oxysporum* race 1.2; bean - *F. oxysporum* f. sp. *phaseoli* (Zvirin et al., 2010; Garcia-Sanchez et al. 2010; Jimenez-Fernandez et al. 2013; Ortiz et al. 2014; Pouralibaba et al. 2016; (Abbasi et al. 2019).

CONCLUSION

The findings of the present study focused on the screening of *Streptomyces griseus* to control the phytopathogenic

fungi *Fusarium oxysporum* f. sp. *lycopersici*. The best antifungal producer, *Streptomyces griseus* from the prawn cultivating soil proved to be capable of protecting tomato plants from *Fusarium* wilting under greenhouse conditions while confirmation that it promoted the plants growth was derived. Resistance process may functional through secondary metabolites by restricting the pathogen invasion and histological changes could be related to the growth regulators and phytohormones production in *S. griseus* treated plants. Hence plant growth promoting *S. griseus* can be used as biocontrol agent and better crop protection. Thus, the finding of present investigation holds a good promise in tomato wilt management.

Conflict of Interests: Authors declare no conflict of interests to disclose.

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Agricultural Communication

On the Quality of Bare-Grained Oats and Influence of its Cultivation Technology Elements on the Yield

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ABSTRACT

The research was carried out with the aim to establish the formation regularity of both yield and its elements, as well as to formulate technological and quality indicators of bare-grained oats under the influence of different sowing periods. The studies were carried out in the conditions of the northern forest-steppe zone of the Kemerovo region (Russia) on the territory belonging to the Kemerovo Research Institute of Agriculture, a branch of the SFNCA RAS in 2018-2019. The soil of the site is leached chernozem, heavy loamy in granulometric composition, of medium thickness. The object of research was the mid-season variety of bare-grained oats Bare-grained. The predecessor is pure steam. Sowing was carried out in three periods: early – on May 4 (when the soil was physically ripe, subsequent ones with an interval of 8-10 days, depending on the prevailing weather conditions), medium - on May 12 and 14, late - on May 20 and 24. Against the background of each sowing period, the seeding rates of 4.0 were studied; 4.5; 5.0; 5.5; 6.0 million crops/ha. It has been established that the optimal sowing time for obtaining high quantitative indicators (yield, number of grains, grain size) of bare-grained oats in the northern forest-steppe of the Kemerovo region is an early period (first decade of May); while a later period (third decade of May) is more promising for such high-quality indicators as protein content, fat in grain, essential and nonessential amino acids, etc. The optimal seeding rate for bare-grained oats at early sowing period is 4.0-4.5 million/ha. At a later period, it is advisable to increase the seeding rate to 5.0-5.5 million/ha.

KEY WORDS: OATS, SEEDING RATES, SOWING TIME, QUALITY YIELD.

INTRODUCTION

Oats are a ubiquitous crop in global agriculture. Consumption of oats in the Russian Federation over the past decade has increased by 10%, amounting to 350-370 thousand tons, or 2.4-2.6 kg per capita (Abashev et al. 2018). In Russia, the sown area of oats slightly exceeds 3.3 million hectares, second only to wheat, barley and corn. In the Kemerovo region in 2020, oat sowing occupied 85.5 thousand hectares, the average yield was 17.6 c/ha, the maximum areas under the crop are occupied by the varieties Phobos, Creole, Altai large-grain. In recent years, because of the more valuable nutritional and feed benefits, producers have taken an increasing interest in bare-grained oats. The region is actively engaged in selection for bare-grain varieties, the

potential yield of which is much higher, up to 60 kg/ha. Bare-grained oats have a clear advantage over varieties of hulled oats, its protein contains a higher content of essential amino acids, a complex of vitamins (B1, B2, E, F), minerals, various biologically active substances with antioxidant properties, which determines its dietary and therapeutic and prophylactic properties. , and also determines its functional, special properties and possibilities of its application (Nikitenko et al. 2015; Sterna et al. 2016; Lyubimova and Eremin 2018; Batalova et al. 2019).

At the same time, bare-grained spring oats make higher demands on growth conditions than hulled oats because of their morphological structure and biological characteristics. One of the reasons for the decline in the yield of oats with modern genetic potential is the insufficient level of production technology (Kononchuk et al. 2017; Polonskiy et al. 2019). Seeding rates, together with optimal sowing times, play an important role in the relationship between agrotechnical measures, which in turn contribute to

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Received 17/10/2021 Accepted after revision 19/12/2021

Published: 31st December 2021 Pp- 1754-1759

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.55>

obtaining high as well as sustainable yields for oats (Chen et al. 2021).

Research of Bobrovsky and Kosyanenko (2013) was carried out in the conditions of the Krasnoyarsk Territory, and showed that the protein content of bare-grained oats is higher than that of hulled oats and depends on the seeding rate. Thus, at a seeding rate of 5.5 million seedlings / ha, its content was 16%, and at 3.5 million seedlings/ha it increased to 17.4% (Bobrovsky and Kosyanenko 2013). Rendov et al. (2018) noted that for the conditions of the southern forest-steppe of the Omsk region, the best terms for sowing bare-grained oats are the second decade of May and the first decade of June with a seeding rate of 4.5 million seed crops/ha. The highest yield of bare-grained oats was obtained during sowing in the first decade of June (2.07 t/ha) (Rendov et al. 2018). Under the conditions of the northern forest-steppe of the Kemerovo region, bare-grained oats produce a higher yield when sown in the second and third decades of May with a seeding rate of 6.0 million seedlings/ha (Isachkova et al. 2018). Batalova et al. (2019) showed the feasibility of increasing the seeding rate from 4.5 to 5 million crops seed crops/ha in the southern part of the Central Non-Black Earth Region, which increases the yield of bare-grained oats up to 4.0 t/ha.

Scherer and Pirozhkov (2018) noted that when sowing bare-grained oats with a seeding rate of 5 and 6 million seedlings/ha, higher quality indicators are formed, so the protein and fat content in grain at these rates is 14.11-15.22% and 4.94-5.30%, respectively, however, at the same time, a decrease in the mass of 1000 grains was revealed. The optimal sowing time in the forest-steppe zone, according to Sartakova, Chumanova and Soldatova (2006) is the second decade of May, however, the best indicators were observed when sowing at an early period with a seeding rate of 5-6 million germinable grains per hectare (Sartakova, Chumanova and Soldatova 2006; Scherer and Pirozhkov 2018; Batalova et al. 2019; McCabe and Burke 2021).

Technologies for the cultivation of spring oats have been developed in many regions of the country; however, in relation to bare-grained varieties, the technologies have not yet been sufficiently developed to realize the potential of cultivated varieties. The purpose of the research is to establish the patterns of the formation of yield and its elements, technological and quality indicators of bare-grained oats under the influence of different sowing periods and seeding rates.

MATERIAL AND METHODS

The studies were carried out in the conditions of the northern forest-steppe of the Kemerovo region in the fields of the Kemerovo Research Institute of Agriculture, a branch of the SFNCARAS in 2018-2019. The soil of the site was leached chernozem, heavy loamy in granulometric composition, of medium thickness. The humus content was 7.8%, the reaction of the soil solution was close to neutral, and pH was 6.0. The object of research was the mid-early variety of bare-grained oats Gavroche, included in the State Register of Breeding Achievements approved for use in the 10th

region in 2014. The duration of the growing season was 65-83 days. Grains were of medium size. The mass of 1000 grains were in between 25-32 g.

The average yield in the West Siberian region was 3.24 t/ha, the maximum yield was 4.57 t/ha and was valued for quality. Protein content were 17.5-20.0%, oil - 7.0-8.0%, sugar - 4.0-5.0%, starch - 62.0-69.0%. The nature of the grain was 540-660 g/l. Splitting of filmy grains was at the level of 0.7%. It was resistant to lodging, germination of grains on the vine, and dusty smut. The predecessor was pure steam. Sowing was carried out in three periods: early on May 4 (when the soil was physically ripe, subsequent ones with an interval of 8-10 days, depending on the prevailing weather conditions), medium - on May 12 and 14, late - on May 20 and 24. Against the background of each sowing period, the seeding rates of 4.0 were studied; 4.5; 5.0; 5.5; 6.0 million crops/ha. Sowing was carried out with a seeder SN-10 Ts, plot area 10 m² in six replicates. The location of the plots was randomized. The harvesting of the experimental plots was carried out with a combine Sampo-130.

During the research, the following methods were used: methodology for state variety testing of agricultural crops; determination of natural weight according to GOST 10840-64; determination of the content of crude fat in grain in accordance with GOST 13496.15-97; determination of nitrogen and crude protein content in grain in accordance with GOST 13496.4-93; determination of raw ash in grain according to GOST 26226-95; determination of calcium in grain according to GOST 26570-95; determination of phosphorus content in grain according to GOST 26657-97; determination of dry matter content in grain according to GOST 31640-2012; statistical processing of experimental data was carried out according to the method of field experiment using the SNEDEKOR software package.

RESULTS AND DISCUSSION

The weather conditions of the crop growing season in (2018) with an early sowing period were unfavorable for the growth and development of the culture in the initial phases of development, which proceeded against the background of low air temperatures (-2.70C to average annual indicators) and a large amount of precipitation (+32 mm), which influenced the decrease in field germination of plants. The period of sprouting and sprouting was marked by increased air temperatures against a background of high humidity (hydrothermal index = 2.0). Grain filling and ripening took place with sufficient moisture supply and elevated air temperatures (hydrothermal index = 1.4) (table 1) (Polonskiy et al 2019; McCabe and Burke 2021).

The prevailing meteorological conditions of the middle and late periods were relatively favorable, the main subperiods of the growing season (seedlings, flowering, ripening) were marked by high air temperatures and a large amount of precipitation (hydrothermal index = 1.4-2.0). The weather conditions of the growing season for plants of the early sowing period in 2019 were characterized by sufficient moisture supply. Elevated air temperatures (+ 0.6 °C to the long-term average) contributed to the early

emergence of seedlings (on average on the 8-10th day). The vegetative phase of the growing season took place at an air temperature and precipitation close to the mean annual values (hydrothermal index = 1.1).

Table 1. Meteorological conditions during different growing seasons of bare-grained oats (2018-2019).

Sowing time	Period	Sowing – seedling			Seedling – ear formation			Ear formation - ripening		
		temperature, °C	precipitation, mm	hydrothermal index	temperature, °C	precipitation, mm	hydrothermal index	temperature, °C	precipitation, mm	hydrothermal index
Early	Many-year	8,2	28	-	14,9	111	1,2	17,3	102	1,2
	2018	5,5	60	-	19,0	154	2,0	17,0	116	1,4
	2019	8,8	10	-	14,7	100	1,1	18,6	103	1,1
Middle	Many-year	11,0	11	0,5	15,2	96	1,3	17,3	102	1,2
	2018	8,1	49	-	19,0	154	2,0	17,0	116	1,4
	2019	10,3	34	1,7	16,0	95	1,2	18,6	103	1,1
Late	Many-year	13,9	33	1,2	17,2	103	1,2	17,6	107	1,2
	2018	13,6	34	1,3	19,8	145	1,8	15,3	143	1,9
	2019	13,4	48	1,8	18,1	118	1,3	18,2	121	1,3

Grain filling and ripening took place under favorable conditions (hydrothermal index = 1.1), which contributed to the formation of a high yield. The water and temperature regimes during sowing of the middle and late period were characterized by low air temperatures against the background of a large amount of precipitation (hydrothermal index = 1.7 and 1.8, respectively), which led to an increase in the sprouting phase. The vegetative and generative sub-periods were relatively favorable for the growth and development of bare-grained oat plants (hydrothermal index = 1.2-1.3). In 2018, on average in terms of sowing, the best results were obtained at an early period (3.11 t/ha), which exceeds the values of other periods by 0.64-0.66 t/ha. Higher yields were noted at higher seeding rates (table 2) (Batalova et al. 2019).

In (2019), a significant influence of the sowing time on the formation of the yield of bare-grained oat varieties was noted (69.4%). In the Gavrosh variety, the average yield at the early sowing period was 4.19 t/ha, with the average - 2.52 t/ha, with the late sowing - 1.97 t/ha. At the same time, with an early and medium sowing period, the best results were obtained at low seeding rates (4 million seedlings per hectare), with a later period - at a rate of 5 million seedlings per 1 hectare. On average, over the years of research, the best yield indicators were obtained with an early sowing period - 3.65 t/ha, which is 1.15 and 1.44 t/ha higher than the average and late terms, respectively.

At the same time, a higher yield at an early period was formed at seeding rates from 4.0 to 5.0 million/ha, and at a later one - at seeding rates of 5.0-5.5 million/ha. The formation of yield in the Gavrosh variety was influenced by such indicators as: productive stalk ($r = 0.8240$ at $R = 0.5140$), grain weight per panicle ($r = 0.5731$ at $R = 0.5140$) and grain size ($r = 0.9474$ with $R = 0.5140$). A significant influence of soil moisture content on the yield of bare-grained oat varieties was revealed. In the Gavrosh variety, high indicators of productive moisture in a meter layer of soil during all phases of growth contributed to a decrease in yield (sowing: $r = -0.7111$; tillering - stem elongation: $r = -0.9449$; ripening: $r = -0.9696$ at $R = 0.5140$) (Batalova et al. 2019).

The sowing time influenced the plant survival rate, i.e., the ratio of the number of preserved plants to harvesting to the number of sown seeds, which characterizes the ability to germinate and plant resistance to unfavorable environmental factors during the growing season. In the Gavrosh variety, the best survival rates in different years of study were noted at early sowing period of 53.7 -56.8%, at middle and late periods, there was a decrease in the indicator - 42.8-44.6% and 41.4-42.9% respectively. At the same time, better survival was observed at lower seeding rates. The high resistance of bare-grained oat plants of early sowing terms to the effects of the prevailing weather conditions determined an increase in the productive stalk of bare-grained oats ($r = 0.5651$ at $R = 0.5140$) in these variants of the experiment. Thus, the number of productive stems on average for two years at early sowing periods was 409 pieces/m² due to better productive tillering, an average term - 403 pieces/m², a late one - 380 pieces/m² (Polonskiy et al 2019).

Table 2. Yield of bare-grained oats (2018-2019).

Seeding rate, mln./ha	2018			2019			2018-2019 (average)		
	early term	middle term	late term	early term	middle term	late term	early term	middle term	late term
4,0	3,22	2,61	2,45	4,27	2,60	1,87	3,75	2,61	2,16
4,5	3,20	2,45	2,16	4,20	2,44	1,94	3,70	2,45	2,05
5,0	3,07	2,58	2,65	4,27	2,49	2,03	3,67	2,54	2,34
5,5	3,28	2,44	2,66	4,09	2,54	2,00	3,69	2,49	2,33
6,0	2,80	2,26	2,34	4,14	2,52	2,00	3,47	2,39	2,17
comparing the terms	3,11	2,47	2,45	4,19	2,52	1,97	3,65	2,50	2,21
HCP ₀₅									
factor A (term)	0,14			0,11			0,13		
factor B (norm)	0,20			0,14			0,17		

The sowing time influenced the morphological parameters of bare-grained oat plants. In the Gavroche variety, taller plants were noted at an early sowing period (83.8 cm), with an average period, the plant height averaged 74.3 cm, and at a later period - 83.6 cm. Due to the existing relationship between plant height and panicle structure ($r = 0.5436 \dots 0.5976$ at $R = 0.5140$), the best panicle morphological parameters were observed in tall genotypes formed in the

Gavrosh variety at an early sowing date. At the same time, with early sowing, loose panicles were formed with the number of flowers in one spikelet 3.0 pcs. At a later period, panicles formed more compact with a large number of whorls (4.7 pcs.), But their shorter length than at the early and middle terms of sowing, and with a higher density (1.26 pcs.) Due to a greater number of spikelets (table 3) (Polonskiy et al 2019).

Table 3. Morphological parameters of bare-grained oat panicles, on average for the sowing period (2018-2019).

Year	Sowing period	Panicle length, cm	Number of whorls, pcs.	Average length of a whorl, cm	Number of spikelets in a panicle, pcs.	Number of flowers in a panicle, pcs.	Panicle density, pcs/cm	Number of flowers in a spikelet, pcs.	Number of grains in a spikelet, pcs.	Winnowing chaff from grain, %	Number of grains in a panicle, pcs.
2018	Early	15,2	4,2	3,6	20,2	50,6	1,33	2,5	1,6	8,6	32,3
	Middle	15,8	4,2	3,8	20,7	51,2	1,31	2,5	1,6	6,0	32,6
	Late	15,8	4,6	3,4	19,8	56,0	1,25	2,8	1,6	3,9	30,8
2019	Early	16,7	4,3	3,9	19,5	66,8	1,17	3,4	2,5	1,9	47,9
	Middle	15,9	4,1	3,9	18,2	55,6	1,14	3,3	2,6	0,9	41,1
	Late	16,0	4,7	3,4	20,3	64,2	1,27	3,2	2,2	0,8	44,7
2018-2019 (average)	Early	16,0	4,3	3,8	19,9	58,7	1,25	3,0	2,1	4,8	40,1
	Middle	15,9	4,2	3,9	19,5	53,4	1,23	2,9	2,1	4,0	38,7
	Late	15,9	4,7	3,4	20,1	60,1	1,26	3,0	1,9	2,4	35,5

These indicators determined the best grain content in the panicle at an early sowing period: 40.1 pcs., While at the middle and late periods the indicator was 38.7 and 35.5 pcs. When analyzing various seeding rates, the best indicators of the panicle structure in the Gavrosh variety were noted on more rarefied crops (4.0 and 4.5 million seedlings/ha) at all periods. The breakdown of hulled grains is an

important indicator for bare-grained oats (McCabe and Burke 2021).

It was revealed that the trait is more pronounced under the condition of low average daily air temperatures and sufficient moisture in the initial phases of plant growth and development ($r = 0.9954$ at $R = 0.9500$). In this regard,

there was a decrease in this indicator from early to late sowing periods. The mass of 1000 grains in bare-grained oats are one of the most important indicators that determine the seed and food value of the variety. The grain size of bare-grained oats varies greatly, both inside the spikelet and inside the panicle, which is largely influenced by the weather conditions of growing. On average, according to

the experiment, the mass of 1000 grains were 22.5 g. The Gavrosh variety showed a significant decrease in the mass of 1000 grains from the early to the late sowing period: 22.5, 21.7 and 21.3 g with HCP05 = 0.6, which determined the level of productivity of the variety under different cultivation conditions ($r = 0.9474$ with $R = 0.5140$) (table 4) (McCabe and Burke 2021).

Table 4. Technological indicators of bare-grained oat grain (2018-2019).

Seeding rate, mln./ha	2018			2019			2018-2019 (average)		
	early term	middle term	late term	early term	middle term	late term	early term	middle term	late term
Weight of 1000 grains, g									
4,0	20,2	19,0	20,5	24,9	23,2	21,5	22,6	21,1	21,0
4,5	20,3	21,7	20,4	24,3	23,0	22,7	22,3	22,4	21,6
5,0	20,4	20,7	20,9	24,9	23,0	21,8	22,7	21,9	21,4
5,5	20,5	20,7	20,7	25,0	22,6	21,9	22,8	21,7	21,3
6,0	20,1	19,8	20,6	24,5	23,2	21,4	22,3	21,5	21,0
comparing the terms	20,3	20,4	20,6	24,7	23,0	21,9	22,5	21,7	21,3
HCP ₀₅									
Factor A (term)	0,9			0,6			0,8		
Factor B (norm)	0,7			0,8			1,0		
Factor C (grade)	0,6			0,4			0,6		
Natural weight of grain, g/l									
4,0	588	561	552	625	634	621	607	598	587
4,5	591	572	550	628	632	626	610	602	588
5,0	588	569	563	629	630	626	609	600	595
5,5	594	568	549	635	637	621	615	603	585
6,0	589	564	537	629	631	624	609	598	581
comparing the terms	590	567	550	629	633	624	610	600	587
HCP ₀₅									
Factor A (term)	4,9			4,6			4,8		
Factor B (norm)	6,2			5,9			6,1		
Factor C (grade)	4,0			3,7			4,0		

Table 5. Biochemical parameters of bare-grained oat grain at different sowing dates, 2018-2019

Seeding rate, mln./ha	2018			2019			2018-2019 (average)		
	early term	middle term	late term	early term	middle term	late term	early term	middle term	late term
Moisture content, %	7,53	6,86	7,63	7,10	7,51	7,65	7,32	7,19	7,64
Mass fraction of crude protein, %	13,88	14,22	15,37	15,22	15,36	15,63	14,55	14,79	15,50
Mass fraction crude fat, %	7,28	7,69	7,64	7,67	7,72	7,93	7,48	7,71	7,79
Mass fraction	3,88	2,39	2,32	2,03	1,97	2,06	2,96	2,18	2,19
Crude ash, %	0,13	0,15	0,10	0,11	0,12	0,12	0,12	0,14	0,11
	0,48	0,48	0,49	0,44	0,49	0,58	0,46	0,49	0,54

The grain size of the Bare-grained variety was influenced by the conditions of moisture supply during the tillering period - going into the tube, with a greater value noted on the crops of the middle term ($r = -0.9317$ at $R = 0.5140$).

Different variants of the experiment also influenced the completeness and density of the grain of bare-grained oats, which are characterized by the full-scale weight of the grain. In the Gavroche variety, the indicator was 600

g/l on average over the experience. There was a tendency to decrease the natural weight from the early to the late sowing period, which is explained by the reduced moisture supply of late crops during the tillering-tube emergence period ($r = 0.9456 \dots 0.9605$ at $R = 0.5140$). The processed technological elements also influenced the quality indicators of grain. Thus, there was a significant excess of the content of crude protein (15.50%) and crude fat (7.79%) in grain at a late sowing period over early and middle periods (Table 5) (Polonskiy et al 2019).

The main advantage of bare-grained oat varieties over hulled oats is the increased content of essential (valine, isoleucine, leucine, lysine, methionine, threonine, tryptophan, phenylalanine) and non-essential (arginine, asparagine, glutamine, glutamic acid, glycine, carnitine, amino acids) ornithine in protein, the total content of which was 3.713 and 9.467% at the early sowing period, with the average - 3.695 and 9.067%, at the late sowing - 3.814 and 9.751%, respectively (Polonskiy et al 2019; McCabe and Burke 2021).

CONCLUSION

The findings of the present study showed that the optimal sowing time for bare-grained oats in the northern forest-steppe of the Kemerovo region to obtain high quantitative indicators is an early period (first decade of May), and a later period (third decade of May) is also promising. At early sowing terms, there are increased indicators of the density of the productive stalk and grain size, which determine the level of yield of the variety. Late sowing periods contribute to a better formation of the morpho-biological parameters of the panicle (length, density, number of spikelets, number of flowers, and number of grains).

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Biotechnological Communication

Gene Expression in Proliferative Diabetic Retinopathy Using RNA-Seq Data: A Computational Study on Saudi Patients

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ABSTRACT

Proliferative diabetic retinopathy is the widespread type of DM which causes chronic as well as progressive alterations at microvascular level, which particularly effects the eye. The main characteristic of this disease is the development of few new blood vessels around the retina of eye as well as at the posterior region of eye segments. For our computational analysis 155 differentially expressed genes calculated through paired t test statistics analysis using the GenePattern platform, of proliferative diabetic retinopathy in Saudi patients were downloaded. Among the 155 genes, 95 were upregulated, and 60 were downregulated. The Annotation Cluster (FAC) tool in the (DAVID) (<http://david.abcc.ncifcrf.gov/home.jsp>) was used to identify biological processes that are abundant in proliferative diabetic retinopathy (PDR). The functions required for response to mRNA splicing, intracellular protein transport, mRNA processing, microtubule cytoskeleton structure, and atrioventricular canal formation are represented by the GO keywords that are abundant in genes. We used the KAAS web server to identify the biological pathways of these DEGs in addition to DAVID functional analysis and found that the majority of the DEGs were associated with important biological processes, with many being classified in metabolic pathways, Spliceosome, Cell cycle, or being involved in the mRNA surveillance pathway. findings are consistent with those of earlier research. To corroborate the predictions stated in this work, which will demonstrate the role enhanced functional processes, experimental validation will be necessary.

KEY WORDS: COMPUTATIONAL ANALYSIS, DIABETES MELLITUS, GENE EXPRESSION, PROLIFERATIVE DIABETIC RETINOPATHY.

INTRODUCTION

Diabetes mellitus is one of the multifactorial diseases and a leading cause of death in world and especially in Saudi Arabia. Proliferative diabetic retinopathy is the widespread type of DM which causes chronic as well as progressive alterations at microvascular level, which particularly effects the eye, along with other body parts. If the disease is left untreated it will grow gradually and ultimately leading to the blindness. Progression of disease is not rapid, but gradual starting from mild alterations, moving towards moderate and ultimately severe proliferative diabetic retinopathy. The main characteristic of this disease is the development of few new blood vessels around the retina of eye as well as at the

posterior region of eye segments i.e., vitreous (El-Bab et al. 2012; Lee et al. 2015; Alharbi and Alhazmi 2020).

The mechanism by which the DM progresses to diabetic retinopathy is not clearly understood and that's why the disease pathology is thought to be complex and unclear. However, a lot of studies has been carried out to examine the disease progression by considering the disease history along with other aspects. It has been suggested that multiple interactive mechanisms are playing an important role, causing the damage at cellular level and adaptive changes, which cause the devastation in this disease (El-Asrar et al. 1998; Sinclair and Schwartz 2019; Alharbi and Alhazmi 2020).

Earlier it was considered that DM and especially PDR is not a prevalent disease at Saudi Arabia, due to healthy diet and routine. However, recent studies have reported that prevalence of disease is increasing in Saudi Arabia as well and the possible risk factors for this progression are supposed

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Received 25/09/2021 Accepted after revision 24/12/2021

Published: 31st December 2021 Pp- 1760-1763

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.56>

to be consumption of more westernized diet leading to increased chances of obesity and ultimately complications of diabetes. Earlier the disease was 23.7% prevalent in Saudi Arabia while by the year (2011), it has reached to increase 30% and increasing day by day with men more affected than females (Ali et al. 2008; Al Dawish et al. 2016; Alharbi and Alhazmi 2020).

Different treatment strategies can be used to treat diabetic retinopathy. Photocoagulation is one of them. Studies have shown that photocoagulation approach causes a decrease in chances of loss of vision by up to 50% (Cantrill 1984). It causes the decrease in visual acuity as well as constricts the posterior visual regions. Intravitreal administration of about 1.25 mg bevacizumab at the time of cataract surgery could be safe as well as protective in preventing the progression of DR and diabetic maculopathy in patients with cataract and diabetic retinopathy (Cheema et al. 2009; Alghamdi et al. 2021).

MATERIAL AND METHODS

155 differentially expressed genes calculated through paired t test statistics analysis using the GenePattern platform, and

identified based on the statistical cutoff of proliferative diabetic retinopathy in Saudi patients with type 2 diabetes were downloaded (Pan et al. 2016). Among the 155 genes, 95 were upregulated, and 60 were downregulated, and has been taken for computational analysis shown in Table 1. For the functional analysis, on the list of differentially expressed genes with a fold change of >1, DAVID (<http://david.abcc.ncifcrf.gov/home.jsp>) functional annotation cluster analysis was done. For analysis, only terms with a value of 0.05 and a count number of 5 genes were chosen. DAVID was used to classify enriched biological themes in the collection of DEGs using the gene ontology (GO) term biological process (BP). The KEGG Automatic Annotation Server (KAAS) (<http://www.genome.jp/kegg/kaas/>) was used to map pathways (Moriya et al. 2007). The amino acid sequences of these DEGs were submitted to the KAAS online site as input, and orthologs were assigned using the single-directional best hit (SBH) technique. KAAS uses BLAST similarity searches against a carefully selected set of ortholog groups in the KEGG GENES database to provide functional annotation of genes in a genome. Genes in the data sets that were mapped to one of KEGG's reference pathways were given a KEGG orthology (KO) number by KAAS (Amoaku et al. 2020).

Table 1. A list of 155 differentially expressed gene selected for analysis (Pan et al. 2016).

Znf207	Utrn	Ctr9	Trps1	Znf80	Cav2	Il33
Smad4	Tardbp	Ythdc1	Usp8	Kcnh3	Loc285847	Adra1d
Sept2	Sfpq	Mia3	Meaf6	Loc100506124	Ramp1	Taar1
Orc2	Cep350	Ubl4a	Fam69b	Rnpc3	Loc100506995	Or4k17
Mtch1	Fam208b	Gpr18	Mgc72080	Tubg1	Prss27	Kdr
Senp1	Ecd	Kctd4	Rbm17	Psat1	Gprc5d	Loc440910
Helz	Coro7	Btbd2	Rqcd1	Pcdhgc4	Nckap5	
Ttc17	Ei24	Tug1	Mmgt1	Dlc1	Hla-Dqa2	
Man2a1	Stard3	C11orf30	Lats1	Ddx1119	Linc00346	
Klhl11	Sytl1	Znf597	Wee1	Cthrc1	Revrn	
Ptpn11	Eif3a	Sec63	Hist1h3i	Rnu4-2	Dyx1c1	
Ssr1	Aste1	Stx17	Sepn1	Dpy19l2	Mfap5	
Dusp11	Chmp6	Vps36	Clock	Ntsr1	Ovch1	
Tmed10	Hnrnpa3	Psmc5	Linc00265	Ifitm10	Pnmt	
Dync1li2	Psimec1	Fam190b	Ppp6c	Acy3	Ppp1r14c	
Slc39a3	Tmem39a	Dtwd2	Loc729852	Fp588	Ccdc144nl	
Slc33a1	Cend3	Rbm25	Paics	Nxph3	Loc100505806	
Arhgef6	Pacs2	Prim2	Loc100652890	Loc100506476	Gs52	
Pap0lg	Pde4d	Znf784	Hsp90b1	Nrxn2	Hrh3	
Hnrnpu	Kiaa2026	Cg030	Eys	Loc100506678	Pp2672	
Ash1l	Calu	Abra	Mob3b	Loc100507144	Cpsf4l	
Loc729082	Ap3m1	Pdcd4	Pnn	Pnmal2	Rtl1	
Usp48	Masp2	Ccar1	Hnrnpa2b1	Bcl6b	Loc100128081	
Zdhc6	Alg2	Sos1-It1	Sdhap2	Tdrd10	Cnpy1	
Utrn	Ddx46	Loc283624	Rab40b	Hcar1	Ugt2a3	

RESULTS AND DISCUSSION

We downloaded the precomputed list of 155 differentially

expressed genes for our computational analysis shown in Table-1 Among the 155 genes, 95 were upregulated, and 60 were downregulated (Pan et al. 2016; Amoaku et al. 2020).

For the functional annotation analysis, the Annotation Cluster (FAC) tool in the Database for Annotation, Visualization, and Integrated Discovery (DAVID) was used to identify biological processes that are enriched in proliferative diabetic retinopathy (PDR) (<http://david.abcc.ncifcrf.gov/home.jsp>). For annotations and GO terms

with statistically significant values from the resultant functional analysis, the name "Biological Process" was utilized. The functions required for response to mRNA splicing, intracellular protein transport, mRNA processing, microtubule cytoskeleton structure, and atrioventricular canal formation are represented by the GO keywords that are abundant in genes in this table (Table 2).

Table 2. Significantly enriched gene ontology (GO) terms detected by FAC in differentially expressed genes. Only those terms which reported a value of ≤ 0.05 and count number ≥ 2 genes were selected for the analysis.

Term	Count	PValue	Genes
GO:0003867 mRNA splicing, via spliceosome	7	0.008898523	PNN, HNRNPA3, DDX46, HNRNPA2B1, HNRNPU, RNPC3, CCAR1
GO:0003807 RNA splicing	5	0.025835922	SFPQ, RBM25, ECD, TARDBP, RNPC3
GO:0000827 G1/S transition of mitotic cell cycle	4	0.03150948	LATS1, PPP6C, PRIM2, ORC2
GO:0006397 mRNA processing	5	0.082803253	SFPQ, RBM25, ECD, HNRNPA2B1, TARDBP
GO:0032754 positive regulation of interleukin-5 production	2	0.089708975	IL33, PDE4D
GO:0010487 gene expression	3	0.041420273	HNRNPA3, HNRNPA2B1, HNRNPU
GO:0036302 atrioventricular canal development	2	0.045173463	SMAD4, PTPN11
GO:0070102 interleukin-5-mediated signaling pathway	2	0.058973318	SMAD4, CTR9
GO:0006906 vesicle fusion	3	0.05992622	STX17, CAV2, SYTL1
GO:0034497 protein localization to pre-autophagosomal structure	2	0.071602905	STX17, PACS2
GO:0006886 intracellular protein transport	5	0.075167601	AP3M1, TMED10, STX17, SYTL1, RAMP1
GO:0000226 microtubule cytoskeleton organization	3	0.08266618	DYNC1U2, WEE1, TUBG1
GO:0043254 regulation of protein complex assembly	2	0.090232937	LATS1, PTPN11
GO:0003807 alternative mRNA splicing, via spliceosome	2	0.090232937	RBM17, SFPQ
GO:0006888 ER to Golgi vesicle-mediated transport	4	0.093438618	DYNC1U2, TMED10, STX17, MIA3
GO:0009888 tissue development	2	0.096360227	TRPS1, CNFY1

For the pathway analysis, we found the biological pathways of DEGs annotated in the current study in addition to DAVID functional analysis. DEG amino acid sequences in FASTA format were put into the KAAS to predict different pathways. There was a total of 154 routes predicted. Table 3 lists the top 20 KEGG pathways, with Supplementary Table S1 providing a comprehensive list of all pathways. The majority of DEGs were discovered to relate to significant biological processes, with many being categorized in metabolic pathways, spliceosomes, or cell cycle, or being engaged in the mRNA monitoring pathway, as seen in these tables (Amoaku et al. 2020).

Recent studies have reported prevalence of Proliferative diabetic retinopathy (PDR) disease is increasing. Proliferative diabetic retinopathy is the widespread type of DM which causes chronic as well as progressive alterations at microvascular level, which particularly effects the eye. The main characteristic of this disease is the abnormal growth of new vessels occurs (Tarr et al. 2013; Safi et al. 2014). Study shows Involvement of angiogenesis, inflammation, and fibrosis in proliferative diabetic retinopathy and Enrichment of genes and pathways related to lymphatic development indicates that targeting lymphatic involvement in PDR progression. Several pro-angiogenic cytokines have been described as being involved in the pathogenesis of PDR, although VEGF is accepted as the most significant cytokine in PDR (Amoaku et al. 2020). The present finding shows significance of mRNA splicing, intracellular protein transport, mRNA processing, microtubule cytoskeleton organization and atrioventricular canal development, and associated with important biological processes, many being classified in metabolic pathways, Spliceosome, Cell cycle or being involved in mRNA surveillance pathway These

are consistent with those of other studies (Korhonen et al. 2021).

Table 3. Top 20 KEGG pathways for DEGs, Number of mapped genes shown in bracket

ko01100 Metabolic pathways (10)
ko03040 Spliceosome (5)
ko04110 Cell cycle (4)
ko05164 Influenza A (4)
ko04144 Endocytosis (4)
ko01110 Biosynthesis of secondary metabolites (4)
ko05205 Proteoglycans in cancer (4)
ko04080 Neuroactive ligand-receptor interaction (4)
ko03015 mRNA surveillance pathway (4)
ko05132 Salmonella infection (3)
ko05200 Pathways in cancer (3)
ko04141 Protein processing in endoplasmic reticulum (3)
ko04151 PI3K-Akt signaling pathway (3)
ko05166 Human T-cell leukemia virus 1 infection (3)
ko04020 Calcium signaling pathway (3)
ko05168 Herpes simplex virus 1 infection (3)
ko05207 Chemical carcinogenesis - receptor activation (3)
ko04510 Focal adhesion (3)
ko04390 Hippo signaling pathway (3)
ko05418 Fluid shear stress and atherosclerosis (3)
ko05014 Amyotrophic lateral sclerosis (3)

CONCLUSION

The findings of the present study have used a Bioinformatics approach to identify the DEGs enrichment indicate the

significance of mRNA splicing, intracellular protein transport, mRNA processing, microtubule cytoskeleton organization and atrioventricular canal development, and associated with important biological processes, many being classified in metabolic pathways, Spliceosome, Cell cycle or being involved in mRNA surveillance pathway The present study's findings are consistent with those of earlier research. To corroborate the predictions stated in this work, which will demonstrate the role enhanced functional processes, experimental validation will be necessary.

ACKNOWLEDGEMENTS

This study was technologically supported by the Bioinformatics and Computational Biology Unit at Department of Biological Sciences, King Abdulaziz University, Jeddah, Saudi Arabia.

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Toxicological Communication

Ecotoxicological Risk Assessment of Paper Mill Effluent Waste Water

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ABSTRACT

Aquatic ecosystem has been reported to be the universal sufferer of pollution caused by direct exposure of industrial discharges which causes severe genotoxic damages to aquatic flora and fauna. Researchers have found that fish have been extensively harmed by such exposure compared to other aquatic fauna. As living organisms directly depend on fish as a food resource, hence the study of mutagenicity induced have been extensively important not only for safety of aquatic organisms but also for safety of other living organisms too. Micronucleus (MN) assay has been continuously used in the evaluation of DNA damage. Mutagenic and genotoxic studies employed this methodology to evaluate possible carcinogenic risk due to exposure to harmful xenobiotics in including aquatic organisms. The aim of this study was to monitor the level of genotoxicity induced in fishes due to exposure to local paper mill effluent by using micronucleus assay as a biomarker. Fish were exposed to different concentrations of PME as 10%, 25% and 50%. Variation of body weight, survival rate and percentage of micronucleated PCEs were analyzed. One-way anova was performed and data were expressed as Mean \pm S.E. Consecutive dose dependent and time dependent increase of toxicity was recorded in PME compared to negative and positive control (Mitomycin C). Our study supported the carcinogenic and chromosomal damage induced in aquatic organisms specially in fishes due to direct exposure of industrial discharges; also, the importance of MN test as an effective indicator for testing genotoxicity in fishes was confirmed..

KEY WORDS: MICRONUCLEUS, MITOMYCIN C, PAPER MILL EFFLUENTS.**INTRODUCTION**

Water pollution day by day has become a global matter of concern. Industrial, agricultural and domestic discharges find their ultimate recipient source as the water bodies leading to pollution in the aquatic environment, which in last few decades in researches have been reported to become a threat to aquatic ecosystem resulting in water quality damage and severe chromosomal abnormalities in aquatic organisms specially in fishes (Porto et al. 2005; Yadav et al. 2010). Thus, Safety of aquatic environment is a major task as water resources covers two third of the globe and living organisms fully depend on it for their livelihood including humans (Krishnamurthi et al. 2003). The country administration has been continuously looking after this serious issue of cleanliness and safety of all water bodies starting from river Ganga upto the smallest stream or pond on which living organisms depends for their livelihood. Because in researches it has come to notice that people are

not concerned for the cleanliness of such water bodies on which they actually depend (Ismail et al. 2014).

As a result, not only the water quality is degrading very fast but also the aquatic ecosystem is in danger including both its flora and fauna (Kohlpoth et al. 2009). Among all of the aquatic organisms, fish have been considered as an important species as it covers the majority of the aquatic fauna and it has been consumed by living organisms especially human beings for their survival (Minovski et al. 2019). So, to test the level of genotoxicity induced by industrial effluents in aquatic organisms, fish can accepted as a good test model. Simultaneously, fish have been successfully used in cytogenetic analysis, being easy to handle and adaptable with the laboratory environment, provides a relatively low-cost method (Hayashi et al. 1997; Singh et al. 2020).

Micronuclei (MN) were first described in the cytoplasm of erythrocytes more than a century ago and were called "fragment of nuclear material" by Howell or "intraglobular corpuscles" in the terminology of Jolly in the late 19th century. Micronuclei (MN) were first described in the cytoplasm of erythrocytes more than a century ago and were called "fragment of nuclear material" by Howell or "intraglobu-

Article Information:*Corresponding Author: jashodeb@gmail.com

Received 19/10/2021 Accepted after revision 28/12/2021

Published: 31st December 2021 Pp- 1764-1770

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.57>

laries corpuscles” in the terminology of Jolly in the late Micronuclei (MN) were first described in the cytoplasm of erythrocytes more than a century ago and were called “fragment of nuclear material” by Howell or “intraglobularies corpuscles” in the terminology of Jolly in the late Micronuclei (MN) were first described in the cytoplasm of erythrocytes more than a century ago and were called “fragment of nuclear material” by Howell or “intraglobularies corpuscles” in the terminology of Jolly in the late Micronuclei (MN) were first described in the cytoplasm of erythrocytes more than a century ago and were called “fragment of nuclear material” by Howell or “intraglobularies corpuscles” in the terminology of Jolly in the late Micronuclei (MN) were first described in the cytoplasm of erythrocytes a century ago and were called ‘ fragments of nuclear material’ by Howell or ‘ Intraglobularies corpuscles ‘ in the terminology of Jolly in the late 18th century and early 1900. Hematologists called these structures as ‘Howell Jolly Bodies’ (Kirsch –Volders et al., 2003)

Micronucleus assay has been successfully used as a mutagenic assay to test the induced genotoxicity in various organisms as fishes. Among the various mutagen tests preferred for bio-monitoring contaminated environments, micronuclei assay (MN) proved relatively simple, reliable and sensitive, and has been used to evaluate the effects of mutagen compounds induced by industrial pollution and domestic discharges in aquatic ecosystem (Al-Sabti and Metcalfe 1995). The use of fish erythrocytes to test the level of toxicity leads to quick results with less chances of suffering of the test organism selected (Minissi et al. 1995; Zúñiga-González et al. 2000; Venier and Zampieron 2005; Singh et al. 2020).

Micronuclei are cytoplasmic chromatin masses that look like small nuclei as a result of lesions at the chromosomes or DNA strains, or at the level of proteins directly involved in chromosome segregation; formation of MN originating from chromosome fragments or chromosome loss events requires a mitotic or meiotic division (Heddle et al. 1983). Micronucleus is composed either of small chromatin fragments which arise as a result of chromosome breaks after clastogenic action, or of whole chromosomes that do not migrate during anaphase as a result of aneuploidic affects (Cavas and Ergene Gozukara 2003). Our work was conducted on the only paper mill situated in Barak valley region, Assam from where industrial effluents after paper composition have been observed to get liberated in local river Barak without proper treatment. During exposure a corrosive smell was also found to get spread in the neighboring atmosphere which can be easily felt by any passer by fellow causing air pollution too. The exposure in river Barak was expected to cause harm not only to water body but also to aquatic life. Before exposure fish were brought to laboratory environment and acclimatized. Fish were divided into groups and exposed to three different concentrations of both Paper Mill Effluent (PME) after proper trial and observation was recorded for three consecutive days (Singh et al. 2020).

Micronuclei (MN) were first described in the cytoplasm of erythrocytes more than a century ago and were called

“fragment of nuclear material” by Howell or “intraglobularies corpuscles” in the terminology of Jolly in the late Besides the average length of the fish, body weight and survivality rate were measured in three replicates. Erythrocyte smears were obtained with heparinized syringes by puncturing the gills on previously washed microscopic slides. The fish remained unharmed and were soon returned to their natural habitat. The slides were air-dried for 24 h, fixed in a 70% methanol solution for 7 min and then dried a further 24 h. Shortly after, they were stained with Giemsa (4%) for 15 min. 3.000 intact erythrocytes were counted from each fish.

Only cells that were clearly visible and isolated under a Zeiss microscope with amplification of 1000 X, were counted. Cells with more than four micronuclei were discarded so as to exclude apoptotic phenomena. Nuclear abnormalities were manifest as changes in the normal elliptic shape of nuclei (Ferraro et al. 2004; Bolognesi et al. 2006). For a detailed description on nuclear abnormalities see previous studies (Ayllón and Garcia-Vazquez 2000; Çavas and Ergene-Gözükara 2003; Çavas et al. 2005). Micronuclei were considered as small inclusions of nuclear material inside erythrocytic cytoplasm. Criteria for identification were a round or oval shape with a flat and well-defined outline, coloration similar to that of the main nucleus and a size from 1/3 to 1/20 in relation to that of the main nucleus (Al-Sabti and Metcalfe 1995; Singh et al. 2020).

MATERIAL AND METHODS

For the sampling site and preparation of water samples, major objective of this current piece of study was to evaluate the acute toxicity of a paper mill effluent of Barak valley region, Assam on the behavioral responses of a regularly consumed freshwater fish *Channa punctatus*. Raw effluent sample was collected from the outlet discharge pipes in the local river Barak in plastic containers. pH was measured on the very collection spot. Effluent sample was brought to laboratory and to prevent further microbial growth was stored at -20°C. For test model, among various water borne organisms fishes have been considered to be efficient model for studying the induced level of carcinogenesis by industries and domestic affairs. Researches have reported remarkable dose and time dependent increase in the induction of micronucleus in peripheral blood of fishes (Chaudhury et al. 2006; Ali et al. 2009; Nwani et al. 2010; Saleh and Alshehri; 2011; Pandey et al. 2014). Around 200 fishes of species *Channa punctatus* were used for the selected bioassays. Fish weighing 15-20 g of weight were selected and before exposure were acclimatized in the laboratory environment for 2-3 weeks. This study was conducted with prior Institutional animal ethics clearance. Fish were given proper treatment and were fed with standard fish food.

For animal exposure and micronucleus assay, after acclimatization fishes were divided into five groups, each group including 40 fishes per aquaria were exposed to three selected concentrations (10%, .25% and 50%, v/v, effluent/distilled water) of the paper mill effluent against negative (distilled water) and positive (Mitomycin C, 2mg/

lit) controls for 24, 48 and 72 hrs. 5 fishes per group of treatment were selected for micronucleus assay and blood erythrocyte smears were obtained. Three slides per sample were prepared. 3000 erythrocytes were counted and scored by a single scorer to eliminate inter-observer variation Using a light-microscope (Leica DMLS) at 1000 magnification where criteria for identification were a round or oval shape with a flat and well-defined outline, coloration similar to that of the main nucleus and a size from 1/3 to 1/20 in relation to that of the main nucleus (Al-Sabti and Metcalfe 1995).

For scoring criteria for micronuclei, following criteria for identification were used (Al-Sabti and Metcalfe 1995):-

- (a) MN must be smaller than one third of the main nuclei,
- (b) MN must be clearly separated from the main nuclei,
- (c) MN must be on the same plane of focus and have same color as of the original nuclei.

For statistical analysis, the data obtained were presented as Mean \pm SE. Data was analyzed using One Way ANOVA and were expressed as percentage frequency for MN test. Significance at different dose levels Were studied by using Graph Pad Prism Software (Graph Pad Inc., san Diego, CA, USA).

RESULTS AND DISCUSSION

Industrial discharges are recognized as one of the major resources of toxic chemicals in the environment. In the present study, the mutagenic potentiality of an effluent from a paper mill industry located in the Barak valley region of Assam, India was assessed by using an in vivo assay in fish system.

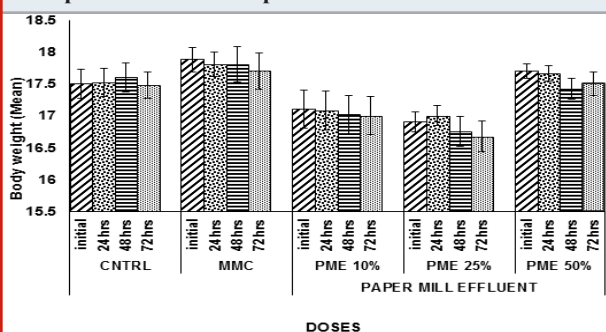
Body weight and Survival rate variation in exposed groups: In current investigation, variation in body weight was measured in fishes exposed to different concentrations of PME. Minute variation in body weight of fishes were recorded in exposed groups compared to negative and positive control. Whereas Remarkable mortality rate was recorded in exposed sets where dose dependent and time dependent death was noted.

Micronuclei Formation: Micronuclei are chromatin masses in the form of small nuclei which appear within the cytoplasm and close to the main nucleus in inter-phase cells. They are originated spontaneously or as consequence of clastogenic and/or aneugenic effects, which generate acentric chromosomal fragments and/or lagging chromosomes during the mitotic anaphase (Fenech 2003). Due the nucleated nature of erythrocytes in fish, the practicality of MN test has gain high relevance in bio-monitoring of aquatic environments, also including assessment of water quality (Xing et al. 2012; Chen et al. 2016).

Table 1. Body weight variation in *Channa punctatus* due to Paper Mill Effluent exposure.

Dose (mg/kg)	Concentrations	Initial	24 hrs	48 hrs	72 hrs
Control	-----	17.5 \pm 0.22	17.52 \pm 0.22	17.6 \pm 0.23	17.48 \pm 0.21
Positive control	2mg/lit	17.88 \pm 0.19	17.8 \pm 0.19	17.8 \pm 0.28	17.7 \pm 0.28
Paper Mill	10%	17.1 \pm 0.31	17.08 \pm 0.30	17.02 \pm 0.30	17.0 \pm 0.31
Effluent (PME)	25%	16.90 \pm 0.16	17.0 \pm 0.16	16.75 \pm 0.23	16.67 \pm 0.24
	50%	17.7 \pm 0.11	17.66 \pm 0.12	17.42 \pm 0.16	17.50 \pm 0.18

Figure 1: Body weight Variation in *Channa punctatus* due to Paper Mill Effluent exposure.



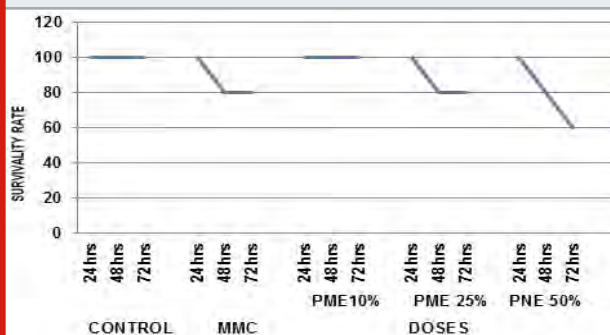
Originally the micronucleus test was developed for application in mammals, it was subsequently modified and used in fish (Schmid 1976; Houk 2007). Later, the analysis of MN was increasingly used for assessing environmental genotoxicity in fish (Al-Shabti and Metcalfe 1995; Ayllon and Garcia-Vazquez 2000; Jha 2000; Oost 2003). The

micronuclei assay is one of the best biomarkers that clearly correlate with pollution load, as it has been shown in a number of studies (Belfiore et al. 2001; Kirch and Sofuni 2003; Cavas and Ergene-Gozukara 2005; Singh et al. 2020).

Several studies throughout years have demonstrated increases in micronuclei frequency in species of marine fish in polluted areas, and their use as genotoxicity markers in accordance with the present study, and also in the laboratory (Al-Sabti and Metcalfe 1995; Hayashi et al. 1998; Ateeq et al. 2002; Teles et al. 2003; Buschini et al. 2004; Cavas et al. 2005). Many researchers have demonstrated the success of using micronucleus test in the evaluation of environmental quality, by using several freshwater fish (Hayashi et al. 1998). Group of researchers (Lopes-Poleza 2004) evaluated the genotoxic effect methylmercury (CH_3Hg^+) in *Hoplias malabaricus*, using chromosomal aberrations (anterior kidney), micronuclei, and DNA damage by the comet assay in erythrocytes (Claxton et al. 2008; Singh et al. 2020).

Table 2. Survivability rate of *Channa punctatus* exposed to Different concentrations of Paper Mill Effluent.

Treatment /DOSE	No. of Fish per aquarium	Concentrations	Survivability (in %)		
			24 hrs	48 hrs	72 hrs
Control	50	-	100	100	100
MMC	50	2mg/L	100	80	80
Paper Mill Effluent	50	PME 10%	100	100	100
	50	PME 25%	100	80	80
	50	PME 50%	100	80	60

Figure 2: Survivability rate of *Channa punctatus* exposed to different concentrations of Paper Mill Effluent.

Just like previous, our study also confirmed the usefulness of the erythrocyte micronucleus as a powerful monitoring tool for detecting genotoxic agents in a coastal environment (Nunes et al. 2015; Singh et al. 2019). Significant increase in the frequency of micronucleated erythrocytes has been observed after exposing *Channa* sp. to various concentrations of industrial effluents. Industrial effluents have already been reported to have genotoxic effects on fishes which arise in the form of micronucleus (Serrano and Montero 2001; Araújo et al. 2006). Present investigation confirmed the importance of erythrocyte micronucleus assay as an effective tool for detecting genotoxic agents.

Table 3. Incidence of micronucleated PCEs in *Channa punctatus* induced by Paper Mill Effluent.

Dose (mg/kg)	Concentrations	Exposure Timing (In hrs)	Total PCE/n	%PCEs With MN (mean ±S.E)
Control	--	24 hrs	5001/5	0.01±0.004
		48 hrs	5002/5	0.03±0.01*
		72 hrs	4017/4	0.04±0.01*
MMC	2mg/L	24 hrs	5020/5	0.07±0.01
		48 hrs	4017/4	0.17±0.02*
		72 hrs	4018/4	0.29±0.01
Paper Mill Effluent	PME 10%	24 hrs	5019/5	0.07±0.01
		48 hrs	5022/5	0.19±0.01*
		72 hrs	5025/5	0.23±0.03
	PME 25%	24 hrs	5021/5	0.27±0.01*
		48 hrs	4017/4	0.35±0.04*
		72 hrs	4011/4	0.37±0.02***, **, *
	PME 50%	24 hrs	5022/5	0.35±0.01***, **, *
		48 hrs	4015/4	0.71±0.03*
		72 hrs	3011/3	0.79±0.02***

When compared PME with Control
 $P < 0.05 = *$, $P < 0.01 = **$, $P < 0.001 = ***$

In present investigation, selected paper mill effluent has been observed to induce MN in the blood erythrocytes of fish. The frequency of micronuclei was significantly higher than in the negative control and Mitomycin C.

There was a significant difference between frequency of micronuclei among the negative and positive control. PME lead to the formation of micronucleus were compared to 10% concentration, 25% and 50% concentration showed

more micronucleus formation in both PME and DS (Singh et al. 2019).

Figure 3: Incidence of micronucleated PCEs in *Channa punctatus* induced by Paper Mill Effluent

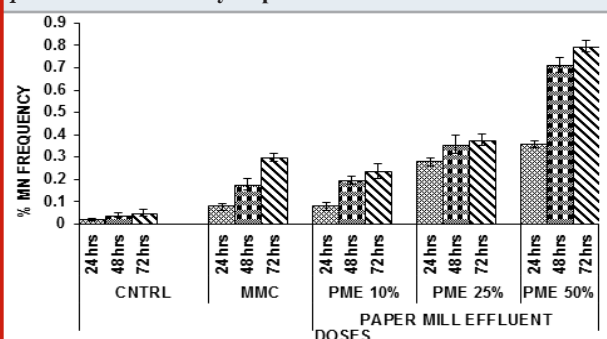
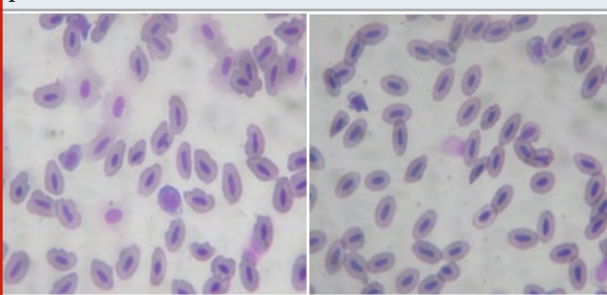


Figure 4: Pictorial depiction of Micronucleus in *Channa punctatus*



Frequency of micronucleated PCEs increased from 24hrs to 48hrs and from 48hrs to 72hrs of time interval. ($p < 0.05$, $p < 0.001$) designating the time dependent increase of genotoxicity. With time the chances of toxicity increase leading to any kind of chromosomal abnormality which may ultimately lead to death of the aquatic organisms in long run. Same happened in our case too (Singh et al. 2020).

CONCLUSION

The findings of the present study suggests that the risk factor graph of pollution of water bodies due to direct industrial exposure is consistently affecting the aquatic ecosystem and human life. Based on previous research reports, current research was conducted using Micronucleus assay as an effective and verified model to monitor the level of induced toxicity which was found up to the mark, as it came out with remarkable results. Cachar paper mill effluent has been visualized to have genotoxic potentials via the tested toxicity parameter as Micronucleus assay. Thus, we hope that we will try to get better details about the mutagenicity caused by applying other parameters and our piece of work will motivate other researchers to work on this issue from other aspects which will attract the attention of government for protection of such water resources from water pollution and make public aware about it, so that such exposures can be prevented and measures will be undertaken to aware people against direct use of untreated water from these polluted water bodies.

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Agricultural Communication

Promising Directions in Strategic Planning of Sustainable Development of Agricultural Territories

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ABSTRACT

Ensuring a stable improvement in the quality and standard of living of the rural population allows preserving the social and economic potential of rural areas. Effective management can ensure the implementation of social control and development of rural areas, the preservation of ecology, which ultimately contributes to the preservation of sustainable development of agricultural territories. The purpose of the study: To determine the directions and effective level of increasing the production of agricultural and livestock products aimed at preserving the sustainable development of agricultural territories. A correlation and regression analysis was carried out in the study to determine the promising directions of the northern districts of the Omsk region (Russia) to increase the effectiveness of strategic planning for the sustainable development of agricultural territories. Sustainable development of the regions, according to the authors, is impossible without increasing the level of well-being, therefore, the authors identified such areas for development as the cultivation of fodder crops, the cultivation of livestock and poultry (for slaughter), the cultivation of poultry for meat and the production of honey. The authors concluded that it is necessary to increase the production volumes of the selected promising areas for the development of agriculture by at least 5% to 13% per year, depending on the direction. There will be an increase in profit only at these values, which is one of the important factors contributing to the sustainable development of the areas allocated for research.

KEY WORDS: AGRICULTURE, CORRELATION, ECOLOGY, REGRESSION ANALYSIS.

INTRODUCTION

The strategic planning tool is the process of collecting information and processing it using various scientific methods to solve analytical problems in the field of strategic management. Since each region has its peculiarities of development, it is necessary to select the most universal tools for strategic planning of the region. It is necessary to identify tools for the development and implementation of regional policy in a fairly large array of tools that are applicable in the framework of strategic planning. The relevance of scientific research is due to the need to develop software tools for strategic planning of the development of agricultural territories. The lack of tools reduces the level of effective management of agricultural territories. In particular, in situations where prompt decision-making on the development of agricultural territories is required to increase their level of sustainable development, solve human welfare problems, preserve the biological environment and reduce

the possibility of environmental threats (Dvoichenkova et al. 2021).

To date, there are many traditionally used tools in the system of strategic planning of socio-economic development of territories, such tools include SWOT analysis, PEST analysis, M. Porter's five forces analysis, BCG matrix, methods of system analysis, etc. (Kopylova 2019). In our opinion, not all the proposed tools can have a proper impact on the socio-economic development of territories in modern conditions despite a considerable number of scientific publications on the strategic planning of territories (Yatsenko et al. 2020; Germanovich et al. 2020). The main purpose of the article is to use correlation and regression analysis as a tool for determining promising directions in strategic planning of sustainable development of agricultural territories.

MATERIAL AND METHODS

We proposed the use of the method of correlation and regression analysis as a tool for determining promising areas in the strategic planning of territories. Correlation analysis and regression analysis were used to study the statistical dependence of several random variables based on sample data.

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Received 13/09/2021 Accepted after revision 25/11/2021

Published: 31st December 2021 Pp- 1771-1776

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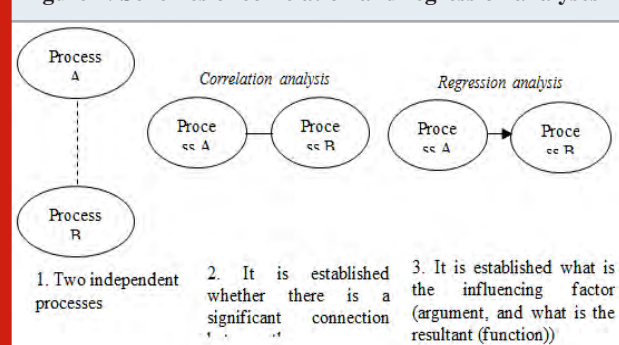
Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.58>

In strategic planning, these methods were used at the stage of developing forecasts of socio-economic development. Correlation analysis allowed inferring the strength of the relationship between data pairs x and y and uses regression analysis to predict one variable (y) based on another variable (x). In other words, in this case, it was possible to identify a causal relationship between the analyzed aggregates (Tereshchenko et al. 2012). Regression analysis distributes the roles between the studied characteristics – the first of them was an argument, and the second is a function. The predicted variable (function) is denoted as y , and the variable used for such prediction (argument or factor) as x .

The characteristic of the constraint force based on the pair correlation coefficient shows that there is no constraint at a coefficient of up to 0.3, the constraint was weak from 0.3 to 0.5, noticeable from 0.5 to 0.7, the constraint is strong from 0.7 to 0.9, and very strong from 0.9 to 0.99 (Baraz 2005). In this study, the method of correlation and regression analysis was carried out concerning two northern districts (Bolsheukovsky and Bolsherechensky districts) of the Omsk region (Russia). The results obtained served as the basis for constructing a linear function, which makes it possible to determine promising directions for each municipality in the field of agriculture (Tereshchenko et al. 2012).

Figure 1: Schemes of correlation and regression analyses



RESULTS AND DISCUSSION

Figure 1 shows that in the first case two completely independent processes do not depend on each other and even more so do not affect each other. The process of correlation analysis was considered in the second case, which allows determining the significance of the interprocess communication. It was established in the third case of regression analysis what is the influencing factor (argument) and what is the resultant (function) (Germanovich et al. 2020). The results of the calculations performed on the example of two northern districts of the Omsk region are presented in Table 1, 2 and Figure 2. The results of the regression analysis of the indicators of the agricultural sector of the Bolsherechensky district are presented in Table 1.

The main indicators of the activity of agricultural enterprises in the northern districts of the Omsk region were laid down as the main indicators underlying the correlation and regression analysis, which are considered in the context of the crop production industry and the livestock industry. When conducting this analysis, it was also possible to determine the most promising industry for them not only in the context of municipalities but in the northern regions and the region as a whole. Based on the results obtained, the linear function will be as follows:

$$y = 46,527.5 - 7.5 * X_1 + 0.41 * X_2 + 20.05 * X_3 - 93.06 * X_4 + 128.50 * X_5 + 2,540.02 * X_6$$

The resulting indicator in the subsequent calculations is the profit indicator of agricultural enterprises in the region. The coefficient of determination R^2 was 0.57, which indicated the reliability of the results obtained since the value was higher than the standard 0.5. The results of the regression analysis of the indicators of the agricultural sector of the Bolsheukovsky district are presented in Table 2.

Table 1. Regression analysis results of Bolsherechensky district

Regression statistics							
Multiple R	0.759273						
R-square	0.576496						
Normalized R-square	-0.27051						
Standard error	13,173.79						
Observations	10						
Analysis of variance							
	df	SS	MS	F	F Significance		
Regression	6	708,730,503.6	118,121,750.6	0.680626	0.685675		
Balance	3	520,645,890.5	173,548,630.2				
Total	9	1,229,376,394					
	Coefficients	Standard error	t-statistics	P-value	Lower 95%	Upper 95%	Upper 95.0%
Y-intersection	46,527.5	242,648.6872	0.191748425	0.860184	-725,689	818,743.9	8,217,289
Technical crops, hectares	-7.50574	13.27819842	-0.565267783	0.611406	-49.7629	34.75142	465.8034
Cattle and poultry for slaughter (slaughter weight), tons	20.05001	50.7424937	0.395132437	0.719164	-141.435	181.5353	1,562.552
Cattle, tons	-93.0692	68.27955297	-1.36306089	0.266151	-310.365	124.2268	1,857.085
Poultry, tons	128.5035	156.167652	0.822856084	0.470901	-368.492	625.4987	3,024.157
Honey, tons	2,540.029	4,445.23048	0.571405547	0.607728	-11,606.7	16,686.74	93,773.51

Based on the results obtained, the linear regression analysis function of the Bolsheukovsky district will be as follows:

$$y = 6,329.22 - 0.79 * X_1 - 10,055.34 * X_2 + 18.16 * X_3$$

The coefficient of determination R^2 was 0.75, which also indicated the reliability of the results obtained since the value was higher than the standard 0.5. The results of the regression analysis and the linear functions obtained on its basis allowed determining the most promising areas for agricultural sectors in the context of each municipality. A

regression analysis method was applied to determine the promising areas of economic activity for the considered districts of the Omsk region, which allowed determining the most significant areas of activity of agricultural enterprises in these districts of the Omsk region, which affect the dependent variable – profit. At the initial stage, the necessary information was collected on the activities of agricultural enterprises in the areas under consideration (Germanovich et al. 2020). The "profit" indicator is used as a dependent variable (Y) – a variable describing the effectiveness of processes (Table 3).

Table 2. Results of regression analysis of Bolsheukovsky district

Regression statistics							
Multiple R	0.870236953						
R-square	0.757312355						
Normalized R-square	0.611699768						
Standard error	652.4313585						
Observations	9						
Analysis of variance							
	df	SS	MS	F	F Significance		
Regression	3	6,641,513	22,213,838	5.200,871,51	0.0537159		
Balance	5	2,128,333	425,666.7				
Total	8	8,769,846					
	Coefficients	Standard error	t-statistics	P-value	Lower 95%	Upper 95%	Upper 95.0%
Y-intersection	6,329.223301	1,820.703	3.476252	0.017,729,92	1,648.9561	11,009.49	1,648.956
Milk, tons	-0.793855302	0.367252	-2.16161	0.083,0178,7	-1.7379059	0.150195	-1.73791
Wool, tons	-1,055.342724	542.5966	-1.94499	0.190,937,015	-2,450.1316	339.4462	-2,450.13
Honey, tons	18.16580847	71.6226	0.253632	0.80,987,871	-165.94594	202.2776	-165.946

Table 3. Results of correlation analysis

	Bolsherechensky		Bolsheukovsky	
	Correlation coefficient	constraint	Correlation coefficient	constraint
Profit	1	-	1	-
Grain and leguminous crops, tons	0.191	Weak	-0.215	Weak
Technical crops, ha	0.044	Weak	-0.152	Weak
Fodder crops, ha	-0.358	average	0.168	Weak
Potatoes, t	0.285	Weak	-0.602	Average
Vegetable and berry crops, t	0.229	Weak	-0.356	Average
Cattle and poultry for slaughter, t	0.048	Weak	0.033	Weak
Cattle, tons	-0.202	Weak	0.049	Weak
Pigs, t	0.210	Weak	0.006	Weak
Sheep and goats, t	-0.340	average	-0.186	Weak
Poultry, t	0.157	Weak	0.249	Weak
Milk, t	0.128	Weak	-0.405	Average
Wool, t	-0.282	Weak	-0.233	Weak
Eggs, thousand pcs.	0.015	Weak	0.171	Weak
Honey, t	0.191	Weak	-0.232	Weak

As part of the identification of promising areas and new projects for the development of agriculture in the northern regions of the Omsk region, it was important to consider their effectiveness. One of the performance indicators

was profit, its growth. The cultivation of poultry for meat and the production of honey, cultivation of fodder crops, the cultivation of livestock and poultry (for slaughter) were identified in the Bolsherechensky district, based on

correlation analysis to assess the impact on profit. We presented the data of the calculation of profit growth as a result of the development of promising areas of agriculture in the Bolsherechensky district of the Omsk region. Figure 2 shows the dynamics of profit generation by agricultural organizations and farms of the Bolsherechensky district as a result of the development of promising areas (Germanovich et al. 2020).

Figure 2: Dynamics of profit generation by agricultural organizations and farms of Bolsherechensky district as a result of development

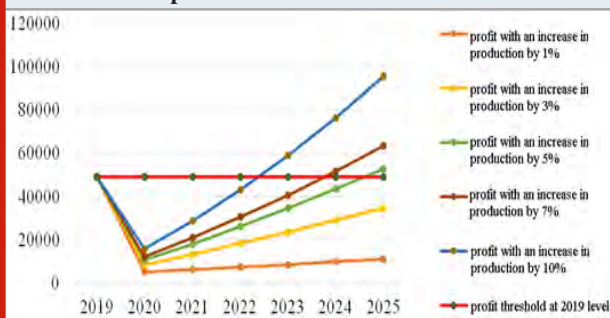
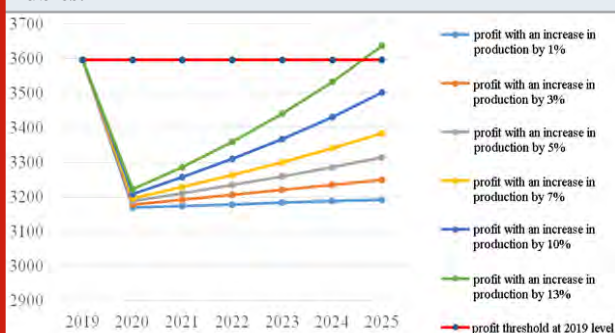


Figure 3: Dynamics of profit generation by agricultural organizations and farms of Bolsheukovsky district as a result of the development of promising areas, thousand rubles.



As a result of the analysis, it can be concluded that, in aggregate, the development of promising areas of agriculture allocated for the district was possible if funds were invested in increasing production in the amount of 7 percent or more. With an increase in production below the threshold value, which was determined by the level of 2019, it was possible not to get a positive result. There was no increase in profit and its increase to the level of 2019 until 2025 with an annual increase in production volumes by 1% and 3%. Thus, it was recommended to increase the production volumes of the selected promising areas of agricultural development by at least 5% per year. Otherwise, there was no return on the invested funds for the development of promising areas. Figure 3 shows the dynamics of profit generation by agricultural organizations and farms of the Bolsheukovsky district as a result of the development of such promising areas as honey production (Piltakyan and Mokrushin 2020).

There was no increase in profit and its increase to the level of 2019 until 2025 with an annual increase in production volumes by 1%, 3%, 5%, 7%, and 10%. Thus, it was recommended to increase the production volumes of the selected promising areas of agricultural development by at least 13% per year. Piltakyan and Mokrushin (2020) determine that the rational use of strategic planning tools in public administration would not only significantly increase the efficiency of using budget funds, but also the effectiveness of strategic planning subjects. Chupina, Zarubina and Simachkova (2020) defined the need to develop effective tools and mechanisms for managing the development of the region, and their implementation in the strategic planning system reflected in strategic documents at the regional level. Shpakova (2019) assessed the level of socio-economic development of the region in the context of the effectiveness of strategic planning and the quality of strategic documents. Thus, the author argued that for further development it is necessary to determine objective potential opportunities, main trends, risks, and, in general, the basis on which this development will be carried out (Shpakova 2019; Chupina, Zarubina and Simachkova 2020; Piltakyan and Mokrushin 2020).

The very assessment of the results achieved should answer the question about the effectiveness of the methods and tools that were used in the implementation of the previous strategic plan. We cannot but agree with Shpakova (2019) that such "work on mistakes" will allow choosing the right tools for the implementation of the new strategic plan. Valentik (2018) identified "points of growth" as the tools of strategic planning at the regional level (Valentik 2018; Shpakova 2019). The author argued that the theory and methodology of strategic planning developed based on the current Federal Law "On Strategic Planning in the Russian Federation" do not define clear contours of such a category as "points of growth". This category would make it possible to actively use the ways to boost the economy of the regions, which defined it as one of the main tasks of the spatial vector of strategic planning in the country (Valentik 2018). Suglobov et al. (2017) highlighted the need to ensure the economic security of the country's regions within the framework of planning the development of the territory. Therewith, it was necessary to use characteristic and accessible tools for this concerning a specific territory (Suglobov et al. 2017).

Rastvortseva (2010) used a multifactorial regression model in her research, which allowed determining the reasons for the deviation of the socio-economic efficiency of individual regions from the average level for the surveyed population (Rastvortseva 2010). Makarova (2019) in her research used the regression analysis method, which allowed determining priority strategic directions for improving the level of economic security of the region (Makarova 2019). The analysis of literary sources allows talking about a broad consideration of strategic planning tools in economics, but despite this, the consideration of correlation and regression analysis as a tool for determining promising directions in strategic planning of territories was not considered in a direct aspect. It should also be noted that correlation

and regression analysis was not used in modern strategic documents at the regional level (Slepov et al. 2021).

As the theoretical and practical results of the study devoted to the problem of strategic planning of sustainable development of agricultural territories show, there was no common point of view among specialists in approaches to defining tools concerning the management of the region. Quite often we observed that the main approaches and techniques used in foreign practice in the formation of regional development strategies are copied without adaptation to the situation in Russia (Kiseleva et al. 2019). We believed that the use of strategic planning tools for regional development is conditioned by the need to analyze environmental factors and select strategic development goals. As a rule, regional development tools allow economic entities to fully explore the prospects for entering other regional markets, as well as assess the risks associated with choosing priorities for regional development and sustainable development of agricultural territories (Slepov et al. 2021).

In our opinion, the use of correlation and regression analysis as a tool for determining promising directions in strategic planning of the northern districts of the Omsk region can be the main one for operational support of the effectiveness of decision-making. Based on the data obtained as a result of the study, we identified areas that have the potential for development and, accordingly, improvement of the socio-economic situation in the region as a result of taking into account the results obtained when developing strategic plans for the municipal district. Thus, the use of this tool would increase the level of sustainable development of agricultural territories of municipal districts in particular and the region as a whole.

The results of the study presented by us show that the scientific determination of promising areas of agriculture, as well as the determination of thresholds for increasing production volumes, will allow obtaining a positive result in the socio-economic sustainable development of the region. We believed that the method of correlation and regression analysis can be used in the development of strategic documents, both at the level of municipalities and at the level of the region (Slepov et al. 2021).

CONCLUSION

The findings of the present study conclude that with the use of correlation and regression analysis tools, it was possible to determine their effectiveness based on calculations carried out to determine the most promising directions in the agricultural sector for the northern regions of the Omsk region. The proposed approach to the strategic planning of the region will allow approaching the identification of promising and priority areas objectively since the analysis of socio-economic development strategies developed in the regions currently does not allow determining the degree of application of various tools in the process of developing strategies.

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Toxicological Communication

Human Health Risk Surveillance of Organochlorine Pesticides in River Water and Fishes from Bangladesh

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ABSTRACT

Organochlorine pesticides (OCPs) are chlorinated hydrocarbons that used extensively in the last century for agricultural purposes. Excess use of OCPs results pesticide residues accumulation in the water and fishes and causes various health problems. The concentrations of Organochlorine pesticides (OCPs) residues in water and fish samples of six most contaminated rivers surrounding Dhaka, Bangladesh namely Buriganga, Turag, Balu, Sitalakhya, Bangshai and Dhaleswari were determined and assessed the possible health risks through consumption of accumulated OCPs in fishes from those rivers. As we know, OCPs are non-biodegradable and thus remain in the environment as pollutants. Therefore, OCPs exposure must be monitored and controlled to reduce the human health risks. Risk assessment is an important procedure to quantify the potential health risks and provides information the risk managers to control the overuse of OCPs. The concentrations of OCPs residues were determined by Gas Chromatography tandem Mass Spectrometry (GC-MS/MS). After sample collection, sample was extracted and analyzed according to the validated method. Several OCPs residues including Aldrin, p,p-DDE, Endrin ketone, p,p-DDD, Endrin, α -Endosulfan, Heptachlorepoxide, p,p-DDT, Endosulfan sulfate and β -Endosulfan were detected in river water and residues including Aldrin, p,p-DDE, Heptachlorepoxide, p,p-DDD, Endrin and p,p-DDT were detected in fish samples. The Health risk index (HI) values of Aldrin, p,p-DDE, Heptachlorepoxide, p,p-DDD, Endrin and p,p-DDT from analyzed three fish species (*Acanthobrama microlepis*, *Barbonymus gonionotus* and *Batasio tengana*) were ranged from 0.036 to 1.696, 0.029 to 2.007, 0.022 to 1.117, 0.0231 to 0.721, 0.019 to 1.597 and 0.019 to 1.205 for Buriganga, Turag, Balu, Sitalakhya, Bangshai and Dhaleswari rivers respectively. OCPs are quantified in river water and fish samples and potential health risks are assessed. This study suggested that, there might have OCPs mediated health risks through long term exposure of OCPs residues from fishes of those polluted rivers. Although, our study provided estimation about the presence of OCPs in water and fishes from six contaminated rivers surrounding Dhaka city but, further studies are suggested to ensure the safety of peoples.

KEY WORDS: BANGLADESH, FISH, GAS CHROMATOGRAPHY, MASS SPECTROMETRY, OCPs.

INTRODUCTION

Organochlorine pesticides (OCPs) are synthetic chemicals with a strong chemical bond between their chlorine and carbon components. OCPs are toxic to the environment, insoluble in water and fat loving pesticides. OCPs are very dangerous for the environment because, they stay around for a long time after using and may exposure to water, soil and animal bodies. OCPs can contaminate the environment either through direct application or waste disposal and rain water.

Although, OCPs are banned in several developed countries because of their long lasting nature but, still they are used in some of the developing countries. Peoples especially from developing countries where OCPs are used regularly are at a higher risk of OCPs mediated health hazards. Peoples can directly uptake the OCPs residues through breathing and ingest OCPs by taking foods like fish, dairy products and other highly fat containing foods (Lee et al. 2020).

As OCPs maintains very strong bonding in the fatty parts so, they can accumulate in the animal or fish tissues and can pass to the human and other animals after consumption of those animals. When human takes OCPs residues through food for long time, they may suffer from serious health problems such as damage of liver, kidney, thyroid gland, bladder and

Article Information:*Corresponding Author: pd-cbirmdp@bcsir.gov.bd

Received 15/10/2021 Accepted after revision 11/12/2021

Published: 31st December 2021 Pp- 1777-1785

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.59>

central nervous system. In agricultural countries, pesticides are used to control of insects, weeds, fungi, bacteria, etc. OCPs are lipophilic in nature with longer half-life. The bioaccumulation and long range transportation ability of OCPs, makes them ideal candidate for contamination of water, air and soil. There has harmful impact of pesticides in aquatic eco systems (De Lorenzo et al. 2001; Frankart et al. 2003; Gupta 2004; Castillo et al. 2006; Aktar et al. 2009). Although, OCPs are used to prevent malaria and typhus but they are banned in the developed countries because of their harmful effects (Aktar et al. 2009). Several pesticides such as DDT, hexachlorocyclohexane (HCH) and aldrin used in some part of Asia due to cheaper price and activity against some harmful organisms. There have several reports on OCPs mediated toxicity in several fish models (Gupta 2004; Yohannes et al. 2014a; Yohannes et al. 2014b; Zhang et al. 2014; Byrne et al. 2015; Cui et al. 2015; Wang et al. 2015; Lee et al. 2020).

Endosulfan was found to responsible for necrosis of focal liver cells (Capkin et al. 2006). Endosulfan sulphate is a toxic OCPs residue. The toxicity was studied in Zebra fish (Lee et al. 2020). Fishes accumulate and concentrate these hydrophobic compounds in their tissues directly from water and the pesticide residues are transferred to the fish consumers directly by trophic food systems. Humans can be affected by OCPs after long time exposure. The poisoning of OCPs may cause headache, nausea, dizziness, vomiting, tremor, lack of co-ordination and mental confusion. The use of several OCPs can cause cancer. DDT is reported as a potential cancer-causing agent in humans according to several researchers (Enan and Matsumura 1998; Diel et al. 2002; Han et al. 2010; Ndebele et al. 2010). Reduction of sperm count and damage of spermatogonial cells and sperm morphology can be occurred because of high endosulfan dose (Ennaceur et al. 2008; Wong et al. 2015).

Higher concentrations of DDE and HCB reduced cell proliferation and produced binucleated cells (Ennaceur et al. 2008; Wong et al. 2015). Bangladesh is called land of rivers. Rivers are very important part of Bangladesh. The rivers provide water for cultivation in the vast majority of areas. Rivers are used for transportation in some areas of Bangladesh. There are about 230 rivers currently flowing throughout Bangladesh during summer and winter seasons. The major river includes Padma, Meghna, Jamuna, Brahmaputra, Karnaphuli etc. All of the rivers are connected to each other and finally falls to the Bay of Bengal. Dhaka, the capital of Bangladesh is surrounded with some rivers. The rivers are: Buriganga River, Turag River, Balu River, Shitalakhya River, Dhaleshwari River and Bangshai River. They supply water to the huge population of Dhaka, Bangladesh. The present condition of these rivers is at very high risk (Li et al. 2020).

The water quality of these six major rivers around Dhaka city is getting polluted day by day and becoming very dangerous for public health. Water from these rivers cannot be used as drinking water. The deteriorating water quality of these six rivers has been a major concern. Rivers are main sources of fish. Bangladeshi peoples prefer fishes on their dishes in almost every day. Fish is one of the importance

sources of protein, vitamin, fat and carbohydrate. Peoples from rural areas get their required protein through consumption of fishes. Fish is considered as one of the most significant indicators of metal pollution in aquatic environment (Rashed 2001). QuEChERS extraction method in combination with GC-MS was developed for the detection of OCPs in shellfish. OCPs analysis method was validated for shellfish and cephalopods (Li et al. 2020; Hwang et al. 2020). A method was developed for the detection of OCPs in potato and sweet potato. A QuEChERS Method with slight modification using N-Doped Graphitized Carbon coupled with GC-MS/MS has been developed for determination of OCPs in tomatoes (Ye et al. 2020; Singh et al. 2020).

In this study, we detected several OCPs residues from water and fish samples from six contaminated rivers (Buriganga River, Turag River, Balu River, Shitalakhya River, Bangshi River and Dhaleshwari River) surrounding Dhaka, the capital of Bangladesh and accessed potential health hazards of peoples who consumed the fishes from those rivers. The limit of detection (LOD) values were appropriate for the detection of OCP residues from both water and fish samples. Considering the problems in pesticide residues detection, this study conducted on quantitative detection of OCPs by GC-MS/MS. Although, LC-MS/MS is preferred method for the detection of OCPs but, GC-MS/MS is more sensitive method (Pico et al. 2020). Excess use of OCPs in agriculture results pesticide residues accumulation in the water and fishes which in turn causes various health problems. This research will helpful for the assessment of the impact of OCPs on public health, agriculture and environment in Dhaka, Bangladesh.

MATERIAL AND METHODS

In this study, both fish and water samples were collected from 6 (Six) most polluted rivers of Bangladesh namely Buriganga, Turag, Balu, Sitalakhya, Bangshai and Dhaleswari. These Rivers are very important as they surrounds Dhaka city. For the sample collection, several sampling points were selected. This study was carried out from November, 2020 to April, 2021. Water samples were collected from the 30-50 cm depth of water surface. Fish samples were collected where the fishing effort is very high. Three fish species from those rivers were collected for this assay: *Acanthobrama microlepis*, *Barbonymus gonionotus* and *Batasio tengana*. Fishes were purchased from the local fishermen. While sampling, fishes of almost same size and weight were selected. Fish samples were stored in polythene bags and transported to the laboratory. For the analysis, fish muscle tissues were selected. A total of 30 water samples and 60 fish samples of three species were collected randomly. After collection, samples were stored in proper condition to maintain the sample quality. OCPs were extracted from river water samples using liquid-liquid extraction (LLE) method (APHA 2002).

OCPs were extracted from river water samples using liquid-liquid extraction (LLE) method (APHA 2002). For the extraction of OCPs from fish samples, 2g of fish muscle samples were dissected. The muscle sample was grounded to fine powder using 5 g of Sodium sulfate. After extraction

with 50 ml of Acetone (2 times), the extracts were filtered and filtrates were again extracted by 350 ml of deionized water, 15 g of NaCl and 40 ml of n-hexane/ethyl acetate (3:2). After organic layer separation, the samples were again extracted using 40 ml of n-hexane / ethyl acetate (3:2) and again the organic layer was collected. The extracts were then passed through the anhydrous sodium sulfate, concentrated and added 20 ml of n-hexane. Finally, the extract was condensed to 1 ml in rotary evaporator (Model: RE100-Pro, DLAB, USA) for GC-MS/MS analysis. In this study, GC-MS system (Model: TRACE 1310, Thermo Fisher Scientific, USA) equipped with Thermo Scientific™ Trace GOLD™ TG-5MS GC Column (0.25 mm X 0.25 µm X 0.25 m) was used. Helium was the carrier gas with a flow rate of 1.2 ml/min. Injection port temperature was 230°C and temperature for GC was from 80°C to 290°C. 2 µL of sample was injected for analysis. Mass Spectrometer (Model: TSQ DUO, Thermo Scientific, USA) was used for MS analysis.

A stock solution of a mixture of 19 pesticides was used as standard solution. Several dilutions were prepared from Standard solution with a range of 5 ppb to 200 ppb. All of those dilutions were injected and analyzed. Blank solutions method was used to determine LOD and LOQ. Signal and noise ratio 3:1 was considered for LOD determination and 10 times of baseline value of blank samples was considered for LOQ detection. The accuracy of the method was validated by recovery performance evaluation. For the recovery performance evaluation, both river water and fish samples were spiked with two known concentrations: 5 µg / L and 10 µg/L for river water and 5 µg / kg and 10 µg/ Kg for fish samples. Then, the samples were extracted and analyzed with the following equation: $P_i = (S_i/T_i) \times 100$ (1); Where, P_i , S_i and T_i stands for recovery percentage; results of control from laboratory and percent recovery of the spiked samples respectively.

Table 1. Data obtained from the Chromatography of multi-standards of 19 OCPs. Pesticides, their retention times, limit of detection (LOD) and Limit of quantification (LOQ).

OCPs	Retention time (Min) RT	Water	Fish		
		LOQ	LOD	LOQ	LOD
		µg/L	µg/ kg		
Aldrin	20.39	0.042	0.012	0.039	0.013
p,p-DDE	24.51	0.067	0.023	0.056	0.029
Eldrine ketone	28.96	0.092	0.032	0.088	0.037
p,p-DDD	25.8	0.032	0.021	0.043	0.038
α-Endosulfan	23.47	0.076	0.023	0.067	0.032
Cis-Chordane	23.61	0.066	0.019	0.063	0.022
Heptachlorepoxide	22.05	0.054	0.028	0.048	0.025
Heptachlor	18.94	0.087	0.026	0.078	0.034
p,p-DDT	27.2	0.053	0.013	0.064	0.039
Methoxychlor	26.92	0.064	0.012	0.048	0.028
Endrin	25.31	0.067	0.018	0.087	0.021
Endosulfan sulfate	27.28	0.054	0.024	0.059	0.036
α-Endosulfan	23.47	0.069	0.021	0.077	0.029
β-Endosulfan	25.67	0.042	0.019	0.054	0.022
Lindane-I	14.23	0.067	0.014	0.062	0.019
Lindane-II	15.48	0.054	0.031	0.048	0.027
Lindane -III	15.76	0.043	0.023	0.038	0.034
Lindane-IV	16.85	0.073	0.038	0.065	0.031
Trans-Chordane	23	0.064	0.027	0.059	0.018
Phthalic Acid	30.54	0.076	0.032	0.066	0.029

The estimated daily intake (EDI) of OCPs is based on the OCPs concentration in the fish and the amount of fish consumed. EDI was calculated by the following equation: $EDI = (C_{OCP \text{ residues}} \times D_{\text{fish intake}}) / B_{\text{Average weight}}$ (2); Here, C_{OCPs} (mg/kg), $D_{\text{fish intake}}$ (kg / person) and B average weight (kg / person) are the OCPs residual concentrations in fish, daily intake of fish and average body weight, respectively. HI is defined as health risk assessment of consumers from the consumption of fishes accumulated OCPs in their tissue.

HI is calculated from estimated daily intake (EDI) and FAO/WHO prescribed acceptable daily intake (ADI) of OCPs using the following formula:

$$HI = EDI/ADI \dots\dots\dots(3);$$

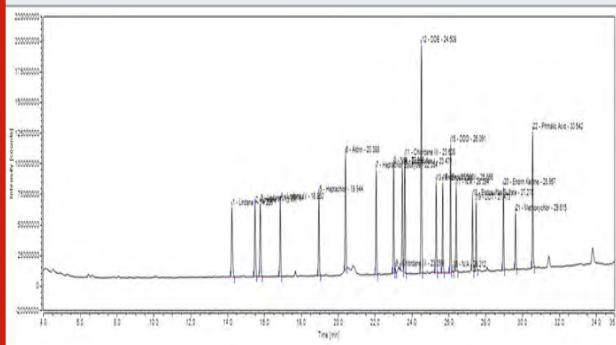
When, calculated HI value is less than 1, the consumers are considered safe from any OCPs mediated health risks (USEP 1986). But, if HI value is higher than 1 then it can be

indicated as potential health hazardous. For data collection, organization and summary preparation, Microsoft Office Excel 2007 and for statistical calculation Statistics 10 software was used.

RESULTS AND DISCUSSION

Method validation: The detection method was validated through recovery evaluation. The spiked river water and fish tissue samples were compared with that of blank samples. The percent recoveries of river water and fish samples were ranged from 87.5% to 99.4% and 87.0% to 100.4% respectively. A linear range was observed from the dilutions of standard solution. The coefficient values (R^2) value from this study was 0.95 to 0.99. The chromatogram showing the corresponding peaks of OCPs in standard solution is presented in Figure 1. The detected LOD values were ranged from 0.012 to 0.032 $\mu\text{g/L}$ and 0.013 to 0.39 $\mu\text{g/kg}$ for river water and fish tissue respectively. Obtained LOQ values were ranged from 0.42 to 0.87 and 0.39 to 0.88 $\mu\text{g/kg}$ for river water and fish tissue respectively. The retention time, LOD and LOQ values of each pesticide are presented in Table 1.

Figure 1: Chromatogram showing peaks of Organochlorine pesticide standards. A total of 19 pesticides were analyzed with different retention times (RT) for each pesticides.



Analysis of samples: The validated method was applied to analyze the water and fish samples from Buriganga, Turag, Balu, Sitalkha, Bongshai and Dhaleswari rivers. All the detected OCPs values in water and fish samples are presented in Table 2 and 3 respectively. Several OCPs including Aldrin (0.834 ± 0.056), p,p-DDE (1.782 ± 0.021), Eldrine ketone (0.273 ± 0.032), p,p-DDD (1.421 ± 0.098), Endrin (1.12 ± 0.068), α -Endosulfan (0.123 ± 0.021), Heptachlorepoxide (0.321 ± 0.082), p,p-DDT, (1.243 ± 0.043), Endosulfan sulfate (0.023 ± 0.078) and β -Endosulfan (0.242 ± 0.098) were detected in Buriganga river water. In Turag river water, OCPs such as Aldrin (0.453 ± 0.076), p,p-DDE (1.267 ± 0.098), Eldrine ketone (0.123 ± 0.072), p,p-DDD (1.236 ± 0.054), Endrin (1.09 ± 0.032), Heptachlorepoxide (0.231 ± 0.067), p,p-DDT (1.076 ± 0.021) and β -Endosulfan (0.175 ± 0.078) were detected.

OCPs such as Aldrin (0.235 ± 0.089), p,p-DDE (1.0231 ± 0.078), Eldrine ketone (0.032 ± 0.092), p,p-DDD (1.007 ± 0.033),

Endrin (0.989 ± 0.034), α -Endosulfan (0.078 ± 0.032), Heptachlorepoxide (0.165 ± 0.082), p,p-DDT (1.243 ± 0.078) and β -Endosulfan (0.08 ± 0.023) were detected in Balu river water. In Sitalkha river water, Aldrin (0.482 ± 0.072), p,p-DDE (0.882 ± 0.065), Eldrine ketone (0.098 ± 0.087), p,p-DDD (0.856 ± 0.075), Endrin (0.776 ± 0.071), α -Endosulfan (0.0945 ± 0.023), Heptachlorepoxide (0.220 ± 0.065) and p,p-DDT (0.605 ± 0.066) were detected. Bangshai river water was contaminated with OCPs such as Aldrin (0.743 ± 0.043), p,p-DDE (0.899 ± 0.034), Eldrine ketone (0.167 ± 0.043), p,p-DDD (0.994 ± 0.033), Endrin (0.907 ± 0.072), α -Endosulfan (0.098 ± 0.032), Heptachlorepoxide (0.182 ± 0.098) and p,p-DDT (0.882 ± 0.023) while Dhaleswari river water was contaminated with OCPs such as Aldrin (0.241 ± 0.053), p,p-DDE (0.832 ± 0.045), Eldrine ketone (0.0743 ± 0.032), Endrin (0.997 ± 0.0632), α -Endosulfan (0.0743 ± 0.021), Heptachlorepoxide (0.098 ± 0.034), p,p-DDT (0.993 ± 0.082).

The ecosystem has been polluted because of use of the OCPs. In African environments Several OCPs including α -HCH, β -HCH dichlorodiphenyltrichloroethane (DDTs), and endosulfans has been detected as most persistent residues (Olisah et al. 2020). Although, the application of OCPs has been reduced worldwide; a recent study in Turkey they have identified OCPs residues in the features of bird species which indicated that, OCPs residues are also present in several wild species (Arikan and Turan 2020). GC-MS has been applied to detect the OCPs residues in Chinese herbal extracts (Hwang and Lee 2000). Several studies have identified OCPs residues in several food items. In a previous study, Aldrin was detected in several milk samples in very low amount. OCPs were detected in Ginseng roots (Wu et al. 2020). In China, OCPs were detected in vegetable oils samples where higher OCPs concentration was detected in sesame oil and lowest concentration was detected in peanut oil (Cui et al. 2020). In Jordan, several OCPs were identified in honey samples (Tahboub et al. 2020; Lobato et al. 2021).

Health risks assessment through consumption of fishes from these rivers: The EDI (Effective daily intake) values analyzed from this study are presented in the Table 5. The HI (Health risk index) values of three fish species from these six rivers were ranged from 0.036 to 1.696, 0.029 to 2.007, 0.022 to 1.117, 0.0231 to 0.721, 0.019 to 1.597 and 0.019 to 1.205 for Buriganga, Turag, Balu, Sitalkha, Bangshai and Dhaleswari rivers respectively. The HI values are presented in Table 6. The HI values of Aldrin is higher than 1 in the fishes of Buriganga and Turag rivers. The HI values of Endrin were detected higher than 1 in the fishes of all of the analyzed rivers. The HI value of Heptachlorepoxide was detected higher than 1 in the fish samples of Buriganga River (Table 6). The analyzed rivers were contaminated with several harmful OCPs and the higher concentrations of OCPs were detected in Buriganga river water while Dhaleswari River was detected with lower OCPs residues. The fish samples from all of those analyzed rivers accumulated OCPs including Aldrin, p,p-DDE, Eldrine ketone, Heptachlorepoxide, p,p-DDD, Endrin and p,p-DDT (Lobato et al. 2021).

Table 2. OCPs residue levels ($\mu\text{g/L} \pm \text{SD}$) detected from Buriganga, Turag, Balu, Sitalakhya, Bangshai and Dhaleswari river water.

OCPs	Blank	Buriganga	Turag	Balu	Sitalakhya	Bangshai	Dhaleswari
Aldrin	ND	0.834 \pm 0.056	0.453 \pm 0.076	0.235 \pm 0.089	0.482 \pm 0.072	0.743 \pm 0.043	0.241 \pm 0.053
p,p-DDE	ND	1.782 \pm 0.021	1.267 \pm 0.098	1.0231 \pm 0.078	0.882 \pm 0.065	0.899 \pm 0.034	0.832 \pm 0.045
Eldrine ketone	ND	0.273 \pm 0.032	0.123 \pm 0.072	0.032 \pm 0.092	0.098 \pm 0.087	0.167 \pm 0.043	0.0743 \pm 0.032
p,p-DDD	ND	1.421 \pm 0.098	1.236 \pm 0.054	1.007 \pm 0.033	0.856 \pm 0.075	0.994 \pm 0.033	ND
Endrin	ND	1.12 \pm 0.068	1.09 \pm 0.032	0.989 \pm 0.034	0.776 \pm 0.071	0.907 \pm 0.072	0.997 \pm 0.0632
α -Endosulfan	ND	0.123 \pm 0.021	ND	0.078 \pm 0.032	0.0945 \pm 0.023	0.098 \pm 0.032	0.0743 \pm 0.021
Cis-Chordane	ND	ND	ND	ND	ND	ND	ND
Heptachlorepoide	ND	0.321 \pm 0.082	0.231 \pm 0.067	0.165 \pm 0.082	0.220 \pm 0.065	0.182 \pm 0.098	0.098 \pm 0.034
Heptachlor	ND	ND	ND	ND	ND	ND	ND
p,p-DDT	ND	1.243 \pm 0.043	1.076 \pm 0.021	1.243 \pm 0.078	0.605 \pm 0.066	0.882 \pm 0.023	0.993 \pm 0.082
Methoxychlor	ND	ND	ND	ND	ND	ND	ND
Endosulfan sulfate	ND	0.023 \pm 0.078	ND	ND	ND	ND	ND
β -Endosulfan	ND	0.242 \pm 0.098	0.175 \pm 0.078	0.08 \pm 0.023	ND	ND	ND
Lindane-I	ND	ND	ND	ND	ND	ND	ND
Lindane-II	ND	ND	ND	ND	ND	ND	ND
Lindane -III	ND	ND	ND	ND	ND	ND	ND
Lindane-IV	ND	ND	ND	ND	ND	ND	ND
Trans-Chordane	ND	ND	ND	ND	ND	ND	ND
Phthalic Acid	ND	ND	ND	ND	ND	ND	ND

*ND: Not detected

Table 3. OCPs residue levels ($\mu\text{g/kg} \pm \text{SD}$) in fish samples from Buriganga, Turag, Balu, Sitalakhya, Bangshai and Dhaleswari rivers.

OCPs	Buriganga			Turag			Balu			Sitalakhya			Bangshai			Dhaleswari		
	A. microl epis	B. goniotus	B. tenaga	A. microl epis	B. goniotus	B. tenaga	A. microl epis	B. goniotus	B. tenaga	A. microl epis	B. goniotus	B. tenaga	A. microl epis	B. goniotus	B. tenaga	A. microl epis	B. goniotus	B. tenaga
Aldrin	0.432 \pm 0.032	0.342 \pm 0.023	ND	0.234 \pm 0.005	0.231 \pm 0.000	0.236 \pm 0.000	0.113 \pm 0.000	0.112 \pm 0.067	ND	0.117 \pm 0.002	ND	0.212 \pm 0.004	0.222 \pm 0.004	0.234 \pm 0.006	ND	ND	ND	ND
p,p-DDE	0.921 \pm 0.004	0.883 \pm 0.003	0.987 \pm 0.002	0.883 \pm 0.004	0.876 \pm 0.003	0.668 \pm 0.002	0.991 \pm 0.002	0.5431 \pm 0.028	0.4321 \pm 0.067	0.675 \pm 0.003	0.432 \pm 0.005	0.453 \pm 0.006	0.456 \pm 0.003	0.118 \pm 0.005	0.556 \pm 0.002	0.342 \pm 0.004	0.267 \pm 0.008	0.121 \pm 0.009
Eldrine ketone	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Eldrine ketone	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
p,p-DDD	0.887 ±0.067	0.785 ±0.086	0.943 ±0.088	0.786 ±0.034	0.675 ±0.043	0.987 ±0.084	0.883 ±0.045	0.885± 0.087	0.342± 0.21	0.386 ±0.047	0.554 ±0.034	0.432 ±0.035	0.118 ±0.029	0.564 ±0.085	0.776 ±0.047	ND	ND	ND	ND
Endrin	0.232 ±0.088	0.156 ±0.082	0.271 ±0.076	0.397 ±0.033	0.143 ±0.022	0.376 ±0.022	0.145 ±0.023	0.253± 0.056	0.112± 0.054	0.153 ±0.042	0.115 ±0.034	0.275 ±0.082	0.364 ±0.068	0.223 ±0.088	0.142 ±0.068	0.143 ±0.087	0.198± 0.543	0.209 ±0.089	0.2
α-Endosulfan	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Cis-Chlordane	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Heptachlor epoxide	0.123 ±0.053	0.231 ±0.021	0.111 ±0.043	0.113 ±0.076	0.110 ±0.032	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Heptachlor	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
OCPs	Buriganga			Turag			Balu			Sitalakhya			Bangshai			Dhaleswari			
p,p-DDT	0.584 ±0.022	0.678 ±0.021	0.384 ±0.032	0.287 ±0.011	0.564 ±0.034	0.484 ±0.037	0.587 ±0.067	0.398± 0.27	ND	0.334 ±0.025	0.467 ±0.039	0.256 ±0.030	0.432 ±0.087	0.223 ±0.068	0.253 ±0.057	0.321 ±0.073	0.232± 0.78	0.353 ±0.093	0.3
Methoxychlor	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Endosulfan sulfate	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
β-Endosulfan	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Lindane-I	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Lindane-II	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Lindane-III	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Lindane-IV	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Trans-Chlordane	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Phthalic Acid	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

The OCPs concentration in fishes from these rivers is presented in Table 3. The average OCPs concentration (mg/kg) in fishes from those rivers is shown in Table 4. Endrin normally stored in the fat tissues and may act as neurotoxin affecting the central nervous system leading to convulsions, seizures, or even death. Although, Endrin is not considered

as mutagen and it has no carcinogenic activity but still endrin is a toxic compound and it may cause serious health problems. Aldrin is a neurotoxic pesticide. It can elevate the neurotoxicity. Aldrin can stimulate the central nervous system (CNS) leading to hyperexcitation and seizures. Consumption of fishes from those rivers may be health

hazardous as HI values of some of the OCPs are above the safety range.

HI values of some of the OCPs are within the range of safety limit indicated that, consumers may not suffer from health risks from those pesticides but, long time exposure of those OCPs may accumulate in the fat tissues and can be hazardous. In addition, there have several

other sources (vegetables, milk, eggs, meat and fruits) from where consumers can exposure OCPs. So, Health conscious peoples should very careful while eating fishes from the rivers contaminated with OCPs. Our current study estimated the level of OCPs in river water and fishes from six contaminated rivers surrounding Dhaka city but, more studies are required to ensure the safety of peoples (Lobato et al. 2021).

Table 4. Mean values of detected OCPs residues in fish tissues.

OCPs	Mean (mg/kg)					
	Buriganga	Turag	Balu	Sitalakhya	Bangshai	Dhalewari
Aldrin	0.000258	0.000233667	0.000075	0.000109667	0.000152	0
p,p-DDE	0.000930333	0.000809	0.0006554	0.00052	0.000376667	0.000243333
p,p-DDD	0.000871667	0.000816	0.000703333	0.000457333	0.000486	0
Endrin	0.000219667	0.000305333	0.00017	6.26667E-05	0.000243	0.000183333
Heptachlorepoide	0.000155	4.95556E-05	0	0	0	0
p,p-DDT	0.000548667	0.000445	0.000328333	0.000352333	0.000302667	0.000302

Table 5. EDI values calculated from the OCPs from the fish samples.

OCPs	EDI						ADI
	Buriganga	Turag	Balu	Sitalakhya	Bangshai	Dhalewari	
Aldrin	0.00017	0.00015	4.9E-05	7.2E-05	1E-04	0	0.0001
p,p-DDE	0.00061	0.00053	0.00043	0.00034	0.00025	0.00016	0.001
p,p-DDD	0.00057	0.00054	0.00046	0.0003	0.00032	0	0.001
Endrin	0.00014	0.0002	0.00011	4.1E-05	0.00016	0.000121	0.0001
Heptachlor epoxide	0.0001	3.3E-05	0	0	0	0	0.0001
p,p-DDT	0.00036	0.00029	0.00022	0.00023	0.0002	0.000199	0.01

*ADI= Acceptable daily intake.

Table 6. Health index (HI) values of detected OCPs in the fishes of six contaminated rivers.

OCPs	HI					
	Buriganga	Turag	Balu	Sitalakhya	Bangshai	Dhalewari
Aldrin	1.696423714	1.536425095	0.493146429	0.721089667	0.999443429	0
p,p-DDE	0.611720748	0.531940614	0.430944226	0.341914857	0.247669095	0.159998619
p,p-DDD	0.573145738	0.536543314	0.462461762	0.300709733	0.319558886	0
Endrin	1.444371095	2.007653905	1.117798571	0.412051238	1.597794429	1.205469048
Heptachlor epoxide	1.019169286	0.325841937	0	0	0	0
p,p-DDT	0.036076401	0.029260021	0.021588855	0.023166923	0.019901198	0.019857363

CONCLUSION

The findings of the present study indicated OCPs concentrations in six contaminated river water and fishes, and assessed the possible human health risks through consumption of those fishes. This study will help to figure out the presence of pesticide residues in the contaminated

rivers surrounding Dhaka city in very small concentration. Consumers' health relies on food safety which is ensured by a safe environment. As these rivers are polluting day by day so, the responsible authorities should monitor the presence of pesticide residues on regular basis for public health safety.

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Biotechnological Communication

Phytoremediation Potential of Flowering Plants in Relation to Copper

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ABSTRACT

Heavy metals such as cadmium, chromium, nickel, mercury, lead, copper, zinc and others are among the priority environmental pollutants. Determination of their content in its main subsystems is an obligatory component in environmental monitoring and certification of agricultural products. On the other hand, all metals are natural components of soil-forming rocks, and some metals are classified as biogenic microelements, and their absence provokes functional disorders in living organisms. This article describes the results of studying the phytoremediation potential of flowering plants in relation to copper ions under laboratory conditions. The following flowering plants were selected as test crops: tansy phacelia, white mustard, small-flowered marigolds and a mixture of cereal grasses consisting of red fescue, perennial ryegrass and bluegrass in a ratio of 40%, 50%, 10%, respectively. Within the experiment, copper ions in concentrations of 2 and 10 maximum permissible concentration (MPC) were introduced into the soil sampled from the territory of agricultural lands (leached medium-thick heavy loamy chernozem with a high level of humus, mobile phosphorus and exchangeable potassium and a low level of nitrate nitrogen, copper in gross and mobile forms). It was found that all plants selected as test crops are capable of accumulating copper ions from the soil to varying degrees, which makes it possible to use them in phytoremediation of agricultural lands planned for organic farming. The ability to accumulate copper ions increases in the following order: white mustard < small-flowered marigolds < tansy phacelia < mixture of cereal grasses. The maximum effect of soil phytoremediation was revealed in the variant with a mixture of cereal grasses. When they are grown, the content of copper ions in the soil with the introduction of 2 MPC decreases by 38.8%, with the introduction of 10 MPC the concentration decreases by 47.8%.

KEYWORDS: ACCUMULATION COEFFICIENT, BIOLOGICAL ABSORPTION COEFFICIENT, COPPER, FLOWERING PLANTS, HEAVY METAL IONS.

INTRODUCTION

Most plants have ability to accumulate copper ions, while this accumulation effect is lower for some more toxic elements, since copper is a biogenic element and is directly involved in vital processes in plant cells; therefore, its concentration in phytomass is controlled by the plant itself (Olshanskaya et al. 2013). Copper is a typical nutrient. It plays an important role in the life of plants, as it participates in the processes of respiration, photosynthesis, moisture balance and other vital functions. Disorders of physiological processes caused by a lack of copper such as photosynthesis and respiration affect the energy metabolism of a plant and negatively affect the processes of their growth and development (Printz et al. 2016; Mosa et al. 2018). Lack of copper in human and animal organisms can lead to disorders of the central and peripheral nervous system, dysfunction of

the immune system, decreased bone density, joint diseases and other negative phenomena. However, the excess intake of copper in living organisms is no less dangerous than its deficiency and causes their poisoning (Bityutskiy 1999; Shtangeeva et al. 2020).

According to the interstate standard in force in the Russian Federation, chemical substances, according to the degree of danger, can be classified as highly hazardous, moderately hazardous or low hazardous for at least three indicators (toxicity, persistence in soil or plants, MPC, migration, impact on the nutritional value of agricultural products). Copper according to this classification is classified as moderately hazardous (GOST 17.4.1.02-83 2008; Wang et al. 2019; Amirova et al. 2019). At the end of the 20th century, the share of techno-genic copper in the environment was 75% and the main sources of its emission are high-temperature processes in nonferrous and ferrous metallurgy, combustion of fossil fuels, ore dressing wastes, copper-containing pesticides used in the practice of intensive

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Received 14/10/2021 Accepted after revision 19/12/2021

Published: 31st December 2021 Pp- 1786-1792

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.60>

agriculture (Yakovchenko et al. 2017; Asati et al. 2016; Shtangeeva et al. 2020).

Intensive agriculture, which has been practiced for a long time on the territory of Russia, has led to the fact that in most constituent entities of the Russian Federation there is a deterioration in the condition of agricultural land, a decrease in soil fertility continues, and their resistance to destruction and the ability to self-regenerate decreases. To preserve and restore the "health" of soils in order to use them in an organic land use system, a scientifically based approach to maintaining and increasing their fertility is required. Therefore, studies of the heavy metals' behavior in the system soil-plant allow scientists not only to assess environmental risks and make environmental forecasts regarding the accumulation of heavy metals in environmental objects, but also to apply new approaches and ways to eliminate them which is especially important in the production of agricultural products. In recent years, there has been a tendency for agricultural producers to switch to organic farming, which provides for high requirements for soil quality (Guidelines for determination of heavy metals 1989; SanPiN 2.1.7.1287-03 2004; Hygienic standard 2.1.7.2041-06 2006; Federal Center for Hygiene and Epidemiology of RosPotrebNadzor 2009; Vodyanitskiy 2011; GOST 12038-84 2016; Musilova et al. 2016; Wang et al. 2019; Amirova et al. 2019).

One of the most promising methods used for the restoration of agricultural land within the framework of organic agriculture can be phytoremediation - a method that is environmentally friendly and, importantly, economically beneficial. Phytoremediation is based on the ability of plants to extract harmful substances and elements from various components of the environment, accumulate them in their cells, tissues and organs without visible signs of oppression or convert them into safe compounds - metabolites (Volkov et al. 2013; Baycu et al. 2015; Yakovchenko et al. 2017; Kirichkova 2017; Poktsepai et al. 2017; Suman et al. 2018; Wang et al. 2019; Amirova et al. 2019; Shtangeeva et al. 2020). The purpose of this study is to determine the phytoremediation potential of some flowering plants in relation to copper ions.

MATERIAL AND METHODS

The study was carried out in the laboratory of the Department of Landscape Architecture of the Kuzbass State Agricultural Academy in the period from 15.04.2020 to 15.05.2020. Ornamental, green manure plants and weeds were test crops to study their hyperaccumulative potential for copper ions: small-flowered marigolds (*Tagetes patula* L.), tansy phacelia (*Phacelia tanacetifolia* Benth.), white mustard (*Sinapis alba* L.), and a mixture of cereal grasses. For the mixture of cereal grasses, the following plants was selected: red fescue (*Festuca rubra* L.), perennial ryegrass (*Lolium perenne* L.) and bluegrass (*Poa pratensis* L.) in a ratio of 40%, 50% and 10%, respectively. The choice in favor of these crops was due to their high ecological plasticity, the availability of seed for agricultural producers, and the simplicity of the cultivation technology.

Before sowing, the bioenergetic potential and laboratory germination were determined in the seeds of the studied crops (GOST 12038-84 2016). For laboratory studies, soil was sampled from the territory of agricultural land, planned in the future to be used for organic farming. Soil samples were taken from the arable horizon. Soil sampling was carried out by the Envelope method (GOST 17.4.4.02-2017 2018). In the soils taken for the study, the main agrochemical parameters and the content of copper ions were determined. Soil analysis was carried out in accordance with the following regulatory documents: mass fraction of organic matter, mass fraction of total and nitrate nitrogen, pH of salt extract, mass fraction of mobile phosphorus and exchangeable potassium, and content of gross and mobile forms of copper. To simulate soil contamination with copper ions, we used aqueous solutions of copper sulfate. The concentration of solutions was prepared based on the MPC for mobile forms of copper in soils (MPC Cu = 3.0 mg/kg of soil), in this experiment we used solutions with conditional concentrations of 2 and 10 MPC (GOST 26483-85 1986; GOST 26951-86 1986; GOST 26213-91 1993; PND F 16.1:2.3:3.11-98 1998; GOST R 54650-2011 2011; GOST R 58596-2019 2019).

The preparation of soils and plants for the control of copper ions in them was carried out in accordance with the method proposed by Lindemann et al. (2008). In accordance with the methodology, one kilogram of soil was placed in previously prepared plastic containers measuring 20×30×10 cm, which was thoroughly moistened before adding 50 ml of a solution with an appropriate concentration of copper ions. Soils from agricultural lands were used as a control group. After that, the studied cultures were sown in three replicates in the prepared soil in accordance with the experimental scheme (Table 1). The control test of the copper ion content in the soil and phytomass of the studied crops was carried out 30 days after sowing the plants using the method of atomic emission spectral analysis using an emission spectrometer with inductively coupled plasma OPTIMA model 2100 DV (PND F 16.1:2.3:3.11-98 1998). Accumulation coefficients characterizing soil remediation were determined as the ratio of the copper content at the end of the experiment to its content in the soil at the beginning of the experiment. Biological absorption coefficients characterizing plants' phytoremediation potential were determined as the ratio of the copper content in plant ash to the total copper content in the soil (Selyukova 2019).

RESULTS AND DISCUSSION

The crop seed quality was determined by the value of such indicators as germination and germination energy. These parameters further determine the most important sowing characteristics, such as the quality of seedlings, their amicability and evenness, which is directly related to the survival of plants in agro-phytocenosis. The observations and measurements of experimental plant samples showed a high bio-energetic potential of the seed, the germination energy and laboratory germination varied within 66-76% and 79-92%, respectively. The germination rate of white mustard seeds in laboratory conditions was significantly

higher than that of other crops. In addition, this culture showed high germination energy, as evidenced by the

highest values of this indicator (Table 2) (Amirova et al. 2019).

Table 1. Scheme of the laboratory experiment

№ option		Test crops		
Control	tansy phacelia	white mustard	mixture of cereal grasses	small-flowered marigolds
2 MPC Cu	tansy phacelia	white mustard	mixture of cereal grasses	small-flowered marigolds
10 MPC Cu	tansy phacelia	white mustard	mixture of cereal grasses	small-flowered marigolds

Table 2. Bioenergetic potential of crop seeds

Crop	Emergence rate, %	Laboratory germination, %	Terms for indicator determination, days	
			Emergence rate	Laboratory germination
White mustard	76±2,1	92±2,6	3	6
Tansy phacelia	72±1,2	90±3,5	4	10
Small-flowered marigolds	66±3,2	83±1,9	3	7
Perennial ryegrass	70±3,1	85±2,8	5	10
Bluegrass	71±2,7	89±2,5	7	21
Red fescue	68±2,5	79±3,4	7	14

Table 3. Agrochemical indicators of the upper horizon (0-20 cm) of the soil sampled for laboratory research

Indicators	Content	Level of concentration
pH of the salt extract	5,7	close to neutral
Mass fraction of nitrate nitrogen, mg/kg of soil	4,2	low
Mass fraction of mobile phosphorus, mg of P ₂ O ₅ per 1kg of soil	127,5	increased
Mass fraction of total nitrogen, %	0,2	increased
Mass fraction of exchangeable potassium, mg of K ₂ O per 1kg of soil	180,1	increased
Mass fraction of organic matter, %	9,1	high

Table 4. Content of copper ions in soil intended for laboratory research in comparison with the Clarke number and APC/MPC

Standard indicators of HM content in soils				HM content in the soil of the experimental plot	
Clarke number in Earth's crust, mg/kg		Total APC, mg/kg	Mobile MPC, mg/kg	Total, mg/kg	Mobile forms, mg/kg
According to Vinogradov (1957)	According to Kabata-Pendias (1989)				
20,0	26,0	132	3,0	25,9±4,4	0,23±0,06
Note. Accuracy indicators (±ΔI at P=0,95)					

The soil that was used in the laboratory experiment was leached medium-thick heavy loamy chernozem with a high level of humus in the upper horizon (0-20 cm) (9.1%), an increased level of mobile phosphorus (127.5 mg P_2O_5 /kg of soil) and exchange potassium (180.1 mg K_2O /kg of soil) and a low level of nitrate nitrogen (4.2 mg/kg of soil). The soil solution reaction was according to hygienic standard 2.1.7.2511-09 2009 and was close to neutral pH 5.7. (Table 3) (Amirova et al. 2019).

When comparing the data on the content of copper total forms in the studied soil with the literature data, it was found that their value was higher than the Clarke number according to Vinogradov, but it is lower than this value given by Kabat-Pendias (Dai et al. 2017; Lia et al. 2018). According to the standards in force in the Russian Federation, the approximate permissible content (APC) of copper total forms in soils, with a soil solution reaction of >5.5 (close to neutral), was 132 mg/kg. In the course of the study, it was found that in the studied soil samples the reaction of the soil solution is close to neutral (pHsol 5.7), and the content of copper total forms is 25.9 mg/kg of air-

dry soil, which was five times lower than the APC value for this type of soil. The content of mobile forms of copper was less than 1.0% of the total one and 13 times lower than the MPC (Table 4) (Shtangeeva et al. 2020).

After introduction of copper ions into the soils taken for the study with concentrations in solutions of 2 and 10 MPC, its content at the beginning of the experiment was 34.0 ± 6.0 mg/kg and 65.9 ± 9.2 mg/kg of air-dry soil, respectively (Table 5). Comparative analysis of soil and plant material samples by the content of copper ions in a laboratory experiment showed that all plants taken for the research (weeds, ornamental and green manure plants) are capable, to one degree or another, of accumulating copper ions from soils (Shtangeeva et al. 2020).

In the options of the experiment when growing white mustard, the lowest value of the accumulation indicator of the studied pollutant in plants on the control was noted (2.5 ± 0.4 mg/kg of air-dry phytomass). The highest value of this indicator in dry phytomass was found in the option with tansy phacelia (7.2 ± 1.4 mg/kg of air-dry phytomass).

Table 5. Comparative analysis of soil and plant material samples by the content of copper ions based on the laboratory experiment

Experimental options		Copper ion content		
		Soil, mg/kg		Plants, mg/kg of dry phytomass end of the experiment
		beginning of the experiment	end of the experiment	
Tansy phacelia	Control	25,9±4,4	19,4 ±3.9	7,2±1,4
	2 MPC	34,0±6,0	21,5±3,6	10,2±1,8
	10 MPC	65,9±9,2	43,1±7,2	21,2±4,2
White mustard	Control	25,9±4,4	23,7±4,1	2,5±0,4
	2 MPC	34,0±6,0	28,2±5,6	5,9±1,2
	10 MPC	65,9±9,2	56,6±9,5	6,7±1,3
Mixture of cereal grasses	Control	25,9±4,4	21,0±4,2	3,7±0,7
	2 MPC	34,0±6,0	20,8±4,7	17,0±4,1
	10 MPC	65,9±9,2	34,4±7,5	38,3±7,7
Small-flowered marigolds	Control	25,9±4,4	21,2±4,1	5,0±1,0
	2 MPC	34,0±6,0	21,4±5,7	10,1±2,0
	10 MPC	65,9±9,2	50,2±9,3	21,5±4,3

Note. Accuracy indicators ($\pm \Delta$ at $P=0,95$)

This indicator was 1.9 times higher than in the option with a mixture of cereal grasses and 1.44 times in the option with small-flowered marigolds. It should be noted that with an increase in the concentration of copper ions in the soil, the accumulating ability of plants to this pollutant increases in all experimental groups. However, the study noted significant differences in the experiment options. Thus, in the option with an increase in the copper ion concentration in the soil (2 MPC), the highest accumulative capacities were observed in plants of the cereal family. The indicator of their accumulative ability in relation to copper ions was more than by 1.6 times higher than in the options with tansy

phacelia and small-flowered marigolds and more than by 2.8 times in the option with white mustard.

In addition, an increase in the concentration of copper ions in the soil (10 MPC) increased the accumulation indicators of this pollutant in the phytomass of cereals more than by 4 times compared with similar indicators in the options with small-flowered marigolds and more than by 2.5 times in the option with phacelia tansy and white mustard. Thus, at low levels of copper ion content in soils (control), the ability to accumulate copper by plants increased in the following order: white mustard - a mixture of crop grasses – small-

flowered marigolds - tansy phacelia; and at concentrations of 2 and 10 MPC, the tendency to minimal accumulation of copper persists for white mustard, but varies for cereals (Shtangeeva et al. 2020).

Their phytomass showed the highest content of the investigated heavy metal - 17.0 ± 4.1 and 38.3 ± 7.7 mg/kg, respectively. Analysis of the results of the copper ion concentration changes in the soil according to the options of the experiment showed that in the control group there is a significant decrease in its content only in the option with tansy phacelia (25.0%). When a copper ion concentration was 2 MPC, its significant decrease in the soil was noted in options with the cultivation of cereal grasses, the studied indicator decreased by 38.8%. In the experiment options with tansy phacelia and small-flowered marigolds, there was observed a decrease in the concentration of the pollutant by 36%. At a copper ion concentration of 10 MPC, a significant decrease in its content in the soil was revealed as well (Shtangeeva et al. 2020).

Figure 1: Phytoremediation potential of flowering plants in relation to copper ions in laboratory conditions (K_{ba} is the coefficient of biological absorption)

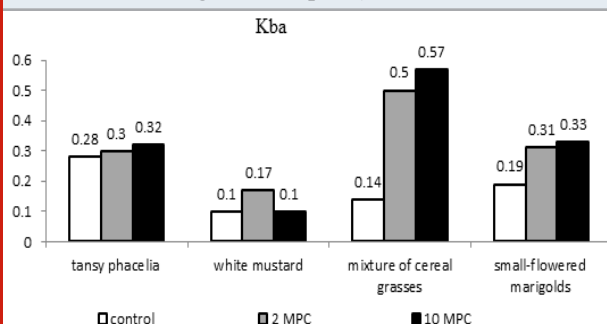
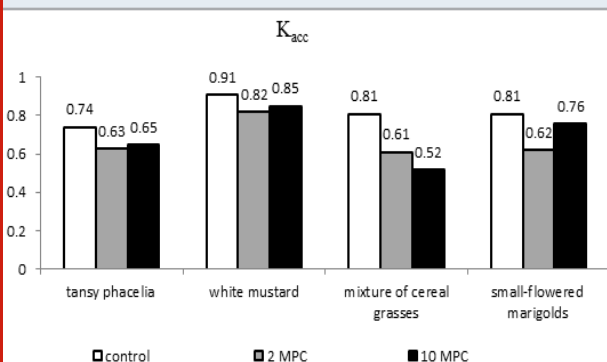


Figure 2: Efficiency of soil phytoremediation in relation to copper ions in laboratory conditions (K_{acc} - accumulation coefficient)



When growing a mixture of cereal grasses, this indicator decreased by 47.8%, when growing phacelia tansy - by 34.6%, and when growing small-flowered marigolds - by 23.8%. In the course of data analysis, a correlation was established between the decrease in the content of the studied copper ions in the soil and their accumulation in plant material, which is quantitatively confirmed by the

coefficient values of biological absorption (K_{ba}) and of accumulation (K_{acc}) (fig. 1-2). Comparative analysis of the values of the biological absorption coefficients of different crops showed that, under laboratory conditions, the ability to accumulate copper ions by plants increases, when the pollutant concentration increases in the soil (Vityaz et al. 2021).

At the same time, it was found that with an increase in the concentration of copper ions in the soil up to 2 MPC, the accumulative capacity of plants in the option with small-flowered marigolds increases 1.5 times ($K_{ba} = 0.3$), and in the option with a mixture of cereal grasses - 3.5 times ($K_{ba} = 0.5$) compared with the corresponding control groups. A further increase in the copper ion content in the soil (up to 10 MPC) leads to a significant increase in the accumulative capacity of plants in the option with a mixture of cereals, where the biological absorption index reaches 0.57, which is 4 times higher than the value of this indicator in comparison with the corresponding control group (Vityaz et al. 2021).

The study revealed that when the copper ion concentration in the soil increases by 2 MPC, the accumulative ability of plants increases in the following order: white mustard < tansy phacelia < small-flowered marigolds < mixture of cereal grasses; at a concentration of copper ions of 10 MPC - the order changes: white mustard < small-flowered marigolds < phacelia tansy < mixture of cereal grasses. According to the analysis of the accumulation coefficient values of the studied crops it follows that with a low (background) content of copper ions in the soil, a significant decrease in its concentration is revealed only in the option with tansy phacelia, which is confirmed by the highest value of the accumulation coefficient ($K_{acc} = 0.74$).

With the content of copper ions in the soil at concentrations of 2 MPC, its significant decrease is found in the option with a cereal mixture and small-flowered marigolds, which is confirmed by the corresponding values of K_{acc} (0.61 and 0.62). When the content of copper ions in the soil at a concentration of 10 MPC, its significant decrease is found in the option with a cereal mixture ($K_{acc} = 0.52$). Thus, the maximum effect of soil phytoremediation was revealed in the option with a cereal mixture, where the content of copper ions in the soil decreases by 38.8% with the introduction of 2 MPC, and with the introduction of 10 MPC - by 47.8%. The results obtained in the course of the study are consistent with the literature data on the high phytoremediation potential of representatives of the cereal family in relation to heavy metals (Vityaz et al. 2021).

CONCLUSION

The findings of the present study found that weeds, ornamental and green manure plants, due to their accumulative capabilities in relation to heavy metals, can be used as phytoremediators to improve the agrochemical parameters of agricultural soils intended for organic farming. When the (background) content of copper ions was low, a significant decrease in its concentration in the soil (25.0%) was revealed only in the variant with tansy phacelia, which is also confirmed by the value of the

accumulation coefficient ($K_{acc} = 0.74$). According to the ability to accumulate copper ions, the tested plants form the following row: white mustard < small-flowered marigolds < tansy phacelia < mixture of cereal grasses.

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Biotechnological Communication

Diversity of Biofilm-Forming Bacteria in Chinnamuttom Harbor of Southeast India

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ABSTRACT

Biofilms are species rich, partially due to highly effective powers of diffusion of the microorganisms and have wide tolerance to marine environmental conditions. Characteristically, the first organisms to respond to and convalesce from stress. The present study aims to isolate and identify the biofilm forming bacterial species, collected from surface water and substratum of the ship hull for four seasons at Chinnamuttom fishing harbor, Southeast coast of India, during June 2015 to May 2016. Among the mean concentration of bacterial isolates of both water and substratum of the station, maximum in monsoon and minimum in summer seasons. Totally 16 isolates were obtained, based on the adherence property, 8 isolates from surface water and 8 isolates from substratum of the ship hull. The samples were plated on Zobell marine agar medium for bacterial isolates of study area. The isolates of *Bacillus* sp., *Flavobacterium* sp., *Pseudomonas* sp., *Aeromonas* sp., *Micrococcus* sp., *Vibrio* sp., *Salmonella* sp., *Staphylococcus* sp., *Shigella* sp., *Klebsiella* sp., *Corynebacterium* sp., *Enterobacter* sp., *Chromohalobacter* sp., *Bacillus* sp., *Escherichia coli* and *Bacillus* sp. were reported in all the seasons at study area. The major diverse bacterial isolates were further characterized through morphological and molecular identification. Based on 16S rRNA gene sequences. Biofilm bacterial isolates were confirmed as *Bacillus* sp., and *Pseudomonas* sp.

KEY WORDS: BACTERIAL ISOLATES, BIOFILM, CHINNAMUTTAM HARBOR, SUBSTRATUM, WATER.

INTRODUCTION

In marine environment, the harbor waters provide a large diversity of living biomass (microorganisms) and complex mixture of prokaryotes and eukaryotes, which of the most, yet identified/or characterized (Boschker 2005; Biao et al. 2020; Antunes et al. 2020). In marine environment due to forceful opposition of space and living, all surfaces living or innate are susceptible to fouling which is directly related to the colonization and adherence of microorganisms leading to the formation of biofilm formation and bacterial adhesion (Chandran et al. 2020). According to Donlan (2002), the suspended particle surfaces tend to collect and concentrate nutrients by hydrophobic interaction and leads to bacterial colonization. These colonization on interactive surface supposed to be one of the microbial survival tactics, for increased nutrients access, toxins and antibiotics resistance, and predation protection (Dang and Lovell 2000; Babich et

al. 2021). For these advantageous, in submerged substratum, the bacteria are quickly colonized. Bacterial polysaccharides and proteins forms biofilms, and provides accessory to other unicellular fouling organisms (Dobretsov et al. 2013; Zheng et al. 2021; Georgiades et al. 2021).

The primarily colonized bacteria move to or transported to a surface and involves in series of steps (Mayer et al. 1999; Guimet and Gomez 2005). In any submerged fresh surface of marine system are predominantly colonized by bacteria and diatoms, such as *Vibrio* spp., *E. coli*, *P. aeruginosa*, *Shewanella oneidensis* and *Bacillus subtilis* to initiate the complex biofouling process. Bacterial colonization transpires by a two-step process beginning with reversible attachment to the substratum followed through irreversible adhesion. Bacterial growth is protected and supported by biofilms through reducing the effects of external hazards and disinfecting the materials in aquatic environment (Paul and Jeffrey 1985; Lee and Newman 2003; Omoike and Chorover 2004; Muhammad et al. 2020).

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Received 15/09/2021 Accepted after revision 19/12/2021

Published: 31st December 2021 Pp- 1793-1799

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.61>

During the expansion of colonization on surfaces, bacteria overproduce extracellular polymeric substances (EPS) (Jayatilake et al. 2017). These polymers, particularly EPS, frequently designated to construct the biofilm matrix, serving as a multi-functional element for immobilization of cells, adhesion on the colonized surface, protection and enabling spatial planning of various species within the biofilm (Carvalho 2018). In some respects, biofilm formation smears to human beings in the remedy process of wastewater, degradation of recalcitrant and aquaculture. In other respects, biofilm processed on heat exchangers, pipelines, ship surfaces and other industrial strategies causes serious problems and consumes large amounts of time and money to removing it. Also, biofilm formed on entrenched materials has been related to microbial diseases. Therefore, control of biofilm formation has been an important topic of interest to date (Lindberg et al. 2001; Balcazar et al. 2015; Carvalho 2018; Krsmanovic et al. 2020). The present study aims to isolate, identify and characterize the biofilm forming bacteria from water and substratum of the Chinnamuttom harbor. Further, the predominant biofilm forming bacterial species of all the four seasons were further characterized through morphological and molecular phylogenesis.

MATERIAL AND METHODS

The biofilm bacterial samples were collected from surface water and substratum of the ship hull in Chinnamuttom fishing harbor, Kanyakumari, Tamil Nadu. All the samples were collected using sterile polythene bags and immediately freeze and brought to the laboratory for further investigation. For the isolation of bacteria, the collected samples were serially diluted and placed over Zobell's marine agar standard spread plates, to isolate the Biofilm bacteria. Plating was done in duplicate and incubated at 37°C for 24–48 hrs. Following incubation, the total number of Colony Forming Unit (CFU) was determined and morphologically different bacterial colonies were selected, purified and sub-cultured on nutrient agar slant supplemented with 2% NaCl. All the bacterial isolates were tested for observance property by inoculating them into sterile seawater containing glass cover slip. The cover slips were removed and stained with 0.4% crystal violet to determine the adherence of bacteria (You et al. 2007). Bacterial isolates which form a slimy layer on the cover slip were selected for further characterization (Yang et al. 2021).

For the morphological and biochemical characterization, the isolated biofilm bacteria characterized using various morphological, biochemical and molecular characters. Gram stain, acid fast stain, spore staining and motility tests were performed for preliminary identification of the isolate (Cappuccino and Sherman 1999). Morphological parameters include colony color and shape followed by biochemical tests as: Indole, Methyl Red, Voges Proskauer, Triple Sugar Iron (TSI) and Citrate utilization were analyzed. (The establishment of the genera and species present in the samples were identified according to their characteristics as outlined in Bergey's Manual of Determinative Bacteriology (Holt et al. 1994; Macfaddin 2000). To study the molecular characteristics, the screened biofilm producing bacterial isolates were identified through

partial 16s rRNA gene sequences analysis. The genomic DNA was extracted using the DNA Kit according to the manufacturer's instructions. 16s rRNA gene sequence was amplified with bacterial universal primers 518F (5'-CCAGCAGCCGCGGTAATACG-3' and the reverse primer 800R (5'-TACCAGGGTATCTAATCC-3'). The purified PCR amplicons were sequenced and compared with the 16S rRNA gene sequences available in the GenBank database using BLASTN tool 2.2.28 algorithm (Zhang et al. 2000; Gwak and Rho 2020).

For the phylogenetic analysis, all the 4-biofilm producing bacterial isolates nucleotide sequences were aligned with the program CLUSTAL W (Narasimman et al. 2021). The aligned complete 16S rRNA sequences were exposed to phylogenetic analysis using the Molecular Evolutionary Genetic Analysis (MEGA) software Version 7.0. The tree was generated with the Neighbor-Joining algorithm and bootstrap for 550 resampling to ensure robustness and reliability of trees constructed. The assembled complete 16S rRNA sequences of eleven distinguishable biofilm producing bacterial isolates were deposited in public sequences repository NCBI Gene Bank using the BANKIT sequence submission tool (Narasimman et al. 2021).

RESULTS AND DISCUSSION

Enumeration of biofilm producing bacteria: The present investigation of biofouling bacterial species for four seasons were isolated from surface water and substratum of Chinnamuttom fishing harbor, Southeast coast of India. The biofilm bacteria were higher in both water and substratum of the ship hull on monsoon as compared to post-monsoon, pre-monsoon and summer. The maximum biofilm bacteria population in water was recorded (31.46×10^4 CFU ml⁻¹) in monsoon followed by (30.4×10^4 CFU ml⁻¹) in post monsoon, (27.2×10^4 CFU ml⁻¹) pre-monsoon and (16.53×10^4 CFU ml⁻¹) summer. Likely, the estimated biofilm bacteria value in substratum was high (38.4×10^4 CFU ml⁻¹) in monsoon followed by (36.4×10^4 CFU ml⁻¹) post monsoon; (31.46×10^4 CFU ml⁻¹) pre-monsoon and (18.46×10^4 CFU ml⁻¹) summer. Among the recorded mean values of biofilm bacterial populations, in both water and substratum of the ship hull shows the higher population in monsoon and lower in 16.53×10^4 summer. The similar mean concentration of maximum bacterial isolates on monsoon and minimum in summer seasons were found (Kim et al. 2016; Antunes et al. 2020).

Identification of biofilm bacteria: In the present study totally 16 bacterial isolates were obtained based on biochemical characterization. The bacteria were identified as *Bacillus* sp., *Flavobacterium* sp., *Pseudomonas* sp., *Aeromonas* sp., *Micrococcus* sp., *Vibrio* sp., *Salmonella* sp., *Staphylococcus* sp., *Shigella* sp., *Klebsiella* sp., *Corynebacterium* sp., *Enterobacter* sp., *Chromohalobacter* sp., *Bacillus* sp., *Escherichia coli* and *Bacillus* sp. (Table 1). Based on the adherence property, 8 isolates from surface water and 8 isolates from substratum of the ship hull were obtained and amplified in all the season samples of Chinnamuttom fishing harbor. Sillankorva et al. (2008), Kreig and Hoit (1984) and hilal et al. (2004) reported *Pseudomonas* sp.,

Vibrio sp., *Aerobacter aerogens* and *Bacillus* sp. from the fouling substratum. Among these bacterial isolates, *Bacillus* sp., were frequently observed (Bhattarai et al. 2006; Bhattarai et al. 2007; Antunes et al. 2020). Among the total 16 identified bacterial species, only four bacterial isolates (Table 2) species were record in all the seasons.

Diverse bacterial isolates in the present study were due to the nutritive water of harbor environment and interaction with substratum. The isolated biofilm bacterial strains in water and substratum samples indicate, that most of the

isolated strains identified as gram negative in nature (Samrot et al. 2021). Among these biofouling bacterial isolates, *Bacillus* sp. is one of the predominant bacterial species followed by *Pseudomonas* sp. and others. In accordance to the present study, occurrence of bacterial strains, *B. subtilis*, *Staphylococcus* sp., *Micrococcus* sp., *Salmonella* sp., *Vibrio* sp. and *Pseudomonas* sp., were the biofilm bacteria involving in 128 types of biofouling process. Based on the hydrographical conditions the biotic organism abundance and biomass occur varies in marine environment (Lee and Newman 2003; Omoike and Chorover 2004; Udhayakumar 2010; Antunes et al. 2020).

Table 1. Morphological and biochemical identification of biofilm bacteria

S. No	Shape	Gram staining	Culture medium	Colony color	Indole	M R	V P	Citrate	Triple sugar Iron				CAT	OXI	Tentative organisms
									A/A	A/K	K/A	K/K			
1	Rod	+	NA	white	-	-	-	-	-	-	+	+	+	-	<i>Bacillus</i> sp.
2	Rod	-	BLA	Yellow	-	-	-	-	-	-	-	+	+	-	<i>Flavobacterium</i> sp.
3	Rod	-	PIA	Green	-	-	-	+	-	+	+	-	+	+	<i>Pseudomonas</i> sp.
4	Rod	-	AE-A	Pale green	+	+	-	-	-	-	-	+	+	-	<i>Aeromonas</i> sp.
5	Cocci	+	NA	Yellow	-	-	-	-	-	-	+	+	+	+	<i>Micrococcus</i> sp.
6	Rod	-	TCBS	Yellow	-	-	+	+	+	+	+	+	+	-	<i>Vibrio</i> sp.
7	Rod	-	XLD	Pink	-	+	+	-	+	-	+	+	-	-	<i>Salmonella</i> sp.
8	Cocci	+	BLA	G-Yellow	-	+	+	-	+	-	-	+	+	+	<i>Staphylococcus</i> sp.
9	Rod	-	XLD	Red	-	-	-	+	-	-	-	+	+	+	<i>Shigella</i> sp.
10	Rod	-	XLD	Yellow	-	-	+	+	+	+	+	+	+	-	<i>Klebsiella</i> sp.
11	Rod	+	BLA	Pink	-	-	-	-	-	-	+	+	+	-	<i>Corynebacterium</i> sp.
12	Rod	-	MCA	Red	-	-	-	+	-	-	-	+	+	-	<i>Enterobacter</i> sp.
13	Rod	-	MARA	White	+	-	+	+	+	+	-	-	+	-	<i>Chromohalobacter</i> sp.
14	Rod	+	NA	White	-	+	-	+	-	+	+	-	-	+	<i>Bacillus</i> sp.
15	Rod	-	EMB	Metallic green	+	+	-	-	-	-	+	+	+	-	<i>Escherichia coli</i>
16	Rod	+	NA	white	-	-	-	-	-	-	+	+	+	-	<i>Bacillus</i> sp.

I – Indole ; MR – Methyl red; VP – Voges proskauer; C – Citrate; TSI – Triple sugar Iron; CAT – Catalase; OXI – Oxidase; A/A – Acid slant acidbutt; A/K – Acid slant alkaline butt; K/A – Alkaline slant acid butt; K/K – Alkaline slant alkaline butt. NA – Nutrient Agar; BLA – Blood Agar; AE- A – Aeromonas Agar; TCBS – Thiosulfate citrate Bile Salt Agar; MCA –MacConkey Agar; EMB – Eosin Methylene Blue Agar; XLD – Xylose lysine deoxycholate agar; PIA – Pseudomonas isolation agar.

Motility test: In this present study, the twelve bacterial strains were confirmed as motile forms with a single polar flagellum or multi polar flagellum, remaining four strains was non-motile forms. The motile nature of all the strains was analyzed by commonly used hanging drop method which confirmed that among the 16 identified bacterial strains 4 strains were motile in nature (Figure 1) (Jain et al. 2020). Bacterial Straining tests: The isolated bacteria were found both cocci and bacilli shaped. Among all the 16 bacterial isolates six gram positive and ten-gram negative bacteria based on absorption of dye in the cell wall of organisms (Table 1). The bacterial strains isolated from water and substratum of the ship hull showed that Gram-positive groups were dominant during all seasons, however, that Gram-negative strains are prevailing on both sites along the harbor environment.

The biochemical test acid fast staining on the 16 isolate showed, bright red to intensive purple, Red, straight or slightly curved rods, happening singly or in small groups, may appear decorated Non-acid fast: blue color (Table 1) and the endospore staining reveals the isolates are non-

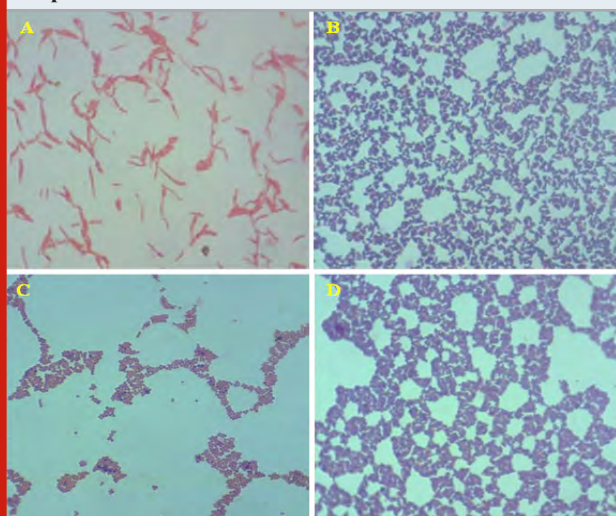
spore form bacteria. The biochemical and straining results revealed that changes in environmental conditions with any season could reflect the capacity of biofilm populations to maintain their diversified functions in an ecosystem. In addition, when observing at the genus/species level within the same phylum, dominances would be expected to change over time. Although several studies indicate that the presence of biofilms may increase the attachment of macro-foulants, the exact interactive mechanisms are yet to be discovered (Kang et al. 2015). *Aeromonas* sp. *Chromohalobacter* sp. *Escherichia coli*, were given the positive results of indole test. Remaining 13 are the negative strains. The 7 bacterial isolates viz., *Pseudomonas* sp., *Vibrio* sp., *Shigella* sp., *Klebsiella* sp., *Enterobacter* sp., *Bacillus* sp., *Chromohalobacter* sp. are positive for indole test and only 4 bacterial strains (*Aeromonas* sp., *Salmonella* sp., *Bacillus* sp. and *Escherichia coli*) showed positive to Methyl red test (Antunes et al. 2020).

Triple sugar test: Table 1 showed the results of Triple Sugar Iron (TSI), from which these strains were isolated, and were alkaline reaction, red colour suggested no

fermentation of sucrose or lactose or both and were Acidic reaction, yellow colour suggested to fermentation of lactose or sucrose. Among all isolates of bacterial strains only five bacterial species were positive to *Voges proskauer* (*Vibrio* sp., *Salmonella* sp., *Staphylococcus* sp., *Klebsiella* sp. and *Chromohalobacter* sp.) (Antunes et al. 2020).

Oxidase and Catalase test: The catalase and oxidase rapid test were for preliminary differentiation of isolated bacterial strains for their hydrogen peroxide decomposition and oxygen utilization capacity (Aggarwal et al. 2020). From the oxidase test the presence of cytochrome oxidase enzyme was confirmed in *Pseudomonas* sp., *Micrococcus* sp., *Staphylococcus* sp., *Shigella* sp. and *Bacillus* sp. Only three bacterial isolates were negative catalase (*Salmonella* sp., *Bacillus* sp. and *Corynebacterium* sp.). The bacterial isolate SS1 was positive catalase and can ferment glucose, maltose and sucrose and reduced nitrates to nitrites. Rajasekar et al. (2007) reported the presence of *Bacillus* sp (Rajasekar et al. 2007; Aggarwal et al. 2020).

Figure 1: Acid fast stained in dominant bacterial strains (magnification 100X). A- Gram-negative rod-shaped bacteria; B- Gram positive rod-shaped bacteria; C- Gram negative cocci-shaped bacteria; D- Gram-positive cocci-shaped bacteria.



Molecular identification: The genus confirmed strains were further subjected to 16s rRNA analysis. The sequenced four strains were similar with *Bacillus amyloliquefaciens*, *Bacillus* sp., *Domibacillus indicus* and *Pseudomonas* sp. All the strains were unique to each other in their nucleotide base pairs. Since the strains were isolated from Chinnamuttam harbor environment the strains were named as *Bacillus amyloliquefaciens*- WS8, *Bacillus* sp., SS1, *Domibacillus indicus* - SS8, *Pseudomonas* sp -WS5 and GenBank accession numbers obtained after submission (Table 2). Based on 16S rRNA gene sequence alignment, the phylogeny tree of biofouling bacterial isolates was assembled with the neighbor-joining method (Dang and Lovell 2000; Babich et al. 2021). The result from the well-supported phylogeny with high resolution inner branches and bootstraps suggests the existence of bacterial strains from *Domibacillusaceae* family. The bacteria of *Domibacillus* species were isolated

and dominated in the sediment and water samples of Indian coast. The strains exhibited genetic similarity with genus and species of the *Domibacillus indicus*. The precise identification of bacterial isolates up to species level is vital since this gives an insight into the bacterial diversity. The identification of biofilm producing, as well as biofilm forming, bacterial isolates in this study was carried out by advanced molecular biology technique based on 16S rRNA gene sequence analysis (Babich et al. 2021).

Phylogeny Tree: A phylogenetic evolutionary tree shows the inferred evolutionary relationships among various biological species. The taxa joined in the tree were oblique to inclined from common ancestor. Unrooted trees proved the sprig lumps about ancestry at all. Figure 1 showed the phylogenetic tree of all sample are not similar sequences.

Based on 16S rRNA gene sequences, biofilm bacterial isolates were confirmed as *Bacillus* sp., and *Pseudomonas* sp. A molecular technique is one of the important techniques involving ribosomal RNA sequence analysis is commonly used to examine evolutionary interactions within different genera of bacterial isolates. Primary and secondary screening of *Streptomyces* strains identified by 16s-rDNA gene sequencing and also it has been confirmed that gene sequence data of specific strain with adjoining a parallel score of <97% represents a new species (Reller 2007). The bacterial isolate WS5 was observed as cream-colored circular colonies on Zobell agar medium. The result showed that non motile rod-shaped gram-negative isolate.

Table 2. Gene bank accession numbers

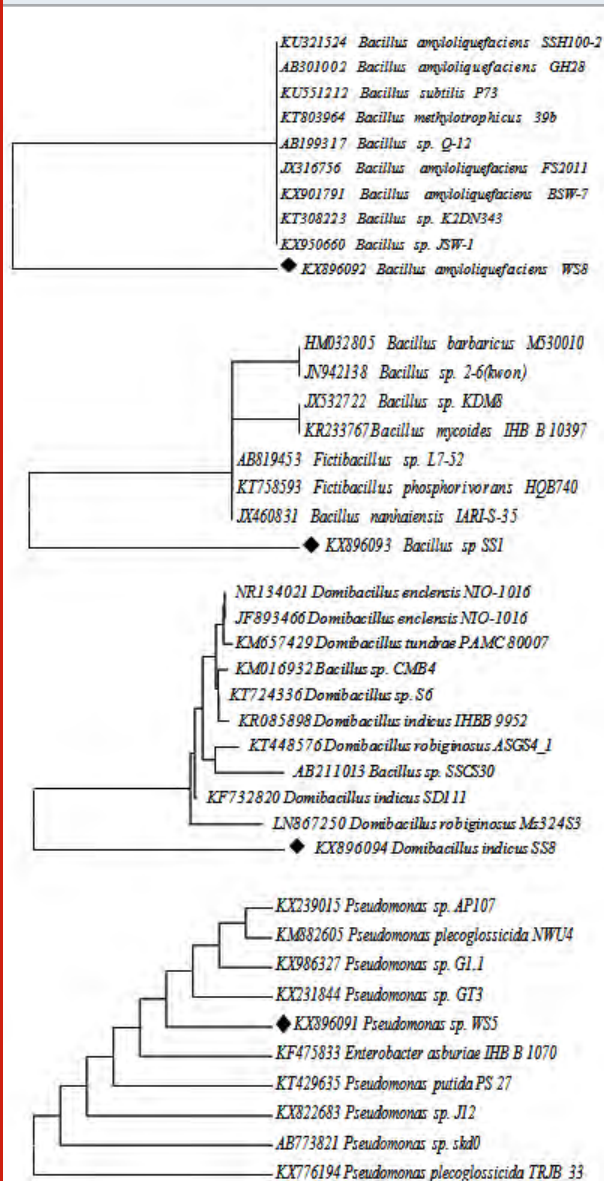
Sample ID	Bacterial isolates	Accession Number
WS8	<i>Bacillus amyloliquefaciens</i>	KX896092
SSI	<i>Bacillus</i> sp.	KX896093
SS8	<i>Domibacillus indicus</i>	KX896094
WS5	<i>Pseudomonas</i> sp.	KX896091

Various biochemical characteristics of biofilm bacteria were used for their identification. In the present investigation, it was found that the bacterial isolate was found to be positive results for citrate utilization, catalase and oxidase and negative results for Indole, methyl red, Voges Proskaur and it can acid and gas production form of negative results. It was reported the presence of *Pseudomonas* sp. Based on amassed whole 16S rRNA gene sequence alignment, the phylogeny tree of these culturable biofilm bacterial isolates was assembled with the neighbor-joining method. This method determines the presence of each bacterial isolate in the phylogeny with bootstrap support. The outcome from high resolution inner twigs recommends the existence of bacterial strains from *Pseudomonadaceae* family and only one from *Enterobacteriaceae* (Reller 2007; Babich et al. 2021).

The strains exhibited genetic similarity with genus and species of the *Pseudomonas* with the cases similarity up to

98% was found. It has been proving that genomic sequence data of separate microbe with a adjacent neighbor displaying a resemblance score of <97% characterizes a new species, the meaning of resemblance scores of >97% is not as clear. The bacterial isolate WS8 and SS8 was perceived as cream color circular colonies on zobell agar medium. The staining report exhibited gram +, rod shaped and motile cells but the strain of SS8 isolates were non-motile. In the present investigation, it was found that the bacterial isolate WS8 was found to be positive for Voges-Proskauer, catalase, oxidase, starch hydrolysis and reduction of nitrate and negative results for Indole production, citrate utilization and hydrolysis of urea. It was reported the presence of *Domibacillus indicus* (Reller 2007; Babich et al. 2021).

Figure 2: Evolutionary relationships of 11 taxa for 4 different Samples *Bacillus amyloliquefaciens*, *Bacillus* sp., *Domibacillus indicus* and *Pseudomonas* sp., using mega 7.0 version Phylogenetic analysis tool.



CONCLUSION

The findings of the present study shows that the increased physical parameters in summer may significantly influence to minimize chemical parameters and inorganic nutrients followed by less bacterial count in water and enhance high bacterial load during monsoon season. Hence, the diverse isolates of 16 various biofilm forming bacterial genera were characterized with morphological and biochemical methods. The study found most of biofilm forming bacteria were rod shaped. The seasonal tolerant and dominant species during study period in both water and substratum were *Pseudomonas* sp., *Bacillus amyloliquefaciens*, *Bacillus* sp., and *Domibacillus* indices, identified and further confirmed through molecular identification. Further investigation of relationship between specific biofilm forming bacterial mass in contrast with macro foulants accumulation and their adherent effect estimation are recommended.

Conflict of Interests: Authors declare no conflicts of interests to disclose.

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Biomedical Communication

Effectiveness of Simulation As a Technique vs Traditional Method on Knowledge and Practice Regarding Basic Life Support: An Experimental Study

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ABSTRACT

Instantaneous institution of Basic life Support (BLS) with Cardiovascular Resuscitation is one of the crucial factors in the survival of cardiac arrest patient. However, ability to learn and retain BLS skill remains questionable. The present study was aimed to compare the effectiveness of teaching BLS skills via simulation technique vs. traditional method to B.Sc. Nursing students and BPT 1st year students of SGT University, Budhera, Gurugram. The investigator prepared the list of all the students from first year B.Sc. Nursing and BPT first year and from that, the investigator selected the samples using simple random probability sampling technique. Before going ahead with data collection, a written consent was taken from the students regarding their willingness to participate in the research study. Then data regarding socio demographic variables was collected followed by a pre -test using knowledge questionnaire and practice checklist. On experimental group, simulation technique was used to teach BLS and control group learnt BLS in traditional way. After 7 days post-test was taken to assess the change in knowledge and practice regarding BLS. Mean post-test knowledge score for experimental group was high i.e., 18.40 in as compared to pre-test knowledge score of 12.90. Mean practice score for experimental group was high i.e., 10.00 as compared to mean pre-test practice score of 4.50. In control group, no significant difference was reported between pre-test and post-test knowledge as well as practice score. This study generates evidence that Simulation is more effective as a technique to teach BLS to student more effective as compared to conventional method.

KEY WORDS: BASIC LIFE SUPPORT, EFFECTIVENESS, KNOWLEDGE, PRACTICE, SIMULATION.

INTRODUCTION

Heart disease being the world's largest killer leads to death of around 175 lakh people in a year. In Indian Scenario, one Indian dies about every 29 seconds because of heart problems. Around twenty thousand new cases of heart problems develop every day. Overall, in India around 90 million of general public suffer from cardiac disease and around one third more are at great risk (Dahiya and Milan 2016). In case of cardiac arrest, an effective BLS can help save life. ABC is the most important aspects in Basic Life Support, which expands to the A-airway, B-breathing and C-circulation. If the circulation fails for three to four minutes, it will cause irreversible cerebral impairment (Lewis et al. 2017). Once a patient progresses into asystole, his probability of survival decreases by seven to ten per cent

for every minute that passes by, until a defibrillator comes for his rescue (Dick-Smith et al. 2021).

In case of a sudden asystole, a prompt and efficacious treatment is very essential to get positive results. With a timely and effective resuscitation, deaths of many patients with cardiac arrests can be avoided (Madams 2003). Basic Life Support acts as a saviour to cease the aggravation of the critical state functioning of brain and heart till the time defibrillation and/or advance life support are provided. With the implementation of two most important actions i.e., recognition of asystole in early stage and instant commencement of Basic Life Support can increase the probability of patient's survival almost twice (Moule 2002). These days, in nursing education, more importance is given to training on Basic Life Support because if a nurse is competent enough to perform Basic Life Support will lead to a significant difference between life and death of a victim (Dick-Smith et al. 2021).

Article Information:*Corresponding Author: deeraman@gmail.com

Received 15/09/2021 Accepted after revision 19/12/2021

Published: 31st December 2021 Pp- 1800-1805

This is an open access article under Creative Commons License,

Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.62>

It is a skill that is very much essential to be taught to the nursing students. It is very much needed that nurses have the skills to assess early for asystole and promptly institute Basic Life Support which includes preserving of respiration and circulation for the casualty prior to arrival of emergency services, or advanced life support service to save the lives of patients who are in danger. Regular training and as well as updates in resuscitation must be imparted to all the nurses. As registered nurses, each and every nursing personnel has an obligation to ensure to remain competent to perform resuscitation. Health is never static rather a dynamic state. It continually changes from minute to minute, day to day and year to year as we grow. Health is considered as a vital factor for everyone because we can appreciate life the most when we are in a healthy state. Historically, health was considered to be the exact opposite of illness. Mere absence of illness or disease was a sign of good health. Today, the new definition of health encompasses not only physical aspects, but social, mental and spiritual dimensions. The most modern definition of health according to WHO is, "Health is a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity" (Regional Office for Africa 2006; Loyola 2010; Süß-Havemann et al. 2020).

The burden of cardiovascular disease (CVD) in India is among the highest globally. The annual number of CVD fatalities in India increased from 2.26 million in 1990 to 4.77 million in 2020 (Murray and Lopez 1997). Coronary heart disease prevalence rates in India have been estimated over the past several decades and have ranged from 1.6% to 7.4% in rural populations and from 1% to 13.2% in urban populations (Gupta et al. 2008). Worldwide the most common cause of death shared by both males and females is due to cardio vascular disease. One of the leading public health problems is sudden cardiac arrest which needs to be managed by Basic Life Support. In Basic Life Support (BLS) management of cardiac arrest patients is such designed so as to sustain sufficient circulation and ventilation, sans medications or any kind of advance medical devices (Parashar 2010; Süß-Havemann et al. 2020).

Under the umbrella of Basic Life Support (BLS) comes recognition of signs of sudden cardiac arrest (SCA), heart attack, stroke, foreign-body airway obstruction (FBAO); and most importantly cardiopulmonary resuscitation (CPR) (Parashar 2010). Being trained to perform Basic Life Support can make a marked difference in the existence of a casualty's life. Nurses perform an assessment for cardiac arrest and accordingly must initiate Basic Life Support and involves maintaining respiration and circulation for the fatality until emergency services, or advanced life support services, arrive. As all nurses have a responsibility for managing the patients competently, they must be offered training and refreshers consistently in resuscitation. Being a registered nurse, it is the self-responsibility of each nurse to ensure to remain proficient to perform resuscitation (Botha et al. 2012). Sequence of actions in Cardio pulmonary resuscitation improves the chances of survival succeeding cardiac arrest. A combined set of coordinated actions is integral to a productive resuscitation following an arrest requires an integrated set of co-ordinated actions

represented by the links in the chain of survival (Süß-Havemann et al. 2020).

In (1962), the American Heart Association (AHA) pioneered in establishing guidelines for Cardiopulmonary Resuscitation and Emergency Cardiovascular Care which commenced the efforts to medically improve CPR and is continued up till now. Research evidences from various clinical studies have been applied recurrently to CPR. The newest development in the CPR guidelines is a change in the BLS sequence of steps from "A-B-C" (airway, breathing, compressions) to "C-A-B" (compression, airway, and breathing). The key to obtain best results which go beyond return of spontaneous circulation is high quality CPR. And the ultimate goal of a revival system of care is to return to a former quality and efficient state of health. Between 2008 and 2030, in a span of 22 years, the global inhabitation is estimated to grow by 20%, from 600.7 million to 800.1 million people. In 2008, if we consider top ten causes for mortality worldwide, other than injuries, five were non-communicable diseases; this will go up to seven out of ten by the year 2030. In fact, CVDs would be the single largest cause of death in the world accounting for more than a 33% of all deaths (Prakash 2009; Lee 2012; Wilson et al. 2020).

In the lifetime of a human being the risk of developing coronary heart disease at the age of 40 is 49% for men and 32% for women. In United States, there is a fatality every minute from coronary heart disease. Due to sudden cardiac arrest, the average number of years of life lost is 15 years. Generally, half of men and around 2/3rd of women who die suddenly from coronary heart disease are asymptomatic before that (Regional Office for Africa 2006; Lee 2012). Every Year, more than 10 lakh heart attacks happen and around 1/4th of people die prior to getting to a hospital. Current studies prove that cardiac arrest is becoming the number one cause of death. In reality, research reports have reported that all heart attacks which occur inside home most likely happen to a family member or friend and number is around 80%. There are more chances that a patient having out-of-hospital cardiac arrest will come out of it safely if there is rapid institution of bystander cardiopulmonary resuscitation (CPR) corresponding to early arrival of BLS trained rescuers and advance equipment. Survival rate increases 2-3 folds if there is rapid occurrence of combination of critical steps viz. bystander CPR comprising airway opening, rescue breathing, and external chest compression along with prompt response from call for ambulance (Wilson et al. 2020).

New Zealand Government, Department of Occupational Health and Safety has given due recognition and importance to CPR and basic emergency care by incorporating these subjects in the school curriculum (albeit as an optional subject) and by providing first aid training at workplace (Eisenberg and Mengert 2001). Competency in Basic Life Support is well-thought-out as a vital skill for health care workers. It is a widely anticipated among community high standards are maintained in nursing education regarding the knowledge and competence in Basic Life Support. Nursing students report that the most nerve racking state that the

nursing students have to cope with after their registration is engaging in both fruitful as well as unsuccessful cardiopulmonary resuscitation and Basic Life Support. An in-depth understanding and proficiency to perform Basic Life Support will put them in an advantageous position for the best survival of the patient whenever it's needed (Inwood 1996; Wilson et al. 2020).

A learning which is regulated by a motivated student himself is better able to support learning the skill in BLS training in students (Süss-Havemann et al. 2020). Competency is essential for effective BLS skills such as the depth of cardiac compression, number of compressions per minute and releasing the pressure off the chest while giving compressions are very efficiently delivered by an automated manikin. Hence the assigning of the training of the psychomotor component of cardiac compressions to a simulation station would be truly effective for student learning (Wilson et al. 2020). Study was conducted with primary objective to evaluate the effectiveness of simulation technique over conventional technique in terms of understanding and competency regarding basic life support among Nursing and Physiotherapy students.

MATERIAL AND METHODS

The study was conducted on 60 students studying in 1st year B.Sc. Nursing and BPT 1st year students in SGT University and fulfilled the inclusion criteria. The investigator employed probability sampling technique using simple random

sampling to draw 60 samples from the study population. The tool used in this study comprised of three sections. Section A consists of Socio demographic variables, Section B consists of Knowledge questionnaire and Section C consists of Observational check list. The data was collected over a period of 4 weeks at SGT University, Faculty of Nursing and Faculty of Physiotherapy Departments, Gurugram, an initial permission was taken from university as well as took grant of a formal permission from the Dean, Faculty of Nursing. All the participants were assured regarding maintaining the anonymity of their identity and confidentiality of the data. Socio demographic data was collected by the investigator after giving explanation to the participants regarding the purpose of the study and written consent was taken from the participants. This was followed by filling up of knowledge questionnaire by the participants and completion of observational checklist by the researcher.

RESULTS AND DISCUSSION

Table no. 1 depicts that 3% students had good knowledge from pre-experimental group, 90% had average and 7% students had poor knowledge whereas from post-experimental group 53% had good knowledge and 47% had average level of knowledge regarding BLS. No student was found having poor knowledge in post-test. From control group, in pre-test, no students had good knowledge while 80% had average and remaining 20% had poor knowledge regarding BLS. In post-test, only 3% students had good knowledge while 80% had average and 17% had poor knowledge.

Table 1. Pre – test and Post-test Level of knowledge regarding BLS among experimental and control group.

Criteria Measure of Knowledge Score				
Score Level	Pre-test Score Experimental Group	Pre-test Score Control Group	Post-test Score Experimental Group	Post-test Score Control Group
Good(19-28)	1(3.3%)	0(0%)	16(53.3%)	1(3.3%)
Average (10-18)	27(90%)	24(80%)	14(46.7%)	24(80%)
Poor(0-9)	2(6.7%)	6(20%)	0(0%)	5(16.7%)

Table 2. Pre-test and post-test level of practice regarding BLS among Experimental group and Control group.

Score Level	Pre-Experimental	Pre-Control	Post Experimental	Post Control
Good(9-12)	0(0%)	0(0%)	22(73.3%)	0(0%)
Average (5-8)	18(60%)	22(73.3%)	8(26.7%)	20(66.7%)
Poor(0-4)	12(40%)	8(26.7%)	0(0%)	10(33.3%)

Table 2 depicts that in pre-experimental group, there is not any student who had good level of practice but 60% students had average and remaining 40% had poor level of practice whereas in post-experimental group 73.3% had good level of practice and 27% had average level. No student was

found having poor level of practice in post-experimental group. Talking of pre-control group, no students had good level of practice, 73% had average and remaining 27% had poor level of practice regarding BLS. In post-control, 67%

students had average level of practice while 33% had poor level of practice.

In above Table 3.1, to compare the knowledge score within the group paired t-test was conducted where results revealed that mean score of post-tests from experimental group is high as compared to post-test from control group.

Table 3.1 Mean pre-test and post-test knowledge score regarding BLS among Experimental and Control Group and corresponding t value

		Knowledge Score				paired t test		
		Pre-test Mean and SD		Post-test Mean and SD				
Group	N	Mean pre- test score	SD	Mean post- test score	SD	df	‘t’value	Result
Experimental Group	30	12.90	2.537	18.40	2.762	29	10.483	Significant
Control Group	30	12.633	2.684	11.63	4.406	29	1.258	Not Significant
Unpaired ‘t’ Test	Df	58		Df	58			
	Value of ‘t’	0.395		Value of ‘t’	7.127			
	Result	Not Significant		Result	Significant			

Table 3.2 Mean Pre-test and Post-test practice score regarding BLS among experimental and control group.

Group	N	Mean Practice Score Pre-test (practice)		Mean Score Post-test (practice)		Paired 't' Test		
		Mean practice score	SD	Mean practice score	SD	df	Value of 't'	Result
Experimental group	30	4.50	1.737	10.00	1.819	29	17.343	*Significant
Control group	30	5.267	1.230	5.27	1.507	29	0.000	Not Significant
	df	58		df	58			
	Value of 't'	1.973		Value of 't'	10.974			
	Result	Not Significant		Result	*Significant			

To compare the mean pre-test and post-test knowledge score between two groups, unpaired t-test was conducted where results showed that mean score for experimental group

was high i.e. 18.40 in post-test knowledge as compared to pre-test knowledge while in control group, pre-knowledge score was high i.e. 12.63 as compared to post-knowledge

score. Hence the Simulation as a technique enhanced the knowledge regarding BLS among B.Sc. Nursing 1st year students. These findings were found congruent with study done by Kose et al. (2019) on nursing students reported results in line with the present study that simulation is effective in improving knowledge and practice of basic life support in nursing students. The findings were also supported by the study done by Daniel and Evangeline (2018) to teach Basic life support to medical students using simulation was found effective and supports the findings of this study (Daniel and Evangeline 2018; Kose et al. 2019).

In Table 3.2 to compare the practice score within the group, paired t-test has been conducted where results revealed that mean score of post-test from experimental group is high as compared to post-test from control group and mean score of post-test was also high from pre-test of control group. To compare the pre and post-practice score between the groups, unpaired t-test was conducted where results revealed that mean score for experimental group was high i.e. 10.00 in post-practice as compared to pre-practice while in control group.

These Findings were found congruent with the study done by Aqel and Ahmad (2014) and Tawalbeh and Tubaishat (2014) obtained statistically significant results for clinical simulation in CPR as compared to other technique and thus supported the findings of the present research and thus created the evidence to promote the use of simulation in nursing education. Recent research conducted by Sandy et al. (2021) reported that there was an increase in clinical simulation of paramedics bridged the lacuna between theory and practice to deal with emergency situations. It was proved by their enhanced satisfaction and confidence level after undergoing simulation training. (Aqel and Ahmad 2014; Tawalbeh and Tubaishat 2014; Sandy et al. 2021).

CONCLUSION

The findings of the present study concludes that Simulation on Basic life support was effective in improving knowledge and practice of B.Sc. nursing 1st year student. In pre- test the experimental group, 3% students had good knowledge and 0% students had good practice, 90% had average knowledge and 60% had average practice and 7% had poor knowledge and 40% had poor practice. While in post-test, of experimental group 53% had good knowledge and 73% had good practice and 47% had average knowledge score and 27% had average practice. This showed that there was significant improvement in knowledge and practice of students.

Conflicts of interests: Authors have no conflict of interests to disclose.

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Agricultural Communication

Substantiation of Priority Areas to Ensure Food Security in the Russian Federation: A System-Cognitive Analysis

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ABSTRACT

Currently, the Russian population's need for vegetables is satisfied due to domestic production only by 87percent (at a rate of at least 90percent), fruits and berries – by 40percent (at a rate of at least 60percent), and milk – by 84percent (at a rate of at least 90percent). The present work clarifies the defining role of agricultural production taking into account the calculated indicators of food security. The analysis of the population's provision with basic foodstuffs has led to the need to strengthen the competitiveness of Russian agricultural producers. The article predicts managerial decisions that would allow improving the performance efficiency of economic entities on the example of the agricultural sector of the Krasnodar Territory. The article presents an automated system-cognitive analysis of the effect of various factors on the efficiency of agricultural organizations. The degree and areas of the influence of various factors were assessed using SWOT analysis based on empirical data, namely, financial and economic indicators of agricultural enterprises of the Krasnodar Territory. The results of the automated system-cognitive analysis confirmed that subsidies for reimbursement of part of the costs of purchasing agricultural machinery and equipment, as well as subsidies aimed at increasing the productivity of farm animals have a significant impact on profits in the production of livestock products. The problem of modernization of agricultural production is associated with the problem of information and consulting services of agribusiness. The article substantiates the necessity of preparing a spiral scheme of the food security model, as well as emphasizes the importance of establishing and developing various service-providing consulting centers.

KEY WORDS: FOOD SECURITY, FOOD SELF-SUFFICIENCY, INNOVATION, STATE SUPPORT, STRUCTURAL TRANSFORMATION OF AGRIBUSINESS.

INTRODUCTION

Food security should be considered as the most important priority task facing Russia. The solution to this problem is mainly associated with eliminating the negative effects of earlier agrarian reforms, updating the economic, technical, and technological potentials of economic entities, and increasing the competitiveness of Russian producers. Despite the implementation of the state policy of import substitution, the risk of reducing Russia's food security remains. The current economic situation in the Russian agro-food market requires urgent measures that can support the agro-industrial sector of the economy. The competitiveness of Russian food producers is weakened due to the dominance

of foreign goods, as well as price disparity (Gaiduk et al. 2017; Gaiduk et al. 2018; Gaiduk et al. 2020a).

Keyzer and Wesenbeeck (2007) point out that "implementing economic, organizational, and legislative measures will ensure an increase in the level of food security, namely, will improve the general conditions for the functioning of agriculture, especially animal husbandry, create prerequisites for sustainable development of rural areas, increase the efficiency of land use and its reproduction, ensure the development of agricultural technologies and increase the competitiveness of agriculture" (Keyzer and van Wesenbeeck 2007). The current research is aimed at developing and substantiating scientific and practical recommendations towards developing managerial decisions and forecasting measures in the agricultural sector of the economy in the food security system. The solution to many problems of food security in various regions is not only

Article Information:*Corresponding Author: bcintermarket@yandex.ru

Received 12/10/2021 Accepted after revision 15/12/2021

Published: 31st December 2021 Pp- 1806-1812

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.63>

theoretical but also, above all, a practical issue (Gaiduk et al. 2020b).

The reasons for the lack of confidence that such security will be ensured are the insufficient theoretical elaboration of the problems, the need to clarify the specific scientific, methodological, and legal support of the comprehensive state measures in the agricultural sector. The works of many researchers are devoted to solving the problems facing agriculture, creating organizational and production structures within the framework of achieving food security (Pingali 2005; Keyzer 2007; Fischer et al. 2008; Alston et al. 2009; Diouf 2009; Campbell 2009; Altieri et al. 2012; Charles et al. 2014; Castro and Chirinos 2015; Buks et al. 2016; Pérez-Escamilla et al. 2017; Cole 2018). According to Cole et al. (2018), "massive food imports into a country with significant production potential destroys not only domestic production, poses a threat of its deficit in the future, but also ruinously affects the overall balance of payments. Food imports are paid for by massive exports of raw materials and energy carriers, and its increase, respectively, will cause the need to increase exports, significantly reducing other import opportunities" (Cole et al. 2018).

MATERIAL AND METHODS

Studying the agricultural sector of the economy in the food security system was carried out on the basis of conducting cluster-constructive analysis of classes and factors, their meaningful comparison, and studying the system of determination of states of the simulated object (Lutsenko and Korzhakov 2011; Loiko et al. 2012).

RESULTS AND DISCUSSION

When ensuring food security, the state should consider qualitative characteristics and ensure the following: effective development of the agribusiness; foreign economic activity in the agricultural sector; income of the population; balanced diet. As a rule, indicators of economic and physical availability of food, threshold values of food independence, and compliance of food products with the requirements of the legislation of the Eurasian Economic Union on technical regulation are used as indicators for assessing food security (Trubilin et al. 2020).

Table 1. Food independence of Russia by main types of activity

Product type	The threshold indicator in the Food Security Doctrine, percent	2015	2016	2017	2018	2019
Meat	not less than 85	88.7	90.6	93.5	95.7	97.4
Food grains	not less than 95	149.0	160.0	171.0	148.0	154.0
Milk	not less than 90	79.9	80.7	82.3	83.9	83.9
Potato	not less than 95	102.1	93.2	91.1	95.3	95.1
Vegetables and cucurbits	not less than 90	86.8	87.4	87.6	87.2	87.7
Fruits and berries	not less than 60	32.5	36.5	33.1	38.8	40.2

Source: Data of the Federal State Statistics Service (2020).

According to the Decree of the President of the Russian Federation dated January 21, (2020), No. 20 "On the approval of the Food Security Doctrine of the Russian Federation", thresholds of food independence for meat and meat products (in terms of meat) are at least 85percent (The decree of the President of the Russian Federation of January 21 2020); for milk and dairy products (in terms of milk) – at least 90percent (Table 1). Currently, the Russian population's need for vegetables is met by only 87 percent due to domestic production, fruits and berries – by 40percent, milk and dairy products – by 84percent. The current economic situation in the Russian food market requires urgent measures that will be able to support the agro-industrial sector of the economy (Trubilin et al. 2020).

The level of self-sufficiency in basic foodstuffs of the country's population is growing (Table 2). This trend is due to the state policy aimed at import substitution, as a result

of the embargo imposed in response to the sanctions of the EU and the USA. Currently, growth in the production of crop and livestock products is unstable. The restrictions on imported products in the context of economic sanctions contribute to the expansion of the market niche of Russian commodity producers in the domestic market due to the growth in production volumes. We consider it necessary to predict managerial decisions that would improve the efficiency of economic entities drawing on the example of the agricultural sector of the Krasnodar Territory (Gaiduk et al. 2017; Trubilin et al. 2020).

It is important to simulate the impact of factors (financial, economic, natural, and energy) on the results of the development of the agro-food sector in the context of food security. It is proposed to solve the problem of decision-making when choosing main paths for increasing the efficiency of the agricultural sector based on automated system-cognitive analysis (ASC-analysis), of the Eidos

system software product. The ASC analysis allows identifying the behavior of a multiparametric system under the impact of factors measured in various types of scales and measurement units. The financial and economic indicators

of 658 agricultural organizations of the Krasnodar Territory for 2019 were taken into account in the calculations (Gaiduk et al. 2017; Trubilin et al. 2020).

Table 2. Indicators of Russia's food security in 2020

Products	Food independence in 2020, percent	Threshold indicator of the Doctrine, percent	Deviation of the actual value from the threshold indicator of the Doctrine
Food grains	167.6	93.1	2.2 times higher
Sugar	99.9	not less than 90	1.8 times higher
Vegetable oil	195.9	not less than 90	2.2 times higher
Meat and meat products	99.4	not less than 85	higher by 14.4 p.p.
Fish and fish products	149.7	not less than 85	1.8 times higher
Potato	86.1	not less than 95	lower by 8.9 p.p.
Milk and dairy products	84.1	not less than 90	lower by 5.9 p.p.
Edible salt	65.9	not less than 85	lower by 19.1 p.p.
Vegetables and cucurbits	87.1	not less than 90	lower by 2.9 p.p.
Fruits and berries	41.2	not less than 60	1.8 times lower

Source: Data from the Final Report of the Ministry of Agriculture of the Russian Federation (2021)

At that, the following factor indicators were used: profit from the sale of livestock products, thousand rubles; profitability of livestock products, percent; revenue from livestock products, thousand rubles; net profit (loss), thousand rubles; return on sales, percent; profitability of core activities, percent; return on production assets, percent; capital-labor ratio, a thousand rubs.; the real volume of capital equipment per unit of labor, thousand rubles; capital-output ratio, rub.; material productivity, rub.; cost recovery coefficient; depreciation coefficient of fixed assets; fixed assets renewal coefficient; fixed assets suitability coefficient; annual labor productivity, thousand rubs.; energy intensity, rub.; hourly labor productivity, rub.; the proportion of arable land in the total area of agricultural land, percent. When preparing a formal model of classes and attributes, classification scales and gradations were used (Trubilin et al. 2020).

In the calculations when carrying out the ASC-analysis, the effect of the following parameters was taken into account: the total cost of livestock production, thousand rub.; annual cow population, heads; average annual pig population, heads; the average annual population of animals in raising and fattening, heads; average annual population of mature hens, thousand heads; the average annual population of young chickens in raising, thousand heads; the main herd of dairy cattle, heads; agricultural land, ha; arable land, ha; costs of the main production, thousand rub.; material costs, thousand rub.; the average annual cost of fixed assets, thousand rub.; the average annual number of employees, people; number of workers employed in agricultural production, people; work effort by workers employed in all industries, thousand staff-hours; energy capacities, h.p.; labor costs, thousand rub.; depreciation, thousand rub.; cost of fixed assets, thousand rub.; commercial and management expenses, thousand rub.; and cost of crop production, thousand rub (Trubilin et al. 2020). Besides, the following

indicators were used when preparing descriptive scales and gradations:

- the amount of support agribusiness producers, thousand rubles;
- subsidies from the budget of the entity of the Russian Federation per employee, thousand rubles;
- targeted subsidies for implementing regional programs on agribusiness development, thousand rubles;
- supporting programs and activities in the field of animal husbandry, thousand rubles;
- supporting short-term lending in the agribusiness sector, including lending to small farms, thousand rubles;
- supporting small businesses (grants), thousand rubles;
- supporting farmers, thousand rubles;
- supporting agricultural consumer cooperatives, thousand rubles;
- subsidies to increase the productivity in dairy cattle, thousand rubles;
- subsidies for compensation of interest rates on investment loans in the agribusiness sector, thousand rubles;
- subsidies for compensation of part of the direct costs incurred for creating and modernizing agriculture, thousand rubles;
- The Federal Target Program "Sustainable development of rural territories for 2014-2017 and the period up to 2020", thousand RUB;
- programs and activities under other state programs, federal target program, and other subsidies with co-financing from the federal budget (except the Ministry of Agriculture of Russia), thousand rubles.
- supporting programs and activities in the field of crop production, thousand rubles;

- supporting programs and activities in the field of crop production per employee, thousand rubles;
- subsidies for providing unrelated support in the field of crop production and seed potatoes production, as well as seeds and vegetables of outdoor growing, thousand rubles.

The results of the ASC analysis confirm that subsidies for reimbursement of part of the costs of purchasing agricultural machinery and equipment have a significant impact on profits in the production of livestock products (the greatest impact of all factors, equal to 3.053). Subsidies, aimed at increasing productivity are in second place in terms of impact (the impact of all factors, equals 2.256). According to the results of the ASC analysis, it was revealed that the profitability of agricultural products, taking into account subsidies, was 25.4 percent. Note that in the first place by the negative impact on performance indicators is the total revenue from the sale of crop products (the impact of all factors is 4.4635). Thus, Russia has become one of the largest importers of meat products. And it is quite obvious that an increase in the share of crop production in the structure of production does not solve, but, on the contrary, aggravates the food problem. Therefore, it is necessary to develop the production of livestock products, thereby getting rid of the structural imbalance in the agricultural sector, and above all, in regions, such as the Krasnodar Territory (Gaiduk et al. 2020).

The pre-reform development model of the livestock industry formulated as "increase in livestock is the increase in production" is excluded today: the market does not forgive the irrational, inefficient, uncontrolled use of production factors. Only modern innovative technologies based on advanced experience and achievements of science and technology, and providing high productivity can be a turnaround for animal husbandry. Depreciation on the main production is in second place in terms of the negative impact on performance indicators (the impact of all factors is 4.626). Currently, measures to improve the material and technical equipment of agriculture are provided for by both federal and regional target programs and projects. The main stake in the issue of technical and technological modernization of agriculture is made on subsidizing investment loans and borrowings. Thus, the excessively high cost of borrowed funds is compensated (Gaiduk et al. 2020).

However, there is another even more acute problem – the excessively high cost of agricultural machinery, which should be solved similarly – by providing subsidies to reimburse part of the costs of purchasing agricultural machinery and equipment. The cluster analysis was carried out in a cognitive space in which one unit of measurement is used for all axes (descriptive scales) – the amount of information, rather than based on the initial variables or the conjugate matrix depending on the units of measurement along the axes. Clustering results do not depend on the initial units of measurement of objects' features (Lutsenko and Korzhakov 2011; Loiko et al. 2012; Gaiduk et al. 2020).

The dendrogram of cognitive clustering of classes reflects the similarity-difference between different groups of

agricultural enterprises in terms of profit. Thus, some of the agricultural enterprises of group I are very similar to group II, which has received the minimum amount of subsidies to the livestock industry, and they form a cluster, which is opposed to the cluster of groups III and IV and a large number of subsidies. In 2020, the amount of financing of the state program of the Krasnodar Territory "Development of agriculture and regulation of agricultural products, raw materials, and food markets" at the expense of the federal and regional budgets amounted to more than 8304.2 mln rubles (Gaiduk et al. 2017). Within the framework of the State Program, funds were allocated for the development of agricultural land reclamation, support for farmers, development of the fisheries complex, agricultural industries, stimulation of investment activity, integrated development of rural areas, etc. However, financing, allocated for the transition of the agribusiness to a new development model is not enough. Studies have shown that integration processes in agricultural production, namely, the addition of industrial processing of milk and meat, contribute to reaching higher efficiency indicators (Trubilin et al. 2020).

It is important to develop a model, whose implementation would give the maximum effect in the regional agribusiness in matters of ensuring food security and self-sufficiency. At the same time, important attention should be paid to the possibility of implementing these measures directly by the region itself. The expediency of using a spiral scheme is explained by the fact that it allows monitoring the food security status more effectively since, in the course of constant circulation of model elements, new problems and threats to the food security of the region will open up. In approximately the same way, new measures of influence should be determined to eliminate these problems in this area and the system of indicators based on which monitoring is carried out should be supplemented (Trubilin et al. 2020). This method should be based on the following areas of activity:

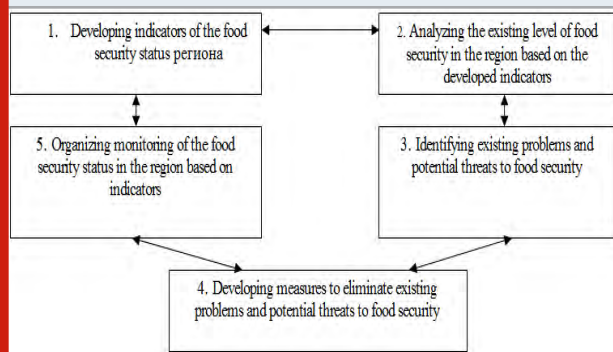
- adjusting the monitoring system of the regional food market as an important component of the state mechanism;
- using operational monitoring services at the local, regional, national, and interstate levels by numerous market participants that will allow free orientation in the price system, and the state will use mechanisms to influence pricing, supply, and demand;
- improving the management system of socio-economic development of agriculture in the region providing control over the impact of the most important economic regulators on the economic results;

The food security model, presented below, should be taken as the basis for the development of measures for active influence in the agribusiness sectors (Fig. 1).

The problem of modernization of agricultural production is associated with the problem of information and consulting services of agribusiness. The lack of unified information space in the field of agriculture makes it difficult for commodity producers to access the necessary information.

Besides, the enterprise executives often do not recognize the important role of the information factor in the development of production, and many of them, due to low qualifications, cannot obtain the necessary information (Sekerin et al. 2021).

Figure 1: Spiral diagram of the food security model of the region



Various consulting centers providing services and agro-consulting agencies have already been established and currently are operating in Russia. At present, it is necessary to unite them into single powerful centers. Functional integration into a single system (service) will require transferring from administration to consulting activities within the framework of the administrative reform of district and regional agriculture administrations. It is necessary to establish their strong cooperation with information and consulting organizations of universities and scientific institutions, as well as private agro-consulting agencies. At the regional level, there are no centers of consolidation of existing economic structures, land users, and owners, necessary for a comprehensive solution to the problem of agricultural development. In our opinion, at this stage of the agrarian reform, it is possible to create a proposed regional service of scientific and consulting services based on agricultural producers (Sekerin et al. 2021).

The development of software for the system of scientific and consulting services for the agribusiness of the region should be determined by the tasks of this system. Its functions can be generically divided into two groups:

1. providing users with information based on which they could make their own decisions (Golubev et al. 2021);
2. consulting and providing to the user decision-making options based on expert assessments and forecasts.

This will allow improving the quality of information and consulting services for agricultural producers and the population of the region will be improved, as well as providing effective training of agricultural specialists and the enhancing effectiveness of scientific research.

Organizational structures of economic management of agricultural sector enterprises are formed independently, depending on the business patterns, organization of production, marketing, and financial activities. The activity

of state and public administration bodies in the regions is manifested in the performance of regulatory, consulting, and orienting (advisory), control, and inspection (supervisory) functions. At that, the main regulation object is the markets of food, resources, and production services for the village. The main objects of support are agricultural producers, and the main objects of inspection supervision are the activities of participants in agricultural production and operators of the food, resources, and production services market. The essential features that characterize the development dynamics of the agribusiness, forming the conditions for ensuring food security are its structure-forming factors (Sekerin et al. 2021). The following measures are necessary to overcome the structural deformations of the agribusiness.

1. Development of the Concept of structural transformation of the agribusiness on an innovative basis, focused on ensuring food security. The main components of this concept should become:

- the essence of innovative transformations in the agribusiness to ensure food security;
- their goals (with disaggregation by strategic and tactical criteria);
- priorities (with a clear definition of their ranking: recognition of achieving social benefits from innovative structural transformations as a major task).

2. Regulatory and legal support of structural transformations in agribusiness on an innovative basis as a condition for ensuring food security.

Within the framework of the Program, it is necessary to develop appropriate regional programs for each region of the country. These programs should be interconnected methodically, structurally, and organizationally in achieving goals and implementation mechanisms. The prerequisite for the program implementation at both the federal and regional levels are:

- determination of the dynamics of effective demand of the country and regions, as well as the elasticity of demand for basic foodstuffs at the appropriate levels;
- determination of rational and critical production volumes of basic products, taking into account the state and dynamics of changes in the production and resource potential of the country and regions in the medium term, as well as the place and role of regions in the system of the national differentiation of labor.

The development and implementation of programs should be aimed at optimizing intersectoral relations and developing models of intersectoral balance within regions, food subcomplexes, and a single economic complex as a whole, as well as solving relevant problems in the field of foreign economic activity. It is necessary to define clear basic provisions of administrative reform to establish and consolidate the powers of national, regional, and local authorities to ensure food security of the country and regions.

3. Determining the Program implementation mechanism, choosing indicative planning for this purpose. Its essence is revealed in the following three mutually dependent components:

- determining socio-economic priorities within the agribusiness in the context of achieving food security of the country and regions;
- forecasting the proportions between the agribusiness sectors, their areas, and product subcomplexes embodied in the system of indicators;
- unbundling plan indicators by industry, food, and regional characteristics and their concord at the final stage.

The basis for the development of indicative plans for the development of the agribusiness of the country and regions, which should be considered as the basis for implementing the socio-economic development strategy of the country and regions, should be indicators reflecting rational and critical production of basic food products at appropriate levels. The development and implementation of indicative plans presuppose taking into account both the total economic and technical effects, social interaction of agribusiness sectors and regions, in particular, the effect of interregional cooperation. In the current context, it is necessary to develop a definite social policy in the agricultural sector. Among these policy goals, first of all, it is necessary to highlight:

- a significant increase in the material well-being of the population, living conditions, and the restoration of social infrastructure in rural areas (for the construction of non-industrial facilities, it is necessary to legislate the use of part of public investments that are planned to be invested in the agribusiness);
- increasing the cost, quality, competitiveness, and mobility of the workforce, optimizing its gender, age, and professional qualification structure;
- stabilizing the rural population size to overcome reducing demographic reproduction in rural areas.

CONCLUSION

The findings of the present study confirmed the need to prepare a spiral scheme of the food security model. This method should be based on the adjusting the monitoring system of the regional food market, using operational services, improving the system of socio-economic development of agriculture, strengthening state management of the reform process and control over the action and influence on the economic results of economic regulators. Functional integration into a single system (service) will require transferring from administration to consulting activities within the framework of the administrative reform of district and regional agriculture administrations. It is necessary to establish their strong cooperation with information and consulting organizations of universities and scientific institutions, as well as private agro-consulting agencies.

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Microbiological Communication

Comparative Investigation on Micro-Structural, Morphological, Optical, Magnetic and Anti-Microbial Traits of Undoped and Nickel-Incorporated Zinc Oxide Nanoparticles

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ABSTRACT

The main objective of the present work is to synthesize pure and nickel doped zinc oxide nanoparticles by facile co precipitation technique. The work is confined to study the effect of various weight ratios (0.3, 0.6, 0.9) % Nickel into Zinc oxide and to witness the drastic changes that occur in its various physical properties such as structural, optical, magnetic from X ray diffraction (XRD), UV visible (ultra violet) spectra, VSM (Vibrating sample magneto meter). XRD analysis reveals the wurtzite hexagonal structure and it is also found that as the doping concentration increases the crystallite size decreases from 4.6 nm to 3.0 nm. SEM results depicts the agglomeration of the particle, the synthesized samples shows both rod and flakes formation when the doping concentration is increased. Morphological changes were analysed TEM (Transmission electron microscope). The enhancement in the optical behaviour were observed and the energy band gap is calculated with the data obtained from UV-Visible spectra and the optical properties shows a tremendous increase as the Ni content increases which proves the sample a suitable candidate for solar cells and photovoltaic devices. Purity of the prepared sample were investigated through EDAX analysis. The hysteresis loop from the VSM analysis elucidate the saturation magnetization and the ferromagnetic behaviour of the sample. X-ray Photoemission Spectroscopy results indicates the presence of several oxygen species adsorbed on the surface. The study is also extended to analyse its anti-microbial effect against *Staphylococcus aureus*. The cell culture dish of the sample showed a notable resistance against *Staphylococcus aureus* when the concentration of nickel is increased and could be extended to pharmaceutical applications in treating several skin infections.

KEY WORDS: ANTI- MICROBIAL, CO-PRECIPITATION, NICKEL DOPING, OPTICAL PROPERTY, PHOTOVOLTAIC DEVICES.

INTRODUCTION

Over few years the usage of nanoparticles based on metals and their oxides have drawn a great interest almost in all the fields. Zinc oxide is a multifunctional II-VI group semiconductor grabbing a centre of attention to researchers due to its fascinating properties like wide band gap of 3.37 eV at room temperature, large exciton binding energy (60 meV), high optical transparency, high surface stability, large electrochemical coupling coefficient and strong excitonic emission etc. It is obvious that both Ni and Zn have the same valence with same structure which is because the ionic radii

of both Ni²⁺ (0.69 Å) and Zn²⁺ (0.74 Å) are very closer (Perna et al. 2020; Gudkov et al. 2021).

It is reported that Ni²⁺ could be easily replaced by Zn²⁺ in Zinc oxide lattice without changing its hexagonal structure, thus making Nickel doped Zinc oxide nanoparticles a promising material in the field of optoelectronic devices with its enormous optical and magnetic properties (Chattopadhyay et al. 2019; Ali et al. 2020). Observing at this point of view, this Zinc oxide which is in the form nanostructured powders could exhibit great efficiency in innumerable performances including gas sensors, solar cells and photo catalyst with high chemical activity. Generally the oxygen deficient off-stoichiometric of Zinc oxide possess a wide n-type conductivity, but a tremendous enhancement in its conductivity is noticed when dopant is added (Akdağ et al. 2016; Anandan et al. 2016; Uma et al. 2019).

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Received 15/09/2021 Accepted after revision 15/12/2021

Published: 31st December 2021 Pp- 1813-1820

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.64>

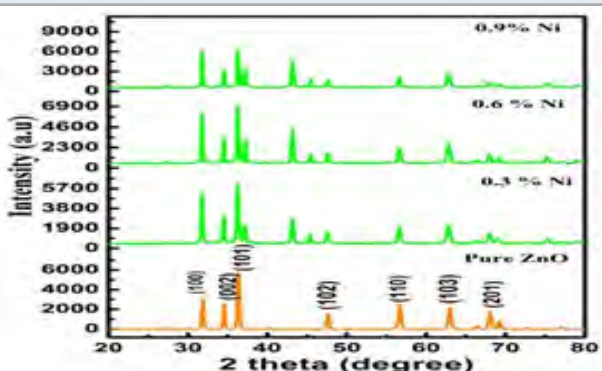
Here in this investigation undoped and nickel doped Zinc oxide nanoparticles were actualized by a very simple yet effective coprecipitation method. The main objective of this work is to study the morphological, optical and ferromagnetic behaviour of undoped and Nickel doped Zinc oxide nanoparticles with variation in the doping concentration (Ahmad 2019; Ali et al. 2020).

MATERIAL AND METHODS

Analytical grade reagents of Zinc acetate dihydrate $\text{Zn}(\text{CH}_3\text{CO}_2)_2$, sodium hydroxide (NaOH), and Nickel chloride (NiCl_2) with purity of 99.7%, were used in the formation of undoped and Nickel doped zinc oxide nanoparticles. During the process, the stemware was well covered in order to prevent contamination and evaporation of ethanol. A solution of 0.5 mole $\text{Zn}(\text{CH}_3\text{CO}_2)_2$ was prepared by dissolving (54.877g) in 450 ml of double distilled water and 0.5 mole of NaOH prepared by dissolving (20g) in 50 ml of double distilled water. The so formed solution of NaOH was introduced into the $\text{Zn}(\text{CH}_3\text{CO}_2)_2$ solution drop-wisely under constant strenuous stirring to avoid cluster formation. The impurities in the precipitate were separated by rinsing repeatedly with double distilled water and annealed at 600 °C for two hours in a muffle furnace to acquire the undoped Zinc oxide nanoparticles.

Nickel doped Zinc oxide nanoparticles were formed by taking equal weight of $\text{Zn}(\text{CH}_3\text{CO}_2)_2$ and NaOH were dissolved in 450 ml of double distilled water. Nickel II chloride ($\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$) were taken in the varying concentration (0.3, 0.6, 0.9 %) was dissolved separately in 50 ml of water and then added to the solution containing Zinc acetate dihydrate $\text{Zn}(\text{CH}_3\text{CO}_2)_2$ and NaOH. The final solution was then allowed to remain in constant stirring for 2 hours. Then left overnight for sedimentation. Then the solution was carefully removed and subjected to centrifuge for further removal of impurity. The powder was annealed at a temperature of 600 °C in muffle furnace for 1 hour.

Figure 1: XRD pattern of undoped and Nickel doped Zinc oxide nanoparticles



For the characterization techniques, the formation of the hexagonal wurzite structure of the prepared particles were determined by X-ray diffractometer. Surface morphology were visualized by both SEM and TEM. The spectra of the photocatalysis absorbance were measured by a UV-Vis

spectrophotometer. Elemental composition, chemical and electronic state exist within the material were found by XPS and EDAX studies. The ferromagnetic behaviour was found from VSM analysis.

RESULTS AND DISCUSSION

XRD Analysis: The hexagonal wurtzite structure of the so prepared nanoparticles was confirmed by the XRD pattern and crystallite size was estimated by Debye-Scherrer method. Figure 1 exhibits the X-ray pattern of undoped and Nickel doped Zinc oxide nanoparticles.

XRD pattern shows that all the obtained peaks of Nickel substituted Zinc oxide nano crystals were incredibly matches well with the Joint Committee on Powder diffraction Standards (JCPDS) for ZnO (Card No. 36-1451, $a = b = 3.2498 \text{ \AA}$ and $c = 5.2066 \text{ \AA}$) which could be indexed as the hexagonal wurtzite structure of Zinc oxide. As the doping concentration was increased the intensity and variation of the position peak were also increased. This changes in peaks position indicates that nickel is substituted in the Zinc oxide lattice without any impurity phase (Danial 2020; Al-Ariki et al. 2021).

But the inclusion of the dopants produced defects in the crystal lattice of Zinc oxide which resulted in its change in size of the sample. The average crystallite sizes (D) of the prepared nanoparticles were calculated by Scherrer's equation

$$D = \frac{0.9 \lambda}{B \cos \theta} \quad (1)$$

where 'D' implies the crystallite size, ' λ ' implies the wavelength of radiation exposed, ' θ ' implies the Bragg angle and 'B' implies full width at half maxima (Belkhaoui et al. 2019; Priya et al. 2021).

It is seen from the above figure 1 that all the prepared nanoparticles have their dominant peak congruent to (102). To calculate the crystallite size three predominant peaks (002), (101), (102) were considered. The crystallite size of undoped and Nickel doped Zinc oxide is tabulated in table 1. It is worthy to note that the diffraction peaks get more intense at the same time becomes narrower as the annealing temperatures was increased indicating the formation of a good crystalline structure. It is also evident that the crystallite size was increased as the concentration of the dopants was raised which proved the impact of the dopants (Wang 2019).

Optical properties: UV-Visible Analysis: Ultra Violet-Vis analysis was used to measure the diminution of a beam of light that occurred after passing through or gets reflected back from a sample surface. Nickel doped Zinc oxide showed higher transparency in the visible regions at around 480 nm and the lowest transparency in wavelength smaller than 380 nm in comparison to the undoped and Nickel doped Zinc oxide nanoparticles. While comparing all samples, Nickel doped Zinc oxide nanoparticles exhibited

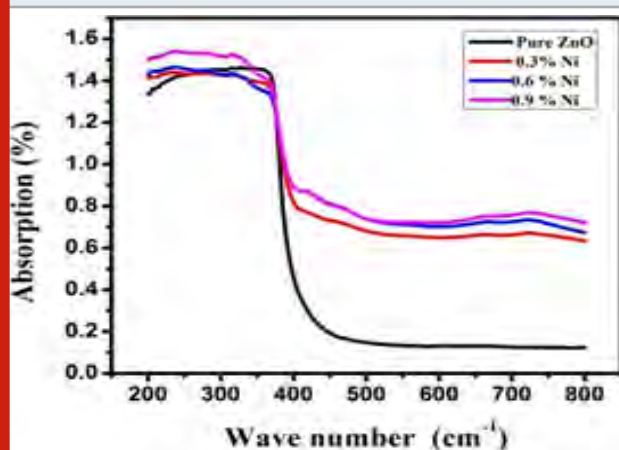
the highest absorption within the wavelength smaller than 400 nm. The absorption spectra of undoped and Nickel

doped zinc oxide nanoparticles is shown in Figure. 2 (Nallusamy and Nammalvar 2019).

Table 1. Crystallite size of pure and Ni doped ZnO nanoparticles

Compound	Annealing Temperature °C	d spacing	θ (radians)	crystallite size(nm)
Pure ZnO	600 °C	2.3333	0.32375	4.68394E-08
Ni (0.3%)		2.3333	0.3235	3.93042E-08
Ni (0.6%)		2.6666	0.2981	3.80981E-08
Ni (0.9%)		2.8808	0.27143	3.04412E-08

Figure 3: Absorption spectra of undoped and Nickel doped Zinc oxide nanoparticles

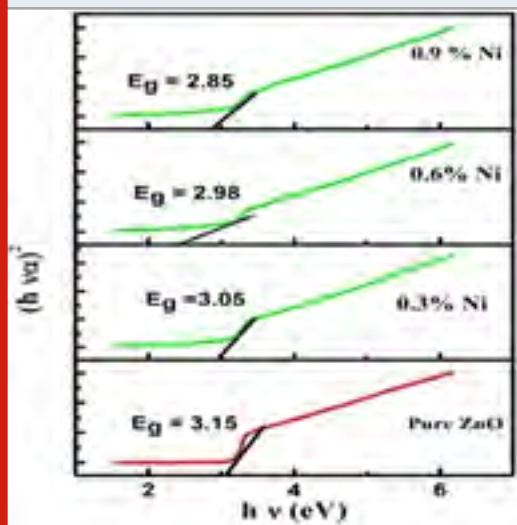


colour exhibited by the semiconductor nanoparticles would be usually due to the to this surface plasmon resonance. Thus by subjecting the sample to UV-Vis spectroscopy upon chromatographic separation, the unique optical properties could be detected and the formation of the nanoparticles could also be found out (Ghosh 2019).

The band gap was calculated by plotting $(\alpha h\nu)^2$ versus the photon energy ($h\nu$), and by extrapolating the intercept of the curve to zero absorption in the photon energy axis which is shown in Figure 3(b). The band gap energies of Nickel doped Zinc oxide nanoparticles were found to be smaller than that of undoped Zinc oxide nanopowder which may be due to the effect of Nickel concentrations (Vidhya et al. 2021).

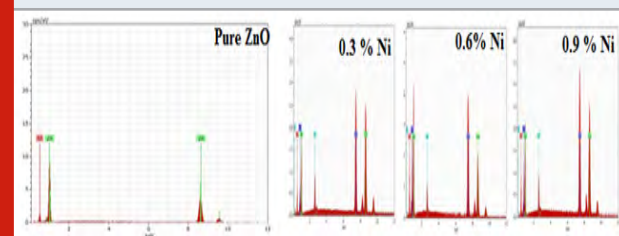
EDAX Analysis: The elemental composition of the synthesized sample of undoped and Nickel doped Zinc oxide was determined by EDAX spectra and shown in Figure4.

Figure 3(b): Band gap of pure and Ni doped ZnO nanoparticles



When the sample was brought under the influence of the electromagnetic field, the electron presents in the valence band experienced coherent oscillation, which in turn triggered the metallic nanoparticles to absorb electromagnetic radiations. This resonance is known as surface plasmons which occur only with nanoparticles and varies accordingly to its formations. Thus the size and the

Figure 4: EDAX of undoped and Nickel doped Zinc oxide nanoparticles

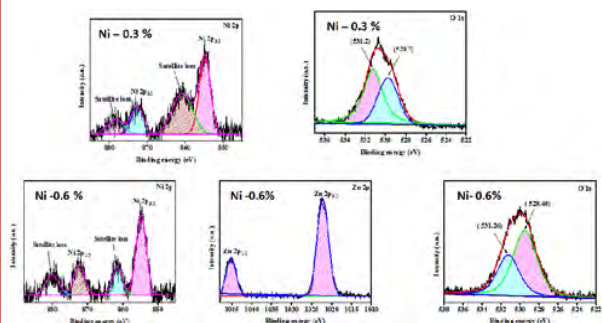


The existence of zinc, oxygen, Nickel and other elements without any impurity was confirmed from the above Figure 4. The pattern showed that the prepared Zinc oxide sample had the elemental composition of Nickel doped Zinc oxide nanoparticles shown in Table 3 (Kumari et al. 2021).

XPS Analysis: XPS analysis is a perceptive characterization approach for reviewing the chemical constitution and the valence states of substances embodied in the prepared nanoparticles. Figure 5 shows the survey spectrum and core level spectrum of Zn and O atoms of Nickel doped Zinc oxide nanoparticles, for 0.3% and 0.6 % concentration respectively. The XPS peak of Nickel doped Zinc oxide nanoparticles is featured in Figure 5.

Table 3. Elemental composition of undoped and Nickel doped Zinc oxide nanoparticles

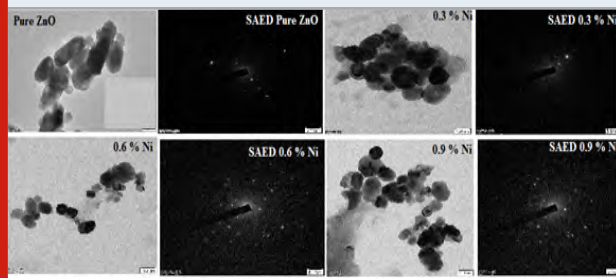
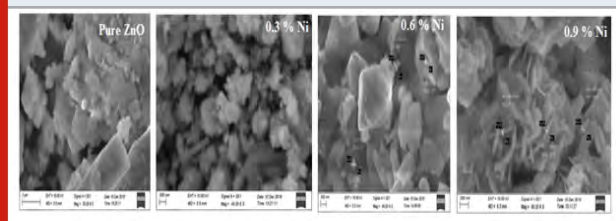
Annealing T° C 600°C	Element	Series	Atomic Weight (Weight %)	Uncertainty Count (Weight %)	K factor (Weight %)
Pure ZnO	O		62.46	28.93	5.57
	Zn		37.54	71.07	1.79
0.3 % of Ni	Zn	K- Series	35.56	51.66	1.52
	O		34.38	12.22	3.96
	Ni		24.13	31.46	0.94
	Cl		5.92	4.66	0.28
0.6 % of Ni		K- Series			
	O		58.50	27.48	6.58
	Ni		21.56	37.55	1.05
	Zn		16.64	27.12	1.05
0.9 % of Ni			3.29	2.91	0.23
	O		38.86	14.50	4.69
	Zn		30.37	46.31	1.47
	Ni		25.37	34.73	1.07
	Cl		5.40	4.47	0.29

Figure 5: XPS peak of Nickel doped Zinc oxide nanoparticles

Doublet peak shown in the Figure 5 which corresponds to the core level spectra of $Zn2p_{1/2}$ and $Zn2p_{3/2}$ ions of ions is shown. The binding energy of $Zn2p_{3/2}$ and $Zn2p_{1/2}$ orbitals appeared at (531.2 eV, 529.7 eV), (531.26 eV, 529.46 eV) which corresponded to ZFLi, ZFNa and ZFK NPs. The doublet peak of the binding energy was transfigured towards the lower energy region when Nickel dopant was added. Thus, the divalent state of Zn ions was confirmed by the difference in the position of the doublet peaks from which the spin split value was calculated (Kazhen et al. 2020; Maleki et al. 2021).

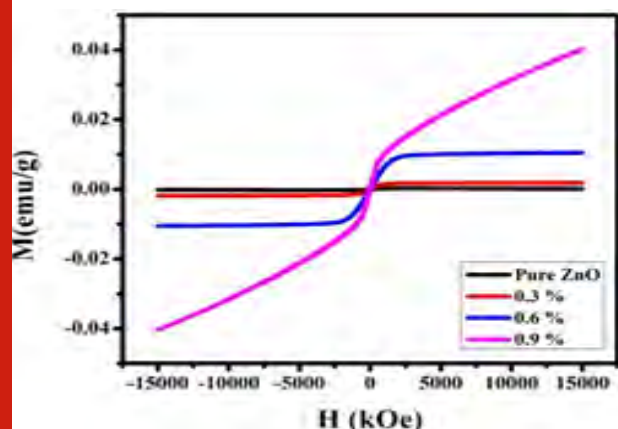
Morphology Analysis: The morphology of $ZnO-xNi$ ($x = 0.3, 0.6$ and 0.9) as-synthesized nanocrystals were investigated with High resolution transmission electron microscopy (HR-TEM) and shown in Figure 6. Selected area electron diffraction (SAED) patterns were also presented. HR-TEM images displayed two types of morphologies, slightly rectangular and cubic. The

nanoparticles were arbitrarily selected from the micrograph in order to measure the particle-size distribution via image J. Figure 6 showcase the TEM images of the Nickel doped Zinc oxide nanoparticles at a concentration of 0.3, 0.6 and 0.9 % by weight. This particle distribution plays a vital role in determining the magnetic behaviour of the sample also provides the information regarding the coupling of spin (Sa- nguanprang et al. 2019; Naskar et al. 2020).

Figure 6: TEM image with SAED pattern of undoped and Nickel doped Zinc oxide nanoparticles**Figure 7: SEM topography of undoped and Nickel doped Zinc oxide nanoparticles**

SEM Analysis: The formation of the particle in the nano range was confirmed from the SEM images shown in Figure 7 which were in good accordance with the particle size estimated from the XRD pattern depicted in the Figure 7 that the particles were spherical in shape.

Figure 8: M-H curve of undoped and Nickel doped Zinc oxide nanoparticles



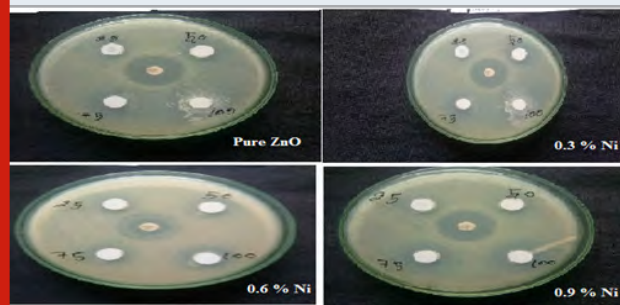
From the results obtained, it was seen that the prepared sample powders were in nano range with a very low dimensions and the size varied with the increase in the Nickel content. It was also observed that while increasing the Nickel doping, the size of nanoparticles was decreased slightly which was already proved by the crystallite size calculation from XRD pattern in above section. Hence the SEM outcomes were in good correlation with XRD pattern, which was well defined that there was a decrease in the particle size as the concentration of the dopants was increased. Zinc oxide when doped with Nickel leads to nanopores which was shown in Figure 7 and it was seen as the doping concentration was increased resulted with the change in the formation exhibiting rod shaped into nano flakes indicated the effect of doping concentration (Beazer et al. 2021; Khan et al. 2021).

VSM Analysis: The M-H loop of the synthesized nanoparticles were shown in Figure 8. The samples pass origin of coordinates, the remanent magnetization (M_r), and coercivity (H_c) were found to be zero. The saturation magnetization, retentivity, and the coercive force values were given in Table 3. It is evident from Figure 8 that a transition from the paramagnetic state to the ferromagnetism state occurred when doping concentration was increased.

Table 3. Various parameters obtained from VSM analysis

Compound	doping concentration by weight (%)	Parameters			
		Magnetization M_s (emu)	Retentivity M_r (emu)	Squarness M_r/M_s	Coercivity H_c (Oe)
Pure ZnO		140.35	122	1.150	654
Ni	0.3	132.45	120	1.103	546
	0.6	127.25	116	1.096	335
	0.9	113.75	108	1.053	203

Figure 9: Zone of inhibition of undoped and Nickel doped Zinc oxide nanoparticles



The ferromagnetism of the Zinc oxide doped with Nickel nanoparticles could arise from two possible sources. One is extrinsic magnetism and the other is intrinsic magnetism. Extrinsic source includes the formation of clusters of transition elements or secondary phase. Exchange interactions come under intrinsic source of magnetism. The probability of ferromagnetism exhibition due to formation of secondary phases and metallic clusters was entirely ruled out in case of Nickel doped sample. It was observed

in the present work, that the oxygen mediated exchange interaction took place among the dopant ions (Ni) leading to the ferromagnetic behaviour and magnetic saturation of Nickel -doped zinc oxide samples (Jeyasubramanian et al. 2019; Satpathy et al. 2021).

It was evident from the XRD analysis that when Nickel was perfectly incorporated into the ZnO lattice. In view of the Ni^{3+} ions substituted into ZnO lattice, the origin of magnetism in the samples was due to the exchange interaction between local spin polarized electrons (such as the electrons of Ni^{3+} ions) and the conductive electrons. Such interaction could lead to the spin polarization of conductive electrons. Consequently, the spin-polarized conductive electrons underwent an exchange interaction with local spin-polarized electrons of Ni^{3+} ions. Thus, after a successive long-range exchange interaction, almost all Ni^{3+} ions exhibited the same spin direction, which resulted in the ferromagnetism of the material (Pallavi et al. 2019; Khalid et al. 2021).

Anti-microbial mechanism: Nickel incorporated Zinc oxide nanoparticles were tested for its anti-microbial

mechanism against gram positive *Staphylococcus Aureus*. Figure 9 shows the antimicrobial action of Nickel doped Zinc oxide nanoparticles for *Staphylococcus Aureus*. The zone of inhibition (mm) of gram positive were ordered in Table 4. which were maintained in nutrient broth.

The analysis of the biological activity of Nickel assisted Zinc oxide nanoparticles were tested for different organisms in sterilized petri dishes adopting agar diffusion technique

for the sample annealed at 600 °C. The nutrient agar ambience was processed by incapacitating at about 121°C. It was then sterilized, aseptically engulfed in petri plates and permitted to densify. Each petri plates were swabbed with the bacterial broth using a sterilized twig. Then the wells were aseptically added with the organic solvent extracts of leaves. The cell culture dish was detected to spot its zone of inhibition incubated at 37°C for 24 hrs. The MIC of Zinc oxide with Nickel was thus calculated.

Table 4. Inhibition zones against different *Staphylococcus aureus*

SAMPLE	Zone of inhibition (mm/ml) of against <i>Staphylococcus aureus</i>				
	25 m/ml	50mm/ml	75 m/ml	100 mm/ml	Dominion
Pure ZnO	16	13	12	15	14
0.3 % Ni	12	15	17	16	15
0.6 % Ni	14	16	19	20	17
0.9 % Ni	16	19	22	26	22

Table 4 indicate the zinc oxide nanoparticles doped with Nickel had moderate activity with the concentration of 75 and 100mg/mL and the following values were observed with respect to the control, against *Staphylococcus aureus*. The growth of all the microbes was inhibited by undoped Zinc oxide and Nickel doped Zinc oxide MCs. An increase in ZOI with the increase in the Nickel doped Zinc oxide -MCs concentration was observed in Figure 9. According to the results, gram-positive microbes, were more sensitive to Nickel doped Zinc oxide -MCs. Then the pure ZnO and becomes still resistant with the increase in the doping concentration. Thus, from the above table it was clear that as the doping concentration was increased the sample showed a drastic improvement in resisting the bacteria *Staphylococcus aureus*. Hence nickel incorporated Zinc oxide could be employed in treating skin diseases (Silva et al. 2019; Khalid et al. 2021).

CONCLUSION

The findings of the present study determines that XPS studies has proved the Nickel dopant to be located exactly into the Zinc oxide lattice that does not alter the structure. The enhanced optical studies further proved that doping Nickel with Zinc oxide makes it a good candidate in the field of optoelectronic devices. Among transition metal, Zinc oxide when doped with Nickel could produce augmented changes without altering the structure leading to many applications. The anti-microbial mechanism of Nickel doped zinc oxide showed a strong resist against gram positive bacteria when the doping concentration was increased from 0.3 % to 0.9 % concentration by weight and could employed making ointment and lotions for treating skin diseases in pharmaceutical applications.

ACKNOWLEDGEMENTS

The study was financially supported by the Research Institute, SRM University, Chennai, Tamil Nadu, India; SEM Facility, Consultancy on Characterization of Nanomaterials

(CCN); Centre for Nanoscience & Nanotechnology, Department of Chemistry, The Gandigram Rural Institute – DU, Tamil Nadu, India and Central Instrumentation Facility, Pondicherry University, Pondicherry, India.

Conflict of Interests: Authors declare no conflict of interests to disclose.

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Ecological Communication

Zooplankton Diversity Indices for the Assessment of Perennial Freshwater Body in Bishnupur, Bankura, West Bengal

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ABSTRACT

The status of health of any aquatic ecosystem is largely dependent on the diversity and density of zooplankton inhabiting the water body. Zooplankton are considered to be the most vital primary consumer in any aquatic ecosystem. At the same time, they also influence to a greater extent in determining the total productivity of an aquatic body. The present study is an attempt to estimate the diversity of zooplankton and to find out the richness, evenness, of the freshwater perennial aquatic body, Lalbundh of Bishnupur, Bankura. The study was carried out during December 2018 to November 2019. The whole period of study was divided into four major seasons viz winter (December 2018 to February 2019), Summer (March 2019 to May 2019), Monsoon (June 2019 to August 2019) and post monsoon (September 2019 to November 2019). Collection of the zooplankton samples and their quantitative analysis was done following the standard procedures. During the whole period of study 18 different species of zooplankton have been identified from the study area. Out of them 7 species belong to the Rotifera group, 6 species belong to Cladocera group, 3 species of Copepoda group and 2 species of Ostracoda group. Rotifera group comprise the dominant of all the groups constituting 39% of the total zooplankton species, followed by Cladocera (33%), Copepoda (18%) and Ostracoda (11%). The range of Shannon diversity index value of zooplankton varies from 2.76 to 2.88. The species richness value varies from 2.45 (winter) to 2.56 (monsoon). Evenness value varies from 0.95 in summer to 0.98 in monsoon. Zooplankton diversity indices values of Lalbundh give a clear indication of light condition of pollution level of the water body. The main reason behind the pollution of this perennial water body may be the floating dead and decomposed macrophytes in it. They should be removed as far as possible at regular intervals so that the pollution level can be reduced and the water body can be more efficiently used for pisciculture practices.

KEY WORDS: DIVERSITY, EVENNESS, PISCICULTURE, RICHNESS, ZOOPLANKTON.

INTRODUCTION

Zooplankton refers to a wide range of very small and microscopically sized aquatic animals. Several complex and interrelating factors including nutrient input, growth and density of aquatic floating and submerged macrophytes, several climatic and physicochemical factors etc. finally put an effect on the density and distribution of zooplankton in an aquatic body (Harikrishnan and Azis 1989; Neves et al. 2003; Manickam et al. 2018). A large number of environmental factors of the habitat of the zooplankton play a very important role in their sustenance and survivability. If there is any sort of major change in the external environmental

factor it will surely make an effect on the dominance, species diversity, tolerance, abundance as well as in the whole community structure of zooplankton inhabiting the aquatic body (Manickam et al. 2018; Sharma 2020).

These zooplankton are well known to act as biological indicator of any natural aquatic water body (Ahamad et al. 2011). Four major zooplankton groups viz. Rotifera, Copepoda, Ostracoda and Cladocera occupy the freshwater ponds and reservoirs (Rocha et al. 1999). Zooplankton are widely accepted as very important organism for fish feeding and nutrition. So, a successful aquaculture management depend to a large extent on the species density, species diversity and abundance of zooplankton in an aquatic body (Ghosh and Biswas 2015; Sharma 2020).

Article Information: *Corresponding Author: tapaszool123@gmail.com

Received 13/10/2021 Accepted after revision 25/12/2021

Published: 31st December 2021 Pp- 1821-1824

This is an open access article under Creative Commons License,

Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.65>

Although a lot of limnochemical study has been done on this water body, but zooplankton diversity study is lacking in which this present study will fill up the lacunae (Santra 2019). The main aim of the present study is to estimate the species diversity of zooplankton and to find out the richness, evenness, of the freshwater perennial aquatic body, Lalbundh of Bishnupur, Bankura. Besides, the study also put an effort to provide an in-depth knowledge about the condition of this perennial freshwater reservoir which can further be used by several government and non-government agencies for piscicultural practices for upliftment the economic condition of this region.

MATERIAL AND METHODS

'Lalbundh' is one of the most important perennial water body of Bankura district. It covers an area of about 32 ha. It is located at Bishnupur sub division which falls under latitude 23°04' and longitude 87°20'. This perennial water body, well known as as 'bundh' by the local people was dug by the rulers of this area to solve the problem of drinking water and irrigation in this area. Presently, this water body is used by several agencies for pisciculture and tourism purposes. The present study was carried out during December 2018 to November 2019. The whole period of study was divided into four major seasons, winter (December 2018 to February 2019), Summer (March 2019 to May 2019), Monsoon (June 2019 to August 2019) and post monsoon (September 2019 to November 2019).

Figure 1: Percentage contribution of different groups of Zooplankton

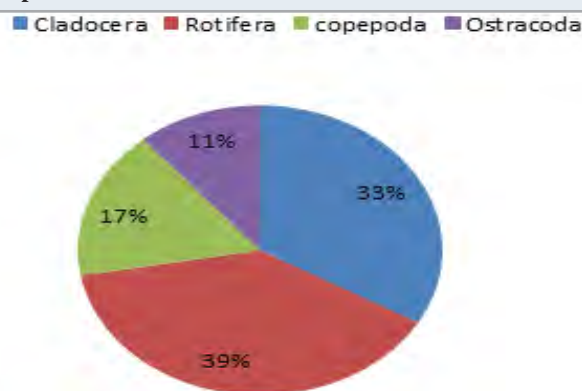
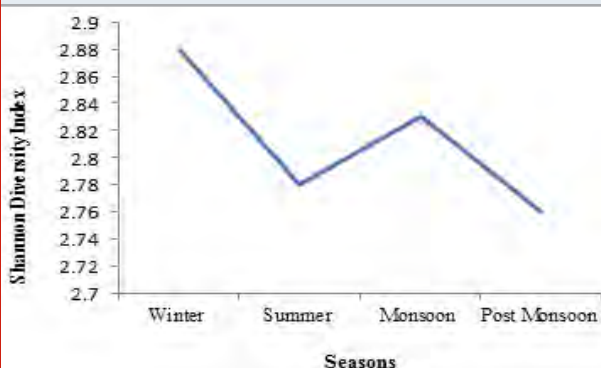


Figure 2: Seasonal variation of Shannon diversity index



Random collection of zooplankton samples was done by filtering a volume of 100L of water through plankton net made up of bolting silk. A suitable glass container was used to preserve the zooplankton samples by fixing it in 5% formalin. The detailed observation and study of the zooplankton was made by using Olympus inverted stereoscopic microscope (MLX-B). The quantitative analysis of zooplankton was made using Sedgwick Rafter plankton counting cell method (Adoni 1985). Identification of zooplankton was done following standard literature key (Battish 1992; Sharma 1998). Three diversity indices were estimated from the collected zooplankton samples viz Shannon-diversity index, evenness and richness. Shannon Diversity index (H') = $-\sum (n_i/N) \log (n_i/N)$ where n_i = Total number of individuals of each group, N = Total number of individuals of all the group (Shannon and Weaver 1963). Evenness index (E) = $H' / \log S$ where H' = Shannon-Weaver Index, S = number of species (Pielou 1966). Species richness index (R): $R = S - 1 / \ln(n)$ where S = number of species and n = total number of individuals observed in the sample (Margalef 1968).

Table 1. Zooplankton diversity of the study area

Sl No	Group	Zooplankton species
1	CLADOCERA	<i>Daphnia carinata</i>
		<i>Daphnia pulex</i>
		<i>Ceriodaphnia cornuta</i>
		<i>Ceriodaphnia reticulat</i>
		<i>Bosmina longirostris</i>
		<i>Moina micrura</i>
2	ROTIFERA	<i>Brachionus angularis</i>
		<i>Brachionus</i>
		<i>quadridentatus</i>
		<i>Brachionus diversicornis</i>
		<i>Brachionus caudatus</i>
		<i>Keratella tropica</i>
		<i>Lecane sp.</i>
3	COPEPODA	<i>Asplanchna sp</i>
		<i>Mesocyclops leuckarti</i>
		<i>Diaptomus pallidus</i>
4	OSTRACODA	<i>Diaptomus denticornis</i>
		<i>Stenocypris sp</i>
		<i>Cyprinotus sp</i>

RESULTS AND DISCUSSION

Composition and abundance of Zooplankton: During the whole period of study 18 different species of zooplankton were identified from the study area. Out of them 7 species belong to the Rotifera group, 6 species belong to Cladocera group, 3 species of Copepoda group and 2 species of Ostracoda group. Rotifera group comprised dominant of all the groups constituting 39% of the total zooplankton species, followed by Cladocera (33%), Copepoda (18%) and

Ostracoda (11%). (Fig- 1). Zooplankton density (Table 2) was found to be highest in winter (1029 Ind/L) and lowest in monsoon (768Ind/L). Similar observations were observed by many other workers (Saba and Sadhu 2015; Sadasivan et al. 2019; Singh et al. 2021).

Diversity indices: Shannon – Weaver diversity index is an acceptable and useful parameter to determine the seasonal fluctuation of zooplankton and quality of water in an aquatic body (Sibel2006). Staub et al. (1970) has described Shannon diversity index (H) < 1 shows heavy pollution, $H=1-2$ means moderate condition of pollution, $H = 2-3$ light condition of pollution and 3-4.5 slight pollution. The range of Shannon diversity index value in the study area varied from 2.76 to 2.88 (Fig-2). This indicates light pollution of the aquatic

body of the study area during the study period. Similar types of observation were made in Ganga River at Arrah (Pandit et al. 2020).

Shannon – Weaver Index (H) was high in winter (2.88). Ali et al. (2003), have also made similar type of observations. High value of Shannon index gives an indication of greater species diversity. The species richness value varies varied from 2.45 (winter) to 2.56 (monsoon). Species evenness value measures the relative abundance of each species in an area. In the aquatic body under study the lowest value of evenness was found in summer (0.95) and highest in monsoon (0.98) (Table2). Similar type of observation has been found on River Seeta (Ramesha and Sophia 2013; Pandit et al. 2020).

Table 2. Seasonal variation of zooplankton diversity indices of the study area

Seasons	Winter (Dec,2018-Feb,2019)	Summer (Mar,2019-May,2019)	Monsoon (June,2019-Aug,2019)	Post Monsoon (Sep,2019-Nov,2019)
Observations				
Cladocera (Ind/L)	454	360	396	441
Copepoda (Ind/L)	103	68	89	102
Rotifera (Ind/L)	450	402	265	400
Ostracoda (Ind/L)	22	24	18	14
Total				
Zooplankton (Ind/L)	1029	846	768	957
Shannon Diversity Index	2.88	2.78	2.83	2.76
Richness	2.45	2.52	2.56	2.48
Evenness	0.96	0.95	0.98	0.96

CONCLUSION

The findings of the present study reveals that zooplankton diversity indices values of Lalbunndh give a clear indication of light condition of pollution level of the water body. The evenness value is quite high in the study area. This suggests that there is a minimum interspecific competition among the species in the aquatic body. Therefore, the compatibility among species is quite high. The decaying macrophytes should be removed from the water body at regular interval to decrease the pollution level and to increase the density of zooplankton so that this large aquatic body can be more efficiently used for pisciculture practices.

ACKNOWLEDGEMENTS

The study was not financially supported by any government or non-government agencies. The author wants to acknowledge Principal of Bankura Sammilani College for extending his support.

Conflict of Interests: The author declares no conflict of interest to disclose.

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Ecological Communication

Economic Availability of Forest Resources in Russia: An Analytical Assessment

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ABSTRACT

The topic of the availability of forest resources is especially relevant for Russia, given the volume of the existing potential of these resources, distributed over a huge area of the country. The purpose of the article is to substantiate the possibilities of using the economic availability of forest resources in the practice of forestry in the Russian Federation. The article presents an analysis of the application of tools based on the economic availability of forest resources. It presents the classification of the forms of forest resources availability and analysis of possible situations that may occur assessing the economic availability of forest resources. The authors analyzed various methods of assessing the economic availability of forest wood resources, formed a criterion of economic availability and considered various conditions of economic availability. The results of calculations of economic availability assessments for the Vilegodskoe forestry located in the southeastern part of the Arkhangelsk region, according to which 19.1% of the analyzed plots were recognized as economically unavailable, are presented. The analysis of the characteristics of economically unavailable forest wood resources in the studied object has been carried out. Proposals have been developed on the spheres of the possible use of the toolkit for the economic availability of forest wood resources. The economic availability of forest resources can be widely used in the forestry practice of the Russian Federation in the areas of pricing and forest exploitation optimization. Its application will increase the level of payments for forest resources, due to the withdrawal of excess profits of loggers, and will also ensure the formation of more realistic forest plans.

KEY WORDS: AVAILABILITY ASSESSMENT OF FOREST RESOURCES, CRITERIA OF ECONOMIC AVAILABILITY, FOREST RENT.

INTRODUCTION

The availability of forest resources is a major factor in the volume of logging. Russia accounts for more than 20% of the world's forest areas, but its share in the global production of round wood is almost 4 times lower than this indicator. One of the current results of the existing level of forest resources economic availability is the low share development of the harvesting permissible volume in mature and over-mature forest stands (allowable cut), which in (2020) in the Russian Federation amounted to slightly more than one quarter (25.12%), while out of 83 constituent entities of the Russian Federation where there is a possibility of felling in mature and over-mature stands, in 21 constituent entities

the development level of the allowable cut was below 5% (including in 12 subjects it was equal to zero).

The concept of natural resources availability is widely used in scientific research, characterizing the existence of opportunities for involving them in economic circulation, which presupposes the availability of resources identified by modern exploration methods that are technically accessible and economically viable for development. Natural resources availability encompasses the complex of relationships related to the operation of natural objects, the development of technological progress, the economy, and society. In the case of different mineral resources, issues of exploration and technical capabilities of exploitation play a significant role. When considering the availability of forest resources, other factors come to the fore. For example, the Alberdi et al. (2016) considers environmental (e.g. protected areas), social (e.g. recreational areas) and economic (e.g.

Article Information:*Corresponding Author: igor_mayorov@bk.ru
Received 20/09/2021 Accepted after revision 15/12/2021
Published: 31st December 2021 Pp- 1825-1831
This is an open access article under Creative Commons License,
Published by Society for Science & Nature, Bhopal India.
Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.66>

profitability) constraints when defining inaccessible forest wood resources (Alberdi et al. 2016; FAO 2020; Medeiros et al. 2021).

The FAO documents indicate both legally protected areas and other reasons as reasons for unavailable for harvesting, such as: physical conditions and terrain, such as steep slopes; remote locations and limited access through lack of infrastructure (roads, etc.) and transport and other factors, such as low productivity, poor stand quality, lack of commercial species, etc (FAO 2020). According to the authors, the forest resources availability has two main forms, which take into account the legislative (institutional form) and economic (economic form) conditions. These forms act as absolute restrictions on the development of wood and non-wood resources. Each of the forms has its own availability criteria and each is influenced by its own factors, which differ both in the nature and mechanism of their impact on the forest resources availability.

The various forms of availability discussed above may be taken into account in these two main forms. For example, the ecological form of availability is taken into account in the institutional, through legislative restrictions that have an ecological character, and various social factors (historical, religious and cultural significance of individual forest areas, energy infrastructure (power grids, pipelines), recreation areas) have a limiting effect on logging for the basis of legislative documents regulating the implementation of social activities (Alberdi 2021).

The institutional form of forest resource availability is based on existing regulatory legal acts (legislation), the various regulations on forest management, which take into account environmental and social aspects, constitute external restrictions on the possibility of involving resources in economic circulation and reduce the potential amount of economically available resources. Therefore, institutional inaccessibility of forest resources should be considered as a synthesis of restrictions reflected in the legislation on the use of forests of different origins (environmental, social causes, institution of ownership, etc.) and taken into account in the management decisions on the involvement of forest resources in economic circulation. It should be noted that institutional restrictions may be absolute, prohibiting forest use in the considered forest area, and relative, narrowing the possibilities for the implementation of certain types of forest use, for example, a ban on clearcutting in forests located in water protection zones (Alberdi 2021; Medeiros et al. 2021).

In case of a prohibition of clearcutting in plantations, the economic availability assessment of forest resources in them is carried out taking into account the additional costs of selective cutting. The economic form of availability determines the possibility of harvesting forest resources, focusing on the economic conditions of forest management, which take into account both individual resource and territorial (logistical) factors, as well as general economic factors that affect the economic availability of forest resources in general.

MATERIAL AND METHODS

At present, there are many definitions of "economic availability (economic unavailability) of forest resources", which differ in approaches to its definition. Analysis of scientific publications allows us to single out a whole set of different interpretations of this concept, according to which the economic availability of forest resources is determined by: establishing the characteristics of the plantations, which determine their economic inaccessibility; the limit level of costs, for the implementation of links between facilities, formed taking into account objective economic and natural conditions of development and ensuring its profitability; qualitative and quantitative condition, as well as territorial location, ensuring during its development the necessary minimum level of profitability; forest resources of positive forest rents; forest resources, the development of which is possible provided that the value of forest rents equals or exceeds the standard value of forest reproduction, conservation and protection (Sokolov 2014; Petrov 2016; Moiseev 2017; Mokhiev et al. 2018a; Pozdnyakova et al. 2018).

Approaches based on the use of plantation characteristics, without cost estimates, were designed to identify areas potentially attractive for exploitation for various purposes, such as justification of the feasibility of logging in particular forests, the formation of calculated forestry, the preparation of management decisions, including in the field of transport network development. For example, when determining the economically accessible design forest area, the researchers propose to exclude certain economically unattractive categories of plantings, such as mature and over-mature forest plantations, with wood reserve ranging from 50 m³ to 100-150 m³; plantations with a high proportion of species that are not in demand on the market, for example, softwood species; plantations with a low percentage of commercial timber yield; deconcentrated logging fund - small areas of mature forest, remote at a considerable distance from the existing road network, so that the construction of even temporary roads does not pay off due to the use of the wood on them (Suhodolov et al. 2012).

Spenser (1986) proposed a method for assessing wood availability based on the use of seven taxation indicators: stock of growing forest, area of the plot, average diameter, height, sawmill stock, percentage of rejected trees, removal distance. Examples of the use of comparative assessment approaches in assessing the economic attractiveness of a forest plot are the establishment of a "class of economic value", formed based on scores of indicators of species-quality characteristics (the ratio of species in the plot/quarter; average stock per hectare; average trunk volume in the bark; age group; type of forest growing conditions) and the formation of rating commodity and transport assessments of wood resources.

The term "transport accessibility of resources", which characterizes the limitation of forest development due to underdeveloped transport infrastructure, is widely used in practice and this accessibility is assessed based on the proximity of the forest areas under consideration in relation

to the existing logging trails and branches (Pochinkov 2015). In the practice of logging, transport accessibility is considered as the most important criterion for the availability of forest resources, and is often identified with the concept of accessibility of forests, including their economic availability (Pilli and Pase 2018; Chumachenko et al. 2018; Verkerk et al. 2019; Ghajar. 2021). The use of the term transport accessibility as an accessibility criterion was also used in world practice. For example, when classifying the world's forest lands into accessible and inaccessible categories, American researchers used a distance of 10 km to forest infrastructure, including waterways (rivers) (Brent et al. 2008).

At the same time, the criterion equal to 30 km was applied to the USA (Brent et al. 2008). However, the transport inaccessibility of forest resources can be seen as a form of economic unavailability, as if the resources were profitable to develop and there are no legal restrictions on their use. Considering the transport inaccessibility of forest resources, two forms were distinguished: high costs of transporting wood and the lack of transport infrastructure. In the first case, these high costs acted as an economic constraint that is limited to the economic availability of forest wood resources, in the second case the lack of transport infrastructure was assessed as a temporary constraint.

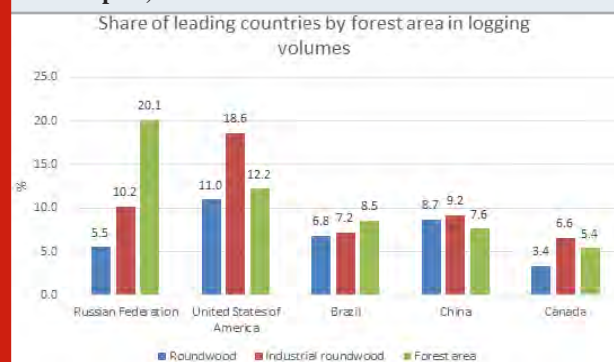
This limitation was eliminated within the time frame necessary for carrying out an appropriate set of measures to create the required road network for the development of forest bases. In this case, the efficiency of forest roads construction, which predetermined the reality of their creation, was justified by the financial results from the development of the existing economically available forest resources, which ensured the appropriate profitability of logging production, taking into account the required costs for maintenance and construction of forest roads (Tretyakov 2014). That is, the accessibility of economically available resources in remote forest areas predetermined the possibility of their transport development in the future. In assessing the availability of European wood resources, researchers, along with Environmental and social restrictions, have taken into account economic constraints, using various factors that may influence the profitability of logging. Accessibility (distance to roads, elevation, and terrain), slope and soil condition, as well as the extent and annual growth of plantings were used as such factors (Alberdi et al. 2020).

RESULTS AND DISCUSSION

The gap between the forest area and the volume of business wood harvested is less significant for Russia, but it was still almost twice, which was much worse than the ratio of the indicators considered for other leading forest countries with the largest forest areas in the world (Figure 1; FAO 2020). To a decisive extent, this situation was a consequence of rather low average values of forest fund characteristics of the Russian Federation (the average tree volume, an average stock of forest stands per hectare, average site class of plantings), which predetermine both the low level of forest resources economic accessibility and the high

importance of the problem of assessing this availability and developing a system of measures to improve it (RAS 2019; Alberdi et al. 2020).

Figure 1: Share of leading countries by forest area in logging volumes in 2020 (Global Forest Resources Assessment 2020: Main report)



The presented methods, based on the use of only qualitative and quantitative characteristics, make it possible to identify forest resources characterized by the most favorable operating conditions, which will positively affect the economic results of their development, but these methods do not take into account the impact of various market factors (prices of forest products, the cost of production factors, etc.), which affects the level of reliability of the results obtained. For example, classifying soft-leaved plantations as economically inaccessible resources was unlikely to be legitimate if there are processing plants in close proximity to them that focus on the consumption of low-quality wood. The remaining approaches to the definition of "economic availability of forest resources" were based on the profitability of their use and were distinguished by the choice of criteria for economic accessibility (relative or absolute indicators) as well as by the set of factors taken into account in its definition (Alberdi et al. 2020).

One of the approaches to determine the economic accessibility of wood focuses on providing the standard level of profitability of logging (Sokolov 2014; Mokhirev et al. 2018b). However, in the conditions of the Russian Federation, when the mechanism of forest land lease is used, this indicator does not take into account additional costs incurred by the entrepreneur under the lease contract related to reproduction, protection and conservation (Bespalova et al. 2019). Considering the above, the authors refer to the economic availability of forest resources as an assessment of the efficiency of their development and involvement in economic turnover on the basis of the economic criterion of availability, determined based on the qualitative and quantitative state of resources, their territorial location, the conditions for transportation, processing, and reproduction of forest resources, the organizational and technological level of production, the state of markets and sectoral institutional characteristics (Dayneko et al. 2021).

Based on this, the following determine the economic accessibility of forest resources:

1. Results of forest inventory work describing the

- qualitative and quantitative condition of forest resources;
2. Forest infrastructure (actual and planned);
 3. The level of wood processing depth, which is determined by both the technologies used and the level of complexity of raw material utilization, which depends on the business process management system and the level of forest sector combination;
 4. Market conditions that determine the price level of forest products depending on the supply-demand ratio;
 5. Industry institutional environment, including innovative development programs, regulatory and regulatory frameworks.

Economic relations between the forest owner (the State) and forest users (tenants, other legal entities, citizens) related to the use and reproduction of forests form the essential basis for the category of economic availability of forest resources. This essential economic relationship was formed by the involvement of forest resources in the management field, one of the conditions of which was to ensure the balance of interests of all actors in forest relations. However, this balance of interests should take into account the environmental impact of these economic processes (Sukhorukova and Pogorely 2017; Tysiachniouk et al. 2021).

Given the existing mechanism of forest leasing, when the forest user is assigned to carry out various forest works, the most adequate was the use as a criterion of economic availability of forest resources - equal or exceeding the value of forest rents of the standard cost of reproduction, conservation and protection of forests. Forest rent refers to the net income generated at the time of forest resources development and calculated as the difference between the market price of final forest products and the total costs of their production, taking into account the receipt of regulatory profit per capital (while the costs of production do not take into account payments for forest resources). The presented criterion for the economic availability of wood resources was most in line with the principles of sustainable forest management, which provides economic opportunities for forest reproduction after logging. The selection and approval of the criterion of economic availability of forest resources was a central issue in the planning of their development, it determined the potential volumes and structure of the forest sector (Sukhorukova and Pogorely 2017; Tysiachniouk et al. 2021).

It should be noted, however, that this criterion of economic availability was formed from the position of the owner, in this case the state, and reflects the potential possibility of involving these resources in the economic turnover, at zero value of their cost. Their actual use by business entities was possible only with consideration of payments of forest resources which they pay to the state. Based on the above, the following conditions of economic availability of forest resources can be defined by the system of inequalities as a criterion of economic availability of wood resources: Forest resources are recognized as economically unavailable based on the existing conditions characterizing the current

organization of forest resources development in terms of technology, technology, economic relations, forest market conditions, etc (Tysiachniouk et al. 2021).

$$r < 0 \quad (1)$$

where r - is forest rent per unit of resource.

Forest resources are recognized as economically available only if they are developed from the perspective of the owner (in which case the value of forest rents will be positive but does not include the costs of forest reproduction, conservation, and protection).

$$r < 0 \quad (2)$$

Forest resources are recognized as economically available according to the conditions of their development and reproduction from the position of the owner.

$$r - C_n > 0 \quad (3)$$

Where C_n is the standard cost for the reproduction, conservation and protection of forests per unit of resource. Forest resources are recognized as economically available by the conditions of their development and reproduction from the perspective of the business structure

$$r - C_n - C_r > 0 \quad (4)$$

Where C_r is payment for forest resources.

Formulated values of the differences between rent and cost can serve as a basis for economic assessments of forest wood resources. Based on the presented approach, calculations were made to estimate economically available forest wood resources for the Vilegodsk forest district located in the southeastern part of the Arkhangelsk region, the territory of which was a typical example of a multi-forest region in which long-term forest exploitation was carried out. Calculations of economic availability assessment were based on forest survey data, which contained information on 9855 plots assigned to final cutting (Tysiachniouk et al. 2021).

The initial structure of the sample information used in the calculations included:

- area,
- forest-forming species,
- economic management (type of felling, type of reforestation),
- in strata - species section,
- average height,
- average diameter,
- class of marketability,
- stock per hectare.

As additional parameters defined in the context of quarters based on cartographic materials and GIS data, such parameters as tax rate (removal distance) the distance of plantations from the existing forest infrastructure are established. The total information, taking into account the species composition of the plots, contained 30521 positions,

for each of which the average diameter, the average tree volume, and the stock for each of the species were taken into account during the commoditization of reserves. More than 85% of the plots were assigned to clearcutting, on which 81.3% of the forest stock was concentrated (Medeiros et al. 2021).

For each plot, an estimate of its economic availability was calculated, and according to the results of these calculations, it was obtained that 1882 plots (19.1%) received a negative assessment (when considering the option of using low-quality wood as a raw material for the production of wood boards). One of the main factors influencing the level of

accessibility was the species composition. Economically unavailable forest resources are mainly represented by plantations, in which the predominant species are spruce, birch, and aspen (Table 1) (Medeiros et al. 2021).

The highest share of economically unavailable timber resources in the total stock of the corresponding species was observed for the following species: alder (all plantations are economically unavailable), aspen, and spruce. The inclusion of spruce plantations in this list is due to various reasons that cause a decrease in the output of commercial products and an increase in the cost of logging and reproduction in these plantations:

Table 1. Characteristics of economically unavailable forest resources of Vilegodsky forestry

Predominant specie	Economically unavailable forest resources					Planting characteristics (percentage of stocks)
	Share in	Plots		Stocks		
	total stocks	Quantity, pcs.	Share%	Volume, thousand m3	Share%	
Birch	15,7%	605	32,1%	1400,8	31,1%	2-4 bonitet class, removal distance is more than 40 km (90%)
Spruce	18,7%	775	41,2%	1661,7	36,9%	Bonitet class 5 and 5A, removal distance is more than 40 km (79%),
Alder	100,0%	22	1,2%	14,6	0,3%	Bonitet class 4, removal distance more than 60 km (14.5%)
Aspen	29,1%	270	14,3%	1090,5	24,2%	1-2 bonitet class, removal distance 10-60 km (100%)
Pine	4,9%	210	11,2%	333,3	7,4%	removal distance over 40 km (92.6%)
Total		1882	100%	4500,8	100%	

— A high proportion of spruce plantations remote from the junction points of plantations, since more than 29% of spruce plantations are located in 5 categories of taxa (removal distance from 60 to 80 km), while for other main rocks this indicator is in the range from 13% to 22%.

— A high proportion of low-bonitet spruce stands in the logging fund, spruce with a bonitet level of 5 and higher (41.3% in terms of reserves) compared to other species, so similar pine plantations form 16.5% of the reserve, birch - 0.2%, and other species of plantations of 5 bonitet are absent.

— A high proportion of soft-leaved wood in spruce plantations (on average almost 30%),

— A relatively high proportion of areas for which artificial reforestation is assigned (11.4% for spruce plantations, 9.8% for birch, 4.4% for pine), which negatively affects the level of economic availability of forest resources, due to the high level of costs for their reproduction.

Accounting for additional costs of payments for forest resources reduced the potential of economically available forest resources for entrepreneurs by 5.8% from 23.9 to 22.6 million cubic meters (Table 2) (Medeiros et al. 2021).

Accounting of payments for forest resources reduces the value of economically available wood from 312 rubles per cubic meter to 255 rubles per cubic meter. This was the amount of rent that forms an additional excess income

of business entities engaged in forest management, which significantly exceeds the value of forest payments. This situation was formed under the conditions of the state setting of prices for forest resources when the price of standing timber in the Russian Federation is an order of magnitude lower than similar prices in neighboring Scandinavian countries. The fact was that these rates do not take into account the economic effect of consuming wood of different quality characteristics, they do not provide much incentive for the integrated use of wood resources, and are not related to reforestation conditions, which largely determine the costs of forest reproduction (Matsuoka et al. 2021).

Economic availability of forest resources was intended to be a tool that allows creating a system of economic incentives aimed at resolving one of the most significant contradictions in the system of forest relations of Russia - the contradiction between the economic interests of the forest resources owner (state) and the economic interests of forest users (tenants). Currently, this category was not used in forest planning practice, which determines the presence of overestimated planned values of forest use indicators in forest plans (Matsuoka et al. 2021). The main purpose of the forest accessibility category is forest management, which includes:

- Establishment of payments for forest resources;
- Determination of the allowable cut taking into account

the economic availability of forest resources;

- Determination of the degree of attractiveness of forest resources from the standpoint of their use;
- Development of incentive systems for the forests use;
- Substantiation of the optimal structure of forest resources consumption;
- Assessment of sectoral and regional forms of the forest

sector development, and the results of activities on the use and forests reproduction.

It should be noted that using a system of special subsidies and subsidies for loggers, you can significantly increase the level of availability of forest resources, just as it happened in Japan (Matsuoka et al. 2021).

Table 2. Indicators for assessing the economic availability of forest resources of the Vilegodsky forestry

Index	Unit	Indicator values		Change index, %
		payments for forest resources are not taken into account	payments for forest resources are taken into account	
Aggregate assessment	mln. rub	6875,4	4963,3	72,2%
Specific assessment	rub / m3	242,3	174,9	72,2%
Assessment of economically available forest resources	mln. rub	7452,9	5764,6	77,3%
Volume of economically available forest resources	thousand m3	23870,4	22599,4	94,2%
Number of stands of economically available forest resources	pcs.	7973	7445	93,4%
Assessment of economically unavailable forest resources	mln. rub	-577,5	-801,3	138,8%
Volume of economically unavailable forest resources	thousand m3	4500,8	5771,8	128,2%
Number of stands of economically unavailable forest resources	pcs.	1882	2410	128,1%

The considered category of economic accessibility of forest resources can be widely used both by state regional and a federal executive authority that form forest policy, and by business structures that develop plans for forest development. In the first case, the role of the economically accessible forest resources category will be to optimize the system of payments for forest resources, to form the calculated cutting area, to set the planned volumes of forest management and forest reproduction, to form the investment program for the development of the forest sector, in the second it will serve as a tool for assessing the effectiveness of management decisions in the field of forest harvesting and wood processing (Matsuoka et al. 2021).

CONCLUSION

The findings of the present study suggests that the formation of assessments of the economic availability of forest wood resources on the basis of the above methodological approaches can be obtained in the framework of forest inventory. Since their assessment takes into account the standard costs of reproduction, conservation and protection of the forest, this will contribute to the formation of continuous sustainable forest management. By stimulating the growth of economically accessible forest resources, the state, as the owner of forests, will ensure an increase in the efficiency of their use while maintaining a balance of interests of participants in the sphere of forest relations.

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Biotechnological Communication

Green Synthesis of Polymer-Capped Copper Nanoparticles Using *Ocimum sanctum* Leaf Extract: Antibacterial and Antioxidant Potential

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ABSTRACT

The advancement of green nanotechnology has piqued the interest of researchers into the environmentally responsible production of nanoparticles. Conventionally used chemical methods for the synthesis of the nanoparticles have shown adverse effect on environment due to the use of highly toxic chemicals. They are also expensive as they utilize costly chemicals as a reducing and capping agent. Use of plant extract can be an environment friendly and cost-effective approach for the synthesis of nanoparticles. Copper is the metal which humans utilize from the ancient time period and it doesn't show any adverse effect on humankind as well as on environment. Leaf extract of *Ocimum sanctum* was employed with CuSO₄ (1:9, v/v) to synthesize stable copper nanoparticles (CuNPs) that were then functionalized with Polyvinyl Pyrrolidone (PVP) polymer. Characterization of synthesized copper nanoparticles was carried out using UV-Visible spectroscopy, X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR) and transmission electron microscopy (TEM). Synthesized CuNPs were subjected against the human pathogenic bacterial strain to evaluate their antibacterial potency. Antioxidative characteristics of CuNPs were analyzed using DPPH free radical scavenging activity. The UV-visible spectra of CuNPs showed unique peaks at 322 and 247 nm indicates the stable formation of nanoparticles. X-ray diffraction pattern suggest the face cubic centered (FCC) structure of copper nanoparticles. FTIR analysis revealed the presence of biomolecules attached on the surface of CuNPs. TEM analysis proven the synthesis of spherical shaped CuNPs with the average particle size of 73.50 ± 1.78 nm. Biosynthesized CuNPs showed maximum zone of inhibition against E. coli which was tends to be 20 mm. 51.48 % of DPPH free radical scavenging activity was observed by synthesized PVP coated CuNPs. As a result, this technology can be employed for the quick and environmentally friendly biosynthesis of stable copper nanoparticles with antibacterial and antioxidant activities with the size range from 10 to 100 nm, implying their potential application in the healthcare, clinical as well as pharmaceutical fields..

KEY WORDS: ANTIBACTERIAL, ANTIOXIDANT, COPPER NANOPARTICLES, OCIMUM SANCTUM, POLYMER FUNCTIONALIZED.

INTRODUCTION

Nanostructures with varying physical, chemical, and electrical properties enable a wide range of applications which includes potent antimicrobial and antioxidative agents, optical and electrical materials. Copper and its alloys, which have increased in value among Nobel/transition metals, have been used efficiently in a variety of applications such as electrical, catalytic, optical, and as an antibacterial and antifungal agent (Bhatti et al. 2015; Ashar et al. 2016; Cao et al. 2016; Iqbal et al. 2017; Khalil et al. 2019; Punniyakotti

et al. 2020). Copper nanoparticles have been described as a potential substitute for gold, palladium, silver, and platinum nanoparticles due to their use as an antibacterial agent, and they also have quality properties in bioscience, biomedical, catalytic, dielectric, imaging, magnetic, and other fields. In terms of benefits, medicinal plants are cost-effective, abundantly available, safe to handle, non-toxic, and this biological technology may be utilised for the industrial scale production of copper nanoparticles (Akbari et al. 2021). In recent years, algae, bacteria, fungi, mushrooms, enzymes and plant leaf extracts have been used to create non-toxic, energy-efficient, cost-effective, and environmentally friendly metallic nanoparticles (Rajeshkumar et al. 2019; Mumtaz et al. 2021).

Article Information:*Corresponding Author: drkap_chem@yahoo.com

Received 17/09/2021 Accepted after revision 21/12/2021

Published: 31st December 2021 Pp- 1832-1838

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.67>

Plants are better platform for nanoparticles manufacturing since they are free of hazardous chemicals and contain natural capping agents. Furthermore, the use of plant extracts lowers the cost of microbe isolation and culture media, improving the cost-competitive viability of microorganism-based nanoparticles synthesis. We report on the fast production of copper nanoparticles utilising *Ocimum sanctum* leaf extract in this paper. Tulsi is an Indian traditional medicinal plant that is high in bio-reduction and stabilisers. Tulsi contains alkaloids, glycosides, tannins, saponins, and aromatic compounds, as well as minerals such as Ca, Mn, Cu, Zn, P, K, Na, and Mg, with tulsi leaves having a higher concentration of Cu than other leaves. It has a Cu content of 12.31 mg/kg (Chowdhury et al. 2008; Dubey and Pandey 2018; Wang et al. 2021).

Urosolic acid is the primary active ingredient of tulsi leaves. Tulsi is employed in ayurvedic medicine due of its therapeutic properties. Tulsi is also an excellent reducer. In an aqueous chemical approach, gallic acid was responsible for the reduction of silver ions into silver nanoparticles (Soundarrajan et al. 2012). *Ocimum sanctum* leaf extracts have recently been employed in the manufacture of silver and gold nanoparticles. Tulsi contains bio-reducers and stabilisers (Vennila et al. 2016). Copper is extremely harmful to microorganisms like bacteria. Lemon fruit extract, Green coffee bean extract, Neem flower extract, *Citrus Paradisi* fruit peel extract, *Hibiscus rosasinensi*, *Ocimum santanum* leaf extract, *Syzygium aromaticum* (Cloves), *Vitis vinifera* extract, *Eucalyptus*, *Cassia alata*, *Centella asiatica*, *Malva sylvestris*, Leaf extract of *capsicum frutescens* and other plant extracts were used to create copper nanoparticles (Hariprasad et al. 2016; Awwad et al. 2021; Ghaffar et al. 2021; Gopalakrishnan et al. 2021; Velsankar et al. 2021; Wang et al. 2021).

MATERIAL AND METHODS

Leaf extract was prepared accordingly method described by Khan et al. 2016. In brief, 5 g of fresh *Ocimum sanctum* leaves were taken and rinsed thoroughly with distilled twice. Leaves were then dried on filter paper to eliminate any remaining moisture. 100 mL of distilled water was added using a measuring cylinder and heated for 10 min. Then the leaf extract was filtered through Whatman's filter paper no. 1 and the filtrate was kept at 4 °C till the use. For the synthesis of copper nanoparticles under continually stirring condition, 25 mL plant extract was added to the 100 mL of 1 mM aqueous $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ solution dropwise. After thoroughly combining the leaf extract with the precursor, the mixture was incubated at 31 °C for 24 h. Continues synthesis of CuNPs was observed by a change in colour from pale green to light yellow. After 24 h CuNPs were then centrifuged at 6000 rpm for 30 min at room temperature. The obtained pellets were then re-dispersed into the deionized water to eliminate any undesired biological elements (Khan et al. 2016).

For the synthesis of polymer functionalized copper nanoparticles in 100 mL of ultra-pure water, 0.2 g of PVP (Polyvinyl pyrrolidone) was dissolved and agitated for 1 h at 80 °C. After that, the solution was gradually added to

the homogenous solution of CuNPs generated from the leaf extract. After 1 h, the mixture's light colour yellowish was converted to a light brown colour. The reaction mixture was allowed to cool for 10 min before being centrifuged at 10000 rpm for 15 min. The precipitates formed were washed with deionised water and dried in a 70 °C oven for 24 h (Aisida et al. 2019). Different typical current techniques were used to characterise synthesised CuNPs and PVP functionalized CuNPs. A UV-visible spectrophotometer (Perkin Elemer USA) was used to confirm the production of CuNPs and polymer functionalized CuNPs (Hulikere et. al. 2019).

FTIR analysis in the 500 - 4000 cm^{-1} range was used to confirm the functional bio molecules associated with the produced CuNPs and PVP functionalized CuNPs. XRD analysis was performed to obtain unique diffraction pattern of synthesized CuNPs using a Rigaku D/max 40 kv X-ray diffraction spectrometer. High resolution transmission electron microscopy was used to analyse the structural morphology of CuNPs (HR- TEM) (Hulikere et. al. 2019). For the antibacterial activity of synthesized CuNPs was carried out via a well diffusion method reported in previous studies with some modifications (Hulikere et. al. 2019). All the test bacterial strains were grown in nutrient broth at 37 °C overnight and adjusted to 0.5 as per McFarland standards. Under sterile conditions, 100 μL of two Gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) and two Gram-negative strains (*Pseudomonas aeruginosa* and *Escherichia coli*) were spread on each nutrient agar plate. A diameter well of 10 mm was punched on the agar plate using a cork borer and the synthesized CuNPs and PVP CuNPs were inoculated in each well. Similarly, 100 μL of streptomycin (1 mg/mL) served as a positive control. Plates were incubated at 37 °C for 24 h and the antibacterial activity was evaluated by measuring the diameter of the inhibition zone using zone scale (HiMedia) (Hulikere et. al. 2019).

For the antioxidant activity, antioxidant properties of synthesized CuNPs and PVP functionalized CuNPs was examined using DPPH method. Ascorbic acid was taken as standard due to its high antioxidant properties. Standard solution of ascorbic acid as well as various concentration (10, 20, 30, 40, 50, 75, 100 $\mu\text{g/mL}$) were prepared. DPPH was prepared by weight of 20 mg was taken and dissolved in 100 mL methanol. 1 mL of various concentration CuNPs and PVP functionalized CuNPs and standard ascorbic acid solution were mixed separately with 1ml of DPPH solution and incubated for 30 min. The absorbance was measured by UV- Visible Spectrophotometer at 517 nm. The free radical scavenging activity was represented as the % of inhibition, calculated by using following formula (Mistry et al. 2021).

$$\% \text{ of Antioxidant activity} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

RESULTS AND DISCUSSION

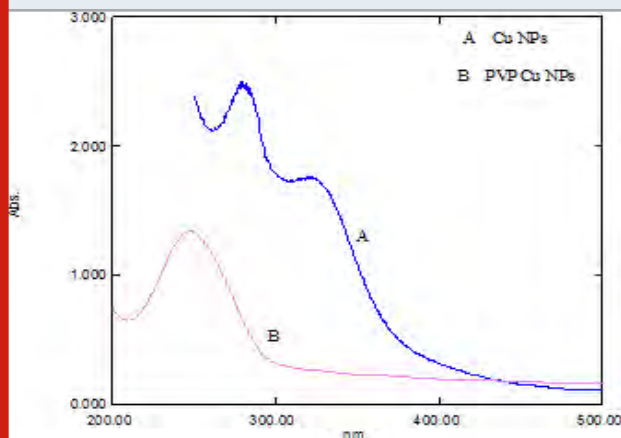
UV-visible spectroscopic analysis: To confirm the generation of CuNPs, the aqueous reduction reaction mixture was tested by a UV-visible spectrophotometer.

The reduction of Cu^{+2} to Cu^{+1} detected by a colour change from light green to brownish was caused by PVP CuNPs excitation surface plasmon resonance (SPR) which finally proved the creation of CuNPs. The observed results are substantially consistent with recent research (Wang et al. 2015; Aswathanarayana et al. 2017). The absorbance peaks of CuNPs and polymer functionalized CuNPs formed successfully are at 322 and 247 nm. According to research, the SPR of most metallic compounds varies with size and shape (Oberdörster et al. 2005; Soylu et al. 2006; Zhang et al. 2010; Aswathanarayana et al. 2017).

Figure 1: Colour Change from light green to yellowish [A] After 24 h [B] After adding PVP solution



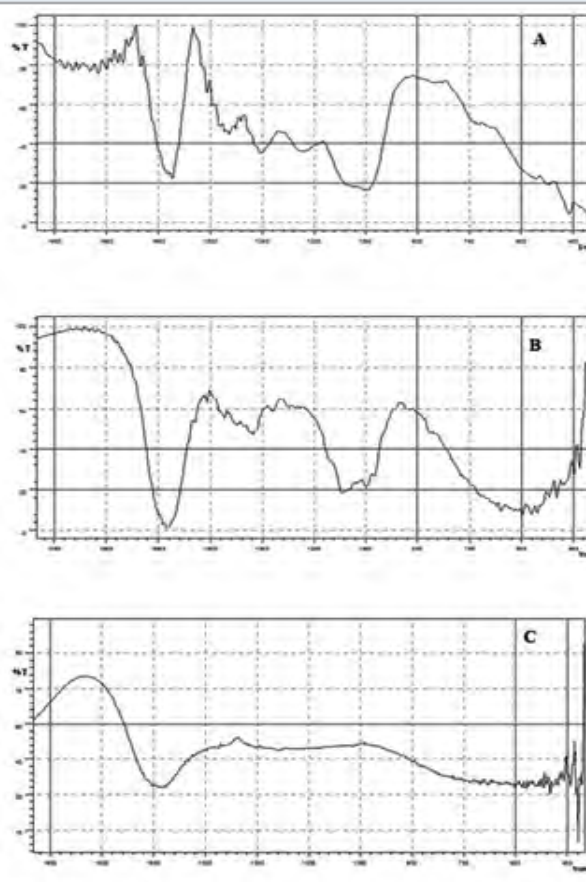
Figure 2: UV Visible spectrum of [A] Cu NPs [B] PVP Cu NPs



Fourier Transforms Infrared Spectroscopy (FTIR) analysis: The FTIR measurements of biosynthesized copper nanoparticles were carried out to identify the possible interaction between protein and copper nanoparticles. Results of FTIR study showed absorption peaks located at about 1600, 1024, 1480, 1377 and 449 cm^{-1} (Fig.3). The band at 1653 cm^{-1} attributed to C=C stretching. In addition, peaks at 1100 and 1700 cm^{-1} corresponds to C-O and C=O stretching, respectively of leaf extracts. Additionally, the observed peak at 610 cm^{-1} correspond to the formation of CuNPs, which is in accordance with previous finding

(Zowlaty et al. 2013; Yugandhar et al. 2017). The band at 1480-1320 cm^{-1} attributed to C-H bending. The band at 1024 cm^{-1} for C-X stretching. These IR spectroscopic studies confirmed that carbonyl group of amino acid residues have strong binding ability with metal suggesting the formation of layer covering metal nanoparticles and acting as capping agent to prevent agglomeration and providing stability to the medium. These results confirm the presence of possible proteins acting as reducing and stabilizing agents (Varghese et al. 2020).

Figure 3: FT-IR Spectrum of [A] Ocimum sanctum leaf extract [B] CuNPs [C] PVP CuNPs



X-RAY diffraction analysis: The XRD pattern of polymer functionalized CuNPs (shown in Fig.4) revealed a well-crystallized sample with prominent diffraction peaks at 2 theta values of 19.64, 41.46, 45.47 and 72.28 corresponding to planes (100), (111), (200), and (311) (Sengottuvelan et al. 2014; Soomro et al. 2014). The bioconjugate between the polymer component and the formed polymer capped CuNPs may be attributed to the modulation of the phase transition by PVP. The Debye-Scherer formula was used to calculate the mean particle size of PVP CuNPs, which is given as,

$$D = \frac{0.9 * k}{\beta \cos \theta}$$

Where D is the crystalline size (nm), k is the X-ray wavelength (0.1541 nm), β represents the angular line full

width at half maximum (FWHM) of the peak (in radians), and θ is the Bragg's angle (in radians) (Guo, 2018). The PVP CuNPs were calculated to have an average particle size of 70.20 nm, which is in good agreement with the HR-TEM average particle size of 73.50 nm (Sengottuvelan et al. 2014; Soomro et al. 2014; Varghese et al. 2020).

Figure 4: XRD pattern of PVP functionalized CuNPs

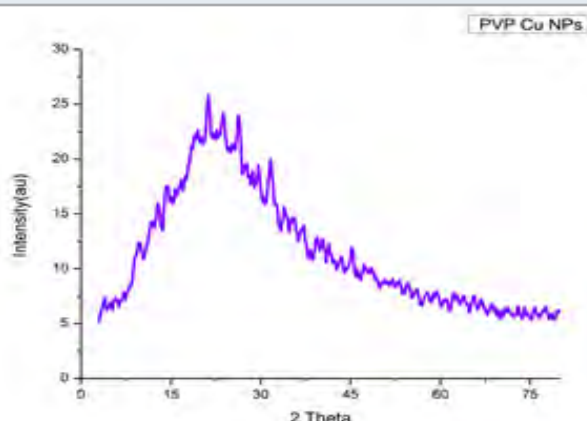


Figure 5: HR-TEM images [A], [B], [C] and SAED image [D] of PVP CuNPs

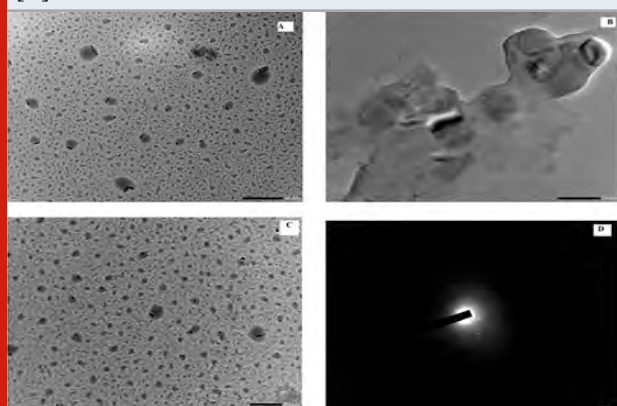
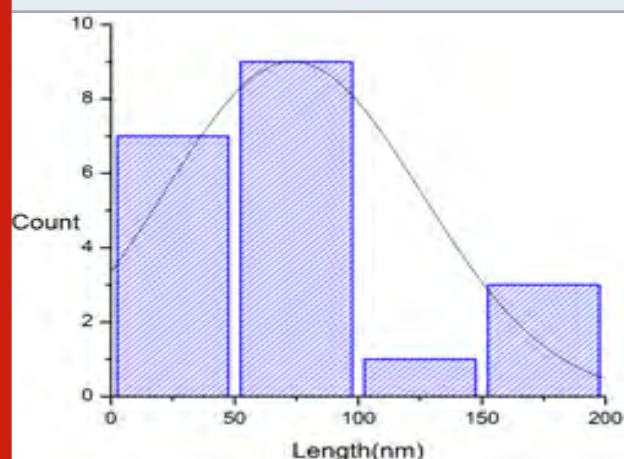


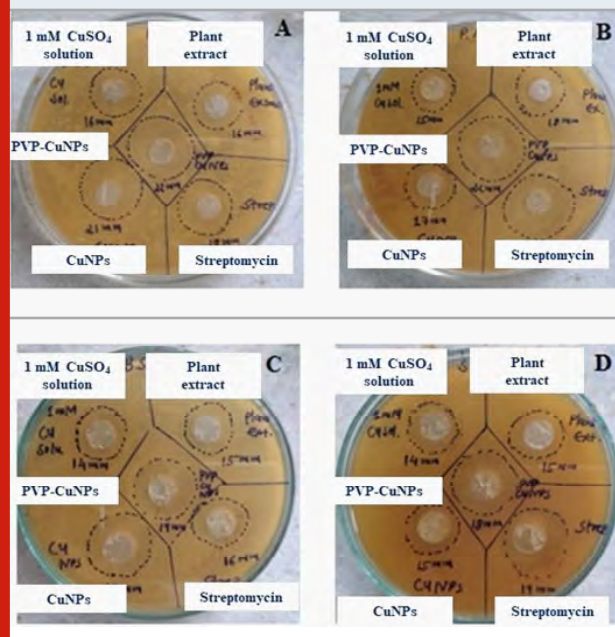
Figure 6: The size distribution curve from the TEM analysis of PVP functionalized CuNPs



HR TEM analysis: The H-7500 model was used for high resolution transmission electron microscopy (HR TEM). HR TEM was used to study the size and morphology. The polymer functionalized CuNPs had a spherical shape uniform size with an average particle size of 73.50 nm. The crystallinity of the biosynthesized polymer functionalized CuNPs was demonstrated using the selected area electron diffraction (SAED) pattern with bright circular spots.

Antibacterial activity of CuNPs and Polymer functionalized CuNPs: The antibacterial potential of CuNPs was assessed by measuring the Inhibition zone of plant extract, Copper nanoparticles and polymer functionalized nanoparticles was summarised in table 1. The PVP CuNPs inhibit growth of *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis* and *Pseudomonas aeruginosa* shown in figure 7. It also shows good activities then the all the organism in comparison with standard drug. Because of its lower concentration activities, PVP CuNPs has a higher possibility of becoming a possible alternative for conventional antibacterial medicines (Mistry et al. 2021).

Figure 7: Antimicrobial study of biosynthesized CuNPs and PVP CuNPs against pathogenic bacteria [A] *Escherichia coli* [B] *Pseudomonas aeruginosa* [C] *Bacillus subtilis* [D] *Staphylococcus aureus*



Antioxidant activity of CuNPs and Polymer functionalized CuNPs: DPPH is a stable molecule that can be reduced by taking hydrogen or electrons and has been commonly used to assess antioxidant activity (Mistry et al. 2021). CuNPs demonstrated effective antioxidant capability, with their radical scavenging capacity rising with concentration. The figure depicts the antioxidant activity of CuNPs, which is around 51.48 %. The percentage of PVP-CuNPs was around 54.54 %. The findings confirmed that

polymer-capped CuNPs have higher antioxidant activity than CuNPs. The antioxidant property of CuNPs is due to

plant component absorption on the copper nanoparticles (Keshari et al.2021).

Table 1. Antibacterial activity of CuNPs and PVP CuNPs

SR.NO.	Organism	Zone of Inhibition (In mm)				
		CuSO ₄ Solution (1 mM)	Plant extract	Cu NPs	PVP-CuNPs	Streptomycin (1 mg/ml)
1	<i>Escherichia coli</i>	16	16	21	22	18
2	<i>Staphylococcus aureus</i>	14	15	15	18	19
3	<i>Bacillus subtilis</i>	14	15	18	19	16
4	<i>Pseudomonas aeruginosa</i>	15	18	17	22	23

Figure 8: Antibacterial zone of inhibition of CuNPs and PVP CuNPs in comparison with standard streptomycin

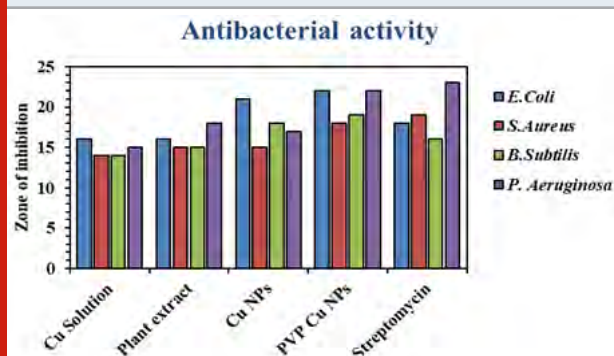
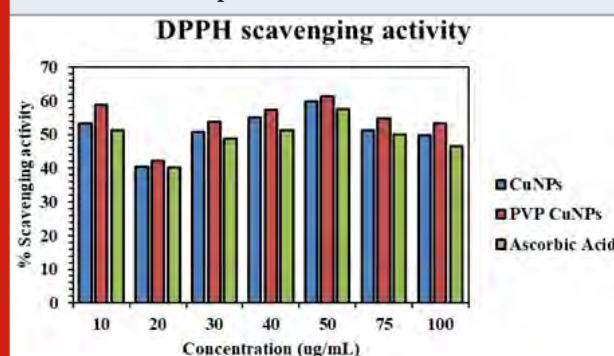


Figure 9: Antioxidant activity (%) of synthesized CuNPs and PVP CuNPs in comparison with standard ascorbic acid



CONCLUSION

The findings of the present study describe an environmentally friendly and cost-effective biological technique for producing polymer-capped nanoparticles with antibacterial and antioxidant activities. Using an extract of *Ocimum sanctum* (Tulsi) leaves and CuSO₄•5H₂O salt solution, copper nanoparticles were successfully produced. The generated CuNPs were further functionalized with PVP to improve biocompatibility without the addition of any harmful or toxic materials. The UV visible confirmed the creation of CuNPs trolls by changing the visible colour

to dark brown after 24 h, with a peak at 247 nm. The FTIR spectra revealed the various functional groups in the *Ocimum sanctum* extract that were responsible for the biogenic synthesis of CuNPs and polymer generated CuNPs. XRD examination confirmed the crystalline nature and average particle size of 70.20 nm of polymer capped CuNPs. HR-TEM imaging microscopy revealed a spherical form with particle sizes ranging from 10 to 100 nm. CuNPs and polymer capped CuNPs shown good antibacterial and antioxidant activities.

ACKNOWLEDGEMENTS

This study was supported by the Department of Chemistry, Hemchandracharya North Gujarat University, Patan, Gujarat, India.

Funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of Interests: Authors declare no conflict of interests to disclose.

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Medical Communication

Biosynthesised Drug-Loaded Silver Nanoparticles: A Vivid Agent for Drug Delivery for Human Breast Carcinoma

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ABSTRACT

The use of nanoparticles as drug carriers has been investigated, and it offers various benefits, including the controlled and targeted release of loaded or associated drugs, as well as enhanced drug bioavailability. They do, however, have certain disadvantages, such as in vivo toxicity, which affects all organs, including healthy ones, and overall disease treatment improvement, which might be undetectable or limited. Silver nanoparticles are being studied more and more due to their unique physical, chemical, and optical properties, which allow them to be used in a variety of applications, including drug delivery to specific targets in the body. Given the constraints of traditional cancer treatment, such as low bioavailability and the resulting usage of high doses that produce side effects, attempts to address these challenges are essential. In this work, Biocompatible Silver nanoparticles (AgNps) loaded with tamoxifen have been prepared using the gelation process. Tamoxifen-loaded green synthesized AgNps are reported to be amorphous. The phytochemicals present in the extract of *Hemionitis arifolia* leaf were responsible for the reduction of silver nitrate to AgNPs. The functional groups existing in the particles were identified with FT-IR analysis. XRD analysis state that the particles were crystalline in nature and arranged in quartzite crystal. Particle size and shape were illustrated from SEM analysis and revealed that the particles were amorphous in nature. UV-visible spectrophotometer showed the band around 440nm which was identified as “surface Plasmon resonance band”. The synthesized AgNps loaded with tamoxifen were significantly effective against Human breast cancer cells. The silver nanoparticle loaded with tamoxifen was found to be inducing apoptotic signals in the selected cells. It inhibits the breast cancer cells even at the lower concentration of AgNPs and TAM-AgNPs. Further apoptotic studies (AO/EtBr and DAPI) reveal that cell death is due to the fragmentation of nuclear material of the treated cells.

KEY WORDS: APOPTOSIS, BREAST CANCER, HEMIONITIS ARIFOLIA, SILVER NANOPARTICLES, TAMOXIFEN.

INTRODUCTION

Cancer is an aggressive phase of cell proliferation in which the cells have a relatively uncontrolled division that results in a gradual increase in dividing cell count (Devi et al. 2012). Cancer is one of the world's leading deaths with an estimated 13.1 million deaths expected in 2030. It is the second most prevalent condition for overall deaths in the world after coronary diseases. There are nearly 23% of deaths in the US and 7% in India. Various cancers are found

based on their tissue location (Parveen et al. 2015). One of the most prevalent cancers in women is breast cancer; about 1.7 million new women are diagnosed with breast cancer worldwide. While substantial progress has been made in breast cancer care in recent decades, existing approaches to therapy remain constrained by a non-specific systemic delivery, inadequate drug concentrations reaching the tumor, and multidrug resistance. The advent of nanotechnology has revolutionized the arena of cancer diagnosis and treatment and nanotechnology in medicine helps to resolve chemotherapy limitations (Su et al. 2021).

Nanoparticles with optimized surface properties can more efficiently migrate within tumor cells delivering a high amount of drugs with significantly reduced toxicity.

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Received 19/10/2021 Accepted after revision 19/12/2021

Published: 31st December 2021 Pp- 1839-1846

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.68>

The applications of Nanotechnology could revolutionize the paradigm of breast cancer treatment in the future (Varadharajaperumal et al. 2017). Metallic nanoparticles have been intrigued for over a century and are now used widely in biomedical sciences and engineering (Pradeepa et al. 2017). Among metal nanoparticles, silver nanoparticles (AgNps), in particular, are attracted to the intensive research interest for important applications in medical industries, catalysis, and surface-enhanced scattering (Chahardoli et al. 2017). AgNps have developed as a superior product in the field of nanotechnology (Nqakala et al. 2021).

AgNps have been used as vehicles for the controlled release of drugs as it is acting as a carrier of drugs and deliver them on a selective basis. AgNps are magic “bullets” that go directly to cells of a particular tissue (Pradeepa et al. 2017). Synthesis of nanoparticles by various physical and chemical methods like laser ablation, lithography, chemical vapor deposition, sol-gel technique, and electro-deposition is very expensive and highly toxic. Bio-material scientists also look forward to improving the synthesis of metal nanoparticles with simple, nontoxic additives, environmentally benign solvents, and renewable materials (Emmanuel et al. 2015). Green synthesis or biological synthesis of nanoparticles is an alternative for the physical and chemical methods of synthesis of nanoparticles (Singh et al. 2017). Biogenic nanoparticles are the organic synthesis of nanoparticles by biological approaches such as the use of enzymes, microbes, fungus, plant, or plant extracts (Soni et al. 2021).

In particular, in recent years, the biological synthesis of plant-based nanoparticles has received far more attention as it is cost-effective and also overcomes the downside of retaining pure microbial cultures as needed in microbial methods. Therefore, green synthesis is the latest approach used for large-scale nanoparticle synthesis (Iravani 2011). Besides, biologically synthesized AgNps have been tested for possible cytotoxic effects in human breast cancer cells (MCF-7) using cell viability, membrane integrity, ROS generation, and DNA fragmentation (Gurunathan et al. 2013). In our research, the Green Nanoparticle Synthesis was performed using the leaf extract of *Hemionitis Arifolia*. *Hemionitis arifolia* (Burm.f.) T.moore. of family Hemionitidaceae is the endemic and widely distributed species on western ghats of India. It has been made use of for the treatment of burns, menstrual disorders, antifertility, and anti-flatulence. *Hemionitis arifolia* frond juice was evaluated for its hypoglycaemic and anti-diabetic properties using rats and has been used to cure burns and a folklore anti-diabetes fern. The medicinal significance of *Hemionitis arifolia* is due to the presence of Alkaloids, Flavonoids, Phenols, Tannins, and Saponins (Sureshkumar et al. 2021).

Tamoxifen is an anti-cancer drug that is widely used for breast cancer, carcinoma, osteosarcoma, and sarcoma of soft tissues. The effectiveness of tamoxifen in the treatment of different forms of cancers is considerably limited by its significant side effects. The initial side effects due to tamoxifen administration include less severe symptoms such as nausea, fatigue, myelo-repression, and arrhythmia, which are typically reversible. Nevertheless, cardiomyopathy

associated with tamoxifen and congestive heart failure has raised considerable concern among health practitioners. The development of nanoparticle-based therapeutic delivery systems is a highly deliberated approach to improving effectiveness and reducing the adverse effects induced by anti-cancer agents such as Tamoxifen (Pradeepa et al. 2017; Sureshkumar et al. 2021).

The objective of the study was to explore a novel Tamoxifen delivery system based on green synthesized AgNps from *Hemionitis arifolia* leaf extract that is environmentally friendly and to characterize the silver nanoparticles using UV Visible Spectroscopy, SEM, XRD, EDAX, and FTIR. This form of Tamoxifen delivery system could offer a fast and convenient way to enable the controlled release of drugs and enrich its chemotherapeutic efficiency. To be the best of my knowledge, no study has been carried out on Tamoxifen-loaded AgNps for stimuli-sensitive drug delivery systems. In this study, Tamoxifen was loaded on green synthesized AgNps to construct drug-loaded nanoparticles as a drug delivery system in MCF-7 breast cancer cells.

MATERIAL AND METHODS

MTT-3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-tetrazolium bromide, Acridine orange, ethidium bromide, DAPI (4', 6-diamidino-2-phenylindole, dihydrochloride) stain, and dimethyl sulfoxide (DMSO) were purchased from Hi-media laboratories Pvt. Ltd. DMEM medium, fetal bovine serum, penicillin, streptomycin, Chitosan (purified viscosity grade 50 cps; MW 150 kDa; deacetylation degree 85%), Fluorescent Isothiocyanate (FITC), Propidium iodide (PI) were purchased from Sigma-Aldrich (USA). The Breast Cancer Cell line (MCF-7) was procured from National Center for Cell Science (NCCS, Pune) and was maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) with 1% antibiotic-antimycotic solution. Cells were grown to 100% confluence at 37°C, with a humidity of 5% (v/v) CO₂ atmosphere. A known number of cells were seeded into tissue culture plates and maintained for further studies. The leaves of *Hemionitis arifolia* were washed several times with water to remove the dust particles and shade dried at room temperature. The dried leaves were made into a fine powder. The leaf powder was weighed and 50g was taken for synthesis purposes. The powder was packed using Whatman No. 1 filter paper (pore size 25µm) (Varadharajaperumal et al. 2017).

The plant aqueous extract was obtained from 5g leaves shade-dried powder dissolved in 100ml of DDW (Double Distilled Water) with boiling 60°C at 20 mins. The extract was cooled to room temperature and filtered using a Buchner funnel (Varadharajaperumal et al. 2017). Silver nitrate was used as a precursor material for nanoparticle synthesis. Silver nitrate was dissolved in de-ionized water under constant stirring. The 50% and 25% of plant extract were prepared with de-ionized water and the volume was made up to 250ml. 50ml of Silver nitrate solution was added along with 20ml of *Hemionitis arifolia* leaves extract under constant stirring. The pH of the mixture was maintained at 6. This mixture of the solution was kept under vigorous

stirring at 60°C for 4-5h in a magnetic stirrer. After this process, a green-yellow precipitate was obtained. This mixture was centrifuged at 2500 rpm for 15 min and the green precipitate was discarded. The supernatant was stirred again at 2500 rpm for 15 min along with distilled water twice. A dark yellow-brown color solid precipitate was obtained. The precipitate was washed with methanol and air-dried (Varadharajaperumal et al. 2017).

UV-Vis spectral analysis was done by using Shimadzu UV visible spectrophotometer (UV-1800, Japan). UV-Visible absorption spectrophotometer with a resolution of 1 nm between 200 and 800 nm was used. One milliliter of the sample was pipetted into a test tube and subsequently analyzed at room temperature. Fourier transforms infrared (FT-IR) spectroscopy of AgNP was performed by using a Nicolet 5700 instrument (Nicolet Instrument, Thermo Company, USA) with the KBr pellet method. Each KBr disk was scanned over a wavenumber region of 500-4000 cm⁻¹. The method of X-ray diffraction (XRD) was used to investigate the crystalline structure of AgNP nanoparticles. The XRD analysis was conducted with a Philips PW 17291 powder X-ray diffractometer with a voltage of 40kV and a current of 25mA. The scanning rate employed was 1min⁻¹ over the 10-80° 2θ range (Varadharajaperumal et al. 2017).

The samples were placed on a polycarbonate substrate and the excess water was left to dry at room temperature. They were then dried in a critical point dryer using carbon dioxide, and sputter-coated with gold in a metallizer, and examined under a scanning electron microscope (JSM5600LV, JEOL, Japan) operating at an accelerating voltage of 20 kV (Varadharajaperumal et al. 2017). Tamoxifen conjugated Silver NPs were prepared by a co-precipitation method with minor modifications. In general, Tamoxifen is virtually insoluble in water but it is soluble in ethanol. The required amount of Tamoxifen was dissolved in 3 ml of ethanol. Then, 100mg of Silver NPs were dissolved in 30 ml of dimethyl sulfoxide (DMSO) and de-ionized water under ultrasonication. Subsequently, the Tamoxifen solution was added to the above mixture and the ultrasonication was continued and allowed to stir for 24 h at room temperature. The pH of the solution was adjusted to 10 by adding KOH solution. The resulting black-colored precipitate was washed with distilled water to remove any unbound drug or any other organic impurities. The resultant drug-loaded Silver NPs were collected using a lyophilizer (Pradeepa et al. 2017).

Tamoxifen-loaded AgNPs (3mg) were dispersed into 6ml of phosphate buffer solution (PBS) and centrifuged at 12,000 pm for 30min. The supernatant was collected to measure the ultraviolet absorption at 280nm. The loading efficiency and encapsulation efficiency of the Tamoxifen-loaded Silver NPs were calculated as follows.

Loading efficiency = $\frac{W_0}{W} \times 100\%$ Encapsulation efficiency = $\frac{W_0}{W_1} \times 100\%$

Where W₀ is the amount of Tamoxifen contained in the Silver NPs, W is the amount of Silver NPs, and W₁ is the

amount of Tamoxifen added into the system. The release behavior of Tamoxifen from nanoparticles was investigated. Tamoxifen-loaded AgNPs were dispersed in PBS (pH 7.4, 5 ml) and transferred into a dialysis bag. The dialysis bag was then immersed in 95 ml of PBS at pH 4.0 and 7.4. The release medium was continuously agitated with a stirrer at 50 g and 37 °C. At predetermined time intervals, 2 ml of the external medium was collected and replaced with the same fresh PBS. The amount of released Tamoxifen in the medium was then determined at 254 nm (Pradeepa et al. 2017).

To evaluate the cytotoxicity of Tamoxifen-loaded Silver nanoparticles the following experiments were carried out quadruplicate wells of confluent monolayers of MCF- 7 cells were grown in 96-well tissue culture plates. Cells were incubated with different concentrations of silver nanoparticles. Then, we examined cell viability, as the ability of the cells to cleave the tetrazolium salt MTT [3-(4, 5-dimethylthiazol-2ol)-2,5diphenyl tetrazolium bromide], Sigma Chem. Co. St. Louis, USA], by the mitochondrial enzyme succinate dehydrogenase which develops a formazan crystal. Each concentration was replicated three times.

The 50% inhibitory concentration (IC₅₀) was needed as the test compound concentration required for reduction of cell viability by 50% was calculated by regression analysis. In the second experiment, plating efficiency was checked with the subtoxic dose of NPs. MCF- 7 cells were used as a control for the anti-proliferation assay. The MCF- 7 cells that were grown on coverslips (1×10⁵ cells/coverslip) were incubated for 6-24h with Tamoxifen-loaded Silver nanoparticles at the IC₅₀ concentration, and they were then fixed in ethanol: acetic acid solution (3:1; v/v). The coverslips were gently mounted on glass slides for the morphometric analysis.

Three monolayers per experimental group were photomicrographed. The morphological changes of the MCF- 7 cells were analyzed using Nikon (Japan) bright-field inverted light microscopy at 40 x magnifications. Approximately 1μL of a dye mixture of 100 mg/mL acridine orange (AO) and 100 mg/mL ethidium bromide (EtBr) in distilled water was mixed with 9 mL of cell suspension (1×10⁵ cells/mL) on clean microscope coverslips. The MCF- 7 cells were collected, washed with phosphate-buffered saline (PBS) (pH 7.2), and stained with 1 mL of AO/EtBr. After incubation for 2 min, the cells were washed twice with PBS (5 min each) and visualized under a fluorescence microscope (Nikon Eclipse, Inc, Japan) at 400x magnification with an excitation filter at 480 nm. The percentage of apoptotic cells was determined using the following formula.

$$\% \text{ of apoptotic cells} = \frac{\text{Total number of apoptotic cells}}{\text{Total number of normal and apoptotic cell}} \times 100$$

MCF-7 cells were grown (1× 10⁵ cells/coverslip) and incubated with Tamoxifen-loaded Silver nanoparticles at their IC₅₀ concentration and then they were fixed with

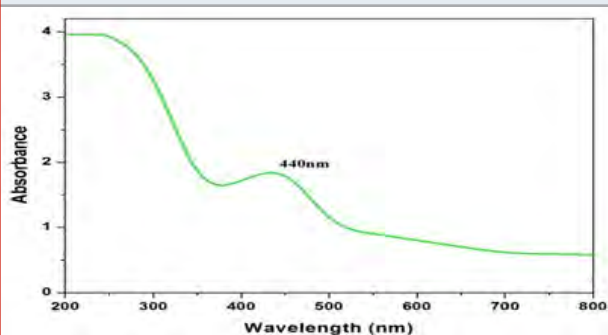
methanol: acetic acid (3:1, v/v) before washing with PBS. The washed cells were then stained with 1 mg/ml DAPI (4, 6-diamidino-2-phenylindole, dihydrochloride) for 20 min in the dark. Stained images were recorded with a fluorescent microscope with an appropriate excitation filter (Pradeepa et al. 2017).

RESULTS AND DISCUSSION

Over the last few decades, several metallic nanoparticles using biomaterials have been extensively developed for use as drug delivery systems (DDS) and other medical applications (Guo et al. 2011). Silver is a versatile metal that has been used in a drug delivery system as nanoparticles because of its biodegradable, biocompatible, and low toxicity (Dodane et al. 1998). The positive amine group of silver gives stability to the nanoparticles with anionic material such as gene, drug, protein, and small molecule by electrostatic interaction. Silver nanoparticles, synthesized from natural plant extracts, are cost-effective, eco-friendly, stable, and safe in cancer treatment (Singh et al. 2017; Bharadwaj et al. 2021).

The AgNPs were prepared by adding 25mL of an aqueous extract with 475mL of 1M AgNO₃. The formation of brown color determined the synthesis of Silver nanoparticles in that solution. The notable color change was observed by visual observation in the Erlenmeyer flask which contains AgNO₃ solution with *Hemionitis arifolia* plant extract. The color of the AgNO₃/plant extract solution changed from colorless to light brown and eventually to dark brown. This color change indicates the formation of AgNPs in the solution. Plant extract without AgNO₃ did not show any color changes. The color variation was interpreted as an indication that AgNPs were formed as a result of the reduction of Ag⁺ ions (Bharadwaj et al. 2021). The formation of Ag Nanoparticles was further confirmed by using UV-visible spectroscopy. The reduction of silver nitrate to silver is examined by the UV-vis spectrum (Figure 1).

Figure 1: UV-Visible spectroscopic analysis of Silver nanoparticles

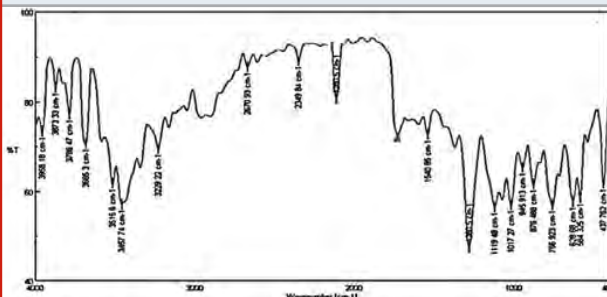


The color change, i.e. light yellow to dark brown, was due to the phenomenon of plasmon resonance, which was the collection of electron oscillation. The band was observed around 440nm which was identified as a “surface plasmon resonance band” and this band was attributed to excitation of valence electrons of AgNPs arranged in the nanoparticles. The shape of the band was symmetrical, suggesting uniform

scattering of spherical shape that also confirms that particle existence and stability (Ma et al. 2021). Thus, from this result, it was concluded that the synthesized AgNPs showed high aqueous stability since a minor reduction in absorbance was observed at 440 nm. A similar type of analysis with almost the same range of results was obtained previously in recent studies of green synthesis of AgNPs using extracts of different plants, such as pomegranate leaves, *Benincasa hispida*, *Prosopis juliflora*, *Allium cepa*, *Parkia speciosa* and *Salvia hispanica* (Bharadwaj et al. 2021).

This suggests that the phytochemicals present in *Hemionitis arifolia* extracted successfully act as reducing and capping agents. Possible biomolecules responsible for the reduction of silver nanoparticle and capping agent of bio-reduced silver NPs through particular bond vibrations peaks coming at defined wave numbers were identified through the FT-IR spectrum (Figure 2).

Figure 2: Fourier Transform - Infrared Spectroscopic analysis of Silver nanoparticles



The peak found around 2670.93cm⁻¹ was C=O. The peak found around 1612.2cm⁻¹ was C=C. The peak found around 1522.52cm⁻¹ was a carboxylic group and N-H binding. The peak found around 2349.84 cm⁻¹ was C-O strong bond. The peak found around 1540.78cm⁻¹ was CH₃. The peak found around 1280.5 cm⁻¹ was O-H deformation. The absorbance peaks located between 1000 and 1600 cm⁻¹ were assigned to the stretching vibrations of hydroxyl groups (O-H) and amine (N-H). The O-H stretching vibration was characteristic of Polyphenols and N-H stretching was attributed to the presence of amino acids and peptides (David et al. 2014). The peak found around 1119.48 cm⁻¹, was C-O-C. The peak found around 1017.27 cm⁻¹ was PO₄-3 stretching. The peak found around 756.923 cm⁻¹ was CH (Anandalakshmi et al. 2016). Through this, the loaded nanoparticles were surrounded by proteins and metabolites such as terpenoids having functional groups. From the analysis of FT-IR studies, it was confirmed that the carbonyl groups from the amino acid residues and proteins have the stronger ability to bind metal indicating that the proteins could form the metal nanoparticles (Hamouda et al. 2019).

X-ray powder diffraction (XRD) is a prompt analytical technique predominantly used for phase identification of crystalline material. It provides information on unit cell dimensions and is used for the identification of unknown crystalline materials (e.g. minerals, inorganic compounds). In the present study, the XRD pattern (Fig.3)

of silver nanoparticles showed that an amorphous pattern of diffraction with the peaks appeared at 2θ values (111), (142), (200) of and (220).

Figure 3: X-Ray power Diffraction analysis of Silver nanoparticles

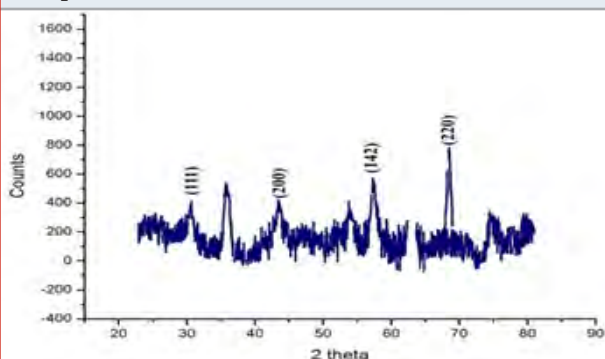


Figure 4: Scanning Electron Microscope Analysis of Silver nanoparticles

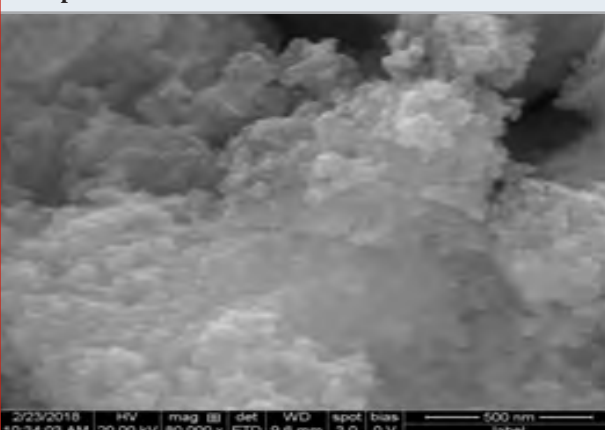
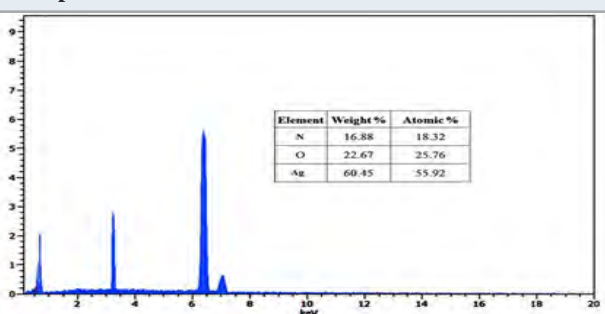


Figure 5: Energy-dispersive X-ray Spectroscopy of Silver nanoparticles



This result corroborated with the XRD analysis reported in previous studies (Garibo et al. 2020). The diffraction patterns showed good agreement with JCPDS(no. 04-0783). Thus, XRD patterns clearly showed the crystalline AgNPs formed by the complete reduction of Ag^+ ions by the aqueous extract of *Hemionitis arifolia*. The other unassigned peak ensued the crystallization of silver nanoparticles along with the organic moieties or impurities that bound to the surface of nanoparticles (Pirtarighat et al. 2019).

FE-SEM images (Fig.4) of synthesized silver nanoparticles at different magnifications provide information on the morphology and particle size of the material. In the present exploration, the size of the particles was 30 nm in diameter and the morphology of particles was nearly like structure due to the electrostatic attraction. The findings clearly show that the synthesised AgNPs material contains fine grain-like particles that agglomerated to form crystals with an almost uniform nearly spherical and a smooth surface. A similar type of findings was previously reported in previous studies (Vinay et al. 2019; Garibo et al. 2020).

The Energy Dispersive X-ray Spectroscopy attachment present with the SEM was known to provide the chemical composition and densely agglomerated area of the Ag nanoparticles at specific locations (Fig.5). Further, the EDX spectrum shows that the Ag atom exhibited a strong signal, along with the Ag atom the small-signal was exhibited from N and O atoms respectively. Ag NPs show a typical absorption peak at approximately 55.2keV due to the SPR phenomenon. Strong signals from the silver observed in EDX spectra confirmed the presence of elemental silver (Vinay et al. 2019; Gomathi et al. 2020).

The drug loading on AgNPs is done and the amount of drug-loaded is shown in table 1. The result indicates that the amount of drug loading depends on the ratio of drug and carrier. The percentage of drug loading increases as the amount of AgNPs is increased and decreases when the amount of drug is increased with a given amount of AgNPs. The amount of drug-loaded per mg of AgNPs has been calculated. The amount of drug-loaded per mg of AgNPs decreases with an increase in the AgNPs and it increases with an increase in the amount of drug. At a ratio of 2:1, the maximum amount of drug is loaded per mg of AgNPs. When the amount of drug is increased, there is no further increase in the amount of drug-loaded per mg of AgNPs. 0.7 mg of Tamoxifen is the maximum of the drug-loaded per mg of AgNPs (Vinay et al. 2019; Gomathi et al. 2020).

Table 1. Drug loading percentage for different ratios of tamoxifen and AgNPs

S.No	Tamoxifen: AgNPs	Drug loading (%)	Mg of Tamoxifen/ mg of AgNPs
1.	1.1	50.6±0.2	0.51
2.	1.2	65.14±0.5	0.33
3.	1.4	71.35±0.6	0.31
4.	2.1	28.5±0.7	0.70
5.	4.1	15.5±0.8	0.62

The pH of the medium and release time plays important role in drug release (Fig 6). The slow and sustained drug release was at pH 7.4 with a release ratio of about $26 \pm 0.58\%$ within 48 h. On the other hand, at lower pH Tamoxifen release rate was considerably faster, with approximately $53 \pm 0.63\%$ (pH 4.0) of the drug released within 48h. At lower pH, protonation of the drug occurred which released chemisorbed drug molecules into the medium.

The surface charge of AgNps turned positive at lower pH, which facilitated the drug release process and blunted the electrostatic interaction of the drug. These results validate drug release behavior from the novel Tamoxifen Delivery System (Vinay et al. 2019; Gomathi et al. 2020).

Figure 6: *In vitro* drug release profile of tamoxifen at different pH

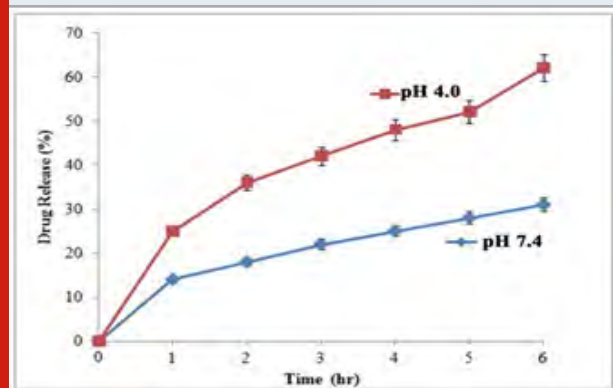


Figure 7: MTT analysis of green synthesized AgNPs (a) and tamoxifen loaded AgNPs (b) on MCF-7 cells

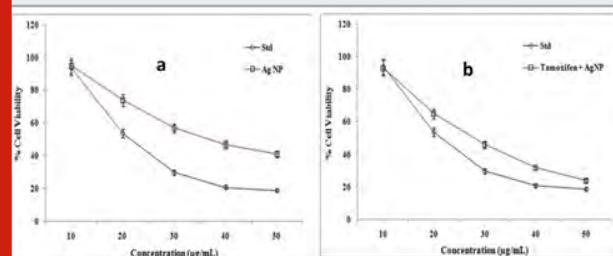
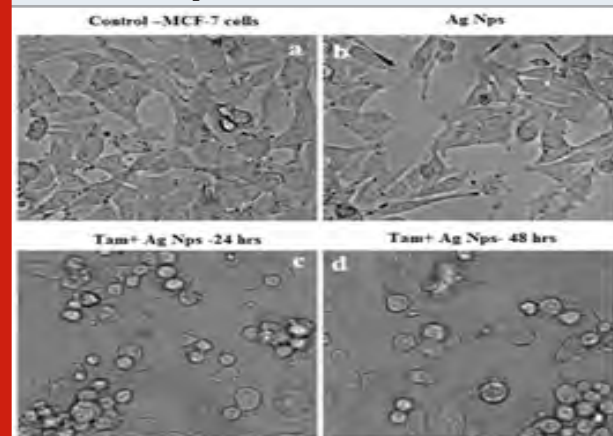


Figure 8: Morphometric analysis of tamoxifen loaded AgNPs on MCF-7 cells; (a). Control (b). Silver Nanoparticles (c). Tamoxifen with Silver Nanoparticles (24h) (d). Tamoxifen with Silver Nanoparticles (48h)



The anti-proliferative potential of the AgNPs and Tamoxifen loaded AgNPs were evaluated by MTT assay (Fig.7). The cell proliferation was inhibited by the nanoparticles in a dose-dependent and time-dependent manner. The IC₅₀ value was calculated from the growth curve obtained by

MTT assay. The IC₅₀ value for AgNps, Tamoxifen, and Tamoxifen loaded AgNPs were 33±1.0, 15±0.5, and 24±1.0 respectively. The Tamoxifen loaded AgNPs showed the greatest effectiveness against MCF-7 cells (Vinay et al. 2019; Gomathi et al. 2020). The phase-contrast micrographs supported the MTT results as there was a decrease in cell density when treated with different doses of chitosan nanoparticles (Fig.8). Significant numbers of cells were found to be formless, detached, and floating at higher concentrations of silver in the treated cells.

Figure 9: Apoptotic analysis of tamoxifen loaded AgNPs on MCF-7 cells; (a). Control (b). Silver Nanoparticles (c). Tamoxifen with Silver Nanoparticles (24h) (d). Tamoxifen with Silver Nanoparticles (48h)

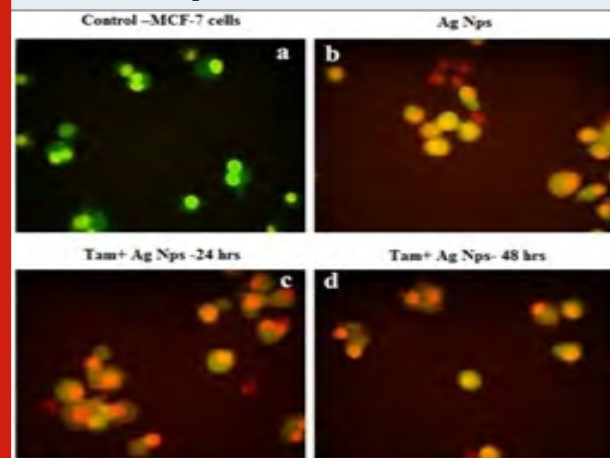
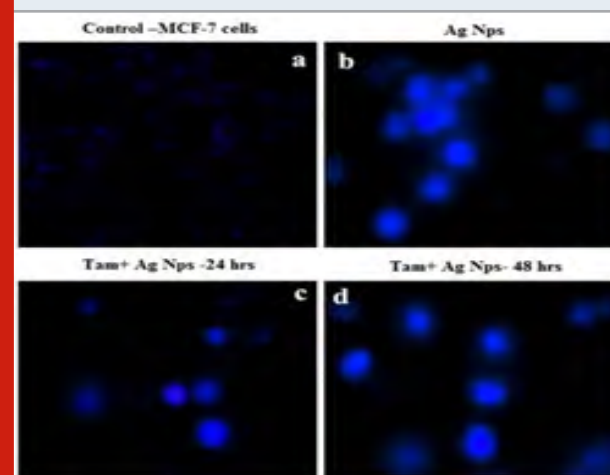


Figure 10: Nuclear fragmentation analysis tamoxifen loaded AgNPs on MCF-7 cells; (a). Control (b). Silver Nanoparticles (c). Tamoxifen with Silver Nanoparticles (24h) (d). Tamoxifen with Silver Nanoparticles (48h)



Based on the overall cell morphology and the cell membrane integrity, necrotic and apoptotic cells were distinguished from one another using fluorescence microscopy (Nikon Eclipse, Inc., Japan). The apoptotic bodies were detected as orange-colored whereas the necrotic cells were observed to be red. A fluorescence microscopic analysis demonstrated that untreated MCF-7 cells were stained with a uniform

green fluorescence (Fig. 9). Similarly nuclear dye DAPI also exhibits nuclear fragmentation in treated cells (Fig. 10). The loss of cell adherence property and severe membrane disintegration were found in MCF-7 cells treated with Silver nanoparticles. This further confirms the particle-induced detachment of cells through activating membrane precipitating proteins. The overall results clearly shows that green synthesized metal nanoparticles can potentially inhibit the proliferation of MCF-7 cells and trigger apoptosis through Bax/Bcl2 and caspase-cascade mediated mitochondrial dysfunction. This research concludes that biogenic metal nano-drug formulations can be utilized for cancer chemotherapy (Jeyaraj et al. 2015; Hamouda et al. 2019; Gomathi et al. 2020).

CONCLUSION

The findings of the present study are in agreement with the hypothesis that the mechanisms of silver nanoparticles toxicity might be related to the proliferative potential of the cell. The antitumor mechanism of Tam-Ag nanoparticles is related to their membrane disrupting and apoptosis-inducing activities. Based on these findings, we confirm that tamoxifen mediated silver nanoparticles act as an effective anti-cancer agent against all highly proliferating cells, especially the cancerous cells. In conclusion, although the application of silver nanoparticles in cancer therapy looks intriguing and exciting, specific tumor cell targeting will be essential. Therefore, further research is needed to be carried out to explain our mechanism of action.

ACKNOWLEDGEMENTS

The instrumentation facilities for this study was provided by the Department of Biotechnology, Sri Kaliswari College, Sivakasi, Tamil Nadu, India.

Conflict of Interests: Authors declare no conflicts of interests to disclose.

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Biomedical Communication

Whole Genome Sequencing and Beta-Lactam Resistant Genes in *Klebsiella pneumoniae* Strains Clinically Isolated from CU Shah Hospital, Gujarat, India

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ABSTRACT

A simple explanation for antimicrobial-resistant opportunistic infections in immunocompromised patients is *Klebsiella pneumoniae* which gradually being associated in insidious infections globally with high mortality rate. Eight hundred fifty-six antibiotic resistant *K. pneumoniae* isolates were collected over 3 years period (from different wards and different specimens) from the Microbiology department of C.U. Shah hospital, whose AST checked by Kirby Bauer disk diffusion method. To study AMR genes, virulome, interference of virulence gene with resistance gene, phylogenomic; 6 clinical isolates were proceeded for whole genome sequencing and bio informatics analysis. *Klebsiella pneumoniae* is a multidrug-resistant (MDR) opportunistic and one of delegate of ESKAPE pathogens groups. This pathogen causes nosocomial infections, urinary tract infections, liver abscesses, wound infections, meningitis. These strains obtain a multidrug resistant phenotype by way of horizontal transfer of ARG transported by either transposons or plasmids. This transfer is generally facilitated by Integrations. In this study antibiotic resistance profile and antibiotic resistance genes analysis as well as virulence gene of *K. pneumoniae* strains were investigated. The study was carried out using 853 clinical isolates collected during 3 years from C.U. Shah hospital of Surendranagar. Antibiotic resistance profile test was carried out by the VITEK 2 against 21 antibiotics. Out of that 6 samples were proceed for DNA extraction, WGS illumina sequencer and analysis of those raw sequences by TORMES pipeline. In this study antibiotic resistance profile included 13 beta lactam antibiotics which classified under 3 class (Penicillin, Cephalosporin, Carbapenem) of beta lactam and in AMR gene study got total 15 different ESBL resistance genes from 6 different *klebsiella pneumoniae* strain. All these genes detected with more than 90% identity by CARD. (TORMES Pipeline) CTX-M-15, NDM-5, OKP-B-6, PDC-2, OXA-1, OXA-181, OXA-362, OXA-50, OXA-9, SHV-1, SHV-11, SHV-187, TEM-1, TEM-150. In this study, we've analyzed the pattern of antibiotic resistance pattern as a phenotypic characteristic and antibiotic resistance genes as genotypic characteristic and co related the results. As multidrug resistance is a worrying matter, constant observation and regular clinical recognition of resistant bacteria are essential to avoid terrible public health incidents. So, our data should be inferred as a warning for need for prevention and control of the MDR *K. pneumoniae* in hospital settings.

KEYWORDS: ANTIBIOTIC RESISTANCE, ARG, KLEBSIELLAPNEUMONIAE, WHOLE GENOME SEQUENCING.

INTRODUCTION

In the treatise Antibiotic resistance, a universal fact is broadly defined which linked with therapeutic failure and increase growth rate in sickness as well as death (Julian et al.

2017). Spreading of resistance in Enterobacteriaceae family is because of swapping the antimicrobial resistance gene amongst the species in hospital environment by plasmid, transposons and integrons like MEGs (Valentina et al. 2019; Kelly et al. 2020).

This can be observed in gram +Ve and -Ve bacteria which is classified by WHO as critical priority pathogens. *K. pneumoniae* is one of the gram -ve pathogenic bacteria which

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Received 15/09/2021 Accepted after revision 21/12/2021

Published: 31st December 2021 Pp- 1847-1854

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.69>

is in red zone of WHO. As per literature total 8 integrons class are available in which first 3 are specifically connected with antibiotic resistance (Sara et al. 2018; Fernanda et al. 2018; Mehdi et al. 2021). *Klebsiella pneumoniae* is gradually intrusive virulent pathogen with antibiotic resistance genes like extended-spectrum β -lactamases (ESBLs), AmpCs and carbapenemases in Enterobacteriaceae family. So, to elaborate the resistome and its mechanism as well as MGEs linked with them and phylogenomic of *K. pneumoniae*, we carried out this study (Hongmao et al. 2021).

MATERIAL AND METHODS

In the present study, total 856 numbers of clinical specimens in the form of different samples (urine, blood, sputum, CSF, swab, pus etc.) from different wards (medicine, surgery, orthopedics, pulmonary medicine, ENT, pediatrics) of C.U Shah medical college were collected, processed and examined at microbiology department of C.U. Shah hospital, Surendranagar from duration of January (2016) to December (2018) (Ethical approval taken-CUSMC/IEC(HR)/APPROVAL-13/2018/1288). By morphological characteristics on MacConkey agar (HI Media) clinical isolates of *Klebsiella* (large mucoid colonies) were identified and isolated. All isolates were further proceeded for gram staining for primary confirmation of the strain. (Clinical and Laboratory Standard Institute, 27th edition). Antibiotic susceptibility testing was performed for all bacterial isolates by modified Kirby Bauer disk diffusion method as per Clinical and Laboratory Standards Institute (CLSI) guidelines.

A suspension equivalent to 0.5 McFarland standards was prepared from single isolated colony. A swab was dipped in suspension and streaked over surface of a Mueller-Hinton agar plate. (Clinical and Laboratory Standard Institute, 27th edition). Obtained Isolates were subjected to susceptibility testing for the following classes of antibiotics: Amikacin, Amoxicillin, Amoxicillin/Clavulanic Acid, Cefepime, cefixime, cefoperazone, cefoperazone/Sublactum, cefotaxime, ceftazidime, ceftriaxone, chloramphenicol, ciprofloxacin, co-trimoxazole, colistin, gentamycin, imipenem, levofloxacin, ofloxacin, piperacillin/tazobactam, tetracycline, tobramycin. (Clinical and Laboratory Standard Institute, 27th edition). The DNA extraction was performed using the HiPurATM Bacterial Genomic DNA Purification Kit MB505. Samples were processed according to kit the instructions for optimum yield. From each isolated DNA sample, 2 μ L was used to determine the 260:280 ratios for DNA quality determination and to estimate the DNA concentration using the Nano Drop 1000 spectrophotometer and Qubit Fluorometer. At least 500 ng of DNA for each sample was sent for further sequencing analysis.

Samples for WGS were processed at the Med Genome Labs Pvt. Ltd., Bangalore 560 099, India. Different qualitative and quantitative control steps were taken for samples at various stages of the library construction (i.e., initial receipt, post-shearing, post-library construction) as per GLP criteria. The sequencing center provided libraries in Fastq format. The raw sequences were assembled and annotated with TORMES. It was an open-source, user-friendly pipeline

for whole bacterial genome sequencing (WGS) analysis directly from raw Illumina paired-end sequencing data. TORMES functioned with all bacterial WGS dataset, despite the number, source, or species. TORMES pipeline is freely available in <https://github.com/nmqijada/tormes>, with a manual how to use it. TORMES pipeline and all the required software and dependencies can be automatically installed by using Conda (Quijada et al. 2019).

RESULTS AND DISCUSSION

The study about this suggest that genes linked with antimicrobial resistance (AMR) are each strong and from different sub populations (Holt et al. 2015; Wyres et al. 2019). So, there is a possibility of a dangerous strains which are highly pathogenic and resistant to all or almost available antibiotics. (Gu et al. 2017; Lam et al. 2019; Mehdi et al. 2021).

Figure 1: From the 3 years data of patients demonstrate the frequency of different type specimen sources and ratio of infected male and Female

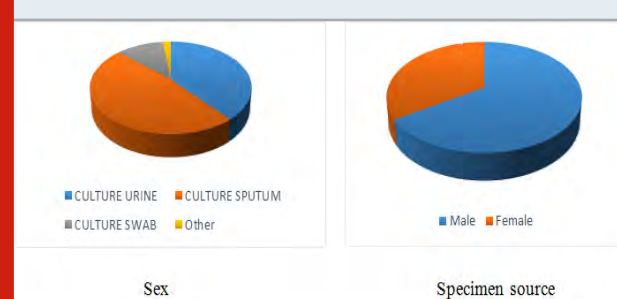
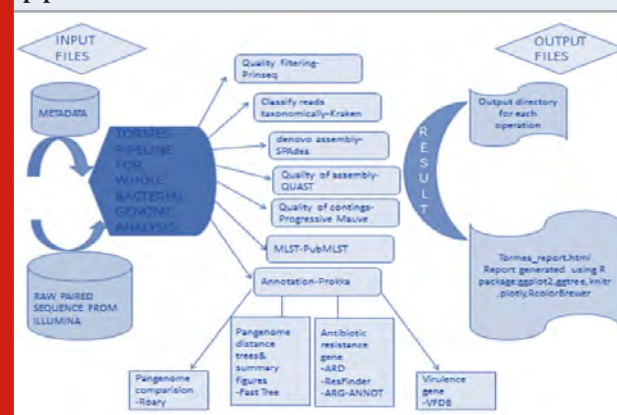


Figure 2: Flow charts show the summary of TORMES pipeline



All the isolates obtained from the samples were detected phenotypically and genotypically as *K. pneumoniae*. All 6 isolates were phenotypically positive for ESBL production. As per the study we found isolate 027 was MDR, 678 was ESBL (+HL AmpC) and carbapenamase positive, 999 was ESBL (+HL AmpC) and carbapenamase positive, 968 was ESBL (+HL AmpC) and carbapenamase positive, 982 and 996 were ESBL positive. Penicillin class antibiotics carried same MIC in all the isolates but variable MIC demonstrated in carbapenem class antibiotics specifically

with Imipenem. Four isolates were non susceptible for cefuroxime, cefotaxime, ceftriaxone and cefepime facing same MIC value (Mehdi et al. 2021).

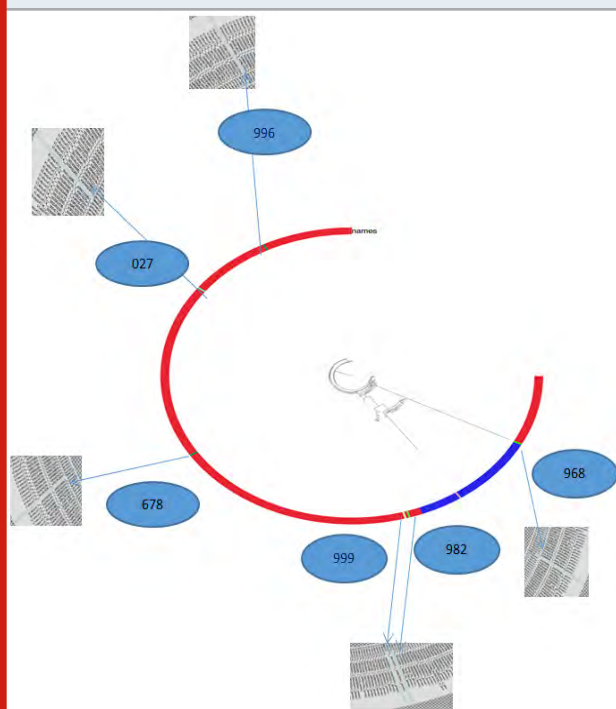
Phenotypic resistance data matched mostly with the genomic results with some exceptions in this study. In this Phenotypic study included 13 beta lactam antibiotics which classified under 3 class (Penicillin, Cephalosporin,

Carbapenem) of beta lactam and in genotypic result got total 15 different ESBL resistance genes from 6 different *Klebsiella pneumoniae* strain. All these genes detected with more than 90% identity by CARD. (TORMES Pipeline) CTX-M-15, NDM-5, OKP-B-6, PDC-2, OXA-1, OXA-181, OXA-362, OXA-50, OXA-9, SHV-1, SHV-11, SHV-187, TEM-1, TEM-150. (Figure-2) (Table 1) (Mehdi et al. 2021).

Table 1. Beta lactam resistance genes present in samples

GENE	Sample1(027)	Sample2(678)	Sample3(968)	Sample4(982)	Sample5(996)	Sample5(999)
CTX-M-15	100	100				
NDM-5		100				
OKP-B-6				99.88		
OXA-1	100					
OXA-181	100	100				
OXA-50			100			
OXA-9	100					
PDC-2			100			
SHV-1					100	100
SHV-11		100				
SHV-187			100			
TEM-1		100				
TEM-150	100					

Figure 3: Phylogenetic diversity



A total of 18 virulence genes were identified in all 6 strains. OmpA, fimE, fimA, ents, fepD, fepC, fepA, entA, entB, entE, fepB, yagV/ecpE, yagW/ecpD, yagX/ecpC, yagY/ecpB, yagZ/ecpA, yagK/ecpR. The search of virulence genes was performed by screening of the draft genome

against Virulence Factors Data Base by using Abricate (Chen et al. 2005; Morales et al. 2021). It is to be noted that these are the genes which are present in all the isolates either resistant or susceptible for different antibiotic. As per VFDB fimE and fimA are type one fimbriae protein and expressed in 90% of clinical *K. pneumoniae* isolates as well as entA, entB and entE are responsible for iron acquisition. Whereas fepA, fepB, fepC, and fepD are siderophore. (Morales et al. 2021).

Phylogenomic and MLST Analysis:

Along with analysis of whole genome sequence, for evolutionary study phylogenomic analysis was carried out for selected *K. pneumoniae* isolates by Fast Tree. Which infers approximately-maximum-likelihood phylogenetic trees from alignments of nucleotide or protein sequences (<http://www.microbesonline.org/fasttree/>). In this study it is done with 365 *Klebsiella* species. (NCBI dataset). *K. pneumoniae* isolates exhibited important phylogenetic diversity (Figure-3), with the whole-genome phylogenetics presenting higher resolution than the MLST typing scheme. For example, K5 (982) was phylogenetically closer to K7 (999) than other K3(968) strains, which were themselves found on different branches. Remarkably, K6 (027), K1 (996) had phylogenetically distant from other strain but they both somewhat closer within the collection.

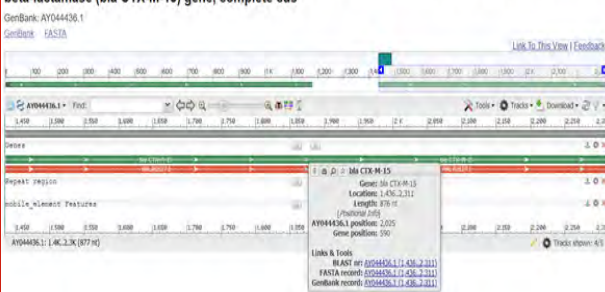
Multilocus Sequence typing (MLST) was performed according to the protocol described by PubMLST database (for *Klebsiella pneumoniae*), Github <https://github.com/tseemann/mlst> in Conda environment on the whole genome assemblies. The MLST profile derived from the following

housekeeping genes: gapA (glyceraldehyde 3-phosphate dehydrogenase), infB (translation initiation factor 2), mdh (malate dehydrogenase), pgi (phosphoglucose isomerase), phoE (phosphorine E), rpoB (beta subunit of RNA polymerase) and tonB (periplasmic energy transducer). (Github <https://github.com/tseemann/mlst>). Molecular mechanisms of resistance and virulence spreading in clinical *K. pneumoniae* strains circulating in hospitals were illustrated and noted to be ornately capable with various factors of resistance, virulence and mobile-genetic elements. Especially, the isolates were MDR to several clinically important antibiotics except for reserved ones such as the colistin. Presence of the MDR strain and the strains which are sensitive to only one or two antibiotics in specimens from the various patients in hospital make this a very perturbing result (Nontombi et al. 2020).

The presence of the CTX-M, TEM, OXA, and SHV ESBL genes in analogous genetic frameworks have been illustrated previously in same and different species or different bacteria from same family in India and global isolates. The presence of these ARGs in the similar genetic environment and on the similar plasmid replicons throughout similar and dissimilar species everywhere in the world effectively indicates the clonal and plasmid-mediated distribution of these ARGs (Reza et al. 2019; Nontombi et al. 2020). Precisely, ISEcp1 plasmid have been revealed to mobilize and facilitate the global spread of CTX-M-15. Including the various CTX-M variants, CTX-M-15 was the major and it was first stated from the Indian subcontinent (Roghieh et al. 2021).

Figure 4: Karim A., et al 2011) (<https://www.ncbi.nlm.nih.gov/nucleotide/15636728/?from=1436&to=2312&report=graph>)

Escherichia coli plasmid-encoded insertion sequence ISEcp1 transposase (tnpA) gene, partial cds; and beta-lactamase (bla CTX-M-15) gene, complete cds



Comparison of gene sequences in the GenBank database revealed homology of the positions 1436 to 2312 in E. coli with CTX-M-15 (100% identity, position 2719 to 3594; accession number AY044436:1436-2312). In our study isolate 027 and 678 which carried this gene were resistant to cefotaxime, ceftriaxone, cefepime/sublactam (all from 3rd generation) and cefepime from 4th generation with ≥ 64 MIC. It was located with the ISEcp1 sequence at upstream of 5' end on the plasmid. (Ramesh et al. 2017; Roghieh et al. 2021).

We reported a similar prevalence of OXA and SHV β -lactamase genes in these isolates, make sure that this apparently chromosomally encoded gene, is almost present in *K. pneumoniae* species. The strong catalog of

ARGs in these isolates indicate the existence of one or more plasmids. OXA-1 plasmid-mediated β -lactamases among *K. pneumoniae* strains indicated the resistance against beta lactam antibiotics especially ampicillin and piperacillin. Several plasmid-encoded beta lactamases are on multi resistance transposable elements. The OXA-1 beta-lactamase gene is part of Tn2603. Comparison of gene sequences in the GenBank database revealed homology of the positions 1400 to 2231 in *Klebsiella pneumoniae* with OXA-1 (100 % identity, position 274 to 1104; accession no. JN420336.1:1400-2231) (Madhan et al. 2014; Roumayne et al. 2019; Nontombi et al. 2020).

Figure 5: (Villa, L., et al 2012) (<https://www.ncbi.nlm.nih.gov/nucleotide/JN420336.1?report=graph&from=1400&to=2231>)

Klebsiella pneumoniae plasmid pNDM-MAR, complete sequence



Figure 6: Tolmasky M.E, et al 1993) (<https://www.ncbi.nlm.nih.gov/nucleotide/M55547.1?report=genbank>)

Klebsiella pneumoniae transposon Tn1331, complete sequence



Figure 7: (Potron et al. 2011) (<https://www.ncbi.nlm.nih.gov/nucleotide/JN205800.1?report=graph>)

Klebsiella pneumoniae strain KP3 plasmid pKP3-A, complete sequence



Gene OXA-9 was originally found within the structure of Tn1331, a multi resistance transposon first isolated from a clinical *Klebsiella pneumoniae* strain. Comparison of gene sequences in the GenBank database revealed homology of the positions 1 to 826 in *Klebsiella pneumoniae* with OXA-9(100 % identity, position 130 to 954; accession no. M55547:1-826). Both OXA-1 and OXA-9 are the part of transposon which is part of plasmid but easily move from plasmid to plasmid and plasmid to chromosome and vice versa which were carrying antibiotic resistance genes (Bojorquez et al. 1998; Navdezda et al. 2019; Katerina et al. 2021).

Figure 8: (Potron et al. 2011)

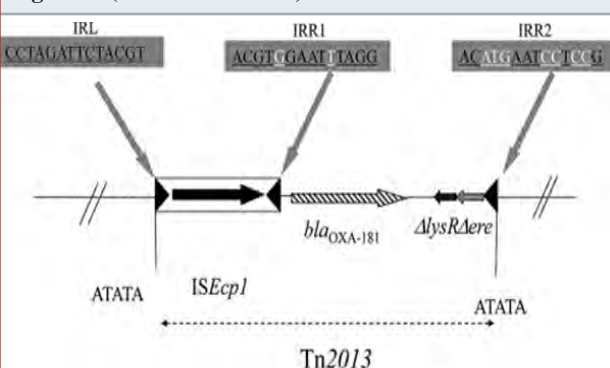
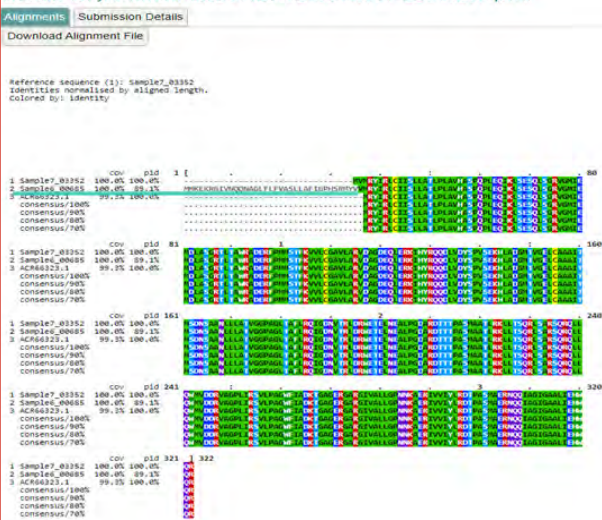


Figure 9: Alignment of gene sequence by clustal omega

Results for job view-I20201129-121651-0767-22819267-p1m



The OXA-181 (Carbapenem-Hydrolyzing Class D β -Lactamase) gene was located on a pKP3-A type plasmid and was linked to the insertion sequence ISEcp1 in *Klebsiella pneumoniae* strain KP3. Comparison of gene sequences in the GenBank database revealed homology of the positions 4141 to 4939 in *Klebsiella pneumoniae* with OXA-181(100 % identity, position 7309 to 8106; accession no. JN205800:4141-4939). In our study we also found the insertion sequence ISEcp1 was identified upstream of the OXA-181 gene (Anais et al. 2011; Katerina et al. 2021).

Comparison of gene sequences in the GenBank database revealed homology of the positions 77 to 938 (isolate-996, 999), in *Klebsiella pneumoniae* with SHV-1 (100 % identity, position 296087 to 296947(996), 254 to 1114 (999); accession no FJ668814:77-938). SHV-1 was present in isolate 996 and 999. Phenotypically isolate 999 show resistance towards all the 3 class of beta lactam antibiotics. But isolate 996 phenotypically susceptible for all beta lactam antibiotics though there was presence of SHV-1. Upstream 5' UTR sequence detected in isolate 996 which was under lined in figure -9 (Isabel et al. 2021).

Hypothetically we can predict this UTR sequence not allow the gene expression.

SHV-11 is a broad-spectrum beta-lactamase found in *E. coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Shigella dysenteriae*. It is differing from SHV-1 only at amino acid position 35, instead of L(Leu) SHV-11 carried Q (Gln). Comparison of gene sequences in the GenBank database revealed homology of the positions 74 to 935 with SHV-11 (99.77 % identity, position 157681 to 158541; accession no. X98101:74-935). It was present in isolate 678 and resistant to all beta lactam antibiotics which used in our study (Anais et al. 2011; Isabel et al. 2021).

Figure 10: (Chen, Y.T et al. 2014) (https://www.ncbi.nlm.nih.gov/nucore/NC_020893.1?report=graph&from=55729&to=57966&strand=true)

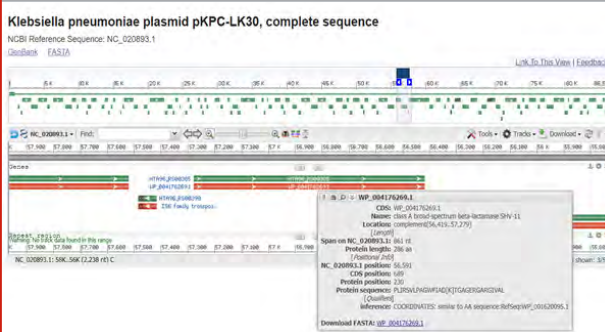
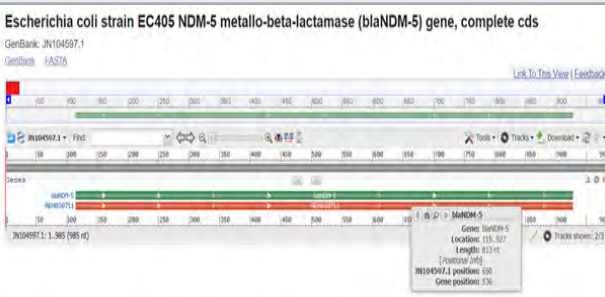


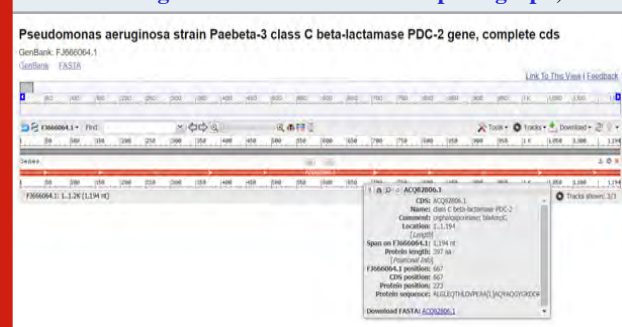
Figure 11: (Hornsey et al. 2011) (<https://www.ncbi.nlm.nih.gov/nucore/JN104597.1?report=graph>)



TEM-1 is the commonly encountered and one of the most well-known beta-lactamase in gram-negative bacteria confirmed resistance against penicillins and generally first-generation cephalosporin. All TEM variants differ in amino acid sequence (in general by one to five substitutions) and many of them differ in resistance phenotype (the degree of

resistance they confer to different antibiotics). In this study TEM-1 was detected in isolate 678 and TEM-150 was in isolate 027 (Salverda et al. 2010; Nadezhda et al. 2021). NDM-5 new delhi metallo beta lactamase was detected in isolate 678 only. It resembles resistance against penicillin, cephalosporin and carbapenem class antibiotics. In the study of Hazim et al. in 2016 and Lauren et al. in 2019 NDM-5 was responsible for the resistance against beta lactam antibiotics. If we compare gene sequence in the GenBank database, it revealed homology of the positions 115-928 in *E. coli* with NDM-5 (100% identity, position 457 to 1569; accession number JN104597:115-928) (Wang et al. 2020).

Figure 12: (Rodriguez-Martinez et al. 2009) (<https://www.ncbi.nlm.nih.gov/nucore/FJ666064.1?report=graph>)



PDC-2 is a class-C ESBL generally present in *Pseudomonas aeruginosa* express resistance against cephalosporin and carbapenem class antibiotics. It is most frequently AmpC-type variant. We detected it in isolate 968. Comparison of gene sequences in the GenBank database revealed homology of the positions 1 to 1195 in *Pseudomonas aeruginosa* with CTX-M-15 (99.58 % identity, position 734 to 1927; accession number FJ666064:1-1195) (Zain et al. 2020).

CONCLUSION

The findings of the present study determine that with the increasing worldwide occurrence of antibiotic resistant pathogens, knowing how bacteria adapt and evolve to drug stress is enormously important. This study showed the distribution of the possible resistome for *Klebsiella pneumoniae* isolates through WGS analysis. The presence of specific AMR genes represents a development towards higher MICs values for β -lactams anti microbials. The results of this work showed the association between phenotypic resistance and the number of exclusive AMR gene sequences. There were difference and similarity in the phenotypic and genotypic resistance between bacterial species. Some specific AMR genes found in these genomes could be potentially invented from horizontal transfer of mobile elements. In general, more studies are needed to search the probable sources of these AMR genes.

Conflict of Interests: Authors declare no conflict of interests to disclose.

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Biomedical Communication

Health and Nutritional Status of Certain Lactating Mothers of Bahawalpur, Pakistan

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ABSTRACT

Diet is the vital part to survive, and balanced diet is the key to better and healthy living. Our daily needs of the body depend upon food to intake for better and proper functioning of vital organs and all organs. These include carbohydrates, proteins, fats, vitamins, and minerals. The amount required for living is much lower than required for the nourishing an offspring. Lactation is an astonishing procedure in which the mother's body makes a secretion that has no immediate advantage to them but may completely support the baby. It was descriptive hospital-based study conducted among lactating mothers visiting Bahawal Victoria Hospital, Bahawalpur Pakistan. Sample size comprised of 200 lactating mothers. The Chi-square statistical test was used to analyze the difference between the groups by SPSS of latest version. The results revealed that groups of Body mass index (BMI), age groups, breast feeding status, working status, health status, family income, family member, mode of delivery, infant age status, parity, energy intake, protein intake, fat intake and carbohydrate intake had statistically significant differences while the educational status had no significant differences. In this study dietary intake pattern of lactating mother was normal because mostly mothers were educated, they have good knowledge of balanced diet.

KEY WORDS: BAHAWALPUR, DIET STATUS, HEALTH STATUS, MILKING MOTHERS.

INTRODUCTION

Nutrition is a significant part of health among more seasoned populace and affects the growth. Pervasiveness of lack of healthy sustenance in this population is increasing and linked with decrease in functional status, disabled muscle function decreased bone density, insusceptible brokenness, paleness, decreased subjective action, poor injury mending and delayed recovery from medical procedures, higher readmission rates for emergency clinics, and death. Older people have often decreased appetite along with a reduction in biological and physiological capacities such as decreased

lean body mass, increases in cytokine and hormonal levels, and improvements in the regulation of liquid electrolytes, slowing the emptying of the stomach and increasing the senses of smell and taste (Mardani et al. 2020). Nutritional Assessment is a method of acquiring and analyzing data in order to make decisions regarding the type and cause of food-related medical problems that affect a person (Anjos et al. 2021). Lack of nutrition is characterized as a disorder in which a lack, excess or clumsiness of energy, food and various supplements cause unpleasant ramifications for body structure, work and clinical outcome (Rajpoot et al. 2020; Leite et al. 2020).

The diet of a breastfeeding mother can have an impact on the composition of human milk. The degree of the maternal diet's influence on milk composition varies per nutrient;

Article Information:*Corresponding Author: wasimape@gmail.com

Received 15/10/2021 Accepted after revision 18/12/2021

Published: 31st December 2021 Pp- 1854-1860

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.70>

RESULTS AND DISCUSSION

In the present study the result of BMI of lactating mothers showed in (figure 2) 2% were underweight 72% were normal weight and 26% were over overweight. Chi-square statistical test revealed that groups had significant ($P<0.0001$) differences in (table 1). The feeding practice among lactating mothers was 88% and 12% was not feeding to their babies due to some health issues. The chi-square statistical test revealed that lactating mothers which were feeding their child had significant high frequency than the lactating mothers which were not feeding their child. The global picture of breast-feeding practice shows that exclusive breastfeeding rates are low comparison to the present study, at about 25% in Africa, 45% in Asia and 31% in Latin America and the Caribbean (Lauer et al. 2004). In present study, the rate of breastfeeding appropriate to infant's age was found 88%. This finding was similar to the study done in the community assessment finding by Essential Service for Health in Ethiopia (ESHE) in Amhara (87%) (Kidane et al. 2008; Ali et al. 2021). But percentage less than the study showed in the previous studies (Seid et al. 2013; Widiyanto et al. 2021).

The 48% lactating mother were government employ 20% working in fields and other 32% were housewife. Chi-square statistical test revealed that groups had significant ($P<0.01$) differences in (table 5 similar results were discovered in the previous studies (Abou-Rizk et al. 2021; Dewi et al. 2021). While the result of study was not in agreement with the study conducted in the previous studies (Susanto et al. 2021). According to disease status 70% mothers were healthy other were suffering from different disease like hypertension, renal stone, diabetes, asthma, HCV and anemia showed in (figure 2). Statistical analysis revealed that groups had significant ($P<0.0001$) differences in (table 6) and group of Healthy lactating mothers had a statistically significant ($P<0.0001$) high frequency (Susanto et al. 2021).

The result of current study was in agreement with the study conducted in the past (Abou-Rizk et al. 2020). While the low percentage found in the previous studies (Widiyanto et al. 2021; Ali et al. 2021). This was due to different geographical area and level of awareness in the resident of this area. The present study showed that 40% lactating mothers belong to families of income level having 5000- 25000 Pkr, lactating mothers 22% had family income between 26000-45000 Pkr, have their small business at their homes and remaining 38% have the government jobs and other income sources and income was more than 45000. Chi-square statistical test revealed that groups had significant ($P<0.05$) differences in (table 7).

Similar result found in the previous studies (Ali et al. 2021). In present study the knowledge and eating habits of mothers were enquired and effect of different socio- economic, cultural and different lifestyles were observed. Regarding socio-economic condition of study population 40% mothers belonged to lower social class and most of their husband were laborers and farmers, 32% lactating mothers was housewives only 1 member was earning and remaining

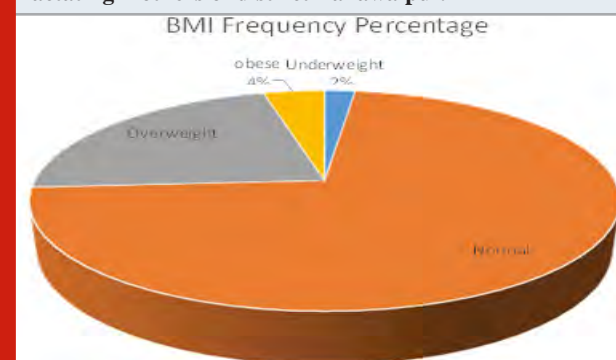
family member were dependent on his earnings. It shows economic conditions of family is affected 68% mothers were working women and their income level was handsome because they were educated and have awareness about balanced diet. So, they were taking as per recommended daily allowances (Ali et al. 2021).

The study explored that groups family members 3-5, 6-8 and 9-12 showed 88%,10% and 2% percentage frequency respectively. The group member 3-5 showed a statistically significant high frequency than others in table (4.8). It showed the awareness about family planning and population direction towards the small family. Similar to this study the result was found in the previous studies (Ali et al. 2021). According to mode of delivery status among lactating mothers 24% delivered their babies by LSCS and 76% by SVD. There was a significant difference between both of the group and SDV had a significant high frequency. The present study not in agreement with the study conducted in the past (Dewi et al. 2021; Susanto et al. 2021). In this study the infant age was one month to eighteen months. The groups of different ages had significant differences. The age group less than 6 months had a statistically significant high frequency than other groups (Susanto et al. 2021).

Table 4.1: The BMI Status of lactating mothers of district Bahawalpur (n=200)

BMI categories	Classification	Number of Subjects	P-Value
Less than 18.5 kg/m ²	Underweight	4	<0.0001
18.5-24.9 kg/m ²	Normal	144	
25.0-29.9	Overweight	44	
30kg/m ² and more	Obese	8	

Figure 2: The percentage frequency of BMI status of lactating mothers of district Bahawalpur.



The prevalence of continued breastfeeding is about 86% for infants 6–11 months of age in the developing world and for children 12–23 months of age, it is about 37% in Latin America, in the Caribbean 70% and 72% in Africa and Asia (Lauer et al. 2004). According to results of parity 32% mother were primary gravida and 68% were multi gravida. The age group Multi parity had a statistically

significant high frequency than other groups. In this study results explored that 74% lactating mothers were taking recommended calories and 18% were taking over calories and remaining 8% mothers were under calories. The recommended calories intake group had a statistically significant high frequency than other groups. The present study was in agreement with the study conducted in the past (Widiyanto et al. 2021; Ali et al. 2021; Taneja et al. 2020). While the result was not in agreement with the previous work conducted in the past (Abou-Rizk, et al. 2020).

Table 4.2: Age wise distribution of lactating mothers of district Bahawalpur (n=200)

Age Groups (years)	Lactating	%age	P-Value
18-25	48	24%	<0.0001
26-32	124	62%	
33-40	28	14%	

Table 4.3: Educational Status of lactating mothers of district Bahawalpur (n=200)

Educational Status	Frequency	%age	P-Value
Uneducated	44	22%	0.3543
Primary and middle	64	32%	
Matric and Intermediate	52	26%	
Bachelor and above	40	20%	

Table 4.4: Breast feeding practice among lactating mothers of district Bahawalpur (n=200)

Status	Frequency	%age	P-Value
No	24	12%	<0.0001
Yes	176	88%	

Table 4.5: Working Status of lactating mothers of district Bahawalpur (n=200)

Status	Lactating	%age	P-Value
Government employs	96	48%	<0.01
Worker	40	20%	
House wives	64	32%	

A nutrition indicator which showed that 2% were underweight 72% were normal weight and 26% were over overweight. Biru and Abeya (2017) showed that in Ethiopia, a significant proportion (19.5%) of respondents were exposed to under nutritional status and suffered from underweight (Biru and Abeya, 2017). The diet is highly

correlated with the welfare of the individual (Mushtaq et al. 2021). The study result from Rural India during 2010 indicated the prevalence of undernutrition among lactating women of 39.7% which is much higher than our study results by Rao et al. (2010) and Ali et al. (2021). The results revealed that 70% lactating mothers were taking normal protein intake and 14% were taking over protein intake and remaining 16% mothers were under protein intake. The normal protein intake group had a statistically significant high frequency than other groups.

Table 4.6: Medical History of lactating mothers of district Bahawalpur (n=200)

Disease Status	Status	P-Value
High blood pressure	8	<0.0001
Renal Stone	4	
Diabetes	20	
Asthma	4	
HCV	4	
Anemia	20	
Healthy	140	

Figure 3: The disease percentage frequency of lactating mothers of district Bahawalpur.

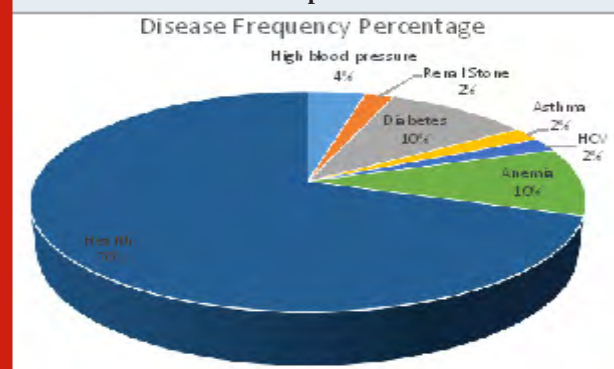


Table 4.7: Family income of lactating mothers of district Bahawalpur (n=200).

Family income in Pkr	Frequency	%age	P-Value
5000-25000	80	40%	0.04
26000-45000	44	22%	
Above than 45000	76	38%	

The result of present study was not in agreement with the study conducted by Widiyanto et al. (2021). The result difference occurred due to different geographical regions and economy and financial status of the people. Results also revealed that the 72% lactating mothers were taking normal fat intake and 26% were taking over fat intake and remaining 2% mothers were under fat intake. The normal fat intake group had a statistically significant high frequency

than other groups. Result reconnoiter that the 85% lactating mothers were taking normal carbohydrate intake and 14% were taking over carbohydrate intake and remaining 1% mothers were under carbohydrate intake (Widiyanto et al. 2021).

Table 4.8: Family members of lactating mothers of district Bahawalpur (n=200).

Family size	Frequency	%age	P-Value
3-5	176	88%	<0.0001
6-8	20	10%	
9-12	4	2%	

Table 4.9: Mode of delivery status among lactating mothers of district Bahawalpur (n=200).

Mode	Frequency	%age	P-Value
LSCS	48	24%	<0.0001
SVD	152	76%	

Table 4.10: Infant age status of lactating mothers of district Bahawalpur (n=200)

Family size	Frequency	%age	P-Value
Less than 6 months	114	57%	P <0.0001
7-12 months	58	29%	
13-18 months	28	14%	

Table 4.11: Parity of lactating mothers of district Bahawalpur (n=200).

Gravida	Frequency	%age	P-Value
Primary	64	32%	0.0003
Multi	136	68%	

Table 4.12: The energy intake of lactating mothers of district Bahawalpur (n=200).

Rank	Energy intake	Lactating	P-Value
Recommended	2500-3000	148	<0.0001
Over	3100-3500	36	
Under	1500-1800	16	

The normal carbohydrate intake group had a statistically significant high frequency than other groups. The result of present study was in agreement with the study conducted

in the past (Abou-Rizk et al. 2021; Ali et al. 2021). While not in agreement with the study conducted by Dewi et al. (2021). A breastfeeding woman requires an additional 500 kcal for the first six months and 400 kcal for the remaining six months. This may be achieved by consuming extra 6-8 pieces of bread every day, for example. Simply eating a more balanced diet should help to cover the increased energy need while the woman breastfeeds. The caloric content of 100 ml of human milk is around 70 kcal. 750 ml of breast milk is produced per day for the first six months following birth. If excess energy needs aren't supplied through nutritional mean, fat reserves will be used instead (Dewi et al. 2021).

Figure 4:

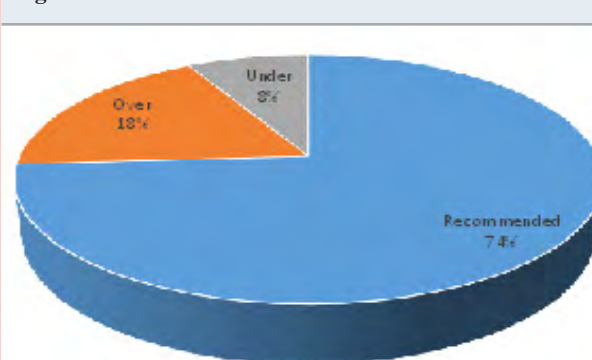


Table 4.13: Protein intake Status In lactating mothers of district Bahawalpur (n=200)

Intake	Frequency	%age	P-Value
Normal	140	70%	<0.0001
Over	28	14%	
Under	32	16%	

Table 4.14: Fat intake among of lactating mothers of district Bahawalpur (n=200).

Range	Frequency	%age	P-Value
Normal	144	72%	<0.0001
Over	52	26%	
Under	4	2%	

Table 4.15: Daily Carbohydrate intake of lactating mothers of district Bahawalpur (n=200)

Range	Frequency	%age	P-Value
Normal	144	72%	<0.0001
Over	52	26%	
Under	4	2%	

When compared to calorie needs, the increase in protein requirements during lactation is minor (Taneja et al. 2020). Protein, on the other hand, will be utilized for energy generation if your energy intake is minimal. Protein-rich meals can help meet the extra protein requirements during nursing (e.g., one egg or 25g of cheese or 175g of milk). If moms do not consume enough protein, the amount of casein in their milk may be lowered. Breast milk contains casein protein, which helps to deliver calcium and phosphate to the infant. It also causes a clot in the stomach, allowing for better nutrition (Susanto et al. 2021).

CONCLUSION

The findings of the present study was concluded that the eating practices among lactating mothers in Bahawal Victoria Hospital Bahawalpur were normal. The socio-economic conditions, education of mother was main reason for taking their balanced diet. The analysis of their intake shows that the nutrients intake of lactating mothers was balanced and sufficient as per their requirements. Nutrition interventions should be geared towards improving both the nutritional knowledge and the nutrient intake of the lactating mothers.

Conflict of Interests: Authors declare no conflict of interests to disclose.

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Biotechnological Communication

Comparative Rheological Properties of Soy Lecithins Produced in Russia

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ABSTRACT

The work is devoted to a comparative study of the features of the structural and mechanical properties of soybean lecithins, produced in Russia, in their relationship with the composition and other characteristics. Considering that the cultivation of transgenic agricultural crops is prohibited in Russia, Russian plant raw materials and products of its processing, including lecithin, are of interest and are in demand in a number of European countries. Despite the fact that the main raspberry raw material in Russia is sunflower, the volume of processing of soybeans grown without the use of genetic modification methods, the main plantations of which are located in the Far East, ranks second after sunflower. Lecithin production technologies in Russia are mostly focused on the production of so-called "raw" liquid lecithins and do not provide for the operation of their subsequent conditioning in order to ensure special characteristics of the composition and consumer properties. Despite this, raw soy lecithins produced by Russian enterprises mostly meet the requirements of GOST 32052-2013 and the European Union E 322 requirements and can be positioned as standard liquid lecithins. At the same time, during the release of such lecithins, problems associated with an increase in their viscosity during storage are periodically observed. This article presents the results of comparative studies of the composition and properties of liquid soybean lecithins of various consistencies and presents conclusions on the factors that determine the features of their structural and mechanical properties.

KEY WORDS: CHEMICAL COMPOSITION, LECITHIN, PHOSPHOLIPIDS, RHEOLOGICAL PROPERTIES AND VISCOSITY.

INTRODUCTION

Lecithins are one of the most demanded food additives obtained as a secondary product in the processing of oilseeds (Shahidi 2005; Considine and Kulik 2006; Hernandez and Quezada 2008; Nieuwenhuyzen and Tomás 2008; Hoogevest et al. 2014; Ahmad and Xu 2015). Lecithins available on the modern market differ in the type of feedstock and in the specific composition and technological and functional properties formed as a result of special technological methods for processing raw lecithin obtained in the process of degumming vegetable oils (Joshi et al. 2006; Nieuwenhuyzen and Tomás 2008; Ahmad and Xu 2015). Another difference in lecithins, the importance of which has increased recently, is the specificity of the origin of the feedstock: with the use of GMOs (genetically modified organisms) or without GMOs (Heiser 2016). The European market prefers lecithin obtained from non-GMO

raw materials (Cabezas et al. 2012; Ahmad and Xu 2015; Schneider 2019). This lecithin is the basis for the production of organic lecithin, the market for which has been actively growing over the past ten years (Cabezas et al. 2012; Ahmad and Xu 2015). Considering that the cultivation of transgenic agricultural crops is prohibited in Russia (Federal Law of the Russian Federation of 17.12.1997 no. 149-FZ), Russian plant raw materials and products of processing, including lecithin, are of interest and are in demand in certain European countries (Fedorova 2019; Sinegovsky 2019).

Lecithin production technologies in Russia are mostly focused on the production of so-called "raw" liquid lecithins and do not provide for the operation of their subsequent conditioning in order to ensure special characteristics of the composition and consumer properties. Despite this, raw soybean lecithins produced by Russian enterprises typically comply with the requirements of GOST (State standard of Russian Federation) 32052 and the European Union E 322 requirements and, therefore, can be positioned as standard liquid lecithins. At the same time, problems associated with an increase in their viscosity during storage are periodically observed. According to the results of some studies, the

Article Information:*Corresponding Author: rosmaplus@gmail.com

Received 19/09/2021 Accepted after revision 25/12/2021

Published: 31st December 2021 Pp- 1861-1870

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.71>

lecithin viscosity is a complex function of the content of the acetone insoluble matter, moisture, minerals, and acid value (Shahidi 2005; Nieuwenhuyzen and Tomás 2008; Ahmad and Xu 2015; Heiser 2016; Sinegovsky 2019).

As a general rule, the higher the content of acetone insoluble matter and moisture, the higher the level of the viscosity of liquid lecithins, and, on the contrary, a high acid value leads to a decrease in the viscosity (Ahmad and Xu 2015). However, in scientific publications, the results of comparative studies of the composition and properties of raw lecithins of different viscosity are not sufficiently presented, which does not allow for the prediction of the quality of finished products of raw lecithins by monitoring the incoming raw materials. This work is devoted to the study of these aspects.

MATERIAL AND METHODS

Samples of raw liquid soybean lecithins produced at Russian enterprises from Russian raw materials were used as the objects of research. In appearance, samples 1 and 2 were a homogeneous viscous liquid, and samples 3 and 4 were in the form of a plastic mass. The rest of the organoleptic characteristics of the lecithin samples

corresponded to the established requirements: color—from yellow to brown; smell and taste—typical for soy lecithin. The determination of the organoleptic parameters, as well as the physicochemical properties, including the content of the toluene insoluble matter and acetone insoluble matter, content of moisture and volatile substances, acid value, peroxide value, color of 10% solution in toluene, and viscosity at 25 °C, was performed in accordance with the control methods regulated by GOST 32052 and corresponding international standards (Federal Agency for Technical Regulation and Metrology 2013).

The determination of the rheological characteristics of lecithins was conducted using a Brookfield rotary viscometer, model LVDV-II + Pro, spindle LV4 (code 64). The composition of fatty acid acyls of the phospholipid complex was determined according to ISO 12966 (European Committee for Standardization, 2015) using a gas chromatograph “Kristall-5000” (ZAO SKB Khromatek Russia), column SOLGEL-WAX 30 m × 0.32 mm ID SOLGEL- WAX × 0.5 μm). The group composition of phospholipids was investigated by high performance liquid chromatography (HPLC) using an “Agilent 1260 Infinity” high performance liquid chromatograph (Agilent Technology, USA), a LiChrospher 100 250x4 mm column, Diol (5 μm).

Table 1. Physicochemical indicators of lecithins

Indicator	Sample 1	Sample 2	Sample 3	Sample 4	Requirements of GOST (State standard of Russian Federation) 32052	Requirements European Union E 322
acetone insoluble matter,%	60.1	62.25	61.47	61.13	No less than 60.0	No less than 60.0
toluene insoluble matter,%	0.30	0.27	0.19	0.16	No more than 0.30	No more than 0.30
moisture and volatile substances,%	0.19	0.19	0.11	0.31	No more than 1.0	No more than 2.0
Acid value, mg KOH/g	25.1	26.4	18,7	16.9	No more than 36.0	No more than 35.0
Peroxide value meq/kg	less than 0.1	1,2	1.0	1.5	No more than 10.0	No more than 10.0
Color of a 10% solution in toluene:						
mg I2	79	58	58	53	No more than 80.0	-
units Gardner scale	11.9	11.0	11.0	10.9	Not standardized	-
Viscosity at 25 oC, Pa * s	6.9	9.3	Over 2000		No more than 12.0	-

This analysis was in accordance with the methods given in the previous studies, as well as by thin layer chromatography (TLC) with subsequent processing of chromatograms by scanning densitometry (Paronyan and Scriabin 2007; Deutsche Forschungsgemeinschaft (DFG) 2007; The American Oil Chemists' Society 2017). When implementing the TLC method, we used high-performance “Sorbfil” plates with a silica gel particle size distribution of 8–12 μm. The solvent system chloroform/methanol/

water (65:25:4) was used as the mobile phase. For the development of chromatograms, a solution of phosphoric-molybdic acid (PMA) in ethyl alcohol with a concentration of 5% was used. The spots obtained were identified using taps and specific tests for individual phospholipid groups. The content of unsaponifiable substances was determined according to the method of (Paronyan and Scriabin 2007).

The composition of unsaponifiable substances was determined by gas chromatography–mass spectrometry using a “Kristall 5000 MS” gas chromatograph and the NIST (National Institute of Standards and Technology, USA) library. The content and composition of tocopherols was determined in accordance with EN 12822 (Federal Agency for Technical Regulation and Metrology, 2015). The content and composition of sterols were determined according to ISO 12228-1 using a “Kristall 5000” gas chromatograph. The content of polyvalent metal ions was determined using an atomic absorption spectrometer “GTA 120 240FS AA” (Agilent Technologies) with flame

and electrothermal atomization, USA. The studies were performed on the equipment of the Research Center of Food and Chemical Technologies of the Federal State Budgetary Educational Institution of Higher Education “Kuban State Technological University” (European Committee for Standardization, 2014).

RESULTS AND DISCUSSION

The results of the study of the main physical and chemical characteristics of lecithin samples, typically normalized for lecithins by domestic and foreign standards, are presented in Table 1.

Table 2. Acid value of lecithins

Lecithin sample	general	Acid value, mg KOH/g	
		due to the presence of free fatty acids	due to the presence of acidic forms of phospholipids
Sample 1	24.4	5.2	19.2
Sample 2	26.4	7.1	19.3
Sample 3	18,7	2.4	16.3
Sample 4	18,6	2.1	16.6

Table 3. Group composition of phospholipids

The name of the phospholipid group	The content of the phospholipid group, % of the total			
	Sample 1	Sample 2	Sample 3	Sample 4
Phosphatidylinositols (PI)	21.6	19.9	13.2	14.4
Phosphatidylcholines (PC)	24.7	21.0	24.3	23.1
Phosphatidylethanolamines (PEA)	26.5	27.2	31.5	29.5
Phosphatidic and polyphosphatidic acids (PA and PPA)	15.3	15.4	13.5	13.2
Di- and phosphatidylglycerols (DPG and PG)	12.0	16.4	17.5	19.8

The analysis of the physicochemical indicators presented in Table 1 shows that, with the exception of the viscosity at 25 °C, all lecithin samples met the requirements, regulated by GOST 32052 and the European Union for the quality of liquid lecithins. The quality of lecithin, characterized by the above indicators, is formed, as a rule, under the influence of technological factors. The acetone insoluble matter characterizes the content in lecithin of the main groups of polar lipids, such as phospholipids and glycolipids, as well as carbohydrates (Van Nieuwenhuyzen and Tomás, 2008). Phospholipids typically make up about 45%, glycolipids—11%, and carbohydrates—4% (Nieuwenhuyzen and Tomás 2008; Ahmad and Xu 2015). Thus, differences between the studied lecithin samples in the values of physicochemical indicators (Table 1) do not allow us to conclude the reasons for the differences in the consistency and viscosity of liquid samples 1 and 2 and plastic samples 3 and 4.

Along with the considered indicators, an important indicator of the quality of lecithins is the acid value, which characterizes both the content of free fatty acids that have

passed into the phospholipid emulsion during degumming in the composition of neutral lipids and the actual content of acidic forms of phospholipids. Research has demonstrated that the acid value of lecithins correlates with the level of their viscosity (Nieuwenhuyzen and Tomás 2008; Van Nieuwenhuyzen 2014). All other things being equal, lecithins with a higher acid value have a lower viscosity than lecithins with a lower acid value. For example, according to early scientific publications, the acid value of liquid commercial lecithins was 24.7–34.4 mg KOH/g, in contrast to plastic samples characterized by an acid value of 21.9–22.9 mg KOH/g (Pardun 1964; Ahmad and Xu 2015; Sinegovsky 2019).

The results of determining the acid value in the analyzed lecithin samples confirm the above position, namely, the acid value of the samples of soybean lecithins No. 3 and 4, having a plastic consistency, ranged from 16.9 to 18.7 mg KOH/g, which is significantly lower (in 1.4 times on average) than the acid value of liquid samples of soybean lecithins No. 1 and 2, which ranged from 24.4 to 26.4

mg KOH/g. To reduce the viscosity of lecithins, foreign companies often use the addition of distilled fatty acids to lecithins, if the value of the acetone insoluble matter allows. This operation is combined with the dilution of lecithins with refined deodorized vegetable oil (Yaron and Letan 1975; Nieuwenhuyzen and Tomás 2008; Nieuwenhuyzen 2014). To determine the content of free fatty acids in lecithin samples, the acid value of their acetone miscella was analyzed in accordance with the recommendations (Shahidi 2005; Nieuwenhuyzen and Tomás 2008; Sinegovsky 2019). The results are shown in Table 2.

As can be seen from the presented data, the difference between the acid value of the samples of soy lecithins No. 1 and 2 and No. 3 and 4 is, on average, 2.8 mg KOH/g, which corresponds to the content of free fatty acids in terms of oleic acid, which is 1.4%. According to the literature, the addition of 2% oleic acid to lecithins results in a decrease in viscosity by half on average. Considering that the difference in the viscosity of liquid (No. 1, 2) and plastic (No. 3, 4) samples of soy lecithins is, on average, more than 250 times,

it is not possible to explain this solely by the influence of a larger amount of free fatty acids (Pardun 1964; Yaron and Letan 1975; Sinegovsky 2019).

Lecithins are structured non-Newtonian systems, the structural and rheological properties of which are determined by the interactions between their constituent components, which are phospholipids, neutral lipids, and minor accompanying substances, including carbohydrates and unsaponifiable lipids. The peculiarities of interactions between phospholipids depend on their group and fatty acid composition, as well as on their chemical composition, since phospholipids in unrefined oils can be represented both by individual molecules (in the form of associates) and complex compounds with unsaponifiable lipids and polyvalent metal ions (Arutyunyan and Kornena 1986; Sinegovsky 2019). Considering this, at the next stage of research, we studied the group composition of phospholipids, the composition of fatty acids, the composition of the accompanying minor components and polyvalent metal ions present in the lecithin samples. The results of the study of the group composition of phospholipids are presented in Table 3.

Table 4. Composition of fatty acids in the studied lecithin samples

Fatty acid name	Fatty acid content, % of the total			
	Sample 1	Sample 2	Sample 3	Sample 4
C16: 0 Palmitic	18.58	17.83	20.94	20.76
C18: 0 Stearic	4.50	4.04	4.40	4.12
C18: 1 Oleic	14.47	14.80	9.49	10.08
C18: 2 Linoleic	54.84	51.39	48.70	54.93
C18: 3 Linolenic	5.67	5.52	7.38	8.11
C22: 0 Behenic	2.04	6.42	9.09	2.01
sum of saturated	25.02	28.28	34.43	26.89
sum of polyunsaturated	60.51	56.91	56.08	63.04

Table 5. Composition of fatty acids of acetone-insoluble polar lipids in the studied lecithin samples

Fatty acid name	Fatty acid content, % of the total			
	Sample 1	Sample 2	Sample 3	Sample 4
C16: 0 Palmitic	22.01	22.51	20.46	21.19
C18: 0 Stearic	4.97	4.32	3.93	3.03
C18: 1 Oleic	10.56	10.17	9.69	7.86
C18: 2 Linoleic	54.11	52.72	57.68	55.94
C18: 3 Linolenic	6.18	5.49	7.60	9.59
C22: 0 Behenic	2.17	4.79	0.64	2.38
sum of saturated	29.15	31.62	25.03	26.60
sum of polyunsaturated	60.29	58.21	65.28	65.53

From the presented data, the samples of liquid lecithins No. 1 and 2 differ from the samples of plastic lecithins No. 3 and 4 by a higher content of phosphatidylinositols and a lower content of phosphatidylethanolamines. At the same time, phosphatidylethanolamines are the predominant group of phospholipids in the samples of plastic soy lecithins. Considering that phosphatidylinositols have pronounced

acidic properties, the data obtained are consistent with the revealed differences in the acid value values of liquid and plastic samples of soy lecithins. The results of studying the composition of fatty acid acyls of lecithin samples, as well as the acetone-insoluble polar lipids included in their composition, are presented in Tables 4 and 5 (Arutyunyan and Kornena 1986; Fedorova 2019).

Our analysis of the data presented in Table 5 showed that the acetone-insoluble polar lipids that are part of the studied lecithin samples contained fatty acid acyls characteristic of the corresponding types of vegetable oils. At the same time, no regularities in the differences in the compositions of fatty acid acyls for liquid and plastic soy lecithins were revealed.

Among the fatty acid acyls of the acetone-insoluble polar lipids included in the samples of plastic soy lecithins No. 3 and 4, a slightly higher content of polyunsaturated fatty acids was observed; however, this difference is not decisive. Nevertheless, this difference is consistent with the data on the group composition of phospholipids (Table 3), namely, with the predominance of phosphatidylethanolamines

in the composition of plastic samples of soy lecithins, which, along with phosphatidylcholines, are characterized by the highest degree of unsaturation of their constituent acyls of fatty acids. At the same time saturated fatty acids predominate in the composition of phosphatidylinositols (Arutyunyan and Kornena 1986; Fedorova 2019).

The results of the study of the minor accompanying substances of lecithins are presented in Table 6. From the presented data, significant differences between the studied lecithins were seen in the content of tocopherols and polyvalent metal ions. The content of tocopherols correlated with the content of polyunsaturated fatty acids in the lecithin samples (Table 4) and generally corresponded to the composition and content of tocopherols in the feedstock.

Table 6. The composition of the minor accompanying substances of lecithins

Indicator	Sample 1	Sample 2	Sample 3	Sample 4
Mass fraction of unsaponifiable lipids,% including:	1.79	0.57	1.12	0.88
Aliphatic alcohols	0.09	0.02	0.48	0.09
Squalene	0.010	0.005	0.010	0.011
Sterols including:	0.287	0.325	0.342	0.377
campesterol	0.067	0.099	0.108	0.118
stigmaterol	0.068	0.082	0.094	0.103
β -sitosterol	0.152	0.144	0.140	0.156
Content of tocopherols, mg/100 g, including:	60.04	75.45	93.32	94.77
α -tocopherol	20.22	7.44	11.25	9.85
$\beta + \gamma$ -tocopherol	60.04	68.01	82.07	84.92
δ -tocopherol	Lack of	Lack of	Lack of	Lack of
Carbohydrate content,%	8.4	5.9	7.7	7.4
Content of ions of polyvalent metals, mg/kg:				
Cu	0.57	0.07	0.22	0.08
Fe	27.40	9.87	Less than 0.1	Less than 0.1
Pb	Less than 0.01	Less than 0.01	0.09	Less than 0.01
Zn	11.09	Less than 0.1	Less than 0.1	1.79

In the samples of liquid lecithins No. 1 and 2, there was a significantly greater number of polyvalent metals, compared to the samples of plastic soy lecithins No. 3 and 4. There was an almost complete absence of Fe (less than the detection limit of the method) in the samples of plastic lecithins No. 3 and 4; however, in the samples of liquid lecithins, this metal was predominant. These data are in part consistent with the data on the phospholipid group composition of the studied lecithin samples (Tables 3 and 4), according to which, in liquid lecithin samples 1 and 2, the content of the acidic forms of phospholipids, including phosphatidylinositols and phosphatidic and polyphosphatidic acids, formed stable complex compounds with polyvalent metals, significantly more than in the plastic samples of lecithins No. 3 and 4. Differences in the phospholipid group composition cannot explain the almost complete absence of Fe in the samples of

plastic soy lecithins No. 3 and 4, although acidic groups of phospholipids present in a smaller amount in the composition of these samples of lecithins, can form stable complexes with Fe (Arutyunyan and Kornena 1986; Bogdanor 1988; Smitss et al. 1988; Ahmad and Xu 2015).

Salts of polyvalent, predominantly divalent metals are used in foreign technologies to reduce the viscosity of standard liquid lecithins (Van Nieuwenhuyzen and Tomás, 2008; Van Nieuwenhuyzen, 2014; Ahmad and Xu, 2015). This technological method is based on the ability of the polar groups of phospholipids to interact with ions of polyvalent metals and to form complex compounds, which provides screening of the polar groups of phospholipid molecules and leads to a decrease in the interaction energy and the ability to

form micelles. The reason for the low content of polyvalent metal ions in the composition of lecithins can be both the characteristics of the raw materials used and the use of special technological methods, consisting in the preliminary treatment of unrefined oil with complex-forming agents that form stable complexes with the polyvalent metals (Bogdanor 1988; Smitss et al. 1988; Ahmad and Xu 2015; Fedorova 2019).

The purpose of such treatment is typically to increase the degree of degumming of phospholipids and to increase the yield of lecithin, although other goals may be pursued, to achieve the desired functional properties of the lecithins (Bogdanor 1988; Smitss et al. 1988; Ahmad and Xu 2015).

Thus, the low content of polyvalent metal ions in the composition of soybean plastic lecithin samples No. 3 and 4 may be one of the reasons for their high viscosity. Analysis of the composition of other related substances in the lecithin samples (Table 7) showed the following. In the composition of lecithins of plant origin, carbohydrates are found in both

free and bound forms (Arutyunyan and Kornena 1986). Bound carbohydrates are present both in the composition of glycolipids and in the form of structural elements of phospholipid molecules. The presence of free carbohydrates is mainly explained by the formation of mixed associates as a result of the appearance of intermolecular hydrogen bonds between the P = O, P-OH, and NH₂ groups of phospholipid molecules and the C = O, C-OH groups of carbohydrate molecules. According to Arutyunyan and Kornena (1986), phospholipids isolated from unrefined vegetable oils may contain free carbohydrates in an amount from 4% to 12% by weight of the extracted phospholipid complex (Arutyunyan and Kornena 1986; Ahmad and Xu 2015; Fedorova 2019).

The content of carbohydrates in the studied samples of soy lecithins generally corresponded to the literature data, while no correlation dependences with their consistency were revealed. A similar conclusion can be drawn with regard to the quantitative and qualitative composition of the unsaponifiable lipids present in the lecithin samples under study (Arutyunyan and Kornena 1986; Ahmad and Xu 2015).

Table 7. Effective viscosity of the studied lecithin samples

Shear rate, s ⁻¹	Effective viscosity value, Pa's			
	Sample 1	Sample 2	Sample 3	Sample 4
0.5	8.4	10.2		
1.0	7.2	9.6	Over 2000	Over 2000
2.0	6.9	9.3		
2.5	7.0	9.3		
4.0	6.9	9.3		
5.0	6.8	9.2		
10.0	6.8	9.1		
20.0	6.7	9.1		
50.0	6.7	9.1		

Figure 1: Rheological flow curves of the lecithin sample No 1 at 25 °C

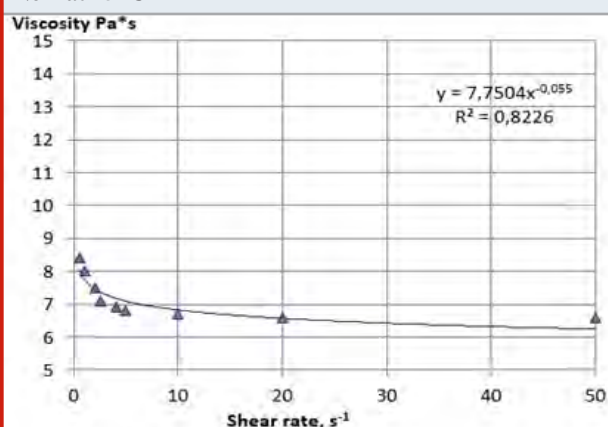


Figure 2: Rheological flow curves of the sample lecithin No 2 at 25 °C

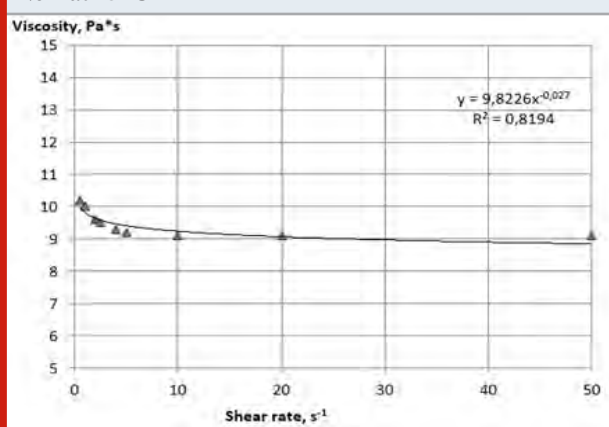


Table 8. Rheological properties of lecithins (Samples 1 and 2)

Indicator	Indicator value	
	Sample 1	Sample 2
Effective viscosity at minimum shear rate (system with intact structure), Pa's	8.4	10.2
Effective (dynamic) viscosity of a system with an extremely destroyed structure, Pa · s	6,7	9.1
Structural destruction coefficient	1,2	1.1

At the next stage of research, the structural and rheological properties of lecithin samples were studied using the method of rotational viscometry. Lecithins with a content of acetone insoluble matter of about 60% are pseudoplastic non-Newtonian systems. Along with the effective viscosity, the structural and rheological properties of non-Newtonian systems are determined by a number of other rheological characteristics, the most complete representation of which is provided by the rheological flow curves with their subsequent mathematical approximation (Evdokimov and Eliseev 2005; Kuznetsov et al. 2005; Sinegovsky 2019).

The rheological flow curves were performed on the basis of experimental data obtained by investigating the samples on a Brookfield LVDV-II + Pro rotary viscometer. Samples of the studied lecithins were placed in a thermostated cell and, after exposure at a predetermined temperature for 30 minutes, a measurement was performed. The range of variation of the shear rate was from 0.5 to 50 s⁻¹. The results of measurements carried out at 25 °C are presented in Table 7. As can be seen from the presented data, the viscosity of the samples of plastic lecithins No. 3 and 4 exceeded the

upper limit of the measuring range of the device, which is 2000 Pa·s, at all shear rates. The rheological curves of the flow of samples of liquid lecithins No. 1 and 2 are shown in Figures 1 and 2. The analysis of Figures 1 and 2 showed that the rheological flow curves of lecithin samples No. 1 and 2 are typical for structured non-Newtonian systems and, therefore, are typical for standard liquid lecithins, which are dispersed systems by their nature with a characteristic macro- and microstructure. The rheological flow curves are described by the Ostwald-de-Ville power equation, which is typical for the flow of pseudoplastic non-Newtonian food systems (Kuznetsov et al. 2005; Sinegovsky 2019).

A fairly rapid decrease in viscosity occurred with an increase in mechanical impacts, due to the processes of destruction of the initial structure of the system and the orientation of the particles of the dispersed phase in the direction of flow, which provides a decrease in the resistance of the system to the displacement of layers relative to each other. The viscosity practically does not change after reaching the limiting shear rates, and the rheological flow curves take the form characteristic for Newtonian systems. The main rheological characteristics of the lecithin samples No. 1 and 2, calculated from the flow curves, are presented in Table 8.

Samples of lecithins No. 1 and 2 were characterized by close values of the coefficient of destruction of the structure, which indicates a similar nature of their structural organization and plastic strength of the structure. The viscosity of lecithins, like other similar non-Newtonian systems, decreases with increasing temperature. Considering this, we investigated the effect of temperature on the structural and mechanical properties of the plastic samples of lecithins No. 3 and 4. The experiment was carried out by thermostating the samples in the measuring cell of a viscometer with a sequential increase in temperature with steps of 1 °C until the viscosity value reached below the upper limit of the measurement range of the device used (less 2000 Pa·s).

Table 9. Rheological properties of lecithins (Samples 3 and 4)

Indicator	Indicator value	
	Sample 3	Sample 4
Effective viscosity at the minimum shear rate (system with intact structure, direct measurement), Pa's	371	88.8
Effective (dynamic) viscosity of a system with an extremely destroyed structure, Pa · s	2.9	4.6
Structural destruction coefficient	128	19
Effective viscosity at the minimum shear rate (reverse measurement), Pa's	76.8	74.4

We found that the temperature that ensured the attainment of a viscosity value of less than 2000 Pa·s was 59 °C for sample 3 and 52 °C for sample 4. To obtain comparable data, rheological flow curves were built at a temperature of 60 °C, which made it possible to carry out measurements for both samples of plastic lecithins (Figures 3, 4). At a temperature of 60 °C, the values of the effective viscosity of the samples of liquid lecithins 1 and 2 were no longer a function of the shear rate and took similar values to 1.5 Pa

s. The main rheological characteristics of the plastic lecithin samples, calculated from the flow curves, are presented in Table 9.

Comparative analysis of the obtained flow curves (Figures 1, 2 and 3, 4) showed that the samples of soy plastic lecithins No. 3 and 4 had a stronger structure even at a temperature of 60 °C compared with the samples 1 and 2 at a temperature of 25 °C. Samples 3 and 4 also demonstrated

thixotropic properties, since, with a decrease in the intensity of mechanical shear action, they demonstrated the ability to partially restore the broken bonds between structural components.

Figure 3: Rheological flow curves of the lecithin sample No. 3 at 60 °C: 1—direct measurement, 2—reverse measurement

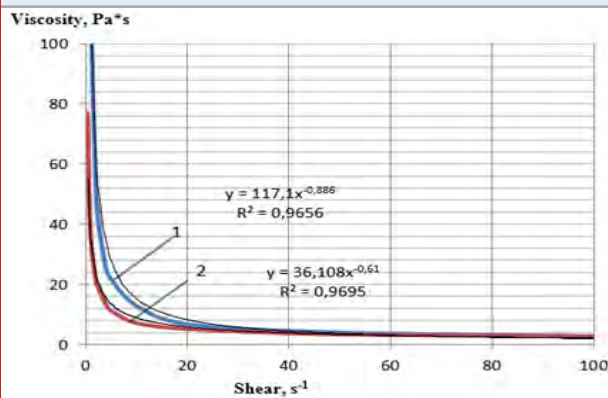
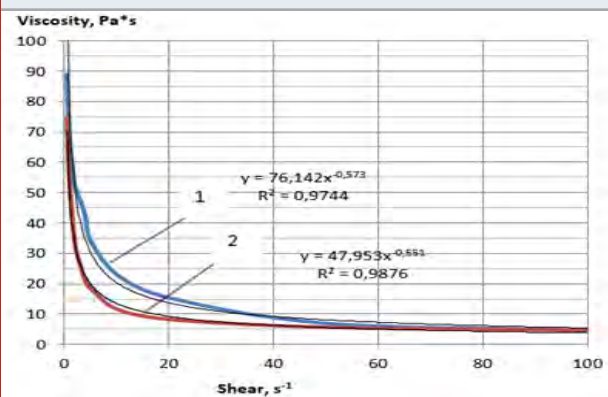


Figure 4. Rheological flow curves of the lecithin sample No. 4 at 60 °C: 1—direct measurement, 2—reverse measurement



The high strength of the coagulation structure and indication of thixotropic properties for the plastic samples of soy lecithins No. 3 and 4 can be explained on the basis of the following concepts. Being surfactants, phospholipid molecules in a non-polar substance (oil) interact with each other through polar groups. Such interactions are based on the electrostatic nature, in contrast to the entropic nature of the interaction of hydrocarbon radicals in aqueous substances (Mittela, 1980). According to the theory of stepwise surfactant aggregation in a non-polar substance, the energy gain from replacing the polar group-carbon interaction with the interaction between polar groups is so significant that, in a non-polar substance, even at low surfactant concentrations (10⁻⁷–10⁻⁶ mol/dm³), the latter form stable aggregates on a small scale, called “premicellar” (Sinegovsky 2019).

Thus, phospholipids, being surfactants, in a non-polar substance are predominantly present not in the form of individual molecules, but in the form of associates and

micelles of various scales, which undergo a number of structural transformations during degumming and subsequent drying. As a result of such transformations, the resulting liquid lecithin becomes a dispersed system with a coagulation structure and consists of micellar aggregates formed by phospholipids and other polar lipids. Such aggregates interact with each other through interlayers of a dispersion medium consisting of neutral lipids (oil) (Sinegovsky 2019).

The more screened the polar groups, the lower the relative polarity of the surfactant and, accordingly, the lower the scale of the formed micelles and the lower the energy of intermicellar interactions. The described mechanism underlies the technological method of reducing the viscosity of lecithins by adding bivalent metal ions to the phospholipid emulsion before drying, for example, in the form of calcium chloride (Nieuwenhuyzen and Tomás 2008; Ahmad and Xu 2015). A decrease in the energy of intermicellar interaction can also be provided by an increase in the thickness of the interlayer of the dispersion medium, or an increase in its relative polarity. In practice, such effects are achieved by adding refined and deodorized oil or distilled fatty acids to lecithin (Yaron and Letan 1975; Nieuwenhuyzen and Tomás 2008; Ahmad and Xu 2015). Thus, an increase in the viscosity of lecithins during storage at a normal level of the mass fraction of moisture and volatile substances (no more than 1%) and, in the absence of intensive oxidative processes, may be due to the following reasons:

- a high content of insoluble acetone substances, and, accordingly, a low content of neutral lipids, which does not ensure the creation of layers of a dispersion medium of sufficient thickness and leads to an enlargement of micelles and an increase in the degree of their interaction during storage;
- a high relative polarity of phospholipid molecules, leading to the formation of a developed coagulation structure with a high energy of interaction of micellar aggregates, which, during storage, leads to their enlargement and, as a consequence, may be accompanied by a partial separation of the dispersion medium. Thus, summarizing the results obtained, we can conclude that the main reason for the increase in viscosity and the formation of a plastic consistency of the samples of soy lecithins No. 3 and 4 is the formation of a developed coagulation structure consisting of micellar aggregates interconnected by strong electrostatic and hydrogen bonds, as a result of the high amount of phospholipids with active polar groups in lecithins at a low content of free fatty acids, which can reduce the activity of such interactions through a "wedging" action.

Soybean liquid lecithins, characterized by similar values of the indicators acetone insoluble matter, moisture and volatile substances, toluene insoluble matter, peroxide value, and color differed significantly in consistency from liquid fluid to viscous plastic. Samples of soy lecithins with a plastic consistency were characterized by a lower acid value of no more than 19 mg KOH/g. Comparative analysis of the group composition of phospholipids of the studied samples of soybean lecithins showed that the samples of lecithins

with low viscosity differed from the samples of plastic lecithins by a higher content of phosphatidylinositols and a lower content of phosphatidylethanolamines. At the same time, phosphatidylethanolamines were the predominant group of phospholipids in the samples of plastic soy lecithins. Acetone-insoluble polar lipids, which were part of the studied lecithin samples, contained fatty acid acyls, characteristic of the corresponding types of vegetable oils. At the same time, no regularities in the differences in the compositions of fatty acid acyls for liquid and plastic soy lecithins were revealed.

Samples of low viscosity liquid lecithins contained significantly higher amounts of polyvalent metals compared to the samples of plastic soy lecithins. There was almost a complete absence of Fe (less than the detection limit of the method) in the samples of plastic lecithins, while, in the samples of liquid lecithins, this metal was predominant. The content of carbohydrates and unsaponifiable lipids in the studied samples of soy lecithins generally corresponded to the literature data, and no correlations with their consistency were revealed. The samples of soy lecithins with a plastic consistency had a stronger structure at a temperature of 60 °C than the samples of lecithins with a low viscosity at a temperature of 25 °C. At the same time, the samples of plastic lecithins had thixotropic properties, as, with a decrease in the intensity of mechanical shear action, they demonstrated the ability to partially restore broken bonds between structural components (Schneider 2019).

CONCLUSION

The findings of the present study suggest that the main reason for the increase in viscosity and the formation of a plastic consistency of the samples of soybean lecithins No. 3 and 4 was the formation of a developed coagulation structure, consisting of micellar aggregates interconnected by strong electrostatic and hydrogen bonds, as a result of the predominance of phospholipids with active polar groups in the lecithins at low contents of free fatty acids, which can reduce the activity of such interactions through the "wedging" action.

ACKNOWLEDGEMENTS

The research was carried out using the equipment of the Research Center of Food and Chemical Technologies of KubSTU (CKp_3331), which development is supported by the Ministry of Science and Higher Education of the Russian Federation (Agreement no. 075-15-2021-679)

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Dental Communication

Effect of Curing Time on the Bond Strength of Orthodontic Brackets Bonded by Light Cure Resin-Modified Glass Ionomer Cement: An *In vitro* Evaluation

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ABSTRACT

This study was designed with the aim to evaluate the effect of curing time on the shear-bond strength of orthodontic brackets bonded using light cure Resin-Modified Glass Ionomer Cements (RMGIC). This class of cement when used for luting orthodontic brackets offers certain advantages when compared to the more commonly used resin cements. Intact natural teeth (premolars) extracted for therapeutic purposes as part of orthodontic treatment was sourced for use in this study. The teeth were equally divided into four groups for testing, Group 1 - brackets bonded with RMGIC and cured for 3 seconds, Group 2 - brackets bonded RMGIC and cured for 6 seconds, Group 3 - brackets bonded with RMGIC and cured for 9 seconds and Control group - brackets bonded with composite and cured for 15 seconds. A high intensity LED light source was used to cure the cements. The Shear-Bond strength of the brackets was evaluated using a universal testing machine. One-way ANOVA test and Tukey multiple comparison tests were done to compare the difference of Shear-Bond Strengths among the groups tested. The average Shear Bond Strength among study groups was 7.64 ± 2.86 MPa. The ANOVA and Tukey multiple comparison tests could not identify a statistically significant difference in Shear-Bond Strengths among the groups. Curing time does not appear to have a statistically significant effect on the Shear Bond Strength of orthodontic brackets bonded using Resin-Modified Glass Ionomer Cements..

KEY WORDS: BONDING, LIGHT-CURE, LIGHT INTENSITY, RESIN-MODIFIED GLASS IONOMER CEMENT, SHEAR-BOND STRENGTH.

INTRODUCTION

Two important discoveries in orthodontics, Buonocore's discovery of the acid-etch technique and Miura and Newman's orthodontic bonding, transformed the field (Rossouw 2010; Graber et al. 2016). These steps simplified orthodontic practice while increasing clinical efficiency and effectiveness. It also aided in increasing orthodontic treatment acceptance and set the way for future improvements in the field. Light-cure orthodontic bonding materials were developed at the end of the 1970s (Eliades 2010). These materials presented a number of advantages over self-cure materials, including the ability to control working time, enhanced bracket placement precision, ease of removal of excess material, reduced risk of field contamination, and the ability to engage the arch-wire right away (Sfondrini

et al. 2002; Sfondrini et al. 2004; Graber et al. 2016; Shen et al. 2021).

Glass Ionomer Cements (GICs) were presented as an alternative to resin adhesives in orthodontics. They were less moisture sensitive, could attach to both enamel and metal, released fluoride, and reduced iatrogenic enamel damage caused by orthodontic treatment (Fukino and Komori 2015; Sidhu and Nicholson 2016). In comparison to resin composites, GICs showed low early mechanical strength and bond strength. Resin components were added to GICs to improve their properties. When compared to conventional GICs, this new material, Resin Modified Glass Ionomer Cement (RMGICs), showed better bond strengths (Owens and Miller 2000; Shen et al. 2021).

Despite being clinically significant, the bond strength was still lower than that of resin adhesives. For polymerization of adhesives, Light Emitting Diodes (LEDs) based on semiconductors produced from gallium nitride (GaN)

Article Information:*Corresponding Author: dr.mathewthomas@gmail.com

Received 25/09/2021 Accepted after revision 26/12/2021

Published: 31st December 2021 Pp- 1871-1876

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.72>

are used. Compared to traditional curing lamps, LED lamps provide a number of advantages (Huang et al. 2017). Increased lamp life, consistent light intensity, impact resistance, lack of heat generation avoiding pulpal damage, a coincidence of peak irradiance of light with camphorquinone, lightweight design and improved ergonomics, low power consumption translating to longer usage between charges and increased battery life, and monochromatic light output are just a few of the benefits (Nicholls 2000; Dunn and Taloumis 2002; Mills et al. 2002; Wiggins et al. 2004; Leprince et al. 2010; Shen et al. 2021).

Various studies utilized Shear-Bond Strength(SBS) as a method to evaluate and assess the strength of bracket bonding systems (Sfondrini et al. 2002; Uşümez et al. 2004; Gronberg et al. 2006; Sfondrini et al. 2006; Bishara et al. 2007; Turk et al. 2007; Yu et al. 2007; Cerekja and Cakirer 2011; Sağır et al. 2013; Graber et al. 2016; Shen et al. 2021). No previous research has employed high-intensity LED curing lamps to cure the luting cements to evaluate the SBS. Furthermore, previous research has only looked at the SBS of resin cements used for luting orthodontic brackets, with no study of the SBS of RMGIC used for luting orthodontic brackets with a high intensity light source. As a result, the goal of this research is to see how curing time affects the shear-bond strength of orthodontic brackets bonded with light cure Resin Modified Glass Ionomer Cement that is cured with a high-intensity LED light source.

MATERIAL AND METHODS

To estimate the sample size, a similar study in the literature was identified. The sample size was calculated using the G*Power statistical power analysis software version 3.0.10 (Heinrich-Heine-Universität, Düsseldorf). A total sample size of 24 was calculated to have 95% power to detect a difference in means between the groups (Dall'Igna et al. 2011). A total of 24 premolars were extracted as part of orthodontic treatment were sourced for the study. The teeth were preserved in a formalin solution for 24 hours following extraction, later these teeth were stored saline until they were to be used in the study (Lee et al. 2007; NawrockaA and ukomska-SzymaskaA 2019). The teeth were then cleaned and mounted onto custom fabricated acrylic mounting jigs. Pumice paste was used to polish the bonding site, etched the teeth for 30 seconds with 37% phosphoric acid, washed for 10 seconds with distilled water, and air-dried for 10 seconds (Gaard and Fjeld 2010; Graber et al. 2016; Shen et al. 2021).

The teeth were randomly divided into 4 groups (n=6). In the Control group, Ortho Solo™ (Ormco Corporation, USA) bonding agent was applied to the etched enamel and light-cured for 10 seconds. Enlight Light Cure adhesive (Ormco Corporation, Orange, CA, USA) was applied to the bracket base of a premolar bracket (Empower® 2 Self-ligating metal bracket; American Orthodontics, USA). A stainless-steel bracket holding tweezer was then used to place the bracket on the tooth, with enough pressure used to allow the extra resin to gather at the bracket's edges. The brackets were cured for 15 seconds using the light cure equipment

after the extra material was removed (Graber et al. 2016; Shen et al. 2021).

The RMGIC material (GC Fuji ORTHOTM LC, Resin-reinforced light cure orthodontic cement; GC America Inc, USA) was mixed and coated on the bracket base in the proportions prescribed by the manufacturer for the study groups. A bracket holding tweezer was used to place the brackets on the teeth, and enough pressure was applied to allow the excess cement to be collected from the bracket borders. The brackets were cured after the excess cement was removed.

Brackets in group 1 were cured for 3 seconds, brackets in group 2 for 6 seconds, and brackets in group 3 for 9 seconds. The manufacturer recommended a curing period of 10 seconds for the RMGIC used in the study. According to the manufacturer's specifications, the light cure unit employed in the study (iLed Curing Light; Guilin Woodpecker Medical Instrument Co., Ltd, PRC) had an overall light output intensity ranging from

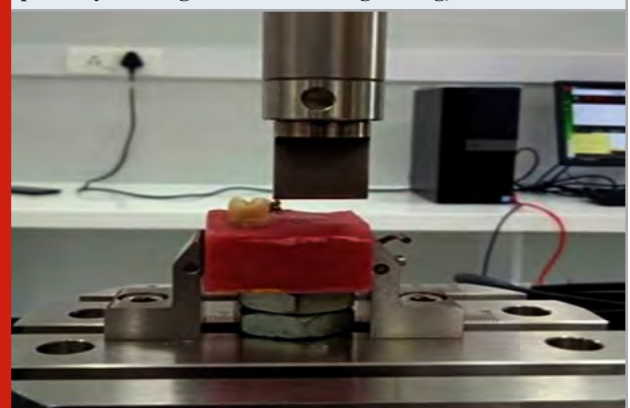
$$1000 \frac{\text{mW}}{\text{cm}^2} - 2500 \frac{\text{mW}}{\text{cm}^2}$$

and a wavelength range of (420nm - 480nm). The TURBO P1 high-intensity mode was used in our research Study, with an with a light output intensity range from

$$2300 \frac{\text{mW}}{\text{cm}^2} - 2500 \frac{\text{mW}}{\text{cm}^2}$$

The teeth were then immersed for 24 hours in distilled water kept at a temperature of 37 °C. At a crosshead speed of 0.5mm/min, the teeth were then subjected to SBS testing on a universal testing machine (Instron® Corporation, MA, USA) (Figure 1). The peak load failure rate was divided by the specimen surface area to calculate SBS values in megapascals (MPa). The SBS values were compared between the groups using analysis of variance (ANOVA) and Tukey multiple comparison tests. IBM® SPSS® Statistics software version 23 was used to tabulate and process the data (International Business Machines Corporation, NY, USA). The study's protocol was approved by the institutional scientific review board.

Figure 1: The SBS of the bonded bracket being tested on the universal testing machine (Photograph taken by the primary investigator taken during testing).



RESULTS AND DISCUSSION

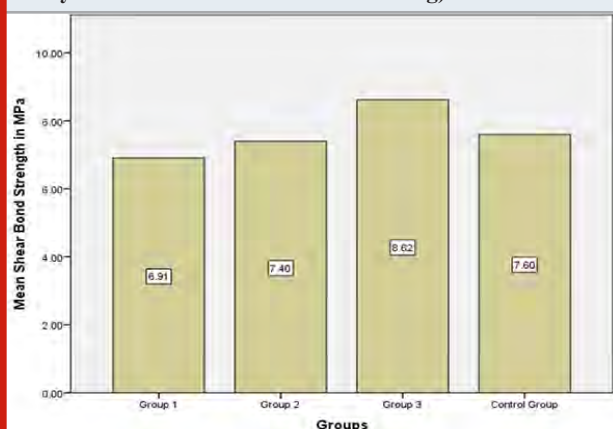
The overall average SBS among study groups was found to be 7.64 ± 2.86 MPa. In Group 1, the average SBS was found

to be 6.91 ± 3.22 MPa. In Group 2, the average SBS was found to be 7.40 ± 2.91 MPa. In Group 3, the average SBS was found to be 8.62 ± 1.67 MPa. In the Control group, the average SBS was found to be 7.60 ± 1.06 MPa (Figure 2).

Table 1. ANOVA test to compare means SBS among the groups. The test shows that the difference of SBS among the groups is not significant statistically.

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	9.308	3	3.103	0.456	0.716
Within Groups	135.991	20	6.800		
Total	145.300	23			

Figure 2: Bar Graph representing the average SBS of the study groups (Graph generated from SPSS post statistical analysis of the data obtained from testing).



The ANOVA test showed that there wasn't a statistically significant difference in SBS among the four groups evaluated in the study (Table 1). The Tukey multiple comparison tests did not identify any statistically significant difference in the SBS among the groups.

The results of the present study showed that there was a meagre increase in the SBS with the increase in curing time, however, the ANOVA and Tukey comparison tests weren't able to determine statistical significance in the SBS among the groups. The increase in SBS with the increase in curing time could be probably due to the increased polymerization that occurs with the increased light-curing time. Such an observation was made by various authors in the case of light cured-resin cements (Uşümez et al. 2004; Mavropoulos et al. 2005; Peutzfeldt and Asmussen 2005; Staudt et al. 2005; Yu et al. 2007; Shen et al. 2021).

The primary objective of this study was to identify whether curing brackets bonded using RMGICs and cured with the use of high-intensity modes on LED curing lamps that have shorter curing cycles would affect the SBS of these brackets. The shorter curing cycles can increase clinical efficiency and reduce chairside time (Omidi et al. 2018).

In our study, we had observed that there was an increase in the SBS, with and increase in the curing time, although the difference wasn't significant statistically. A 3 and 6 second curing times can offer a balance of both significant SBS for clinical usage and reduced clinical chairside bonding time. Our observation regarding intensity was similar to the results obtained by Cerekja and Cakirer, and Par et al. (Cerekja and Cakirer 2011; Par et al. 2020).

Although resin composite enjoys significant popularity, RMGICs still has its niche in orthodontics, especially in cases where the acid-etch technique isn't effective such as enamel hypoplasia, dental fluorosis, and amelogenesis imperfecta (Choo et al. 2001; Wiltshire and Noble 2010). RMGICs are used in the indirect bonding techniques of lingual fixed appliances (Komori et al. 2010; Komori et al. 2013). Recently studies that were conducted to study the feasibility of using RMGICs to bond lingual attachments such as buttons, and also the bonding of fixed lingual retainers are showing promising results (Baysal and Uysal 2010; Alkhateeb and Al-Sheakli 2013). The benefits of the use of RMGICs such as its advantages acting as a fluoride reservoir could help in the prevention of decalcification, reduced enamel damage during removal post debonding (Bishara and Ostby 2010). Improved accuracy being pursued through the techniques of indirect bonding and customized fixed appliances could utilize RMGICs for bonding (Miyashita et al. 2017; Shen et al. 2021).

Also, a study conducted by Mota et al. identified increased anti-microbial activity and reduced plaque accumulation adjacent to orthodontic brackets bonded with RMGICs during the initial stages of treatment (Mota et al. 2008). Although the average SBS of the study group seems low, according to Reynold's, an SBS of 6 - 8 MPa is adequate for normal clinical use (Eliades and Brantley 2000; Brantley and Eliades 2001). The lower SBS in the laboratory testing can be a misrepresentation as it does not translate into clinical performance and failure (Graber and Vanarsdall 2000). Silverman et al. reported a bond failure of 3.2% for brackets bonded with RMGICs in an 8-month long in-vivo study (Eliades and Brantley 2000; Brantley and Eliades 2001). Enamel preparation, oral environment, humidity,

moisture control, and bracket design could also play a role in SBS (Cacciafesta et al. 2004; Graber et al. 2016; Proffit et al. 2018).

A Limitation of our investigation is its in-vitro nature which cannot reproduce the complex interaction occurring in the oral environment. Numerous authors have pointed out variabilities in laboratory results and clinical observations (Sifakakis and Eliades 2017). Another limitation is the crosshead speed of the universal testing machine set 0.5mm/min. This according to Eliades and Brantley although is generally used does not accurately correspond to the in-vivo scenario due to higher speeds in clinical conditions (Eliades and Brantley 2000). With all these limitations we can surmise that RMGICs deliver the best of both worlds in terms of clinical performance and can be considered as a suitable alternative to resin composites. The shorter curing cycle with high-intensity LEDs can help reduce chairside time and improve efficiency. Although we have to always take into account the enormous amounts of research and improvements occurring in the field biomaterials which could still advance and evolve our practice and delivery of care to our patients (Shen et al. 2021).

CONCLUSION

The findings of the present investigation indicate that curing time has no statistically significant effect on the shear bond strength of brackets bonded using resin modified glass ionomer cements. RMGICs can offer significant advantages and decent clinical performance when used for luting orthodontic brackets. The shorter curing cycle offered by the high-intensity LEDs can help in reducing clinical chairside time, improving clinical efficiency and aid in the delivery of better care to orthodontic patients.

Conflict of Interests: Authors declare no conflicts of interests to disclose.

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Dental Communication

Radiographical Measurements of Overeruption in Unopposed Posterior Teeth Among Selected Population of Saudi Arabia

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ABSTRACT

The suspended replacement of extracted teeth may disrupt the structural integrity of the whole dental arch, particularly in posterior maxillary or mandibular tooth/teeth. This phenomenon often has consequences of masticatory deficiency and temporomandibular joint disorders. This work aims to assess and measure the overeruption of posterior teeth from panoramic radiographs (OPGs) and its relation to gender, age, tooth type, arch, sides, and cause of tooth loss. A total of 100 OPGs were included in this *in vitro* study. The level of posterior overeruption was measured in mm using the panoramic digital radiograph machine and its software systems. Other variables such as gender, tooth/teeth involved, arches, and side were considered. All parameter values were inputted to a software program and assessed. P value < 0.05 was considered as statistically significant. Males accounted for 63% of the OPGs. Age groups 18–30 and 31–45 years had the highest frequency and percentage (34%). Approximately 74% of the patients got their tooth/teeth extracted 4–6 years ago. The 0.7–1.2 mm overeruption subgroup had the highest proportion of 41%. The rate of overeruption in mandibular molars was 36%, which was the highest among the subgroups. Males had higher percentage than females in ≥60 and 31–45 age groups (84.65% and 76.5%, respectively), 4–6 years post-extraction time (85.7%), with significant differences, also, mandibular molars (85.0%) and mandibular arches (74.3%) were the higher among males. The highest level of overeruption was 0.7–1.2 mm, and mandibular molars were the most affected type. Significant differences between genders were detected in age groups, years after tooth loss, and arch side but not in cause of tooth loss, supra-erupted tooth type, and arch type parameters.

KEY WORDS: EXTRACTION, OCCLUSAL INTERFERENCES, OVERERUPTION, PANORAMIC-RADIOGRAPH, POSTERIOR- TEETH.

INTRODUCTION

Overeruption is defined as the degree of excess tooth movement starting from the cusp tip to the carve passing

over the canine and posterior teeth. The overeruption of maxillary or mandibular premolars and molar tooth/teeth can be considered as one of the common clinical findings in day-to-day dental practice. Postponing the replacement of lost teeth often leads to the extrusion of the contrasting tooth into the edentulous space, which in turn could result in masticatory insufficiency and temporomandibular joint disorders (Craddock 2010; Gupta et al. 2014). When a

Article Information:*Corresponding Author: drmoaleem2014@gmail.com

Received 28/09/2021 Accepted after revision 11/12/2021

Published: 31st December 2021 Pp- 1877-1884

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.73>

prosthesis, either fixed or removable, is planned on the opposing edentulous arch, the function of the posterior occlusion must be re-establishing through a comprehensive dental treatment plan (Craddock and Youngson 2004; Craddock et al. 2007; Mahoorkar et al. 2010; Shillingburg et al. 2012; GPT-9 2017; Ahmari et al. 2020).

Over-erupted tooth/teeth can be categorized into three types according to the degree of overeruption extending from the occlusal plane: mild (over-erupted tooth ranges 0.1–1.5 mm), moderate (1.6–3.5 mm), and severe (exceeds 3.5 mm) (Compagnon and Woda 1991; Craddock and Youngson 2004; Newman et al. 2018). Another classification is according to the amount of reduction required from the over-erupted tooth/teeth: conservative, semiconservative, and nonconservative (Ahmari et al. 2020). The most frequently extracted teeth are the first permanent molars either in the maxillary or mandibular arch, followed by the premolars in the maxillary arch (Djemal et al. 2004; Craddock et al. 2007; Tiago et al. 2016; Patil et al. 2016; Baeg et al. 2016; Salazar et al. 2018). Marcus et al. (1996) and Craddock et al. (2007), mentioned that mandibular posterior teeth are more likely to be removed than maxillary posterior teeth; with aging, posterior teeth would probably be extracted and missed in both sides (Marcus et al. 1996; Craddock et al. 2007; Shillingburg et al. 2012; Basutkar et al. 2018; Ahmari et al. 2020).

In relation to the level of over-erupted tooth/teeth (in mm), Craddock et al. (2007) found that the average volume of over-erupted tooth/teeth ranges is 1.68–3.99 mm for the teeth without antagonism, 1.03 mm for mandibular arch, and 1.91 mm for maxillary arch. Kiliaridis et al. (2000) recorded that 24% of unopposed teeth had more than 2 mm overeruption among 82% of the examined subjects without antagonist teeth. Other studies calculated the occlusal change of unopposed teeth within the border of 2 mm (Kiliaridis et al. 2000; Craddock et al. 2007; Faggion et al. 2011; Ahmari et al. 2020).

After examining their overeruption from OPGs, Moaleem et al. (2021) reported no significant differences in overeruption values among different gender or age groups. Significant difference was found between the types and sites of teeth, with a relatively high ratio observed in molar teeth, mandibular arch, and young age group. Other studies have investigated the relationship of tooth positional interferences following posterior tooth loss during different mandibular arch movements (Craddock et al. 2007; Craddock et al.; 2007b; Craddock 2008). This study aimed to measure and compare the level of posterior tooth overeruption from OPGs and its relation to gender, age group, tooth type, arch, sides, and cause of tooth loss. The hypothesis was as follows: no differences in the level of tooth overeruption exist between genders in relation to other parameters measured from the OPGs.

MATERIAL AND METHODS

A total of 100 OPGs were collected from private dental centers in Jazan, SA, for this cross-sectional radiographic study. All OPGs were obtained from the pool of patients

who reported for the replacement of missing posterior maxillary or mandibular molars. The supra-erupted teeth selected from these OPGs were unopposed posterior teeth in the maxillary or mandibular arches. This cross-sectional study was approved by the Research and Ethics Committee at the Authority of Medical Sector in Jazan City and was conducted in accordance with the guidelines of the World Medical Association Declaration of Helsinki. Non-randomized clinical study design was conducted among OPGs collected from the last 2 years. The sample size of approximately 100 participants was verified based on a G*Power software ([http:// www.gpower.hhu.de/en.html](http://www.gpower.hhu.de/en.html)) with the self-assurance amount altered at 90%, power adjusted at 85%, and a reasonable outcome amount. This study included patients who attended private dental clinics and had their OPGs taken.

Inclusion criteria were as follows: aged 18 years or above from both genders; a minimum of one supra-erupted posterior maxillary or and mandibular tooth; presence of posterior and anterior vertical stops; the extracted teeth should have been in the posterior zone of any arch and had been removed in the past 4 years or more; and have not received any previous dental treatments (prosthodontic or orthodontic). Radiographic evaluation and demographic data of subjects were collected as followed. First, individual, and demographic information was collected, such as gender, age groups (18–30, 31–45, 46–59 and ≥ 60 years), years after extraction (4–6 and ≥ 6 years), and cause of tooth removal (dental caries, periodontal disease, failure in root canal treatment [RCT] and others such as periapical pathosis or dental trauma).

A tooth was classified as extracted due to periodontal disease when it satisfies the eighth criterion of Russell's periodontal index, i.e., the presence of considerable mobility in accordance with Miller's mobility index (1995) (McCaul et al. 2001). However, a tooth was labeled as requiring extraction due to dental caries when the clinical crown was broken down by caries and is non-restorable (Gossadi et al. 2015). A failed RCT occurs when retreatment cannot be made due to any reasons under any present condition (Meshni et al. 2018).

Overeruption is defined as the movement of a tooth or teeth above the normal occlusal plane (GPT-9 2017; Kim et al. 2016). The involved over-erupted teeth were recorded as molars and/or premolars, left or right side, and maxillary or mandibular arch. Overeruption values were recorded, measured, and registered into three scales (0.1–0.6, 0.7–1.2, and >1.3 mm) (Craddock and Youngson 2004; Craddock et al. 2007; Kim et al. 2016; Moaleem et al. 2021). This part concerned with the measurements of the extent of over-erupted teeth of participants based on their OPGs. The panoramic digital radiograph machine was a tomography x-ray system model PaX-Flex3D (Germany) operated at 30% magnification.

Supra-eruption was calculated by drawing a straight line joining the tips of buccal cusps between the canine and the adjacent or the last tooth of arch on the over-erupted tooth/teeth side. The amount of tooth structure outside or lower

than this line was considered as overeruption (in mm) and was calculated as shown in Figures 1 and 2 (Craddock et al. 2007; Kim et al. 2016; Moaleem et al. 2021). Figure 1 shows the over eruption in mandibula and maxillary arch in right or left side of both arches, and Figure 2 displays a bilateral overeruption in the right and left sides of maxillary or mandibular arch. All measurements for overeruption level were performed by a qualified investigator (Al M.M).

Data were tabulated and summarized in an Excel sheet (Office 2010) on a personal computer. Descriptive statistical analysis including mean, frequency, percentages, and standard deviation (SD) was performed using SPSS software program version 21 (Chicago, Illinois, USA). Assessments and associations among different genders, age groups, and types of overeruption tooth were determined using Chi-square test. $p \leq 0.05$ was considered as significant. Informed consent was obtained from all participants included in the study. Moreover, the study acquired the ethical approval from the ethical committee at the College of Dentistry, University of Jazan on 20/10/2020.

RESULTS AND DISCUSSION

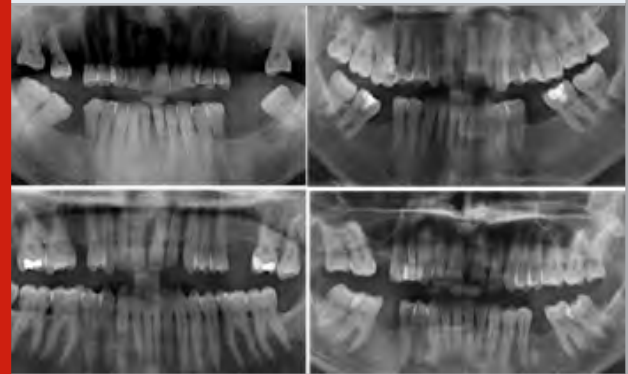
A total of 100 over-erupted tooth/teeth in the posterior area of both arches were involved in this in vitro study. The values of overeruption (in mm) from the OPGs were calculated after 30% magnification reduction. Figure 1 offers the descriptive statistics of the subjects, including the gender, age groups, years after tooth loss, cause of tooth loss, arch, and side involved in overeruption. The means and SD for gender, age groups, years since tooth loss, causes of tooth loss, arch, and sides were 1.370 ± 0.485 , 1.775 ± 0.779 , 1.275 ± 0.449 , 2.220 ± 0.773 , 2.030 ± 0.810 , and 1.550 ± 0.500 , respectively. Most of the OPGs were obtained from male subjects (63%) and patients aged 18–73 years. The highest frequency and percentage were observed in the 18–30 and 31–45 age subgroups (34%) and in the 4–6 years post-extraction subgroup (74%). Other pieces of demographic information are presented in Statistics 1.

Figure 1: Unilateral overeruption in maxillary or / and mandibular arch in the right or left side



With respect to the overeruption level, 41% of the cases had 0.7–1.2 mm with mean and SD of 0.725 ± 0.64 . Slight differences were recorded in the over-erupted cases and tooth types between mandibular (36%) and maxillary (30%) molars with mean and SD of 2.780 ± 1.088 (Table 1).

Figure 2: Bilateral overeruption in maxillary or mandibular arch in the right or left side



Variable	Subgroups	Percentage (%)
Gender	Males	63%
	Females	37%
Age groups (years)	18–30	34
	31–45	34
	46–59	19
	≥ 60	13
Years since tooth loss	4–6 years	74
	>6 years	26
Cause of tooth loss	Periodontal disease	13
	Dental caries	60
	Failed RCT	19
	Others	8
Arch	Maxilla	31%
	Mandibula	35%
	Both arches	34%
Side	Right	45%
	Left	55%

Table 2 shows the association of overeruption with gender groups and other parameters. Males accounted for 52.3% and 50.0% of the ≥60 and 31–45 age groups, respectively. Females accounted for 85.6% and 76.5% of the 46–56 and 18–30 age groups, respectively. The subgroup of 4–6 years post-extraction represented the highest males and females with 85.7% and 54.1%, respectively. In relation to the side, the left side was recorded for 74.3% of males, and the right side was recorded for 51.1% of females. These parameters showed significant differences with gender at p value > 0.050.

Among the males, dental caries, and failure RCT were the most frequent cause of tooth removal with 68.4% for both parameters. For females, other causes and periodontal

diseases accounted for the highest percentages with 62.7% and 53.8%, respectively. Maxillary and mandibular molars were the most frequent in males and females with 20 and

16 cases, respectively. Mandibular arches accounted for the highest percentage of 74.3% among males, and both arches were recorded among females (44.1%). All these parameters showed non-significant differences with gender.

Variable	Subgroups	Number (n)	Percentage (%)	Mean	Standard deviation
Gender	Males	63	63%	1.370	.485
	Females	37	37%		
Age groups (years)	18–30	34	34	1.775	.779
	31–45	34	34		
	46-59	19	19		
	> 60	13	13		
Years since tooth loss	4–6 years	74	74	1.275	.449
	>6 years	26	26		
Cause of tooth loss	Periodontal disease	13	13	2.220	.773
	Dental caries	60	60		
	Failed RCT	19	19		
	Others	8	8		
Arch	Maxilla	31	31%	2.030	.810
	Mandibula	35	35%		
	Both arches	34	34%		
Side	Right	45	45%	1.550	.500
	Left	55	55%		

Statistics 1. Descriptive of demographic data of subjects (OPG=100)

Table 1. Overeruption descriptive of amount (mm) and type of tooth and presence of occlusal interferences included in this study (OPG=100)

Variable	Subgroups	Number (n)	Percentage (%)	Mean	SD
Overeruption from OPG (mm)	No overeruption	8	8	0.725	0.464
	0.1–0.6 mm	29	29		
	0.7–1.2 mm	41	41		
	>1.3 mm	22	22		
Over-erupted tooth type	Maxillary Premolar	14	14%	2.780	1.088
	Maxillary Molar	30	30%		
	mandibular premolar	20	20%		
	Mandibular Molar	36	36%		

Table 3 shows the relation of the overeruption level (in mm) to gender and tooth type. No significant differences between the overeruption degree and the two parameters with p values of 0.117 and 0.217. For gender, the highest percentages were obtained for the amount of overeruption at 0.1–0.6 mm (40.5%), followed by >1.3 mm (29.7%). Maxillary molar and premolars had the highest percentage in the 0.7–1.2 mm subgroup (49.9% and 36.1%, respectively), mandibular premolars had the highest number in 0.1–0.6 mm subgroup (55.0%), and molar teeth had the highest frequency in the 0.7–1.2 mm subgroup (36.1%). Assessment was performed on the interclass correlation coefficient for the measurement of supra-eruption (in mm) on the OPGs. Intra-examiner reliabilities were determined by

drawing a Bland–Altman plot 7 for continuous data. The two measurements fell between 96% and 98% confidence interval bands (Table 4) (Bland and Altman 1986).

When the edentulous space is in the posterior part of the mouth, replacing an extracted tooth is important to patients to maintain the state of dynamic balance with the teeth supporting each other. After a tooth loss, the teeth adjacent to or opposing the edentulous space frequently over-erupt, tilt, and move into the extraction site. The severe overeruption of unopposed teeth into the edentulous space results in an insufficient place to replace the missing tooth. Hence, the tooth opposing the edentulous space must be replaced (Shillingburg et al. 2012). This study aimed to

evaluate and measure the overeruption degree of posterior teeth from OPGs and its relation to gender, age, tooth type, arch, sides, and cause of tooth loss.

Results showed 0.7–1.2 mm as the most frequent overeruption level and mandibular molars as the most frequent tooth type for overeruption. These results agreed with previous studies (Craddock et al. 2007; Kim et al. 2016; Moaleem et al. 2021). The null hypothesis was partially rejected because of the significant difference in age groups,

years after tooth loss, and right or left side of the mouth between genders. Other parameters such as cause of tooth loss, type of over-erupted teeth, and maxillary or mandibular showed no significant differences between genders; hence, the null hypothesis was considered as partially accepted. Assessment on intra-examiner reliability to evaluate overeruption by Bland–Altman plot test score was recorded between 96% and 98% confidence interval bands (Table 4). The values indicated respectable and acceptable reliability in this radiographic study (Moaleem et al. 2021).

Table 2. Association between the frequency and % of variables in relation to different gender groups by Chi-square or Fisher's exact test (n=100)

Parameters		Males N %	Females N %	Total N and %	P-Values
Age groups	18–30	17 (50.0)	17 (50.0)	34	0.018*
	31–45	26 (76.5)	8 (23.5)	34	
	46–59	9 (47.7)	10 (52.3)	19	
	≥ 60	11 (84.6)	2 (15.4)	13	
Years since tooth loss	4–6 year	54 (85.7)	20 (54.1)	74	0.007*
	>6 years	9 (14.3)	17 (45.9)	26	
Causes of tooth loss	Periodontal disease	6 (46.2)	7 (53.8)	13	0.200
	Dental caries	41 (68.3)	19 (31.7)	60	
	Failed RCT	13 (68.4)	6 (31.6)	19	
	Others	3 (37.5)	5 (62.7)	8	
Supraerupted tooth/teeth	Maxillary premolars	9 (64.3)	5 (35.7)	14	0.106
	Maxillary molars	17 (56.7)	13 (43.3)	30	
	Mandibular premolars	17 (85.0)	3 (15.0)	20	
	Mandibular molars	20 (55.6)	16 (44.4)	36	
Side	Right	22 (48.9)	23 (51.1)	45	0.012*
	Left	41 (74.5)	14 (25.5)	55	
Site (Arch)	Maxilla	18 (58.1)	13 (41.9)	31	0.261
	Mandible	26 (74.3)	9 (25.7)	35	
	Both arches	19 (55.9)	15 (44.1)	34	

*Significant differences

Table 3. Association between the frequency and % of gender and over-erupted tooth in relation to amount of overeruption in mm by Chi-square or Fisher's exact test (n=100)

Variable	Overeruption from OPG (mm)	No Number (%)	0.1–0.6 mm Number (%)	0.7–1.2 mm Number (%)	>1.3 mm Number (%)	P values
Gender	Males	2 (3.2)	21 (26.5)	25 (40.5)	15 (29.7)	0.117
	Females	0 (0.00)	11 (29.7)	15 (40.5)	11 (29.7)	
Over-erupted tooth	Maxillary Premolar	0 (0.00)	4 (28.5)	7 (49.9)	3 (21.3)	0.217
	Maxillary Molar	3 (10.0)	6 (20.0)	14 (46.7)	4 (13.2)	
	Mandibular Premolar	0 (0.00)	11 (55.0)	6 (30.0)	3 (15.5)	
	Mandibular Molar	0 (0.00)	10 (27.8)	13 (36.1)	13 (36.1)	

The effects of gender on the prevalence of periodontal disease are not clearly understood, and research offers conflicting evidence on this matter. In the present study, significant differences between genders were detected in

age groups, years after tooth loss, and side of the arch but not in cause of tooth loss, supra-erupted tooth type, and arch type parameters. Such comparison between the degree of overeruption and gender has never been reported. This

finding might be related to the differences in the bone type of maxillary and mandibular arches between genders, muscle activity, cause of teeth extraction, and genetic factors. Previous studies in Jazan concluded significant differences

between genders and dental caries, with mandibular first molars accounting for the highest number and percentage (Moaleem et al. 2016; Moaleem MM. 2017; Noman et al. 2019; Moaleem et al. 2021).

Table 4. Overeruption interclass correlation for OPGs

	Intraclass Correlation Coefficient for PRs						
	Intraclass Correlation ^b	95% Confidence Interval		F Test with True Value 0			
		Lower Bound	Upper Bound	Value	df1	df2	Sig
Single Measures	.997a	.996	.998	955.937	99	99	.000
Average Measures	.999c	.998	.999	955.937	99	99	.000

Similar parameters percentages were recorded in the current radiographic study. Kim et al. (2016) conducted a clinical study on the use of OBG for the implant replacement of missing teeth. The results showed that the majority of the extracted teeth were mandibular molar teeth 27 out of 35 extracted teeth replaced by implant), followed by maxillary molars with six extracted sites and one each for maxillary and mandibular premolars (Moaleem et al. 2021).

To some extent, this finding was in agreement with the present finding, that is, mandibular molars were the most frequent with 36/100, followed by maxillary molars with 27/100. The previous study recruited only a few patients, and their main objective is to implant replacement for the missing teeth. Meanwhile, the current work aimed to measure the overeruption of posterior teeth. However, marginally similar results were found regarding the number of over-erupted premolar teeth in both arches (Kim et al. 2016; Moaleem et al. 2021).

In the presence of <2 mm overeruption distance, prosthetic replacement, or implant placement with their superstructure prostheses of missing teeth can be performed without addressing the super-erupted opposing tooth. This study determined that the lack of treatment for the super-erupted tooth will have no negative effects on the implant or the opposing tooth. Most of the over-erupted values were between 0.7 and 1.2 mm, which are in parallel with previous results (Faggion et al. 2011; Kim et al. 2016). Craddock et al. 2007 recorded a mean overeruption value of 1.68 mm and observed >1 mm overeruption for 68% of cases and >2 mm in 27% of cases; for the control group, 8% of cases had overeruption of >1 mm and none had supra-eruption of >2 mm (Moaleem et al. 2021). Gupta et al. (2014) investigated 153 patients with supra-erupted opposing teeth and observed the clicking of the TMJ in 106 patients (69.3%). The drifting of teeth adjacent to the missing molars was also a frequent finding (133 patients). Clicking was observed in 96 patients (72.2%) out of these 133 patients.

Once the tooth begins to shift in angle, the vector of force tends to increase tooth tilting and thus induce a different bio mechanical effect on the mandible (Obrez and Gallo 2006). These results agreed with concept of tightly locked occlusion, a kind of secondary change associated with supra

eruption, drift, or tipping, and tilting of the posterior teeth associated with TMD (Wang et al. 2007; Moaleem et al. 2021). Livas et al. (2016) conducted a radiographic study on 65 patients and found no significant eruption in unopposed mandibular second molars bonded with fixed sectional retainers compared with the molars partially occluded with antagonists lacking fixed retention. Therefore, fixed retention should be measured to limit tooth overeruption in unopposed molars. This study also indirectly revealed that the first molar tooth was the most frequently extracted among the samples; this finding agreed with the current results and other previous studies (Craddock et al. 2007; Faggion et al. 2011; Moaleem et al. 2021). In addition, significant differences were observed between genders, which also coincided with the current findings. However, the number of maxillary molar teeth was higher than that of mandibular ones. This result might be related to their study objectives.

In this study, 8% of the examined OPGs had no overeruption. Such status requires only observation with no treatment. A similar pattern of no overeruption of unopposed posterior teeth was previously reported. Craddock and Youngson (2004) recorded over 30 samples, and Craddock et al. 2007b noted no over-eruption in 27% of their samples. Moaleem et al. (2021) verified zero over eruption of the unopposed posterior teeth in 7 (11%) out of 65 samples (Moaleem et al. 2021). This radiographic study has some limitations, such as its cross-sectional design, small number of OPGs, and failure to collect data from different clinics. Further randomized clinical research using dental cone-beam computed topography with long follow-up period is recommended to confirm these results.

CONCLUSION

The finding of the present study suggests that the highest amount of overeruption was 0.7–1.2 mm, and mandibular molars were the most affected type. Significant differences between genders were observed in age groups, years after tooth loss, and arch side with overeruption. No significant differences between genders were detected in the overeruption ratio among cause of tooth loss, supra-erupted tooth type, and arch type. The acceptability of the

study method was confirmed by the good relation of the intra-examiner reliability and the good confidence interval band based on the Bland–Altman plot.

Acknowledgements: For this study, the sample collection was supported by Professor Mohammed M. Sobhey.

Conflict of Interests: Authors declare no conflict of interests to disclose.

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Environmental Communication

Population Status and Habitat Requirement of Endangered Migratory Waterfowl of Patisar Lake, Bahawalpur, Pakistan

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ABSTRACT

Patisar lake is an important wetland for migratory birds in mid-winter which is located in the center of the Lal Suhanra National Park of Bahawalpur, Pakistan. In this study Patisar lake has been explored to find out the endangered bird species. We used a map, a pencil, a notebook, a watch that is used to show seconds and binoculars for the census. Point counting system is used for the monitoring of birds and their habitat requirement was also measured. Result of the study indicated that these population belonging to 6 different orders, 10 different families, 18 genera and 32 different species of waterfowl were observed (1) *Podicipediformes* (*Podicipedidae*; *Tachybaptus iridipennis*), (2) *Pelecaniformes* (Family: *Phalacrocoracidae*; *Phalacrocorax iniger*; *P. carbo*; Family: *Anhinga*; *Anhinga melanogaster*), (3) *Ciconiiformes* (Family: *Ardeidae*; *Ardea cinerea*, *A. purpurea*, *Ardeola grayii*, *Bubulcus ibis*, *Egretta alba*, *E. garzetta*, *E. intermedia*), (4) *Anseriformes* (Family: *Anatidae*; *Anas acuta*, *A. crecca*, *A. platyrhynchos*, *A. strepera*, *A. penelope*, *A. clypeata*, *Aythya ferina*, *A. fuligula*, *A. nyroca*), (5) *Gruiformes* (Family: *Rallidae*; *Fulica atra*, *Porphyrio porphyrio*, *Gallinula chloropus*) and (6) *Charadriiformes* (Family: *Recurvirostridae*; *Himantopus himantopus*; Family: *Charadriidae*; *Vanellus indicus*, *V. vanellus*, *V. leucurus*, *Charadrius dubius*; Family: *Scolopacidae*; *Tringa tetanus*, *T. nebularia*, *Actitis hypoleucos*; Family: *Laridae*; *Sterna aurantia*). According to They all preferred fresh water layer of the lake and marshes near the lake as their habitat. It was concluded that Patisar lake is a prodigious natural resource for the migratory waterfowl and the number of waterfowl declining with the passage of time.

KEY WORDS: BAHAWALPUR, ENDANGERED, MIGRATORY BIRDS, PATISAR LAKE, WATERFOWL.

INTRODUCTION

Pakistan is bordered to the west by Iran and Afghanistan, while China is from the north and India is to the east by its huge neighbors. The country is divided by the Indus River, which flows throughout the country and through the vast Indus delta in the south, before entering the Arab Sea. There is a rich diversity of bird habitats in Pakistan from the dry alpine and tropical temperate western Himalayan forests to the Baluchistan and Sind deserts. A wide range variety of

birds is present in Pakistan because of their broad habitat diversity (Ahmad et al. 2020). Pakistan's bird fauna is an interesting mixture making the blending species from the Palearctic region and Oriental region. For the migration of birds, Pakistan is an important crossroad. A large number and a great variety of birds pass through Pakistan in the autumn and also in spring which majorly heads towards and from the subcontinent of India and also towards East Africa. To spend the winter season many species of the birds stayed in Pakistan (Grimmett et al. 2008; Ahmad et al. 2020).

In pursuit of good conditions, thousands of birds belonging to many species pass across Pakistan and stay temporarily on their way to other destinations in the Indian subcontinent

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Received 18/10/2021 Accepted after revision 23/12/2021

Published: 31st December 2021 Pp- 1885-1889

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.74>

at various lakes and water reservoirs. Pakistan provides many migratory birds with enticing wetlands in the winter seasons each year. In principle, for migratory birds, it serves as a central Asian flying route. Wetlands from the northern mountains to the southern coast of Siberia is used as habitats for waterbirds (Ali 2016). It is estimated that approximately 1 million birds are migrating over a distance of approximately 2800 miles by International Migratory Bird Route Number 4. Mainly the bird species that migrate from Siberia and towards the Pakistani regions are Cranes, Teals, Houbara bustards, Geese, Spoonbills, Pintails, Mallards, Pelicans, and Waders (Umar et al. 2018). The Patisar lake protected area is home to many animal species. These include over 160 birds, including griffon vulture, houbara bustard, hen harrier, marsh harrier, laggar falcon, Indian sparrow hawk, kestrel, Egyptian vulture, barn owl, shrike, and wheatear.

These bird species do have an important part to play with. A large body of water in the center of the park, the Patisar Lake is suitable for watching birds (Waris et al. 2014). Previous studies conducted in Lal Suhanra National Park was conducted and observed that between October and December (2015), 7,443 birds from 74 species belonging to 35 family members and representatives of 16 orders had been seen while in 2001 only 40 bird species were found (Maan and Chaudhry 2001; Jambhekar et al. 2021). Patisar lake is the abandoned reservoir on the edge of the Cholistan Desert, situated at the Lal Suhanra National Park, 25 km east of Bahawalpur and 10 km south of the Sutlej River. Patisar lake which was originally maintained as a stopping point for irrigation which is fresh and then slightly brackish, ponds which are supplied by canals, streams, and springs for special purposes (Mishra et al. 2021).

The lake was initially constructed as a storage reservoir provides irrigation water during water scarcity times but is no more used for this purpose. So, it was constructed as a water reservoir, this lake (1,935 hectares) was a significant wetland as a wintering site to most waterfowl (Mishra et al. 2021). The lake water is filled by the Bahawal Canal's Desert Branch, and it also gets excess water from nearby irrigated land. The lake is permanent year-round with a stable water level with an average depth of 4.5 m, a maximum depth of 6.0 m, and a pH value of 6.8. The annual rainfall is roughly 150-200 mm, as well as the relative humidity, is 25-72%. In January the mean minimum temperature is 11.5°C, and in June the mean maximum is 37°C (extremes of 1°C and 49°C have been recorded). The lake contains large reed beds as well as an extensive growth of floating and submerged aquatic vegetation (Mahmood-ul-Hassan and Muhammad 2011). Today, though, the swamp supports numerous reed beds, watertight as well as floating vegetation, so that for the majority of birds it has lost its habitat function. (Tatu and Anderson 2017; Mishra et al. 2021).

The lake is a vital wintering ground for the various migration waterfowl. The findings were migrating to nineteen percent of the total birds. The lake, which is the home to numerous birds, has been modified in order to minimize the avian population of this region for recreational activities. They could have been brought here during the winter season by

the right temperature, food supply, less predation danger as well as favorable conditions (Khan et al. 2018). Over the past three decades, the number of migratory birds coming in from Siberia has declined drastically. According to a survey conducted during the winter season, some 150,000 birds, including local and migratory waterfowl, were reported to have landed at 12 aquatic 'stopovers' which is 70 percent lower than in (2017-2018) (Mishra et al. 2021).

Migratory birds start arriving in Pakistan just at end of August as well as begin choosing to leave in February. The birds are mostly moved by March. As Siberian temperatures fall below -30 degrees Celsius, the birds make their way to warmer climates. The migratory bird path is called 'Flyway Four' in Pakistan, or the 'Natural Road.' The migrating birds are flying across Siberia, over Afghanistan, and then into Pakistan, in which they are monitoring the Indus River on the Sindh route (Ahmad et al. 2020). Therefore, we studied the population status and habitat requirement of endangered migratory waterfowl at Patisar lake, Bahawalpur.

MATERIAL AND METHODS

Patisar lake located at 29°20'41.2"N 71°56'21.0"E, which is situated in the middle of Lal Suhanra national park, Bahawalpur. Patisar lake was a large water body that was perfect for birds watching. In the middle of the winter lake become home to 10,000 to 30,000 ducks as well as common coot. The study was conducted during the 2019-2020. The Patisar lake was located about 47.4 Km away from Bahawalpur city. For the identification and selection of Endangered species of waterfowl from the Patisar lake almost every bird that has been observed was noted. To identify which of the bird was endangered IUCN Red list has been concerned.

So, from the IUCN Red list of endangered species all those waterfowl that are endangered have been described. Point counting system was used for the monitoring of birds. Perhaps the most effective and data-rich method for bird counting was the point counting process. In forested environments or in difficult terrain it was the preferred form. At points, one of the observers will observe all of the birds seen or heard at a fixed distance in multiple points standing in one position. At a certain point, the same method was repeated several times. Points – setting of counting stations were randomly, but they were not be close together at a minimum distance of 200 m.

As a relatively large number of points were expected from each research plot, point counting was not an effective technique for small areas of analysis. Counts shall be rendered at every station for a fixed time. Each bird we saw or heard was registered. Counting was done longer than 10 minutes in areas with a very rich bird fauna or where species are hard to detect or to recognize. For equipment, we used a map, a pencil, a notebook, a watch that was used to show seconds and binoculars for the census. In the field with plastic tape or streamers the routes and points are marked on a survey map to ensure that in the coming years there are similar points. Based on the distance between points and the travel method, the time available to census

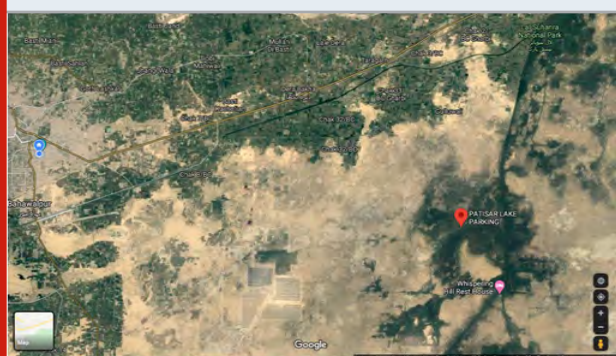
one-point count routes were typically not more than four hours a day. For field work, the number of points protocols are very different, but we preferred an unlimited distance point counting system. Each bird (no matter how far) we detected at every station in 5 minutes, by sight and by the sound. Although main focus of the study was to record Endangered bird species but we recorded all birds of the Patisar lake.

The data sheet consisted of specie name and 3 columns (0-25 meters, 25-50 meters and > 50 meters with number of birds observed) of distance and total number of a single specie observed. Each bird that has been observed was place in according to the distance, hence by this method we were able to count total number of a specific bird species observed from a single point station.

RESULTS AND DISCUSSION

Patisar lake situated at the center of Lal Suhanra National Park district Bahawalpur contain a variety of bird's biodiversity because it acts as an important wetland for the migratory birds. In this study Patisar lake has been researched to find out what species of migratory birds inhabit this lake. So, we performed a well detailed study on Patisar lake of Lal Suhanra National Park specifically that runs in the center of the park. 29°20'41.2"N and 71°56'21.0"E.

Figure 3. 1: Location map of Patisar lake



Throughout Pakistan, birds being intensively hunted as well as captured for feeding and sport decreases local populations, however the overall status of the species was considered to be stable. Non-domesticated birds have become a major component of human diets, and migratory bird numbers have been found to be declining due to predation. Dalmatian pelican (*Pelecanus crispus*), a Pakistani winter visitor, has shown a decrease in their population due to poaching and disruption. Similarly, black-headed Ibis was a seasonal and intermittent migrant to Pakistan during the year who experience extreme risk of hunting and habitat destruction, mainly through irrigation and cultivation within their habitats forage and breeding. Painted stork was Pakistan's local winter tourist facing a pronounced decrease in population size because of over-hunting (Umar et al. 2018). The survey of Patisar lake in the mid-winter and the waterfowl populations was recorded and it has been observed that population of waterfowl population has declined drastically from the previous years. During the

study and data collected from all point stations a total of 67 waterfowl population was recorded (Ahmad et al. 2020).

Result of the study indicated that these population belonging to 6 different orders, 10 different families, 18 genera and 32 different species of waterfowl. The endangered Waterfowl observed in the Patisar lake has been described in detail in the following (Table. 1). Results showed the decline in the endangered species and the population from the previous conducted by Akbar et al. (2006). Patisar lake for waterfowl population. They spent 10 years exploring Patisar lake for waterfowl population and found that waterfowl population has been decreased drastically from 1996 to 2005 in the mid-winter every year.

They found total of 10,142 waterfowl belonging to six orders which are: Podicipediformes, Pelecaniformes, Ciconiiformes, Anseriformes, Gruiformes, Charadriiformes and the families Family: Podicipedidae (*T. iruficollis* spp.), Phalacrocoracidae (*Phalacrocorax iiniger* spp.; *P. carbo* spp.), Anhingidae (*Anhinga melanogaster* spp.), Ardeidae (*Ardea cinereal* spp., *A. purpurea* spp., *Ardeola grayii* spp., *Bubulcus ibis* spp., *Egretta alba* spp., *E. Garzetta* spp., *E. intermedia* spp.), Anatidae (*Anas acuta* spp., *A. crecca* spp., *A. platyrhynchos* spp., *A. strepera* spp., *A. Penelope* spp., *A. clypeata* spp., *Aythya ferina* spp., *A. fuligula* spp., *A. nyroca* spp.), Rallidae (*Fulica atra* spp., *Porphyrio porphyrio* spp., *Gallinula chloropus* spp.), *Recurvirostridae* (*Himantopus himantopus* spp.), Charadriidae (*Vanellus indicus* spp., *V. vanellus* spp., *V. leucurus* spp., *Charadrius dubius* spp.), Scolopacidae (*Tringa tetanus* spp., *T. Nebularia* spp., *Actitis hypoleucos* spp.), Laridae (*Sterna aurantia*). In agreement to present study, they observed a massive decrease in the waterfowl population. In 1996 two thousand seven hundred and forty-four waterfowl were observed but they decreased to the number of one hundred and fifty-nine in 2005. Field study between October and December 2015 at Bahawalpur National Park Lal Suhanra (LSNP) to analyze its vertebrate diversity (Mishra et al. 2021).

This research was initiated to assess the status and environmental harassment of the National Park fauna. The data were obtained by method of counting points. There were 7443 birds with 74 species belonging in the study to 35 families and described 16 orders. Seasonal birds distributed by year about 76%, 7% were breeders in summer, 4% were migrants in transients while 5% were rare and 8% winter migrants were common. The LSNP's main threats to wild animals are hunting, erosion, timber harvesting, damage of human activities and habitat destruction. Increased flows and natural reservoirs are also a significant hazard for recreational purposes. Current research habitat for the survival of this biosphere reserve special animal species should be given due attention (Khan et al. 2018; Mishra et al. 2021).

Patisar Lake as a habitat there are various types of aquatic oat habitat. The hypopycnal layer was a phenomenon consisting of a fresh water layer, floating on the saline lake water, a historically rich duck habitat. Each has a substantial and persistent fresh water influx. A big source of feed for

ducks was Patisar lake itself, where they spent much of their time in the past. Ordinary ducks including mallards, green-winged teal, and gadwalls usually eat flies. The lagoons are a valuable habitat for waterfowl, particularly those close to a source of fresh water. It forms where brackish water was kept behind sediment shores and also shelters the windy days from rough lake waters. However, open water does not tend to provide useful duck environments, other

than the marshes themselves. Fresh open water provides swimming and drinking areas while marsh vegetation offers food and clothes. Besides the lake itself, riverbeds are the most important habitat for waterfowl as they also combine the ecosystems alluded to above with plenty of fresh water. Since the ducks are dependent on fresh water and do not tolerate well the salt. Hyperpycnal stratification, which by far provides the most important duck habitat on Patisar Lake (Ahmad et al. 2020).

Table 1. Population status of endangered waterfowl recorded at Patisar lake during 2019-2020.

Order	Family	Specie	0-25m	25-50m	>50m	Status
Podicipediformes	Podicipedidae	<i>Tachybaptus ruficollis</i>	0	1	3	EN
Pelecaniformes	Phalacrocoridae	<i>Phalacrocorax niger</i>	1	0	1	EN
		<i>Phalacrocorax carbo</i>	2	1	0	EN
	Anhingidae	<i>Anhinga melanogaster</i>	1	2	1	EN
Ciconiiformes	Ardeidae	<i>Ardea cinerea</i>	0	3	1	EN
		<i>A. purpurea</i>	1	0	2	EN
		<i>Ardeola grayii</i>	1	1	2	EN
		<i>Bubulcus ibis</i>	0	1	3	EN
		<i>Egretta alba</i>	1	1	2	EN
		<i>E. garzetta</i>	1	0	1	EN
		<i>E. intermedia</i>	1	1	3	EN
Anseriformes	Anatidae	<i>Anas acuta</i>	0	0	2	EN
		<i>A. crecca</i>	1	1	2	EN
		<i>A. platyrhynchos</i>	2	2	1	EN
		<i>A. strepera</i>	0	2	2	EN
		<i>A. Penelope</i>	1	1	3	EN
		<i>A. clypeata</i>	0	1	2	EN
		<i>Aythya ferina</i>	1	2	0	EN
		<i>A. fuligula</i>	3	2	1	EN
Gruiformes	Rallidae	<i>A. nyroca</i>	1	1	2	EN
		<i>Fulica atra</i>	2	3	2	EN
		<i>Porphyrio porphyrio</i>	0	1	2	EN
Charadriiformes	Recurvirostridae	<i>Gallinula chloropus</i>	0	2	1	EN
		<i>Himantopus himantopus</i>	1	1	3	EN
	Charadriidae	<i>Vanellus indicus</i>	2	1	1	EN
		<i>V. vanellus</i>	1	2	2	EN
		<i>V. leucurus</i>	3	1	3	EN
		<i>Charadrius dubius</i>	1	2	1	EN
	Scolopacidae	<i>Actitis hypoleucos</i>	2	1	2	EN
		<i>Tringa tetanus</i>	2	2	1	EN
		<i>T. nebularia</i>	1	1	1	EN
Total	Laridae	<i>Sterna aurantia</i>	3	1	1	EN
	10	32	36	41	54	

CONCLUSION

The findings of the present study determine that in the Patisar lake of Lal Suhanra National Park of Bahawalpur a total population belonging to 6 different orders, 10 different families, 18 genera and 32 different species of waterfowl.

They all preferred fresh water layer of the lake and marshes near the lake as their habitat. It was concluded that Patisar lake was a prodigious natural resource for the migratory waterfowl and decline occurred with time.

Conflict of Interests: Authors declare no conflict of

interests to disclose.

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Agricultural Communication

The Logistic Function of Cooperation in the Agro-Food Supply with Digitalization in the Russian Federation

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ABSTRACT

Currently, digital solutions are increasingly penetrating consumer cooperation. The need for the use of innovative technologies is increasing to improve the efficiency of logistics of consumer societies of the Centrosoyuz of Russia. Consumer societies have a solid opportunity to combine their economic activities into a single system based on a digital platform and become the undisputed leader in the segment of national food security. Operational processes are developing and improving in the digital economy simultaneously with the development of scientific and technological progress, the introduction of innovations, and the modernization of production. Cooperative formations participate in replenishing the resources of agricultural products to ensure the food security of the country. Consumer societies of the Centrosoyuz of Russia play a significant role in providing a guaranteed channel for the sale of agricultural products and raw materials for small agricultural businesses. Various approaches to the participation of consumer cooperation in food security are revealed in the conditions of the digital economy with the industrialization of production. Therewith, the relevance of consumer cooperation in replenishing food supplies is increasing. Its role in the development of small agricultural business in rural areas is revealed, the need for more complete use of the resources available in consumer cooperation in the organization of procurement activities in the field of procurement of agricultural products and raw materials in the households of the population, individual entrepreneurs and peasant farms is argued. The use of the achievements of the digital economy in the system of consumer cooperation is considered as one of its practical applications in modern conditions to increase competitiveness in the domestic and international food market, improving the quality of life of the working population of rural areas.

KEY WORDS: AGRICULTURAL PRODUCTS, CONSUMER COOPERATION, DIGITAL ECONOMY, LOGISTICS, PROCUREMENT.

INTRODUCTION

The concept of the digital economy originated in the 90s of the 20th centuries, and it developed in Russia mainly through the interaction of large IT organizations in the field of cooperation based on cooperative and integration ties, including the creation of projects to train new competencies for working in the digital economy. More and more attention has been paid in recent years to the new economy in the agro-food sector (Nabieva and Tkach 2019). Decree of the President of the Russian Federation No. 204 "On National goals and strategic objectives of the development of the Russian Federation for the period up to 2024" was issued on May 7, 2018, in the interests of fulfilling the tasks of organizing the active development of digital technologies in the economy and social sphere in Russia. The Russian

Government adopted the federal program "Digital Economy of the Russian Federation" for the real implementation of the provisions formulated in the decree. The program includes the main directions of the digital development of the economy, the use of information technologies in social and economic activities, allowing to increase the efficiency of the organization of entrepreneurial activity in production and the quality of life, including using the advantages of cooperation and integration (Suglobov and Tkach 2020).

The National Program on the Digital Economy was approved and accepted for execution on June 4, 2019, by the decision of the Presidium of the Council under the President of the Russian Federation (Protocol No. 7). The program includes federal projects: on the legal regulation of the digital environment, on training personnel to work in the digital economy, on the creation and development of information infrastructure, the elaboration of methods and means of information security, the development of digital technologies, the definition, and consolidation of

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Received 21/10/2021 Accepted after revision 15/12/2021

Published: 31st December 2021 Pp- 1890-1894

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.75>

digital public administration mechanisms (Karmanov et al. 2021).

The program formulates the main goals, sets tasks, develops directions, and determines the timing of state support measures to provide the required conditions for the progressive dynamic development of the digital economy in the country. The management of the program is entrusted to departments and management structures of federal authorities by Decree of the Government of the Russian Federation No. 234 of March 2, 2019 (Karmanov et al. 2021). The purpose of the work is to characterize the logistics of purchasing activities of the consumer cooperation of the *Centrosoyuz* of Russia in the field of food security in the digital economy. To reveal the potential and role of small agricultural businesses as a resource base for the work of procurement offices and consumer cooperation points. To determine the main directions of procurement logistics and types of agricultural products produced in small forms of management.

MATERIAL AND METHODS

The research methodology was based on scientific methods for organizing the work of consumer cooperation in the creation of food resources for the food security of the state in the digital economy.

RESULTS AND DISCUSSION

The role and place of purchasing and marketing activities of consumer societies in the creation of agro-food resources for the food security of the state in the digital economy have been revealed. Reserves have been assessed and proposals have been developed to modernize the procurement system to improve the economic situation of small agricultural businesses, households, and farmers. Proposals have been developed for the rational use of agricultural products and raw materials in rural areas with the specialization of production. The organizational and legal forms of farming in rural areas have been characterized and proposals have been formulated to improve the efficiency of small agricultural businesses using the advantages of consumer cooperation. The ways of improving logistics to ensure a guaranteed sales channel for agricultural products produced in small forms of management, their processing, and sale have been proposed (Dudin et al. 2020).

The digital economy is considered as the economic activity of economic entities based on digital technologies and interacting with e-commerce in the development and sale of digital goods or services. Data is provided in digital form and is the main factor of production in all areas of socio-economic activity (Ragulina et al. 2018; Dudin et al. 2020). The digital economy is divided into online business and the digital activities of enterprises. It affects the functioning sectors of the production, economic and social activities of the infrastructure of the national economy, including the organizational structure of enterprises and associations based on cooperation and integration, the logistics of wholesale and retail trade in agro-food products.

Competition between manufacturers and suppliers of goods increases when using the advantages of the digital economy in market conditions. With the deepening of the social division of labor, specialization of production, expansion of cooperation, and integration, the digital economy blurs the lines of the territories of individual regions, districts, territories, republics, and states, lowers and levels the national characteristics of countries. The development and improvement of logistics in the transportation system break down all kinds of barriers in the promotion of goods from the manufacturer to the consumer (Zavyalova et al. 2020; Krivova et al. 2021; Vasyukov et al. 2021).

The digital economy contributes to improving the efficiency of partners in various fields of activity, changes the point of view on the feasibility of real movement of goods in space, determines a decrease in a load of vehicles, shopping and distribution centers, a reduction in the flow of consumers to retail outlets and the burden on the transformation of territories and rural settlements. With the socio-economic development of society and the expanded mobilization of natural and economic resource potential, the digital economy expands its activities and increases its importance, affecting various spheres, both production and the life of the population, including education, healthcare, etc. The national program "Digital Economy of the Russian Federation" provides for accelerated development of digital technologies in practice, both in the economy and the social sphere, providing conditions for high-tech business, increasing the country's competitiveness in the global market, strengthening Russia's food security and improving the comfort of people's lives, including in rural areas (Krivova et al. 2021).

The program provides for the creation of a regulatory system in each field of activity in the country based on a flexible approach, the removal of barriers to the development of modern technologies and business related to the use of innovations, as well as the creation and implementation of electronic document management in many areas of socio-economic activity. The program outlined the main areas accumulating legal regulation of production and economic activities, staffing and education, the development of research competencies and certain groundwork in various fields, publicists' infrastructure, and national information security of the state. The Program provided for the accelerated development of large value chains, where large operations were integrated based on cooperation. The organization of timely purchases, prevention of spoilage and loss of products, and their delivery for processing to storage facilities with the subsequent sale to the consumer was given a significant place in the system of promoting food products from producer to consumer (Krivova et al. 2021).

A responsible role was assigned to the procurement, processing, and sale of products, the work of procurement offices, and consumer cooperation points. Thus, in (2019), the producers of consumer societies of the *Centrosoyuz* of the Russian Federation purchased from representatives of small agricultural businesses over 55.0 thousand tons of meat, 218.2 thousand tons of raw milk and dairy products, 37.2 thousand tons of potato, 45.7 thousand tons of vegetable

products, 25.9 thousand tons of fruit and berry products. The analysis of the purchasing activity of consumer societies of the Centrosoyuz of Russia testifies to the successful work of cooperators in this field in several regions of Russia. For example, consumer societies of the Volga Federal District in 2019 purchased agricultural products and raw materials from agricultural producers for 12,668 million rubles, that was, 56.2% of the all-Russian volume of purchases by consumer societies of the Centrosoyuz of Russia (Vasyukov et al. 2021).

This work was carried out in large volumes in the consumer cooperation of the Republic of Tatarstan, where agricultural products worth 8,158 million rubles were purchased in (2019), which is 64.4% in the Volga Federal District. In

terms of its activities in this area, the Union of consumer societies of the Republic of Tatarstan compares favorably with the unions of consumer societies of other subjects of the Russian Federation (Table 1).

Practice showed that as the number of animals increases, as a rule, production increased in parallel. This pattern was also observed in small agricultural businesses in the Republic of Tatarstan, where the number of almost all types of livestock and poultry increased every year during the period under review (2015-2019). In parallel, the volumes of milk production, production of cattle and pigs' meat, poultry and sheep meat, rabbit meat, and honey in farms of small agricultural business of farmers and households of the population, among individual entrepreneurs grew (Vasyukov et al. 2021).

Table 1. Purchases of agricultural products and raw materials by consumer societies of the Volga Federal District of the Russian Federation, 2019

Consumer unions of the subjects of the Russian Federation	All agricultural products, rubles. million	Agricultural products by type, tons				
		Meat	Milk	Potato	Vegetables	Fruits
Centrosoyuz of the Russian Federation	22,559	52,493	218,907	33,486	40,758	24,489
Volga Federal District	12,668	24,850	197,743	11,920	14,020	8,639
Share in Russia, %	56.2	47.3	90.3	35.6	34.4	35.3
Tatar	8,158	16,078	161,666	5,996	3,311	1,469
The share of RT in the Russian Federation, %	36.2	30.6	73.9	17.9	8.1	6.0
The share of RT in the FD, %	64.4	64.7	81.8	50.3	23.6	17.0

Source: Centrosoyuz (2020)

Table 2. Trend of livestock production by farms of the population of the Republic of Tatarstan

Indicators	Years					2019 in % by 2015
	2015	2016	2017	2018	2019	
Livestock and poultry for slaughter, in live weight, thousand tons	130.0	128.6	127.5	126.9	126.2	97.1
Milk, thousand tons	581.5	569.3	554.0	552.5	546.0	93.9
Eggs, million pieces	305.2	304.5	305.4	307.7	307.4	100.7
Honey, t	9,863	8,997	7,728	7,780	7,342	74.4

Source: Statistical collection (2020)

In terms of the volume of agricultural products produced, agricultural organizations were followed by households of the population, which included personal subsidiary plots in rural areas, households of citizens in rural settlements, horticultural and market-gardening non-profit associations, as well as individual entrepreneurs. These representatives of small agricultural businesses, which were one of the forms of management, carry out the production and

processing of agricultural products by the personal labor of family members to meet personal needs. Representatives of small agricultural forms include horticultural and market-gardening non-profit associations of citizens acting voluntarily. In the Republic of Tatarstan, farms of the population in 2019 raised 126.2 thousand tons of livestock and poultry for slaughter, 546.0 thousand tons of raw milk and 307.4 million eggs were produced, 7,342

tons of honey were obtained (Table 2). The production of livestock products in the Republic of Tatarstan was carried out at the highest rates in peasant farms from (2015 to 2019), where the actors are united by kinship or have material and technical means in common ownership. Farmers, together with their participation, carry out production activities, provide processing, transportation, storage, and marketing of agri-food products.

A separate place in agri-food activity was occupied by individual entrepreneurs for the production of agricultural products. As a rule, such economic entities include individuals engaged in entrepreneurial activity without forming a legal entity from the moment of its state registration following the Civil Code of the Russian Federation and who have

declared the types of activities classified to agriculture in the Certificate of State Registration according to the OKVED, Russian Classification of Economic Activities. In the Republic of Tatarstan, peasant farms, including individual entrepreneurs, increased the production of agricultural products from (2015 to 2019). In this category of farms, the cultivation of livestock and poultry meat increased from 15.9 to 23.2 thousand tons, that was, by 45.9% during the specified period. Raw milk yields increased from 140.2 to 174.7 thousand tons, that was, by 24.6%. Particularly high indicators were achieved in the production of eggs – from 9.8 to 29.7 million pieces, or more than 3 times. Beekeeping has become widespread in small agricultural businesses. Tatarstan farmers received 285 tons of honey in 2019 (Table 3).

Table 3. Trend of livestock production by peasant farms* of the Republic of Tatarstan

Indicators	Years					2019 in % by 2015
	2015	2016	2017	2018	2019	
Livestock and poultry for slaughter, in live weight, thousand tons	15.9	17.8	19.0	20.9	23.2	145.9
Milk, thousand tons	140.2	139.1	157.9	155.1	174.7	124.6
Eggs, million pieces	9.8	11.4	17.4	24.6	29.7	303.1
Honey, t	484	507	428	380	285	58.9

Source: Statistical collection (2020) *including individual entrepreneurs

Agri-food products produced by agricultural producers of small agricultural businesses were purchased by procurement enterprises of the Centrosoyuz of Russia and supplied for processing. At the industrial enterprises of the consumer societies of the Centrosoyuz of Russia, various types of food products were usage and confectionery products, canned meat, vegetable and fruit, various kinds of beverages and other food products. In 2019, 252 thousand tons of bread and bakery products were produced at the enterprises and workshops of the Centrosoyuz of the Russian Federation, 3.3 thousand tons of sausage and sausage products, 21.3 thousand tons of confectionery, 25.3 msc of canned food, 3.5 million dl of soft drinks.

A significant part of the purchased agri-food products by organizations and enterprises of consumer cooperation was spent and sold through the logistics system in the field of public catering. Various dishes and semi-finished products are prepared from harvested agricultural raw materials in a wide range. In 2019, food products worth 13.0 billion rubles were sold through the public catering system of the consumer cooperative of the Centrosoyuz of the Russian Federation. The catering system produced food products worth 11.2 billion rubles, that is 86.2%. Through its activities in the procurement system, consumer cooperation participates in the formation of the country's food resources, ensuring food security, which is the primary task of the leaders of any state (Dudin et al. 2020).

In the digital economy, the issue of ensuring food security is the focus of attention of the federal authorities of the country and the subjects of the Russian Federation. A positive role in the conditions of the digital economy is played by cooperation in the agro-food complex to ensure sustainable food supply of the population with food products in the necessary assortment according to medical nutrition standards. The Government of the Russian Federation was instructed to develop a set of measures to implement the provisions of the food security doctrine by Presidential Decree No. 20 of January 21, 2020. In this regard, at the federal and regional levels, an assessment is being carried out and measures are planned to improve the food supply of residents of Russia, regulatory legal acts related to food security issues are being developed, practical activities in the regions of Russia, including by organizations of consumer societies of the Centrosoyuz of the Russian Federation, are being noted (Dudin et al. 2020).

It is important to ensure the effective development of agricultural production in the conditions of the digital economy, including the functioning of the individual sector in rural areas, to form and maintain the state of the material base of agriculture at the proper scientific and technical level, including crop and livestock industries that supply food resources to provide the population with food products. Therewith, it is necessary to ensure the development of both the entire agriculture and all its categories in the right

proportions. The directions of agricultural production should ensure that the rational structure of the population's diet is filled with the main types of agri-food products, including considering the possibility of exporting and importing products. Special attention should be paid to ensuring the effective functioning of all types of small forms of agricultural producers (Dudin et al. 2020).

Consumer societies and unions are a ready-made organizational and economic form and a reliable partner in the system of formation of food resources in the digital economy. Procurement enterprises and points of consumer societies perform the necessary logistical function in the system of saturation of the food market with domestic food products. Consumer cooperation will perform the functions of servicing producers of agricultural products, supplying the rural population with various types of food and industrial goods in a market economy (Vasyukov et al. 2021).

CONCLUSION

The findings of the present study suggests that the procurement enterprises and purchasing points in the system of formation of food resources act as the leading edge of consumer societies of the Centrosoyuz of Russia in ensuring the national food security of the country. Procurement offices and consumer cooperation points, enterprises, and public catering points of consumer societies of the Republic of Tatarstan act as a reliable channel for the sale of products by representatives of small agricultural businesses. Consumer cooperation of Tatarstan, as well as Russia as a whole, using the existing production and intellectual potential, plays a significant positive role in the formation of food resources, strengthening food independence and security of the state.

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Toxicological Communication

Identifying Air Pollution As a Major Threat to Newly Grown Ecotourism Spot in Baranti, Purulia, West Bengal, India

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ABSTRACT

Air pollution is a notable worldwide warning to human health. Every year, air pollution is accountable for more than five million death, out of these 91% occur in lower-middle-income countries. In addition to this, various respiratory & cardiovascular diseases, lower productivity and increased mortality are also related to air pollution, that's why it's often called a silent or invisible killer. However, Ecotourism generates opportunities for tourists wishing to enjoy the natural environment without destructing or disturbing its habitats. It is increasingly considered instrumental in helping the local socio-economic sustainable development and also as a means for generating revenues with the object of preserving the local traditional culture and craft. Environmental ambient quality must be considered as a crucial aspect in the predetermining process of prospective tourists and tourism destinations. The present study site Baranti, in Raghunathpur subdivision of Purulia district, West Bengal, India is situated in a splendid location within Baranti Lake and Baranti Hill, both has emerged as a fast-grown ecotourism spot over the last 7-8 years. The present study was carried out through survey questionnaire method from May 2020 (01.05.2020) - November 2020 (30.11.2020) on different categories of respondents like local people, hotel-resort owners & staff, local businessmen and tourists and simultaneously during this period, the air quality was also measured in respect of temperature, humidity, PM 2.5, PM 10, particles and CO₂ through Temtop M2000C Air Quality Monitor at the said site. In the observation, ambient air quality was measured at three hours intervals on a day every week and 10 readings were taken from each site at a distance of 10 meters apart and the mean values were considered for statistical analyses. It was found that average PM 2.5, PM 10 & CO₂ in ambient air in the study site were 64.26 ug/m³, 89.43 ug/m³ & 701.66 respectively therefore unexpectedly the ambient air is not only polluted but also moderate to unhealthy in respect of the said parameters as judged by the yardstick of Air Quality Guideline Levels laid down by the WHO. A well-defined management plan is required for controlling and minimising the said pollution with the interference of the Government and other agencies for the sustainable growth and development of the said ecotourism spot.

KEY WORDS: AIR POLLUTION, AMBIENT AIR, ECOTOURISM, MANAGEMENT PLAN, SUSTAINABLE DEVELOPMENT.

INTRODUCTION

Ecotourism fulfils the tourists' wish for experiencing the natural environment, of course without causing any damage to it or causing any disturbance to its natural environment. It is a sort of tourism that includes a visit to natural areas that are comparatively undisturbed. It is often considered to be an alternative in miniature form to standard mass tourism conducted on a commercial basis. It may have

manifold purposes like educating the travellers, providing funds for the conservation of ecology, boosting up economic development and making the local communities politically powerful, or nurturing different cultures (Acott et al. 1998; Fletcher et al. 2012; Nataraja et al. 2014; Chow et al. 2019; Ramyar et al. 2020).

From the year 1980s, environmentalists started considering ecotourism as a veritable prospect, for posterity may go to places that are comparatively out of human intervention. In this connection it may be mentioned that in China owing to air pollution domestic tourist arrivals in the neighboring cities have significantly reduced. Therefore, air pollution has negative effects on the tourism industry not only in the

Article Information:*Corresponding Author: saha.buruniv@gmail.com

Received 14/10/2021 Accepted after revision 24/12/2021

Published: 31st December 2021 Pp- 1895-1900

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.76>

short run but in the long run (Dong et al. 2019; Striphas 2019; Tang et al. 2019; Zhou et al. 2019; Koondhar et al. 2020). In this context, it may further be said that air pollution is a formidable factor in determining human health. It has been found in various studies that there is a close relationship between air pollution and its adverse impact on the health of the people at large. Contaminated air is considered one of the crucial components leading to many diseases such as cardiovascular, respiratory diseases such as asthma, bronchiolitis, Chronic Obstructive Pulmonary disease (COPD), lung cancer, central nervous system dysfunctions and cutaneous diseases. CO₂ particles are mainly responsible for air pollution. The particles are named as PM categorised into 2.5 µm (PM 2.5) or 10 µm (PM 10) CO₂ and particles are toxic for human health proved by epidemiological and toxicological studies (Koondhar et al. 2020).

Particulate air pollutants not only negatively affect human physical health but also mental health (WHO Regional Office for Europe 2013; Braithwaite et al. 2019; Payne-Sturges et al. 2019; Sun and Zhu 2019; Almetwally et al. 2020; Manisalidis et al. 2020; Yang et al. 2021). Since decades it is well known that Purulia, West Bengal, India as a place of natural magnificence, significantly consists of numerous magical places including Baranti hills and reservoir, Joychandi hills, Doldanga, Saheb bandh, Ayodhya hills, Gajaburu hills, Surulia and Murugama dam, Cheliama to indicate a few that are hotspots of interest. The places have charmed tourists who enjoy hill trekking, who ardently love rock-jock, who enjoy migrant bird watching, camping, historic sites and above all enjoying nature's beauty. In the recent past, the tourist attraction was very limited due to so many causes (Yang et al. 2021).

However, recently many of these limitations have been controlled and they have marked the origination of the tourism industry. As an outcome, sustainable development and progress of the socio-economic condition of the local people have been begun. But air pollution may have a significant negative impact on tourist destinations of the location as well as on the economic condition of the local inhabitants (Bhaya and Chakrabarty 2016; Patra et al. 2018 2019; Churchill et al. 2020; Palit and Saren 2021). The aims and objectives of the study are to collect first-hand information and acquire knowledge through surveys among respondents of different categories regarding a newly grown, fast-growing ecotourism spot namely Baranti, Purulia of West Bengal, India and study the air pollution that is a veritable threat to the sustainable development of the fast-growing ecotourism spot.

MATERIAL AND METHODS

Baranti is a small village situated at the bottom of Baranti hill and on the bank of Muradi dam (also called Baranti dam) and the Machkhandajore River (commonly called Baranti River), in Santuri Block, Raghunathpur subdivision of Purulia, West Bengal, India. Geographically it is located at 23.5879660 N latitude and 86.8371590 E longitude, situated 263 km and 36 km away from Kolkata and Asansol respectively. Asansol, Paschim Bardhaman is the nearest

city cum major trade hub to Baranti. Muradi, South Eastern Rly is the nearest railway station 6 km away from the Baranti village. The total population of this village is only about 300. Muradi dam was completed in 1991 as part of Ramchandrapur Medium Irrigation Project. The maximum height of this dam above the foundation is 15 m and it's an earthen dam 899 m long with a concrete spill. The study site, Baranti, is situated in Purulia district of West Bengal, India, previously named as the district of Manbhum, is the first step of the gradual descent from the elevated plateau of Chotanagpur proper (plateau in eastern India) to the plains of lower Bengal and also a chunk of the Ranchi peneplain. It's composed of Precambrian rocks, aged more than about 540 million years.

The valleys and hills compose most part of the district surrounded by the west Ranchi and Hazaribagh, into the north by Hazaribagh & Santhal Parganas, into the south by Singhbhum of Jharkhand state and to the east by Paschim Bardhaman, Bankura and Paschim Midnapore of West Bengal state (Coupland 1911; Mahato 2010; Raha and Gayen 2021; Palit and Saren 2021; Paul and Ganguly 2021). The present study was based on a survey from May 2020 (01.05.2020) to November 2020 (30.11.2020) on local people, hotel-resort owners & staff, local businessmen and tourists etc. An ideal questionnaire related to ecotourism issues, problems and associated matters was prepared. After that from the respondents, an idea about this newly grown small ecotourism spot was considered.

Simultaneously, during the study period the air quality of Baranti, Purulia was measured in 6 different points through Temtop M2000C Air Quality Monitor. Data on Temperature, Humidity, PM2.5, PM10, Particles and CO₂ of these sites were taken into consideration for the analyses. The observation was carried out at three hours intervals on a day every week. 10 readings were taken from each site at a distance of 10 meters apart and the mean values were considered for statistical analyses.

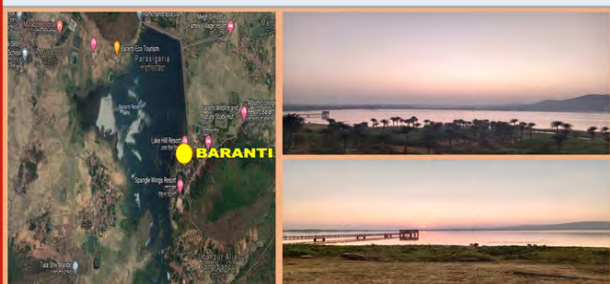
RESULTS AND DISCUSSION

In the present study both primary and secondary data have been used to fulfil the objectives and analyse the impact of air pollution on ecotourism of this area.

Figure 1. Geographical location of Baranti, Purulia (source: <https://www.vectorstock.com>, <https://www.google.com/maps/place/Purulia>)



Figure 2: Satellite view of Baranti, Purulia (source: <https://www.google.com/maps/place/Purulia>)



Primary data was collected through the survey that was carried out on 200 considered respondents on the basis of an ideal questionnaire format with a view to getting information and knowledge of the prospect of ecotourism in the area as well as the interest of the local people and other matters related to it. The secondary data was generated from the basic primary ones collected from the field study for statistical and explanatory analyses. Table 3. shows $F > F_{\text{Crit}}$, reject the null hypothesis. This is the case $10.3343 > 9.5520945$. Therefore, reject the null hypothesis viz. Baranti ambient air and WHO air quality guideline values are not equal.

Table 1. Status of average ambient air in respect of Temperature, Humidity, PM 2.5, PM 10, Particles and atmospheric CO₂ at 6 spots of Baranti, Purulia during June 2020 to November 2020

Temperature (°C)						Humidity (%)					
Average June	Average July	Average August	Average September	Average October	Average November	Average June	Average July	Average August	Average September	Average October	Average November
41	39	39	34	32	28	79.5	80	82.5	78.1	76	74
PARTICLES per L						Atmos. CO ₂ ppm					
Average June	Average July	Average August	Average September	Average October	Average November	Average June	Average July	Average August	Average September	Average October	Average November
7354	7547	7254	7741	8201	8758	699	702	690	695	709	715
PM2.5 µg/m ³						PM10 µg/m ³					
Average June	Average July	Average August	Average September	Average October	Average November	Average June	Average July	Average August	Average September	Average October	Average November
59.6	62	58.2	65.2	68.5	72.1	10.3	11.2	10.3	89.3	98	106.5

The ambient air quality in Baranti, Purulia was considered and it was found that the air quality here is moderate as well as unhealthy for the local people and tourists as compared to WHO Air quality guideline values (Table 1-3, Figure 5-9). Our study site is Baranti, a small village in the district of Purulia, West Bengal, India. It mainly consists of tribal people and people belonging to sub-altern class. A major portion of the people here earn their living by means of the Government Project '100 Days Job'. Besides, some of them are engaged in biannual irrigation activities and they cultivate paddy and vegetables. Again, some others engage themselves in pisciculture in dam. Only a few of them are employed in Government or other sectors. Prior to 2010-2011, only 2 small guesthouses or farmhouses were

there but they were not well known. From 2011 onwards, 40 resorts have been set up in the area and they are much in demand now. Even the Govt. of West Bengal has built a resort here. Without booking in advance, it is very difficult to avail of these resorts, especially during the peak season for tourism. It actually explains the popularity of the spot. Local people are directly or indirectly dependent on tourism. They run and maintain the resorts and the tourists' transport is also looked after by them. The local market and shops have been flourished by the tourists (Raha 2015; Paul and Ganguly 2021).

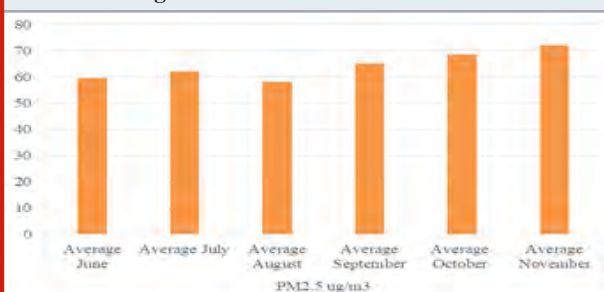
Figure 3: Ambient air quality studied points at Baranti, Purulia



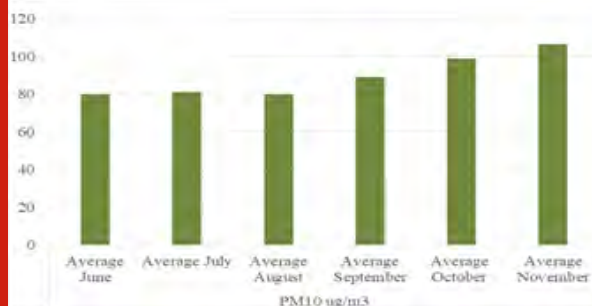
Figure 4: Ambient air quality of Baranti was measured at different spots through Temtop M2000C Air Quality Monitor



Figures 5, 6, 7 & 8. Bar graphs of PM 2.5, PM10, Particles and atmospheric CO₂ respectively in ambient air of Baranti, Purulia during June 2020 to November 2020



Figures 6



Figures 7

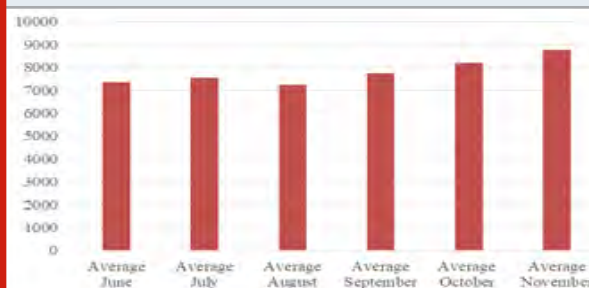


Figure 8

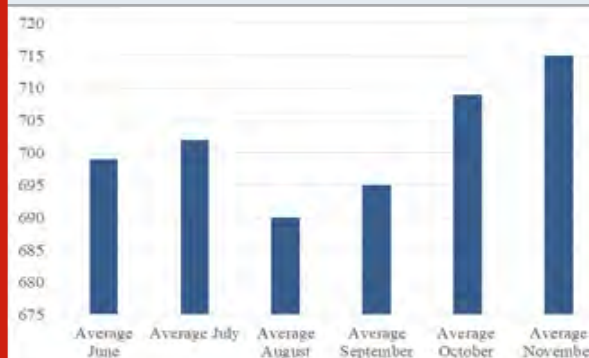
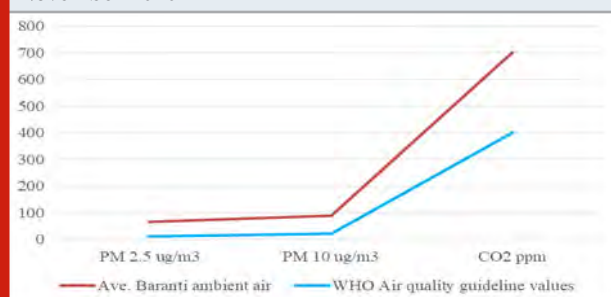


Table 2. During June 2020 to November 2020 average PM 2.5, PM 10 and atmospheric CO₂ in ambient air of Baranti, Purulia as compared to WHO Air quality guideline values

Parameters	Ave. Baranti ambient air	WHO Air quality guideline values
PM 2.5 ug/m ³	64.26	10
PM10 ug/m ³	89.43	20
CO ₂ ppm	701.66	400

Figure 9: Graph of average PM 2.5, PM 10 and atmospheric CO₂ in ambient air of Baranti, Purulia as compared to WHO Air quality guideline values during June 2020 to November 2020



The Govt. of West Bengal has improved the infrastructure of the area. The socio- economic condition of the area has also developed on a massive scale by dint of tourism. The local traditional folk culture has also been revamped by the tourists. Tribals' handicraft etc. have also become popular. Local people demand for a well-equipped green park and greeneries at the site. They also demand that a circular road be set up surrounding the park and more improved transportation system be ensured as well as number of vehicles for tourists be increased. Last but not least they also demand that a Development Authority be set up for proper planning and execution of ecotourism promotion (Mishra 2019; Palit and Saren 2021).

Table 3. ANOVA: Single Factor shows average PM 2.5, PM 10 and atmospheric CO₂ in ambient air of Baranti, Purulia an compared to WHO Air quality guideline values during June 2020 to November 2020

ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	340218	2	170109.005	10.3343	0.04513	9.5520945
Within Groups	49381.7	3	16460.5714			
Total	389600	5				

A comprehensive study was made during the last 6 months from May'20 to November'20. Unexpectedly it was noticed that the month wise average ambient air at the said side is contaminated in respect of PM 2.5, PM 10, Particles and CO₂ parameters (Table 1-3; Figure 5-9). After analyses it was also found that the ambient air quality of the said study site is very much polluted as judged by the yardstick

of Air Quality Guideline Levels laid down by the WHO (Table 2, Figure 9).

Tourism in Baranti has an enormous effect bearing on the socio-economic improvement of the district. Many important parameters are instrumental in the flourishing of the tourism industry in the past few years. Tourism

business leads to job opportunities which help enhance income sources as well as approaches to greater education and access to better health for the local inhabitants. They do not require to migrate to other region in search of job. But here the study revealed that air-pollution at an alarming rate appears to be a major cause of concern and a great threat to tourism (Mishra 2019; Banik and Mukhopadhyay 2020; Palit and Saren 2021).

Figure 10: The local traditional folk culture: Santali dance at Baranti, Purulia



Figure 11: The local traditional folk culture: Chhau dance at Baranti, Purulia

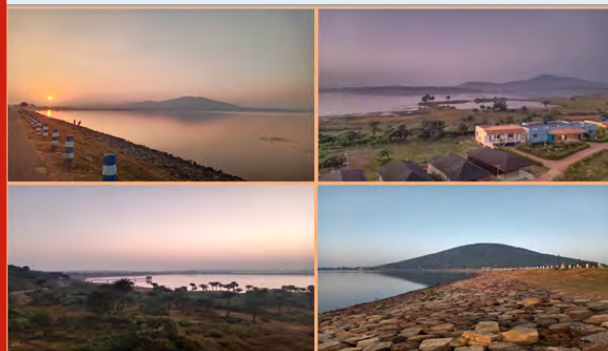


Figure 12: Display of Tribals' handicraft to tourists for sale at Baranti, Purulia



Tourism does not merely mean learning or knowing facts about various locations. It gives us access to the unique cultures and styles of living of others and leaves an indelible impression on one's mind. The overall progress in tourism

Figure 13: Some attractive glimpses of Baranti, Purulia ecotourism spot



also influences the comprehensive growth of the region and also of the inhabitants. But in such a fast-growing ecotourism spot as Baranti situated in the midst of backward areas peopled by the Tribals, air pollution at an alarming rate is a veritable threat.

CONCLUSION

During the study, it was found that average PM 2.5, PM 10 & CO₂ in ambient air in the study site were 64.26 ug/m³, 89.43 ug/m³ & 701.66 respectively therefore unexpectedly the ambient air is not only contaminated but also moderate to unhealthy in respect of the said parameters as judged by the yardstick of Air Quality Guideline Levels laid down by the WHO. A proper management planning for controlling and minimising the said pollution with the interference of the Government and other agencies is the need of the hour for ensuring sustainable development of the area and making it more eco-friendly that will help in boosting up the economic condition as well as folk-culture of the local people. The preservation of nature & its beauty and controlled contamination of the environment leads to the encouragement of tourism.

ACKNOWLEDGEMENTS

The authors are grateful to Head of the Department, Botany, The University of Burdwan, West Bengal, India for Laboratory facilities.

Conflict of Interests: The present study will make the local people, hotel-resort owners & staff, local businessmen and tourists, Government and other authorities / organisations aware of the ambient air pollution and in this context the authors do hereby declare that there is no conflict of interest with the above said categorised persons & authorities / organisation.

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Dental Communication

Phytogenic Synthesis of Nanoparticles from the Extract of *Achyranthes aspera*-A Panacea for Dental Health Care

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ABSTRACT

Achyranthes aspera belongs to Amaranthaceae family and is found in barren and arid regions in India. The plants are categorized as a weed in Ceylon, Asia, Africa and Australia but tribal communities in India have known its benefits for topical treatments of many ailments from Vedic civilizations. The leaf, stem and root aqueous extracts were used for monometallic zinc and bimetallic copper-zinc nanoparticle synthesis and testing antibacterial activity against the diseases causing drug resistant bacterial pathogens viz. *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Bacillus subtilis* using standard methods. Microbes confirmed that the plant contains bioactive compounds that exhibit measurable antimicrobial activity against standard pathogenic bacteria. The nanoparticle synthesis was confirmed by visual color change and UV spectroscopic evaluation of solution. The different plant parts showed different absorption peak at 328 nm, 298 nm for roots and stem while 395 nm for leaves in case of zinc nanoparticles while bimetallic Copper-Zinc nanoparticles resulted in peaks at 402.0, 400.8 and 402.0 nm for leaf, root and stem respectively. The synthesized nanoparticles of zinc and copper zinc from all three plant parts were further tested for their as cariogenic potential against cariogenic pathogen *Streptococcus mutans* which resulted in significant zones of inhibition. The plant has antimicrobial literature using organic solvent extracts where researchers reported negligible activity of different plant parts while nanoparticle synthesis resulted in potent zone of inhibition. The bimetallic Cu-Zn will enhance its potential to be used in mask and PPE kits for safety concerns.

KEY WORDS: A. ASPERA, ANTIMICROBIAL, NANOPARTICLES, PHYTO-CHEMICAL, ZONE OF INHIBITION.

INTRODUCTION

The present investigation deals with the synthesis of monometallic Zinc and bimetallic Copper- Zinc nanoparticles using leaf, stem and roots as plant part from *Achyranthes aspera* L. belonging to Amaranthaceae family and is a considered invasive weed species. It is perennial stiff erect herb of 2m height commonly known as “Apamarga” and is available in India as roadside weed (Figure-1) (Jain et al. 2006). It bears elliptical, ovate, and broad leaves. The inflorescence is 8-30 cm with single red or white colored flowers. The root paste has anti-fertility attributes. The decoction is prescribed to terminate pregnancy while the ethanolic extracts have been reported to have spermicidal activity. The methanolic extracts of leaves have antitumor activity. The shoots and seeds were commonly used to treat asthma; hypertension and diabetes (Zafar 2009; Paul et al. 2010).

The leaf extracts have hypoglycemic thyroid stimulating properties. Biological or green route synthesis has overcome the toxicity concern which has to be taken into account while chemically synthesizing nanoparticles. Methods like sol gel, gas spraying, and pulsed laser deposition, wet chemical and electrochemical methods have inherent limitations which limits them in nanoparticle processing. The primary benefit of plant-based nanoparticle synthesis is its non-toxic mechanism. Zinc nanoparticles have wide range of application in cosmetics, paintings, glass industry, and packaging industry. The chemical methods implemented for zinc nanoparticle synthesis needs to have special expertise and authenticated instruments which make the process more sensitive and expensive (Pandey et al. 2014; Jadoun et al. 2021).

In comparison, combustion method is single step method which can be carried out under normal conditions without much expertise. Moreover, the process is eco-friendly. Zinc nitrate used as precursor is economical in comparison to gold and silver nanoparticles synthesis. Zinc oxide is most commonly used metal oxide for gas

Article Information:*Corresponding Author: sunitakhatak2019@gmail.com

Received 19/10/2021 Accepted after revision 29/12/2021

Published: 31st December 2021 Pp- 1901-1908

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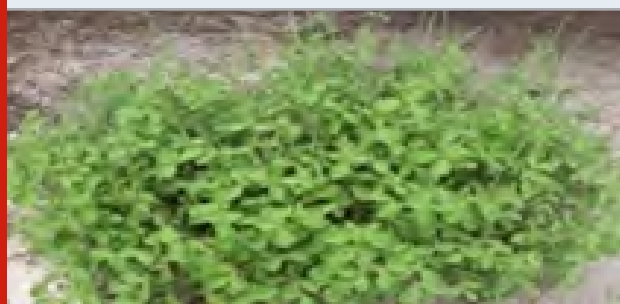
Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.77>

sensing. Large surface area results in fast response and has multipurpose applications due to corrosion resistant potential in biomedical healthcare. Economic benefits and high corrosion resistant property make zinc nanoparticles synthesis more versatile than silver and gold nanoparticle synthesis (Narayana et al. 2018).

Although literature is studded with synthesis of nanoparticles using *Achyranthes aspera* but major focus was on synthesis of silver and gold. It was assessed the toxicity and stability of gold nanoparticles synthesized using *Achyranthes*, which in turn was pH dependent. The visual color change from dark brown to dark purple revealed the nanoparticle synthesis which was further confirmed by a peak at 540 nm. The synthesized nanoparticles were in the range of 50-80 nm and had spherical morphology as confirmed by SEM and TEM. Cytotoxicity assay resulted that a concentration of 100 µg/ml is applicable to be used for nanomedicine development. IRapid green synthesis of nanoparticles was reported by using aqueous leaf extract of *Achyranthes aspera* (Amaladhas et al. 2013; Tripathi et al. 2016). The disc diffusion method was used to test sensitivity of bacterial strains against standard pathogens similar to present study. Praveena et al. (2014) reported silver nanoparticle synthesis using silver nitrate and plant leaves extract of *Achyranthes aspera*. The antibacterial activity represented a significant advancement in nano-material field. The silver nanoparticles synthesized from this plant displayed 100% efficacy against *Aedes aegypti* (Praveena et al. 2014; Sharma et al. 2021).

The present investigation exploited all three plant parts of *Achyranthes aspera* in the nanoparticle synthesis. In addition to zinc, bimetallic Cu-Zn nanoparticles were also synthesized using all three plant parts and tested against all standard pathogens. Significant zones of inhibition were obtained which attracted the potential of bimetallic nanoparticles over monometallic particles. Cu-Zn nanoparticles have been previously made from toddy palm plant which exhibited antibacterial, antitumor and antioxidant activity (Merugu et al. 2021).

Figure 1: *Achyranthes aspera* plant from UIET, Kurukshetra University Campus.



MATERIAL AND METHODS

Plant leaves, stem and roots of *Achyranthes aspera* were collected from roadside of university campus itself and identified from Botany Department of Kurukshetra

University of Kurukshetra, Haryana, India. The various human pathogenic microorganisms were purchased from Microbial Type Culture Collection (MTCC): Institute of Microbial Technology (IMTECH), Chandigarh; which included two gram-positive bacteria: *Staphylococcus aureus* (MTCC-3160) and *Bacillus subtilis* (MTCC-121) and two Gram-negative bacteria: *Pseudomonas aeruginosa* (MTCC-2295) and *Escherichia coli* (MTCC-5704), along with one cariogenic bacterium namely *Streptococcus mutans* (MTCC-497). The test tubes were incubated at 4 °C in the refrigerator for further studies.

The leaves stem and roots of healthy *Achyranthes aspera* plants were selected from road side to be used as reducing agents using zinc nitrate salt and copper sulphate penta hydrate to synthesize monometallic zinc and bimetallic copper zinc nanoparticles. The leaves and roots were rinsed and washed with distilled water after prior wash with tap water 3-4 times. The thoroughly washed plant materials were chopped and grounded to fine powder after drying. 10 gram of powder was soaked in 100 ml de-ionized water in one 250 ml flask. The flask was kept on top of hot plate for 30 to 45 minutes at 80 °C. The green colored extract from leaves, stem and brown colored extract from roots were filtered using Whatman filter paper no-1. The filtrate was used for synthesis of nanoparticles separately for different plant parts (Le et al. 2020).

Single step economical method was employed for zinc oxide nanoparticles synthesis under sterile conditions. Aqueous plant extracts from all three plant parts namely leaves, root and stem were prepared along with 100 ml of aqueous solution of zinc nitrate hexahydrate (0.1 M) and mixed in a ratio of 1:2 (PE: Zinc nitrate hexahydrate solution). Zinc nitrate solution (25ml) was added drop wise into of plant extract (12.5ml) and solution was incubated in water bath at 70°C for complete one hour. Visual observation via color change of original solution to cream colored solution and taking absorption maxima at the wavelength range of (300-600) confirmed the synthesis of zinc nanoparticles. The nanoparticles were extracted by heating on hot plate till a dried extract is obtained and then further heated at 70°C using heating mantle until get converted to fine powdered form. The nanoparticles were further kept in hot air oven for one hour at 110 °C for calcinations (Ramesh et al. 2014).

Approximately 5 gram of copper sulphate pent hydrate (20 mM) and 6 gram of zinc nitrate (20 mM) were dissolved in 90 ml of triple de-ionized water and incubate at ambient temperature in water bath till solution become homogeneous. The dissolved copper zinc solution (90ml) was added drop wise into plant extract (10ml) and incubated in water bath at 70 °C for complete one hour. Visual observation via color change of original bluish colored solution to dark green colored solution and taking absorption maxima at the wavelength range of (300-600) confirmed the synthesis of Cu-Zn nanoparticles. The antimicrobial activities of plant extracts were evaluated by agar well diffusion assay using standard protocol (Perez et al. 1990; Minal and Prakash 2016).

RESULTS AND DISCUSSION

Antimicrobial Propensity: Antimicrobial analysis basically explains the measurement of zones of inhibition which depicts the cleared zones where plant extract has shown its inhibitory action. The monometallic zinc and bimetallic copper zinc nanoparticles synthesized using three different plant parts viz. leaf, stem and roots from *Achyranthes aspera* at three different volumes of extracts 50, 75 and 100 µl.

Against Gram negative strains: Zones of 13, 16 and 17mm were observed at three volumes of extracts using zinc nanoparticles while prominent zones of greater size of 31, 32 and 34 mm were obtained using bimetallic copper zinc nanoparticles using leaf as plant part against *E. coli*, a gram-negative pathogen. The difference in monometallic and bimetallic zones was remarkable and depicts the efficacy of bimetallic nanoparticles over monometallic even when most of the researchers have reported the selective inhibition of only gram-positive bacteria preferred over gram-negative strains. Similiar to our study, stem was exploited to synthesize zinc and copper zinc nanoparticles produce equivalent zones of 14, 16, 18 mm in zinc nanoparticles and again showing dominant zones of inhibition of 34, 34 and 36 mm using bimetallic copper zinc nanoparticles (Bordbar et al. 2020).

Similarly roots resulted in zones of 13, 17 and 18mm using zinc nanoparticles while the copper zinc nanoparticles producing zones of bigger size of 29, 31 and 33 mm again showing influence of two nanoparticles working together against *E. coli* (Fig-2). The zones have been explained and described in correspondence to three different concentrations of 50, 75 and 100 µl respectively. Metallic nanoparticles hold huge application in detection of pathogenic bacteria. A group of nanotechnologists synthesized zinc nanoparticles using zinc nitrate hexa- hydrate similar to present investigation along with zinc chloride and zinc acetate and further reported antimicrobial efficacy of these nanoparticles. The UV visible spectral analysis revealed peaks at 278, 374 and 378nm respectively which confirms the synthesis was a success. The antimicrobial assay resulted in higher zones of inhibition against *E. coli*, and *S. aureus* using agar well diffusion assay which corroborate well with our studies (Getie et al. 2017; Bordbar et al. 2020).

Similar results were observed considering another gram-negative bacteria *P. aeruginosa* but the zone size was lesser in diameter in comparison to *E. coli*. Zinc nanoparticles synthesized using leaves resulted in zones of 14, 16, and 18 mm while in comparison copper zinc bimetallic extracts produced zones of 19, 24, and 25 mm at three different volumes of extracts. Zinc nanoparticles have been found to be very effective against cancer less lines in multiple studies. Similar pattern was observed using stem as reducing agent in nanoparticle synthesis of zinc giving zones of 13, 16 and 17 mm while bimetallic particle extract resulted in zones of 21, 25 and 27 mm at three different extract volumes. While root as plant part were used to synthesize monometallic zinc and bimetallic copper zinc nanoparticle

resulted in similar zones with very minor differences of 14, 17 and 22mm using zinc nanoparticles and zones of 21, 23 and 25 mm using bimetallic nanoparticles (Chandran et al. 2019).

Table 1. Antimicrobial Activity of Zinc nanoparticles against selected pathogens.

Pathogen	Volume of Extract (in µl)	Zone of Inhibition (in mm)		
		Leaf	Stem	Root
<i>E. coli</i>	50	13	14	13
	75	16	16	17
	100	17	18	18
<i>B. subtilis</i>	50	14	-	15
	75	16	14	18
	100	18	16	20
<i>S. aureus</i>	50	13	14	-
	75	17	16	-
	100	18	17	20
<i>P. aeruginosa</i>	50	14	13	14
	75	16	16	17
	100	18	17	22

Table 2. Antimicrobial Activity of Cu-Zn nanoparticles against selected pathogens.

Pathogen	Volume of Extract (in µl)	Zone of Inhibition (in mm)		
		Leaf	Stem	Root
<i>E. coli</i>	50	31	34	29
	75	32	34	31
	100	34	36	33
<i>B. subtilis</i>	50	28	25	27
	75	30	26	33
	100	32	28	35
<i>S. aureus</i>	50	27	35	35
	75	30	36	37
	100	36	38	38
<i>P. aeruginosa</i>	50	19	21	21
	75	24	25	23
	100	25	27	25

The zones of inhibition are although greater than what observed using simple methanolic extracts of plants and aqueous extracts which did not result in any zone of inhibition but are significant enough to be considered as potent agents against *P. aeruginosa* as shown in Table-1 and 2. The methanolic extract of the plant has been found to exhibit strong anti-tyrosinase activity *in vitro* (Sinan et al. 2020).

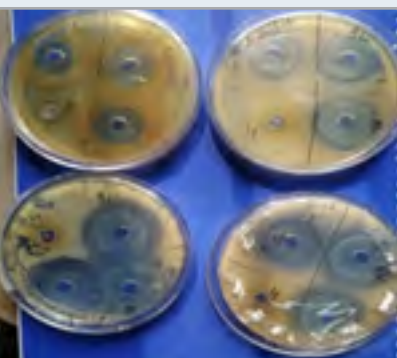
Against Gram Positive strains: In consideration of gram-positive bacterial pathogens the literature revealed that nanoparticles have been more inhibitory in action

as compared to gram negative bacteria. In the present investigation *Bacillus subtilis* and *Staphylococcus aureus* were taken as standard pathogen to study the propensity of both monometallic and bimetallic nanoparticles as inhibitory agents. In case of *B. subtilis* zones of 14, 16, and 18 mm were reported considering leaves for zinc nanoparticles while zones of 28, 30, and 32 mm were produced using bimetallic copper-zinc nanoparticles (Beg et al. 2020).

Figure 2: Bimetallic Cu-Zn nanoparticles synthesized using roots of *Achyranthes aspera* at three different volumes against four standard pathogens.



Figure 3: Bimetallic Cu-Zn nanoparticles synthesized using leaves of *Achyranthes aspera* at three different volumes against four standard pathogens.

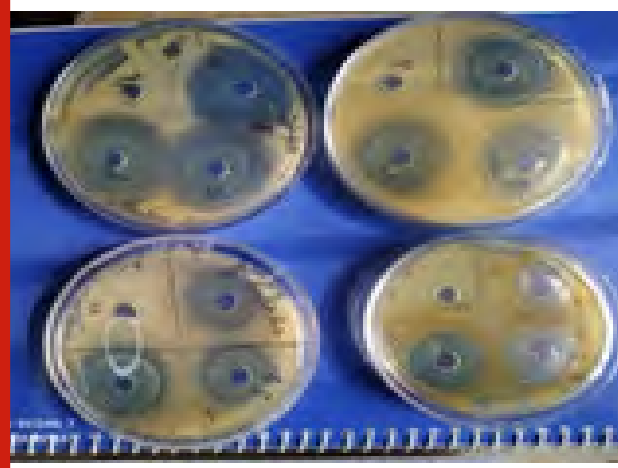


Taking stem as plant parts equivalents zones of 14 and 16 were observed at only two higher concentrations of 75 and 100 μ l while lesser concentration of 50 μ l was unable to produce any zone of inhibition. In comparison bimetallic stem used copper-zinc nanoparticles resulted in significant zones of 25, 26, and 28 mm at three different volumes of extracts of 50, 75 and 100 μ l respectively (Fig-3,4). Roots comes out to be most significant plant part against *Bacillus subtilis* resulting in zones of 15, 18 and 20mm using zinc nanoparticles while zones of 27, 33 and 35 respectively at three different volumes of extracts. The roots of the plant have been reported in literature to consist of ample of phytochemicals to be considered for its prominent activity

against bacterial pathogens. Moreover, the phytochemicals present in the plant have been found to display strong anti-cancer properties (Beg et al. 2020).

Staphylococcus aureus has a long history of being a prominent pathogen in majority of disease outbreak in India and world. The resistant strains developed are considerably resistant to almost most of the antibiotics developed in regard. The medicinal plants need to be exploited by scientific community to fight against such resistant strains emerging in day-to-day life. The zinc nanoparticles and bimetallic nanoparticles synthesized using leaves resulted in zones of 13, 17, 18 mm and 27, 30 and 36 mm respectively at three different volumes of 50, 75 and 100 μ l. While stem parts used to synthesize zinc and copper zinc nanoparticles resulted in zones of 14, 16 and 17 mm and zones of 35, 36 and 38 mm respectively at three volumes of extracts (Fig-4).

Figure 4: Bimetallic Cu-Zn nanoparticles synthesized using stem of *Achyranthes aspera* at three different volumes against four standard pathogens



In comparison to these two plant parts the root extracts again proved their efficiency over other plant parts resulting in zones of 20mm at only at 100 μ l considering zinc nanoparticles, while zones of much bigger size area of 35, 37 and 38mm using copper- zinc nanoparticles. The results in investigating the propensity of roots to be exploited against *S. aureus* are the need of the hour. Zinc oxide nanoparticles were synthesized using flower, leaves and stem plant extracts from *Passiflora* (Manokari and Shekhawat 2016). The bioreduction completes in 2 hours with resulting in strong peaks at 332 nm from leaves, 296 nm from stem and 326 nm from flower extracts similar to present research outputs. The process can be further scaled up for large scale synthesis of nanoparticles (Duraimurugan et al. 2019).

The zinc nanoparticles synthesized are affected by the type of leaf extract used and have superior photocatalytic activity for Methylene Blue dyes degradation which can be implemented as photo catalyst in waste water treatment in textile industry. A group of investigators evaluated

structural optical and photo catalytic properties of zinc oxide nanoparticles obtained using by simple plant extracts of *Achyranthes aspera* and *C. quianensis* as reducing agent using zinc nitrate as precursor (Duraimurugan et al. 2019).

Figure 5: Mono metallic - Zn nanoparticles synthesized using leaf root, stem and thorns of *Achyranthes aspera* at three different volumes against *S. aureus*.



Table 3. Antimicrobial activity of Zinc and Cu-Zn Nanoparticles against Cariogenic pathogen (*Streptococcus mutans*)

Pathogen (<i>S. mutans</i>)	Volume of Extract (in μ l)	Zone of Inhibition (in mm)		
		Leaf	Stem	Root
Zn Nanoparticles	50	18	18	-
	100	20	20	24
Cu-Zn Nanoparticles	50	23	22	19
	100	25	24	26

Cariogenic propensity: The literature survey and the ancient knowledge of using roots by tribal people in gum pain and as toothbrush have inspired us to explore the potential of leaf, stem and root extracts of zinc and copper zinc nanoparticles against a cariogenic pathogen namely *Streptococcus mutans*. Although the zones were not in a range as obtained against *S. aureus* but considerable activity was reported resulting in zones of 18 mm and 20 mm at two different volumes of 50 and 100 μ l using leaf and stem while a bigger zone was observed in comparison to both plant parts of 24 mm at a higher concentration of 100 μ l using roots as plant part (Table-3) in zinc nanoparticles. While in comparison bimetallic copper zinc nanoparticles resulted in zones of 23 mm and 25 mm using leaves, zones of 22 mm and 24 mm using stem which were almost comparable (Jaisankar et al. 2020).

While roots as plant part resulted in zones of 19 mm against *Streptococcus mutans* at 50 μ l volume of extract while a zone of 26 mm at 100 μ l volume of extract using bimetallic copper zinc nanoparticle. Some microbiologists evaluated antibacterial activity of *Achyranthes aspera*

extracts against *Streptococcus mutans* taking stem and roots as plant part at four different concentrations of 2.5%, 5%, 7.5 % and 10 % which in turn resulted in significant zone of inhibitions (Yadav et al. 2016). In case of roots MIC assay for root, a minimum zone of 14 mm was observed at 2.5% at a concentration of 100 μ l and 250 μ l, while a maximum zone of 21 mm at 10% and a volume of 250 μ l. While in case of shoots a minimum zone of 12 mm was observed at a concentration of 2.5% at a volume of 100 μ l, while a maximum of 16 mm at 10% and at a volume of 250 μ l in comparison to standard chlorhexidine which resulted in a zone of 19 mm at 2% and at a volume 10 μ l. Both root and stem resulted in a MIC value at 2.5% concentration. The plant's juice is applied on the gums to relieve the pain and is being used since immemorial by people of India and Nepal as a toothbrush after thorn removal (Jaisankar et al. 2020).

A group of researchers reported the major key route of these effective zones of inhibitions or antibacterial activity is owned by different phytochemicals like alkaloids and tannins and further revealed tannin being water soluble the aqueous extract from roots of plant is due to tannins components (Kumar et al, 2009). Ethyl acetate extracts of *Achyranthes aspera* resulted in significant zones of inhibitions against *S. mutans* in comparison to other solvent system and thus have cariogenic potential (Jebashree et al. 2011). A group of researchers elucidated the importance of medicinal plant extracts in cariogenic activity as an important natural alternative to prevent microbial growth in oral infection disease. They investigated nineteen medicinal plants using leaves and fruit extracts against four cariogenic bacteria (Trivedi et al. 2018; Jaisankar et al. 2020). The extracts were prepared using organic solvents methanol, ethyl acetate, hexane and distilled water against *L. acidophilus*, *L. casei*, *S. mutans*, and *S. aureus* using agar well diffusion method. The plant proves out to be a natural alternative to prevent oral diseases (Trivedi et al. 2018).

Figure 6: Bimetallic Cu-Zn nanoparticles using plant leaves showing green colored solution in comparison to Cu-Zn solution



Confirmation of Zinc oxide and Cu-Zn nanoparticles:

The visual change in the color of the colloidal solution from greenish to light cream color confirmed the synthesis of zinc oxide nanoparticles which was further confirmed using UV-spectral analysis in range of (300-600) which resulted in an optical absorption band peak at 328 nm and 298 for roots

and stem while 395 nm for leaves for zinc nanoparticles. Zinc nanoparticles were synthesized using leaf extracts of *Hibiscus rosa-sinensis* using different concentrations of zinc nitrate. They observed a peak at 269 nm which confirmed the zinc nanoparticle synthesis (Sindhura et al. 2013). While zinc nitrate was used to synthesize zinc nanoparticles using olive (*Olea europaea*) leaf extracts (Hashemi et al. 2010; Saeed et al. 2021).

The zinc nanoparticle synthesis was confirmed by UV-visible spectroscopy observing a peak at 370 nm and further revealed spherical particles with average size of 41 nm, which well corroborate with our results. The color change associated with Cu-Zn Nanoparticle is displayed from blue colored solution to reduced dark green colored solution (Figure-6) and absorption peaks at 402.0, 400.8 and 402.0 for leaf, root and stem respectively further confirmed the synthesis of Cu-Zn nanoparticles not been an alloy but as one solution giving one peak similar to Minal and Prakash results (Minal and Prakash 2016) (Figure-7,8,9). The Zinc nanoparticles synthesized from this plant have been found potent to eliminate poultry pathogenic bacterium (Saeed et al. 2021).

Figure 7: Bimetallic Cu-Zn nanoparticles synthesized using plant leaves with absorption peak at 402.0nm.

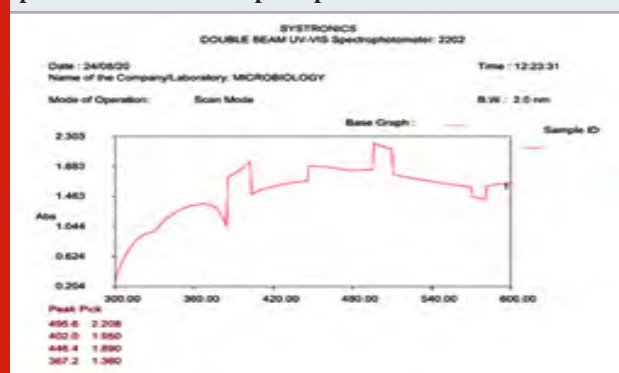
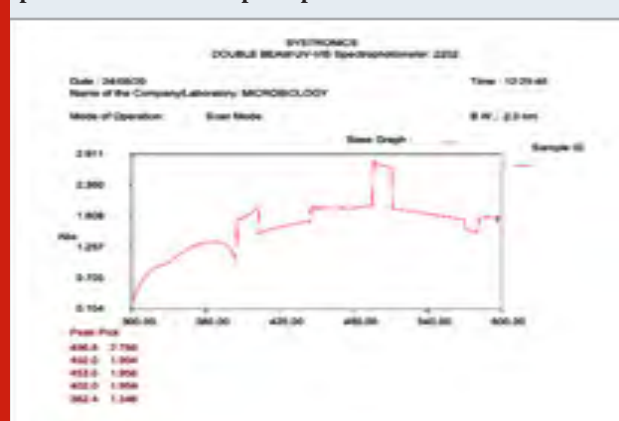


Figure 8: Bimetallic Cu-Zn nanoparticles synthesized using plant stem with absorption peak at 402.0nm.

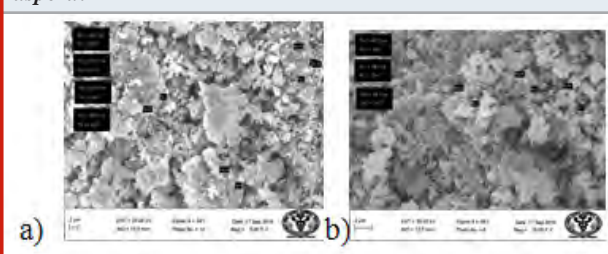


The structure (Morphology) and size of nanoparticle synthesized was determined using SEM analysis for Zinc nanoparticles. Mixed surface morphology was observed

Figure 9: Bimetallic Cu-Zn nanoparticles synthesized using plant roots with absorption peak at 400.8nm.



Figure 10: SEM images of zinc nanoparticles agglomerates observed in root (a) and stem(b) using *Achyranthes aspera*.



using SEM images by 9 Hitachi VP-SEM SU1510. The Zinc oxide nanoparticles showed agglomeration with mixed morphology structure with irregular shapes (Fig-10).

CONCLUSION

The findings of the present investigation exploited a roadside weed having immense potential as cariogenic alternative along with suitable material to synthesize nanoparticles. All plant parts even inflorescence was used to act as antimicrobial agent. Evaluation of antimicrobial activity of asper against *B. subtilis*, *E. coli*, *P. aeruginosa* and *S. aureus* gave significant zones of inhibition. It can be assumed that the Zn nanoparticles have a higher antimicrobial activity even at the lower concentration. Lower concentrations of plant extracts result in reduction of zinc ions but cause aggregation of nanoparticles as shown in SEM images. The present research depicted the susceptibility of bacterial pathogens to nanoparticles synthesized using asper. The zinc nanoparticles mixed with textiles were used to combat the viral infection in present pandemic and bimetallic Cu-Zn that will further enhance the potential to be used in mask and PPE kits for safety concerns.

ACKNOWLEDGEMENTS

This study was technically supported by the University Institute of Engineering and Technology, Kurukshetra, India.

Conflict of Interests: Authors declare no conflicts of interests to disclose.

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Biomedical Communication

Isolation of Antibiotic Resistant Plasmid Deoxyribonucleic Acid from MDR Diarrheal Pathogens

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ABSTRACT

Cholera is an acute infectious disease in countries with poor sanitation. The main clinical symptom of cholera is gastroenteritis and a symptom of the disease includes mild to moderate dehydration, vomiting, fever and abdominal pain. Antibiotic drug resistance in *V. cholera* has become a serious problem mostly in developing countries and multi-drug resistance to different antibiotics as well as Tetracycline, Ampicillin, Kanamycin, Trimethoprim, Sulphonamides Streptomycin and Gentamicin. Multi-drug resistant *V. cholera* showed resistance against three or more contrasting classes of antibiotics for recent decades. Fifty diarrheal samples were collected from the different hospitals in and around the Tirupur district. 25 positive *V. cholerae* were isolated. The isolates were characterized morphologically and biochemically. The confirmed strains were taken to decide susceptibility patterns by the disc diffusion method. *Vibrio cholerae* isolated in this study was subjected to an antibiogram against 10 commonly used antibiotics. All the tested isolates showed maximum resistance to Erythromycin (97%) and minimum resistance was noted in Trimethoprim (50%) respectively. Electrophoretic examination of plasmid DNA was carried out by Agarose gel Electrophoresis on 0.7%. More than 90% of isolates showed were taken for plasmid isolation. The molecular weights of the fragments were evaluated by using a 10,000 bp DNA ladder and it was determined to be 1500 bp respectively. It is crucial to know the sensitivity and resistance pattern of any bacterial species for intercession an effective drug of choice in future..

KEY WORDS: ANTIBIOTIC SUSCEPTIBILITY TEST, DIARRHEA, PLASMID, VIBRIO CHOLERAEE.

INTRODUCTION

Cholera known as a global 'disease of poverty', is a bacterial gut infection producing liquefied diarrhea that can speedily lead to severe dehydration and prove fatal within hours, if untreated. Cholerae still continues to be the foremost cause of human infection, especially in developing countries that shortage of access to guarded drinking water and proper sanitation. It remains global intimidation and is one of the crucial pointers of social development. Presently World Health Organization (WHO) guesstimate, 1.3 - 4 million cholera cases globally every year and an estimated 21,000 - 143,000 deaths transpire (Sophia lonappan et al. 2020). Nevertheless, the role of these islet in *V. cholerae*'s life cycle, predominantly the 26-kb VSP-II, remains badly understood (Murphy et al. 2021). The life-threatening diarrheal disease occurs in infestation form in many developing countries and it is one of the major public health

loads with a high morbidity and mortality rate around the globe. Though important trouble has been made to identify the factual number of cholera cases, 90% - 95% of cases of cholera remain unheeded due to deficient surveillance systems and poor socio-economic conditions (Uddin et al. 2018; Anna et al. 2021).

Vibrio spp. infections generally do via the consumption of weakened seafood or exposure to polluted water and tend to be self-limiting (Anna et al. 2021). Over the years, a number of antimicrobials such as tetracycline, fluoroquinolones and azithromycin have been effectually used in the treatment of cholera patients (Das et al. 2020). The antibiotic resistance of *V. cholerae* strains has become a global warning problem, failing to treat the life of the patient. Recurrently MGEs play a central role in microbial evolution, ration as an apparatus by which hereditary material can be transferred, disseminated, and rearranged, allowing for rapid alteration to new and changing environments. At present, *V. cholerae* O1 was common among cases over five in a metro clinic in Bangladesh (Hamasalih et al. 2020).

Article Information:*Corresponding Author: sarathkgm2010@gmail.com

Received 26/09/2021 Accepted after revision 15/12/2021

Published: 31st December 2021 Pp- 00-00

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.78>

Because its significant factors associated with antibiotic-resistant may be actionable in identifying patients with a high probability of multidrug-resistant (Stephanie et al. 2021). The soaring pathogenicity of *Vibrio* strains and general weakness of hospitalized patients make it necessary to assess the presence of strains in diarrheal samples. So hence the present research has made an attempt to point out the purpose of the susceptibility test were two folds; to guide the choice of the antibiotic treatment for the individual patients and provide scrutiny data to monitor resistance trends including the epidemiological data are to provide the resistant gene linked with plasmid.

MATERIAL AND METHODS

In this study, 50 diarrheal samples were collected from various hospitals in and around Tirupur District. The Isolation and identification of *Vibrio* species from the watery diarrhea sample were based on Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar plates at 37 °C for 18-24 hours aerobically followed by staining and biochemical test which includes IMViC, Gram's staining, Motility, Starch hydrolysis, Triple sugar iron, Ornithine Decarboxylase, Arginine Dihydrolase, Lysine, Decarboxylase and Gelatin test it is described by (Talukder et al. 2019).

Antibiotic Sensitivity Testing (AST) was performed by disc diffusion method using the antibiotics which include Ampicillin (10 mcg), Bacitracin (10 unit), Clindamycin (2 mcg), Erythromycin (15 mcg), Piperacillin (30 mcg), Ticarcillin (75 mcg) and Trimethoprim (5 mcg) respectively. The discs were gently placed into the MHA agar plates and the plates were incubated at 37 °C for 24 hours. At the end of the incubation, the diameter of the inhibition zone was measured and calculated using a standard chart and detected the antibiotics as sensitive, intermediate, resistant to *V. cholerae*. Isolation of plasmids was performed by (Holmes and Quiley 1981). The electrophoresis probe of the plasmid DNA was carried out by Agarose Gel Electrophoresis on 0.7%. The molecular weight of the fragments was evaluated using a 1kb DNA ladder.

RESULTS AND DISCUSSION

Among the 50 diarrheal samples were collected from different Hospitals and Primary Health centers from rural areas of Kangayam around Tirupur city. The isolates were confirmed by using TCBS agar. Further, the identification of *V. cholerae* isolates was done by a standard biochemical test (Fig I & II, Table I). The confirmed isolated were further taken to antimicrobial sensitivity test by disc diffusion method (Fig – III and Table -II). Erythromycin showed 97% and Trimethoprim showed minimum resistance (54%). The maximum resistant pattern percentage (97%) was noted in strain no. NSKVC24 and the minimum resistance percentage (54%) were noted in strain-no NSKVC26 (Table - II). Electrophoretic probe of plasmid DNA was prepared and carried out by Agarose gel electrophoresis on 0.7%. Two isolates (NSKVC24 and NSKVC26) which showed more than 90% resistance against tested antibiotics were selected for plasmid isolation. The molecular size of the

plasmid DNA was calculated to be 1500bp (Fig - IV) using a 1kb DNA ladder (Hamasalih et al. 2020).

Figure 1: *V. cholerae* on TCBS Agar

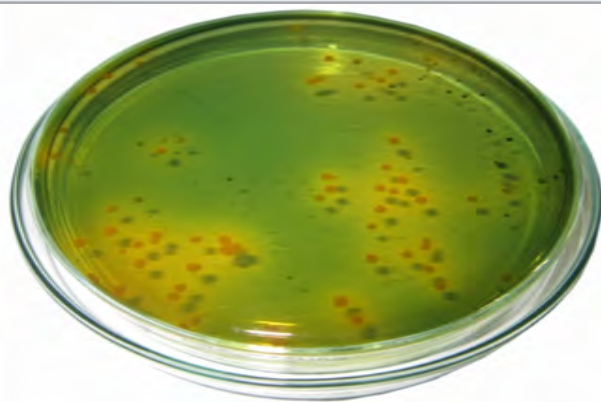


Figure 2: Biochemical Test

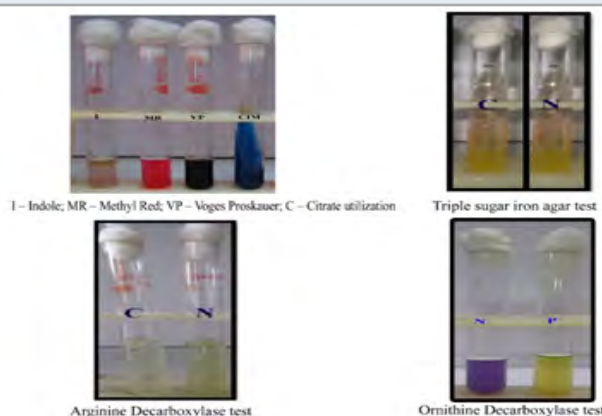
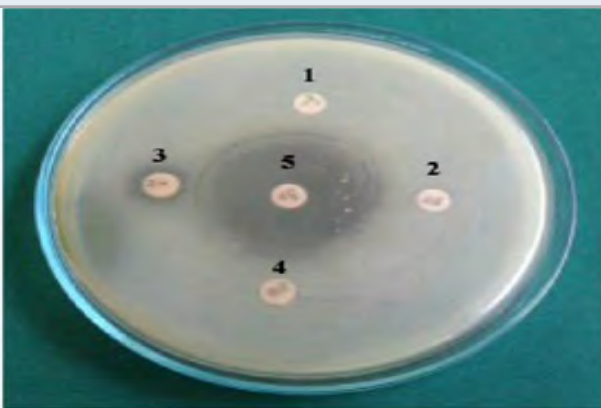


Figure 3: Antibiotic Susceptibility Test



Globally, *V. cholerae* is recognized as an important bacterial pathogen contributing to hospital infection. Despite the use of potent antibiotics still, a high mortality rate exists in cases of cholera infection. In the present study, an antibiotic sensitivity test was done and the highest percentage of resistance was noted against the antibiotics tested, this is due to the development of antibiotic-resistant gene in the plasmid of *V. cholerae*. Resistance of *V. cholerae* to

antimicrobials is generally thought to be chromosomal in the inception and rarely plasmid-mediated because it is not very clear whether this pattern of plentiful resistance of *Vibrio cholerae*, to many antimicrobials, means that the organisms are obtaining more of plasmid-mediated resistance or is the mass use of antimicrobials responsible for the selection of resistant strains. These facts clearly highlight the need for new novel therapeutic agents have become necessary (Stephanie et al. 2021). Malla et al. (2014) have been isolated 98 *Vibrio cholerae* from 877 stool specimens and recorded the 11.17% of the isolates were isolated. They used the biochemical tests: IMViC, triple sugar iron test, oxidation/fermentation test, Urease test, Nitrate test to identify the isolates and the *Vibrio cholerae* isolates were identified as belonging to the serogroup O1. E1 Tor biotype and Ogawa serotype (Malla et al. 2014; Stephanie et al. 2021).

Table 1. Biochemical characterization of *V. cholerae*

S. No.	Reaction	Result
01.	Grams Staining	Negative
02.	Indole	Positive
03.	Methyl red	Negative
04.	Voges proskauer	Positive
05.	Citrate	Positive
06.	Motility	Positive
07.	Starch hydrolysis	Positive
08.	Triple sugar iron agar	Negative
09.	Ornithine Decarboxylase	Positive
10.	Arginine Dihydrolase	Negative

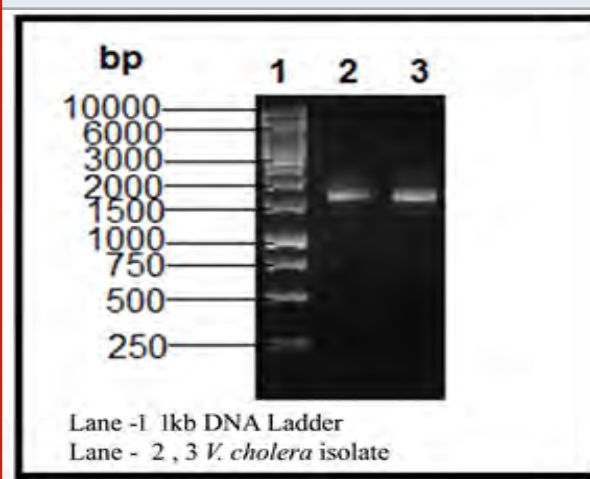
Table 2. Antibiotic resistant percentage of *V. cholerae*

S. No.	Name of the antibiotics	% of resistance
01.	Ampicillin	90 %
02.	Bacitracin	69 %
03.	Clindamycin	84 %
04.	Erythromycin	97 %
05.	Piperacillin	52 %
06.	Ticarcillin	90 %
07.	Trimethoprim	50 %

But in the present investigation has isolated 50 *Vibrio cholerae* from 100 stool samples. Similar biochemical tests were used. According to the Guidance of WHO the *Vibrio cholerae* O1 Serogroup was identified in this study. Adeleye et al. (2010) have been isolated *Vibrio* spp. from the seafood, 10 antibiotics were used for antimicrobial susceptibility test by disc diffusion method on Mueller Hinton Agar. Plasmid DNA extraction was done using the alkaline lysis method of the Birnboim and Dolly method. Only four of the isolates move plasmid DNA, *V. alginolyticus* carried plasmids of molecular weight 25.00 kbp and 9.416 kbp each. Two other

isolates of *V. mimicus* harboured very small plasmids of molecular weight 4.361kbp each. 10 antibiotic discs were used for the antimicrobial susceptibility test; the dissimilar method was used in this test. The extraction of plasmid was done by the method of boiling techniques (Holmes and Quigley 1981). In this study above 50%, resistant strains were selected for plasmid extraction. All the above 50% resistant two isolates were showed plasmids born both are were harbored one fragment of molecular weight like 1500bp respectively (Stephanie et al. 2021).

Figure 4: Plasmid DNA isolation of *V. cholerae*



A total of 250 different samples from poultry and poultry environments were examined and a total of 24 (9.6%) samples were detected as positive for *Vibrio cholerae*. Out of a total of 50 samples 8, 12, 3 and 1 samples were detected positive for *V. cholerae* respectively in the cloacal swab, intestinal fluid, egg surface and hand wash of the chicken handlers. The highest positive samples 24% for *V. cholerae* detection was found in the case of intestinal fluid followed by the samples of the cloacal swab (16%) and egg surface (6%) and the hand wash of chicken handlers was the lowest. Antibiotic susceptibility pattern of *V. cholerae* has been conducted against selected 11 antibiotics and agar plates, All the 15 isolates of *V. cholerae* (designated as Vc1, Vc2, Vc3 and so on up to Vc15) were found to the 100% resistant to Erythromycin, Ampicillin, Kanamycin, Penicillin, Tetracycline, and Rifampicin and 60% of the strain resistant to the Cephalexin, Streptomycin (Hamasalih et al. 2020).

100% cases of tested strains were found sensitive to Norfloxacin, Gentamycin, and Neomycin (Sule et al. 2019). Similarly in the present investigation, 10 antibiotics were used for antibiotic susceptibility patterns. The highest percentage of resistance was shown in Erythromycin (97%) and the highest susceptibility was shown in Erythromycin (96%) followed by ampicillin and ticarcillin (90%) (Sule et al. 2019).

CONCLUSION

The findings of the present study has indicated that *Vibrio cholerae* strains were found to develop resistance day

by day to the currently used antibiotics. Presence of a large number of Multi-Drug Resistant isolates it makes necessitates curbing irrational use of antibiotics to prevent further spread of drug resistance. This example needs some novel therapeutic drugs in future to beat this sort of dreadful disease.

Acknowledgements: This study was financially supported by the Chikkanna Government Arts College.

Conflict of interests: Authors declare that have no conflicts of interests to disclose.

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Technological Communication

Effect of Exogenous Factors on Transformation Processes in the Russian Agro-Industrial Complex

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ABSTRACT

The purpose of the research is to study the trends of transformation of forms of management in the Russian agro-industrial complex in the conditions of preserving the general contradictions of the development of small and large forms of management. The main objective is to analyze the Russian agro-industrial complex. The subject of the study is the forms of management and the trends of their development. The last two decades are considered as the main time horizon of the study. The study made it possible: firstly, to reveal a stable tendency towards the predominance of large farms in the structure of production and the dynamics of output volumes given the existing institutional factors; secondly, to determine scenario forecasts (conservative, basic and optimistic) of the main trends in the further development of small and large forms of management; thirdly, to show the influence of the phenomenon of a global pandemic on transformation processes in the Russian agro-industrial complex. The relevance of this study is since during the thirty years of the market transformation of the national system of agrarian relations, significant changes have occurred among the main economic entities: especially in the issues of the evolution of the content, motivation of activity, sustainability factors and classification criteria for economic entities engaged in the production of agricultural products. These processes are reflected in the solution of common strategic problems: issues of ensuring both national food security and global food security issues.

KEY WORDS: AGRARIAN RELATIONS, AGRICULTURAL HOLDINGS, FORMS OF MANAGEMENT, INSTITUTIONS, PEASANT FARMS.

INTRODUCTION

According to established approaches, economic entities in the system of agrarian relations of the Russian economy, regardless of the specifics of their activities, the size of the total number, the volume of revenue, are called forms of management (FM). However, the attitude to this category among researchers is ambiguous: when, for example, FM is associated with different methods of management. As a rule, attention is focused on two main FM in Western literature: farms or large agricultural corporations. Farms that have their specifics in each country are considered as the main market entity that ensures competitiveness in food markets (Knickel and Renting 2000; Bachev 2001; Meurs 2005).

The general trends in the agro-industrial sphere of the economy include quite obvious concentration processes that contribute to the reproduction of the stable nature of the model of imperfect competition: when large agricultural corporations are special cases of oligopolies in the markets of

agricultural products. Therewith, it is logical to assume that, according to the general features of imperfect competition markets, large players with a large share of market power in the industry have an indirect influence on the vector of economic policy on the part of the state and general trends in the transformation of large and small forms of management (SFM). The enlarged integrated formations are characterized by an ultra-high concentration of land resources: from several hundred thousand hectares to several million hectares (Goncharov et al. 2016; Shagaida 2020).

It is noteworthy that in matters of concentration of land resources, these are global processes: for example, in (2021), B. Gates was included in the list of the largest landowners in the world, whose sphere of interests in business for many decades had been far from agricultural issues. However, for Western agricultural holdings, it is more often characteristic that they are more often engaged in processing of produced crop, livestock, poultry products: as, for example, in Brazil. The complexity of the study of Russian agricultural holdings is that they remain out of the focus of official statistics, which makes it difficult to study their specifics and economic effects based on comparative analytical approaches. More and more scientific and analytical publications have

Article Information:*Corresponding Author: maksimova.tp@rea.ru

Received 19/10/2021 Accepted after revision 24/12/2021

Published: 31st December 2021 Pp- 1913-1918

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.79>

appeared recently about Russian agricultural holdings (Shagaida 2020; Uzun 2020).

Therewith, a certain phenomenon remains that agricultural holding, as before, are not taken into account separately in the Russian economy by official statistics. A significant degree of uncertainty, from a theoretical point of view, is added by the fact that a generalized approach is also absent in English-language translations of FM, which can be represented as "forms of economic management"; "forms of farming" or laconically "farms" (forms of farms or agricultural holdings), "forms of business" (forms of doing business or forms of entrepreneurship) and even "lifestyles" (way of life). Our position is that the Russian specifics are more objectively reflected by the translation options from Russian into English: "forms of business" and "lifestyles", since, firstly, the concept of a form of doing business is broader than the concept of, for example, the forms of agricultural holdings; secondly, the use of a variant of the "way of life" concept allows focusing on the specifics of farming on the land. When considering the essential characteristics of modern FM, and hence the specifics of their transformation, uncertainty is already inherent from the very beginning, when the problem of identifying FM remains relevant: for example, as an object of state support (Uzun 2020).

METHODOLOGY

In the context of those problems that remain open when clarifying the substantive aspects of FM as subjects of production, it is important to adhere to a comprehensive systematic approach to the subject of the problem under study, including the definition of the main determinants of transformation, the selection of dominant components from them and the identification of possible trends for further development. This methodological approach also involves the use of the principles of verifiability: when analyzing the economic effects of production activities of different FM and identifying the causes of recessions (or rises) in the agricultural sector of the economy as a whole. In addition, studying the peculiarities of the transformation of FM in the national system of the agro-industrial complex, we guided by the methodological principle that Academician of the Russian Academy of Sciences that has repeatedly expressed that any scholar should have the full right to express his/her opinion, without claiming that he is the only owner of the truth (Uzun 2020).

Monopoly in science inevitably leads to its death. Abalkin (2009) ranked: price disparity, the fight against monopolies, and the creation of a competitive environment among the most serious problems in the field of the agro-industrial complex (Abalkin 2009). These problems remain relevant decades later. General scientific methods allow approaching the clarification of the dual, often burdened with a complex of contradictions, nature of the manifestation of the essential characteristics of FM both as the main subjects of production activity and as objects of their further reform.

It is also important to choose the dominant theoretical paradigm for defining the concept under study. Thus, if we

consider the FM, from the point of view of the political-economic approach, it is logical to consider SFM from the standpoint of the small-scale way of life. At the beginning of market reforms, this approach seemed justified when there was a transformation of land ownership relations and the emergence of new FM: agricultural enterprises (AE). Currently, this approach requires dialectical adjustment, since different FM continue to develop in the conditions of unresolved problems on land relations (Uzun 2020).

If we adhere to the mainstream (or the prevailing concept) of the basic principles of the neoclassical theory of market economy, which continues to dominate the basic disciplines of economic theory and is based on the principles of rational behavior, profit maximization, and personal utility, then it is logical to assert that the FM, as economic entities, perform the functions of firms in the market of goods and services. In this case, FM (as firms) supply food markets and, consequently, changes in the volume of supply and the supply itself (or shifts in the entire supply curve: an increase/decrease in volumes at the same prices) are determined by the same factors that are studied in the supply theory with the neoclassical approach. In the resource markets, the role of FM in the simplest model of circular flows is changing: they demand the main groups of resources (first of all: land, labor, and capital). Finally, from the position of the institutional approach that is quite widespread at present, each type of FM can be considered as a local institution at the micro-level with its built structure (Uzun 2020; Maksimova et al. 2020).

In recent years, the authors have increasingly preferred the methodology of institutional analysis in the context of studying the peculiarities of the influence of formal and informal institutions, the specifics of the impact of which on the transformation processes can be traced most clearly on the example of the agro-industrial sphere of the national economy. It does not exclude the synthesis of an institutional and political-economic approach, since the nature of land ownership and land use relations remains an important dominant in the field of agrarian relations. In general, the use of an institutional approach allows: firstly, establishing the relationship between the resource support of the investment process and the innovations being introduced when improving the ways of organizing the production process using effective contracts; exploring the possibilities of using smart contracts in the new conditions of the digital economy; secondly, carrying out a comparative analysis of the potential opportunities of horizontal integration (for example, in models of production cooperatives) and vertical integration (in models of modern Russian agricultural holdings and agro-industrial clusters), including the use of features of the spatial development of rural areas; thirdly, developing options for scenario modeling of long-term trends in the development of small and large FM, searching for optimal development scenarios (Maksimova 2020; Maksimova et al. 2020).

Special attention should be paid to the emphasis on informal institutions, which are of particular importance at the micro-level of rural territories, and the effects of their impact determine the nature of the activities of SFM in the agro-

industrial sphere of the Russian economy as a whole. A clear example is agricultural enterprises in the Russian economy (AE), as one of the types of small businesses that differ not only from the Western model of farms but also from the "Chayanov" model of peasant farms that was historically established in the pre-Soviet period, based mainly on the use of family labor (Nort 1997). Therewith, the "Chayanov" model of peasant economy also significantly lost its original features during the decades of the Soviet period. When peasant and landowner farms were transformed into collective farms and state farms based on the principles of the common use of means of production and land use, on the principles of collective economic activity (Maksimova et al. 2020).

Logically, there is a transformation of the mentality and motivation of management in the new institutional conditions: from personal interest to the general. However, some signs of the stability of peasant farms, experience, and management practices were preserved at the micro-level of private farm holdings (PFH) in rural areas. Another radical "breaking" of the established economic practices took place by the beginning of market reforms in the last decade of the twentieth century: the reorganization of collective farms and state farms and the return to the practice of SFM. However, it is necessary to take into account that the transition to market methods of management was carried out on a different, transformed platform of the mentality of economic entities.

The fact that a new type of economic entity has been formed in rural areas over the decades of market transformation helps to understand and realize the use of the case method, which in recent years has been increasingly used by analysts in various fields of activity, and in the context of the development of digitalization, including the use of various variants of Internet technologies, this method is logically considered as complementary to the method of field research when studying intermediate results and features of the transformation of different FM (Maksimova et al. 2020).

In this regard, it is worth recognizing the positive effects of professional communities in social networks when studying and analyzing various specific situations: especially when economic entities maintain their professional blog as an exchange of experience, diary entries about their economic activities, share their problems, and opinions on ways to solve them. In modern conditions, this method, when the main information flows are transmitted through Internet technologies, acquires a separate meaning: in fact, the economic entities themselves recognize that Internet technologies become an additional assistant not only for professional communication but also for the exchange of experience, as well as a promotion of their products according to the model: "from the manufacturer to the buyer", for finding suppliers and partners. A detailed analysis of such case situations with feedback opportunities becomes not only complementation but sometimes a substitute for field research (Maksimova 2020).

Analysis of the study: The results of the study of the specifics of FM based on the methodological approach

of comparative analysis of the variability of theoretical approaches and practical realities indicated that there is no unity of approaches. Thus, there are following main types of FM: agricultural organizations (AO), AE, individual entrepreneurs (IE), and individual farms (IF), including personal subsidiary farms. The same approach was used during the first and second All-Russian agricultural censuses (Federal State Statistics Service 2016). The analysis of approaches in the economic literature allows distinguishing a more extended "line" of the typology of modern FM from the position of one or another selected criterion (Chayanov, 1989). Thus, from the position of such a criterion as "the use of hired labor", it is logical to distinguish commodity farms that use hired labor, and family farms that continue to remain the basis for the stability of the rural way of life at the micro-level of rural territories. Interestingly, family farms do not have a clear legal status, and therefore they are not singled out separately by official statistics (Sokolnikova 2021).

The peculiarities of the transformation and evolution of different FM will also be influenced by such a factor as the nature of the use of labor resources from the position of territorial affiliation (the work of local villagers or migrant labor) and the position of the temporary factor (the use of hired workers permanently or the use of seasonal workers). Another important criterion is size. There is its specificity: if the official statistics rank the size of enterprises (economic entities) according to the specified numerical parameters of the number of employees, then the numerical parameters of land areas and acreage are more important in the agricultural sphere of the national economy. For example, large and medium-sized agricultural enterprises can own or lease from several hundred hectares to several thousand hectares of land area and more than 60 employees, receiving all types of rent. Agricultural enterprises with the size of the cultivated land area in the range of up to 100 hectares and with a small number of employees are already referred, as a rule, to SFM (Sokolnikova 2021).

The following criteria are also relevant: the prevailing goals of functioning, which become decisive in the observed processes of deurbanization, which, in particular, are manifested in increased demand for suburban life, suburban farms, and the evolution of economic interests towards the production of eco-friendly horticultural products, vegetables. The criterion of legal registration of economic entities remains relevant: state and municipal enterprises; joint-stock companies; limited liability companies; individual entrepreneurs; peasant (farmer) farms; agricultural production cooperatives (APC), etc. This criterion is of paramount importance in terms of issues of monitoring and control by tax institutions. The presented variants can also be dialectically supplemented. Thus, it is logical to use the criterion of "forms of land ownership and land use" in the system of agrarian relations. Considering separately farms, activities of which are based on a certain nature of land ownership (having land in ownership) or farms activities of which are regulated by the nature of land use (lease relations) could contribute to the theory of rent relations in modern conditions (Sokolnikova 2021).

When analyzing individual FM, the criteria for determining them remain unspecified: for example, there are many questions when clarifying the AE, both from the point of view of conceptual approaches and in connection with some "innovations" in the regulatory framework. For example, in (2020), there were innovations on the part of tax institutions to cancel the registration form of the IE-head of the AE, in which the prefix "head of the AE" was proposed to be removed. This caused a very mixed reaction in professional communities since such an approach would lead to a distortion of the formal signs of the identity of the AE and the possibility of developing a farming lifestyle at the micro-level of rural territories: including an increase in obvious risks when providing targeted state support to farmers. Such regulatory changes form the content of asymmetric information for state institutions when considering issues of targeted support for farmers (Sokolnikova 2021).

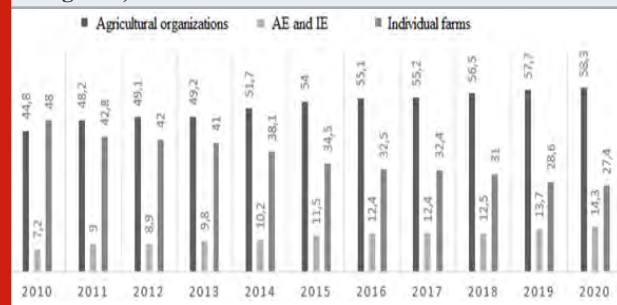
When studying the specifics of the transformation processes of different FM, obvious signals from government agencies are also of interest. Thus, at the end of 2020, the Ministry of Agriculture of the Russian Federation approved a list of system-forming organizations of the agro-industrial complex: 88 organizations are represented in this list together with JSC Rosagroleasing. Of these: 16 – in the field of crop production; 31 – in the field of animal husbandry; 33 – in the field of food and beverage production; 7 – in the field of fishing. Interestingly, most of these enterprises are large agricultural holdings, which include from one to several dozen farms with the legal status of an LLC or JSC. A quantitative analysis of such enterprises as part of agricultural holdings indicates that about 600 economic organizations with the status of LLC, CJSC, etc. are represented in the group of companies in the field of crop production and animal husbandry; 317 – in the food sector; 67 – in the field of fisheries. One more peculiarity: there is practically no AE in the structure of such backbone organizations (agricultural holdings) (Kabanenko 2019).

For comparison: according to the All-Russian Agricultural Census-2006, 36.1 thousand agricultural organizations, 174.8 thousand AE and IE (78% of them are AE and 22% are IE), 23.5 million PFH, and other individual farms were represented in the system of the Russian agro-industrial complex (Uzun 2005). For example, in the field of crop production in the Stavropol Territory, the agro-industrial holding "Eco-culture" is defined as such a system-forming enterprise, which includes 11 farms with the legal status of LLC and one farm with the legal status of CJSC. There are three largest agricultural corporations in the Krasnodar Territory – these are the Agrocomplex Group of Companies: JSC "Agrocomplex Firm" named after N.I. Tkachev", GC Progress: LLC Progress Agro, GC AFG National: LLC AFG National, each of which, in turn, includes up to 10 FM with the legal status of LLC and CJSC.

The current market situation can be considered as an institutional direction of market development through the promotion of large FM, supported by the state (Kabanenko 2019). In turn, a comparative economic analysis of official statistics data shows that despite the orientation at the initial stage of market transformations to SFM, in fact, the share

of AE in the structure of agricultural production, although increasing, is significantly lagging behind agricultural organizations (Figure 1) (Uzun 2020).

Figure 1: The structure of agricultural products by the main FM (in actual prices; as a percentage of farms of all categories).



A comparative analysis over a longer time interval (using 5-year time slices) shows another paradox of the results of market transformation in the agro-industrial sphere: the share of AO in the total structure of agricultural production decreased by only 15% over the 30 years of reform, and the share of IF remained almost unchanged, having decreased by only 1% (Table 1). In addition, at the beginning of the market transformation in 1990, AO were represented by collective farms and state farms, and by (2021), these are enterprises, sometimes even larger, both in terms of land area and revenue (Uzun 2020).

Hence, the obvious question is whether it was worth carrying out radical reorganizations of existing FM if the institutional conditions for creating a competitive environment for the development of small businesses in the form of AE and IE were not created, including, for example, institutions for the implementation of land ownership rights. We are not ready to give an unambiguous answer to this difficult question within the framework of this article and believe that this is the subject of further research: including, possibly, based on using the methodology of cyclical development from the position of criteria for the validity of production concentration and the use of positive effects of the scale of production in the agro-industrial complex (Uzun 2020).

Compiled by the author based on official statistics (Ministry of Agriculture of the Russian Federation 2021). If we carry out a comparative analysis over the past five years, when the dominant character has increasingly begun to manifest itself from exogenous factors, including geopolitical factors and the phenomenon of sanctions confrontation, it can be argued that there are no significant changes in the transformation processes. This is confirmed by the preservation of leading positions in the production structure of large FM and the growth rates of AO and AE are almost at the same level (the share of AO in the production structure increased by 4%, and AE – by 3% against the background of a decrease in the share of personal subsidiary farms in the total production structure) (Figure 2) (Maksimova et al. 2020).

Compiled by the author based on official statistics (Ministry of Agriculture of the Russian Federation 2021). The analysis

of quantitative indicators of the dynamics of output volumes also indicates that the growth rates are significantly higher

for agricultural organizations, which, form the basis of large FM, including agricultural holdings (Figure 3).

Table 1. The structure of agricultural products by the main FM for 1990-2020 (in actual prices; as a percentage of farms of all categories)

FM	Years						
	1990	1995	2000	2005	2010	2015	2020
Farms of all categories							
Of these:	100	100	100	100	100	100	100
AO	73.7	50.2	45.2	44.6	44.8	54.0	58.3
AE and IE	...	1.9	3.2	6.1	7.2	11.5	14.3
PFH and individual farms	26.3	47.9	51.6	49.3	48.0	34.5	27.4

Figure 2: The structure of agricultural products by the main FM in 2015 and 2020

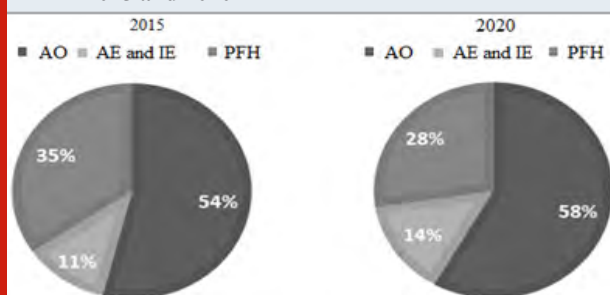


Figure 3. Dynamics of agricultural output volumes for the main FM (in actual prices; as a percentage of farms of all categories)

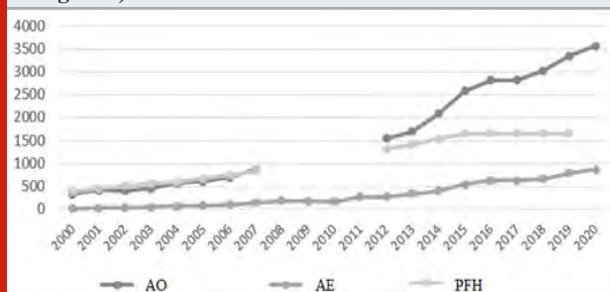
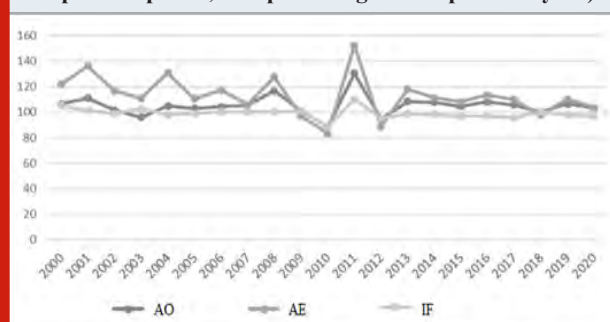


Figure 4: Indices of agricultural production by main FM (in comparable prices; as a percentage of the previous year)



Compiled by the author based on official statistics (Ministry of Agriculture of the Russian Federation 2021). Therewith, if relatively slow growth rates are observed in the first

decade of the 20th century, then a rapid upward leap is evident in the second decade. It seems that these indicators are an indirect sign to confirm the hypothesis not only about the remaining dominant features of large FM but also about their predominant development in the last decade. The "gaps" observed on the graph are evidence of the lack of integrated official statistics data for these years (Maksimova et al. 2020). The analysis of agricultural production indices (without highlighting the analysis of crop production and animal husbandry) for the main FM over the past 20 years indicates the following:

- there is a clear predominance of the growth rates of manufactured products in AE, compared with AO and PFH (Figure 4);
- until 2013, the amplitude of fluctuations in the production indices had been expressed most acutely in AE, while the development rates for AO and IF have a more smoothed character;
- on the one hand, common peak points of decline in 2010 are characteristic for all FM, which once again indicates the importance of the natural and climatic factor in the agro-industrial complex (in particular, with an abnormal drought in 2010);
- there are general peak points of growth in 2011.

Compiled by the author based on official statistics (Ministry of Agriculture of the Russian Federation 2021). It should also be noted that the indices of agricultural production in IF among the general trends are significantly inferior to AO and AE. However, the general nature of the "wave-like" dynamics of agricultural production indices remains stable for AE and AO (Maksimova et al. 2020). Thus, following the results of decades of market transformation, the problems in the system of agrarian relations are stable, which remain obvious internal challenges for the development of a long-term economic policy for further transformations in this area of the economy:

– firstly, it is the lack of unified approaches to identifying the essential characteristics of FM, in general, and determining their unified typology;

– secondly, these are obvious trends in the consolidation of FM into whole integration associations, which are still

not considered by official statistics, and it becomes difficult to carry out a comprehensive analysis of the volumes and production structure of functioning "de facto" agricultural holdings;

– thirdly, the output volumes and their dynamics prevail among agricultural organizations, which is logical to consider as indirect signs of a stable concentration of agricultural production.

A significant feature of recent years is the degree of influence of exogenous factors on the development of the entire agricultural sector of the national economy as a whole, and individual processes of transformation of economic entities. This is also manifested in the constant change of the internal institutional environment, since state institutions react quite quickly to many external factors, constantly developing, changing, and improving both the instruments of state support and dialectically changing the instruments of regulation. Therewith, despite the obvious "signals" about the priority attention "from above" to large market players, there is hope that external shocks as a protracted process of a global pandemic can have a positive effect on the sustainable development of SFM. This is primarily due to increased consumer demands for farm products to maintain and preserve the quality of life. Hence, it can be assumed that the vector of attention will shift to SFM not only from consumers but also from state institutions, ensuring a balance of interests between small and large FM in the long-term period of development while maintaining the dominant position of large farms (Uzun 2020; Maksimova et al. 2020).

CONCLUSION

The findings of the present study suggests that when developing a long-term economic policy for the further transformation of the main economic entities, the forecast of the following scenario modeling options is logical: a conservative scenario that assumes further concentration and oligopolization in the agricultural sector of the national economy; the basic scenario, according to which the existing proportions in the structure of agricultural production are maintained by large and small FM, which, in general, currently ensure the fulfillment of the main targets for food security; the optimistic scenario assumes that the combination of the phenomenon of the impact of the global pandemic with the improvement of state support tools for SFM will increase the level of competitiveness in the production of agricultural products.

Conflict of Interests: Authors declare no conflict of interests to disclose.

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Medical Communication

Effectiveness of Hold Relax Proprioceptive Neuromuscular Facilitation Technique in Total Knee Arthroplasty

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ABSTRACT

Proprioceptive Neuromuscular Facilitation (PNF) is a popular flexibility training, which involves stretching and contraction of the target muscle group. PNF stretching is effective among athletes for improving passive range of motion, flexibility, and performance. Patients who undergo total knee replacement surgeries exhibit pain, swelling, decreased range of motion, muscle weakness, and stiffness. This study aimed to investigate the effect of the hold relax PNF technique to improve the balance and muscle strength in subjects with a bilateral knee replacement. Sixty four patients were selected based on inclusion/exclusion criteria. The patients were allocated into two groups by random allocation. The experimental group A (n=32) received the Hold Relax PNF technique with conventional treatment whereas, the control group B (n=32) received only conventional treatment as a part of the Home Exercise Program (HEP). Outcome measures used for evaluation included the Visual Analogue Scale (VAS), Manual Muscle Testing (MMT), and Berg Balance Scale (BBS) Score for measuring pain, muscle strength, and balance respectively. Students t-test showed significant differences between variables mean scores from two groups after three weeks of intervention. There was a statistical significant ($t(32) = 2.38$, $p = 0.02$) change in the mean scores of VAS in the experimental group (mean difference pre-post=3.19) as compared to control group (mean difference pre -post=3.19) with Cohen's $d = 2.744588$. Similarly for MMT($t(32) = 0.415$, $p = 0.005$) and BBS scores ($t(32) = 2.628$, $p = 0.035$). The result of the study suggests that the Hold Relax PNF technique is more effective than conventional treatment alone in decreasing pain as well as increasing balance and muscle strength in bilateral knee replacement patients.

KEY WORDS: PNF(HOLD RELAX), KNEE ARTHROPLASTY, HOME EXERCISE PROGRAM.

INTRODUCTION

Total knee arthroplasty (TKA) procedures have expanded dramatically in last one decade due to aging population with rising lifestyle diseases, resulting in increased prevalence of Osteoarthritis, especially in Knees. The exponentially growing health insurance linked with increasing penetration

of private healthcare has led to changes in payment policies which has caused post-TKA rehabilitation primarily been transferred to home health and outpatient settings. Knee arthroplasty is a surgery that replaces the weight-bearing surfaces of the knee joint to relieve pain and impairment, most typically been used to treat osteoarthritis apart from other knee illnesses including RA and psoriatic arthritis. Knee replacement surgery can be conducted in two ways: partial or total. A knee replacement might be more accurately termed as knee resurfacing because only the surfaces of the bone are replaced (Lauermann et al., 2014, Varacallo, Luo, and Johanson, 2021).

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Received 25/10/2021 Accepted after revision 30/12/2021

Published: 31st December 2021 Pp- 1919-1923

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.80>

Whether comparing rehabilitation received in different venues or analyzing different physical therapy (PT) procedures, numerous studies have indicated that rehabilitation is related with improved post-TKA results. Following total knee arthroplasty, the mainstay of rehabilitation is achieving maximal feasible knee range of motion (ROM) and achieving specified functional goals (TKA) (Koca et al.,2014; Yang et al.,2019; Han et al.,2021). ROM is one of the most important outcomes after TKA since patients with less than 105° of knee flexion have difficulty going up and down stairs or getting out of a low chair, and is a major sign of patient discontent (Han et al.,2021).

Literature review suggests that different strategies and techniques are being used for improving range of motion in comparison to other strategies used primarily on healthy or elderly individuals (Oh 2013; Rhyu, Kim and Park, 2015; De Carvalho et al.,2017). The Proprioceptive Neuromuscular Facilitation technique (popularly called as PNF) is one such popular technique been used to increase ROM and flexibility (Hindle et al.,2012). Originally developed by Dr. Herman Kabat in year 1940, the technique mainly works on the principle of raising neuro-inhibition mechanism for releasing muscular spasm and elongating muscle length, or increasing neuro-excitation mechanism for boosting muscle strength. It comprises of 3 main techniques namely, Hold Relax (HR), Contract Relax (CR), and Contract Relax Agonist Contract (CRAC) (Gonzalez, Gomez and Garcia, 2012).

The effect of PNF stretching on both joint restriction improvements is less well understood. Likewise, balance impairment, which is again one of the major problems encountered by the patients after knee replacement is due to the weakness of quadriceps muscles, which is further a

consequence of the direct trauma from the surgical incision. The hold relax PNF technique is one of the most popular techniques and helps to develop muscular strength and endurance joint stability, mobility, neuromuscular control, and coordination all of which are aimed at improving the overall functional ability of people (Hindle et al., 2012; Koca et al.,2014; De Carvalho et al.,2017). However, not many evidence-based researches are present, where the Hold Relax PNF technique has been used in patients with total knee arthroplasty (Kim Kang and Kim,2018; Han et al.,2021). Hence, the present research study aims to explore the application of PNF stretch for changes in pain, muscle strength, and balance and therefore on the overall functionality of the lower extremity following total knee arthroplasty.

MATERIAL AND METHODS

The study design was a randomized controlled trial (pretest-posttest experimental control group design), where the patients were recruited from Rehabilitation Center, College of Applied Medical Sciences, Majmaah University located at Al-Majmaah city. Necessary approval was taken from the Institutional review board, Majmaah University. The study was commenced in Sept 2020 and was completed in May 2021. A total of 64 subjects between the age of 42 to 73 years were included in the study who fulfilled the inclusion criteria of patients diagnosed with OA knee as well as undergoing bilateral TKR. The exclusion Criteria included knee flexion more than 110 degrees or thigh girth more than 55 cm, poor cognition, CNS or PNS neuromuscular disease, received operation in recent 3 months, joint infection and loosening, revision total knee arthroplasty, or complication during follow up. Those who met the study's eligibility requirements and gave their consent to participate were finally chosen to participate in the study.

Table 1. Showing treatment protocol for experimental and control group.

Week 1. <ul style="list-style-type: none"> •Active quad contraction •Safe independent ambulation with a walker •Passive knee extension to 0° •Knee flexion to 90° or greater •Control of swelling, inflammation, bleeding. 	Week 2. Same as phase 1 <ul style="list-style-type: none"> • Straight leg raising test • Hip abduction and adduction
Week 3 <ul style="list-style-type: none"> • VMO recruitment during quad sets and SLR • Terminal knee extension 45 to 0° • Hamstring curls • Knee flexion to at least 115° 	Hold Relax PNF Treatment (Group A) <ul style="list-style-type: none"> •Hold relax technique to start from Day 7 of TKR up to 3wks. •Muscle under Treatment (Quadriceps or hamstrings muscle) •Few seconds (6 - 10 second /10 repetitions) is given for quads muscle as well as 10 repetitions is given for hamstring muscle/day •Patient position: Patient is sitting on high bed knee at 90 flexion •Back should be in a straight position •Foot should not touch on the ground
Group A (Hold Relax PNF + Conventional Home Exercise Program)	Group B (Conventional Home Exercise Program)

The minimum sample size of 60 participants was required using statistical power of 80%, the effect size of 0.20, and a level of significance of 0.05 to measure the effectiveness of the technique used in the study. In the present study, 32 patients were recruited who fulfilled the inclusion criteria subjects were then randomly assigned into experimental (n=32) and control groups (n=32). While the Experimental group A(n=32) received PNF along with conventional treatment, the control group B, (n=32) received only conventional treatment. The outcome measures used for evaluation were Manual Muscle Testing (MMT) and Berg Balance Score (BBS) were used for measuring muscle strength and balance respectively.

The Hold relax PNF technique is one of the key PNF techniques. In this method, the muscle under treatment is lengthened to the point of limitation or to the extent that is comfortable for the subject. The subject then performs a pre-stretch, end range, an isometric contraction for 5 to 10 seconds followed by voluntary relaxation of the tight muscle (Oh 2013). The Conventional treatment protocol which was the Home exercise Program (HEP) was illustrated in Table 1 (Minshull et al., 2014; Kim et al 2018). The protocol was same as described in an earlier published research, (Alaca et al 2015). The physiotherapist sat in front of the patient on a low-height stepper with one hand placed on the lower quadriceps muscle as well as another hand placed on the lower tibia anteriorly. Command was given to the subject to perform knee extension against resistance and was asked to hold for 6 to 10 seconds followed by relaxation as per detailed protocol given in Table 1.

Statistical analysis: Using the Statistical Package of Social Science (SPSS) version 22.0, the data were checked for normality assumptions using the Kolmogorov–Smirnov test (Chicago, IL). The normally distributed data were then analyzed for mean, SDs, t-values, and p values to conclude group comparisons. The unpaired t-test score was used to compare participant characteristics and demographic data between groups.

Table 2. Showing baseline characteristics of the patients in both group A and B

Characteristics	Group A (n =32)	Group B (n =32)
Gender (M: F)	22: 10	18: 14
Mean Age	65.54(7.2)	65.06(6.87)
Mean BMI	29.54(4.53)	31.22(5.88)
Baseline Active Flexion	41(11.89)	48(12.44)
Baseline Passive flexion	48(14.53)	52(13.54)
Baseline Knee Circumference	41.56(3.31)	42.74(2.94)

RESULTS AND DISCUSSION

The baseline characteristics values for the demographic characteristics for all the participants are depicted in Table 2 with no significant differences observed between the two groups in terms of baseline demographic data and clinical

findings (Table 1, $p \geq 0.05$). The treatment for both groups continued for 3 weeks with readings measured for all the outcome variables at 0 weeks (Baseline line) and 3rd week (Table 3, 4 and 5).

Table 3. Showing VAS Score between group A and group B

VAS Score	Group A	Group B	T score(p-value)
Week 1	6.68(0.54)	6.83(0.79)	NS
Week 3	2.77(0.75)	4.61(0.58)	2.38(0.02*)

Visual Analogue Scale (VAS) readings were measured for both the groups at Week 1 followed by 3 weeks of intervention in both groups. It was observed that while significant improvement was observed in both groups but on intergroup comparison Group A fared better than group B ($p=0.02^*$) (Table 3). The outcome measures of Manual Muscle Testing for both the legs for both the groups at week 1 and Week 3 are depicted in Table 4. Regarding the MMT measurement between the two groups A and B, about both the limbs (Right and Left leg) it was observed that over the 3 weeks both groups witnessed a significant change in the muscle strength, however, group A demonstrated a greater gain in strength in both rights (T score = 1.8 and 0.33, $p < 0.05^{**}$) and left leg (T-score = 1.031 and 0.4, $p < 0.05^{**}$) respectively (Table 4).

In Table 5, within a group as well as an intergroup comparison for groups A and B were measured for the BBS scale. The table shows a statistically significant difference over the 3 weeks both within the groups (T value (Gp. A, t score = 24.04 and Gp. B=10.22, $p < 0.05^{**}$) as well as between the groups with Gp. A showing significant improvement in BBS as compared to Group B (t score = 2.628, $p < 0.05^{**}$) (Table 5).

The purpose of the present study was to investigate the effectiveness of the hold relax PNF technique to improve balance and muscle strength in subjects with a bilateral knee replacement. The result suggests that there was a significant improvement in PNF group A when compared with the conventional group B. Visual analog Scale (VAS), Manual muscle testing (MMT), and Berg Balance Score (BBS) were found to be statically significant in both the groups, however in the PNF group A, there was a significant decrease in pain along with a significant increase in MMT and BBS score than the conventional group as their mean and standard deviations suggested ($p < 0.05^{**}$, Table 3, 4 and 5).

The results of the study draw an analogy from the study conducted by De Carvalho et al. (2017) where two exercise protocols were compared for postural balance among elderly women. They found the PNF group showed a significant reduction in most of the stabilometric parameters. Similarly, the BBS score in the experiment group was also found better than the control group thus concluding that women in the PNF group demonstrated better improvement in postural static stability using evaluation of various stabilometric parameters. Likewise, significant improvement was

also noticed for dynamic balance using functional test performance.

It is suggested that the hold relax PNF technique more effectively increases muscle activity with decreased muscle fatigue. This could be because during hold relax PNF, isometric contraction of the muscle causes autogenic

inhibition, which inhibits - motor neurons of contracted muscle and synergist in interneuron and activates antagonist by transmitting tendon tension to the anterior horn of the spinal cord via 1 b afferent nerve fiber (Minshall et al., 2014). Concentric contraction generates reciprocal inhibition, which activates -motor neurons of contracted muscles and synergists while inhibiting antagonists during agonist contraction (Cha, Cho and Choi, 2014).

Table 4. Showing the manual muscle testing (MMT) score for both groups A and B

MMT			Group A (n=32)		Group B (n=32)		T score (p-value)
			Mean	SD	Mean	SD	
WEEK 1	Right Leg	F	3	0.365	2.8*8	0.342	NS
		E	2.31	0.479	2.63	0.5	NS
WEEK 3	Right Leg	F	3.94	0.443	3.75	0.447	1.192(p=0.008**)
		E	3.75	0.447	3.69	0.602	.333(p=0.062**)
MMT			Group A n=32		Group B n=32		T score (p-value)
			Mean	SD	Mean	SD	
WEEK 1	Left Leg	F	3.06	0.443	3.06	0.25	NS
		E	2.44	0.629	2.69	0.479	NS
WEEK 3	Left Leg	F	3.94	0.443	3.75	0.577	1.031(p=0.043*)
		E	3.81	0.403	3.75	0.447	.415*(p=0.005**)

**significant at 0.05 level

Table 5. Showing Berg balance scale (BBS) score for both groups A and B

BBS (Within Group Comparison)	Group A (n=32)		T-Value	Group B (n=32)		T score (p-Value)
	Mean	SD		Mean	SD	
WEEK 1	16.88	4.787	NS	16.83	4.745	NS
WEEK 3	39.06	4.739	24.04**	33.19	7.583	10.220** (p=0.006**)
Between Group Comparison						
At Week 3	Group A(n=16)			Group B (n=16)		T score (p-value)
	39.06	4.739		33.19	7.583	2.628** (p=0.035**)

** significant at 0.05 level, mean and SD of BBS (week 1 and week 3) for both groups A and B

The findings gain credibility by yet another study conducted by Kim, Kang, and Kim(2018) in Korea where PNF exercises were found to improve range of motion, pain, and functional activity among TKR patients. However, the present study differs from the Korean study using the outcome measures since the authors measured the improvement by objective indices including the MMT and BBS scores. In yet another study by Rhyu, Kim, and Park (2015), a six-week elastic

band exercise program using proprioceptive neuromuscular facilitation (PNF) was evaluated to measure isotonic strength of abductor's muscles in the lower extremity. It was found that PNF-based band exercises significantly improved the peak explosive muscular power in the isotonic contraction of the lower limb abductors. It was hypothesized that the PNF facilitates certain physiological changes which potentiate the functioning of both the central and

peripheral vestibular system and thereby are responsible for the therapeutic results.

The perception of articular position and mobility is critical for maintaining strength and balance as reduced articular sensitivity contributes to balance impairments in both the aged and osteoarthritic individuals (Jette et al. 2020). These imbalances are caused by decreased articular mobility. Both pain and decreased proprioception have an adverse effect on the joint increasing the risk of falling. In the present study, using hold relax PNF Technique in the experimental group, had brought out significant change ($p=0.035^*$) in Berg Balance Score(BBS) thereby proving its efficacy post arthroplasty (Table 5). This suggests that PNF might improve both neuromuscular control as well as joint stability bring better control in both static and dynamic balance (Gong 2020). There are some limitations to this study. The trial was limited to the Rehabilitation Department of Majmaah University and generalizability of the study cannot be justified. The study used only bilateral TKR patients and therefore evaluating the effects of the PNF techniques in unilateral cases arthroplasty especially in terms of strength, balance and proprioception cannot be emphasized clearly.

CONCLUSION

The data of the study conclude that the addition of the hold relax technique of PNF to the conservation exercise program decreases pain, improves range of motion and balance in patients with bilateral TKR as compared to conservative exercise program alone.

ACKNOWLEDGEMENTS

The authors are grateful to the Deanship of Scientific Research, AlMajmaah University via group No. RGP-2019- 35 for funding through Vice Deanship of Scientific Research Chair.

Declaration of Interest Statement: The authors report no conflicts of interest.

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Biomedical Communication

Assessment of Hand-Washing Habits Among Secondary School Students in Saudi Arabia

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ABSTRACT

The health effects of infectious diseases affecting school-aged children continue to be a source of concern in the majority of low- and middle-income nations, owing to poor personal hygiene habits and insufficient sanitary facilities in public primary schools. Schoolchildren and primary school pupils are at increased risk due to a lack of attention to a fundamental yet crucial aspect of personal hygiene, such as continually washing hands with soap under running water, and even due to a lack of information of correct hand washing with soap practice. In recent years, public health concerns about communicable diseases such as respiratory infection and pneumonia have increased. The majority of school students are more exposed to infectious risk factors as a result of their frequent engagement in unhealthy behaviors. The purpose of this study is to determine the handwashing habits of Saudi Arabian secondary school students. The study employed a cross-sectional design in which an online survey was used to assess handwashing habits. Boys and girls from Saudi Arabia's secondary schools were included in the population. Statistical software was used to synthesize and analyze the data collected. The findings indicate that there was a relatively high level of commitment to hand washing. Additionally, findings indicated that the primary reason for skipping hand washing was a lack of recall. When it comes to handwashing materials in schools, the majority of participants prefer water and soap. The study discovered that the majority of secondary school students are unaware of the dangers associated with improper handwashing. Regression analysis revealed that there are significant predictors of handwashing commitment. Factors influencing secondary school students' handwashing habits include their region, academic year, gender, age, and parent's education. Thus, hygiene education programs in schools and communities should be implemented to raise awareness about the importance of hand hygiene.

KEY WORDS: HANDWASHING, HYGIENE, SECONDARY SCHOOL STUDENTS.

INTRODUCTION

Due to poor personal hygiene practices and insufficient sanitary facilities in public primary schools, the health consequences of infectious diseases affecting school-aged children continue to be a source of concern in the majority of low- and middle-income countries. Due to a lack of attention to a fundamental but critical aspect of personal hygiene, such as constantly washing hands with soap under running water, and even due to a lack of knowledge about proper hand washing with soap practice, school children and primary school pupils are at a greater risk (Bolon 2011; Delea et al. 2020).

Inadequate awareness, practice, and attitudes toward personal hygiene, such as soap-washing, contribute significantly to the high prevalence of communicable diseases and can have long-term negative consequences for students as a whole (Bolon 2011; Delea et al. 2020). Many communicable diseases cause illness and death worldwide (Organization 2014). Infectious diseases are a major cause of morbidity and mortality among school-age children worldwide (Starke 2017). Hand hygiene is a simple, effective, and inexpensive way to prevent the spread of infectious diseases (Cevizci et al. 2015). Schools are high-risk areas for infection because children work and play in close quarters. They may come across contaminated objects and surfaces. Infectious diseases can spread quickly in schools (Cutler 2010). Hand washing is the most cost-effective way to prevent infection spread from feces, body fluids, and inanimate objects (Besha et al. 2016; Ahmed et al. 2021).

Article Information:*Corresponding Author: zhasan@ksu.edu.sa

Received 11/09/2021 Accepted after revision 19/12/2021

Published: 31st December 2021 Pp- 1924-1930

This is an open access article under Creative Commons License,

Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.81>

Hand washing is especially important for children and adolescents, as unwashed hands can spread infections. Decreased absenteeism is another benefit of proper hand washing (Besha et al. 2016). Saudi students make up a significant portion of the total population, which was 31,742,308 in 2016, with 7277317 students. Only 1860116 (961888 males, 898228 females) are secondary school students (Cahyani 2017). Secondary students are regarded as an important cultural foundation due to their age, maturity, and knowledge. This section of society wants to be aware of and educated about hand-washing practices to improve their lives, families, and communities (Ahmed et al. 2021).

Overcrowding in a classroom increases the risk of infection and other health issues. Furthermore, the number of students in a classroom can affect the environment, resulting in poor ventilation and disease spread. The OECD estimated the normal range for students per class not to exceed 21.4 in public schools and 20.5 in public-private schools (Indicators 2009). However, in one region, Jizan city, the number of students per class exceeded the OECD recommendation by 24.6 at the intermediate level and 40.9 at the secondary level (JEC Report 2017; Ahmed et al. 2021).

As such, the purpose of this study was to ascertain the hand washing habits of secondary school students in Saudi Arabia. The specific objectives are as follows: (1) to ascertain the most frequently used substances for hand washing by secondary school students in the preceding month; (2) to ascertain the most frequently cited reasons for skipping handwashing in schools among secondary school students; and (3) to ascertain demographic variables as predictors of secondary school students' handwashing habits (Ahmed et al. 2021).

MATERIAL AND METHODS

This study was employed as a descriptive correlational research design to examine secondary school students' handwashing habits and the factors that influence them. We have looked for significant associations between the independent and dependent variables. This study was conducted at the secondary school level. The schools, which are either exclusively for boys or girls, are located throughout Saudi Arabia. By the end of the first half of this year, the Kingdom of Saudi Arabia will have a total population of 34.22 million people (Ahmed et al. 2021).

Participants were chosen based on inclusion criteria. The schools of the participants should be recognized as state-owned and located in Saudi Arabia. As secondary school students, they are between the ages of 15 and 18 and enrolled in schools for the 2019–2020 academic year. The primary and elementary schools were excluded. The total sample size required for the study was calculated using the 2018/2019 records at a 95% confidence level, a 5% margin of error, and a population size of 1,057,205 (Cahyani 2017). Using Rao soft software to calculate the sample size, the total sample size required for this study is 386 subjects with a response distribution of 25%. However, 699 students were surveyed to ensure that the sample was representative and

to reduce margin error. With this sample size, the level of confidence increased to 95% and the margin of error was 3.71% (Ahmed et al. 2021). Due to the current COVID-19 pandemic situation, data collection was conducted online. Restrictions on visiting schools were implemented, and the use of paper surveys is likely to contribute to the spread of infection. The researchers created an account on an online survey platform (such as surveyparrow.com) and then converted the tools to an online format. The first step was to validate the tool on a subset of students prior to conducting the final data collection via pilot study.

Changes were made to the questionnaire in response to the students' responses and reactions. The time required for students to complete all sections of the survey was estimated to be between 3-5 minutes. Permission was obtained from the responsible authorities at the Faculty of Nursing College of King Saud University and the responsible persons at the Ministry of Education to conduct the study online. Prior to data collection, each school's principal was consulted. The questionnaire included nine questions and was divided into three sections. The first section contained six questions requesting demographic information. This section included information about the participant's gender, age, education level, place of residence, and parents' educational level.

The second section contained two questions in which respondents were asked about the handwashing substances they used in the previous month and their personal reasons for not washing their hands. The third section contained a single question in which students were asked about their handwashing habits or frequency of handwashing in various situations. These components were quantified using a five-item Likert scale based on their frequency of occurrence: never, rarely, occasionally, mostly, and always. The instrument was adapted from previous research (Pati et al. 2014; Adriaenssens et al. 2015). The author revised the content and validity as necessary, particularly to ensure easier comprehension in Arabic. Additionally, a pilot study with 62 students from various grades was conducted three days prior to the final data collection time to evaluate the reliability.

Permission to use this tool was obtained from the author, who generously granted it. The tool was originally written in English and then translated into Arabic. The tool was translated from English into Arabic because the respondents in this study spoke Arabic as their first language, and then it was translated back from Arabic to English to ensure conformity with the original. To determine the content validity of each question, a panel of three expert professional staff members from a nursing college was selected. Each reviewer used a separate form provided for this purpose to record both criticism and suggestions for improvement. As a result, each reviewer provided both feedback and suggestions for improvement. Others were omitted and some were replaced based on the experts' objective analysis of the items, primarily due to their clarity and consistency, particularly on items 3 and 2 (Waltz et al. 2010).

The major changes include response coding and question formulation to avoid leading questions and complex

sentences, which had no effect on the questionnaire's reliability. The content validity test was used to determine whether the Arabic version appears to be relevant to the study's objective and the concept of handwashing among school students. The Arabic version received an overall rating of 0.83 from the judges, indicating that the content is valid (Waltz et al. 2010). A pilot test was conducted to assess reliability using ten students randomly selected from two schools that were not included in the main study subjects.

The primary objective of the pilot study was to assess the questionnaire's internal consistency in light of the tool's intended use among Saudi Arabian school children. The data from the pilot study were analyzed using SPSS version 22. The total average for all questions was calculated and added to the statistical analysis; the results indicated a Cronbach's alpha of 0.71. This score indicates that the tool is suitable for use by secondary school students in Saudi Arabia. Due to pandemic restrictions, this study followed an online survey. As a result, most young people in Saudi Arabia have access to it via WhatsApp, Snapchat, and Twitter. Second, because all schools were closed due to precautionary measures to control and limit the spread of COVID-19, most students were at home. They broadcasted at lunchtime and at night, when most people are at home. It was done to reduce sampling bias in the analysis.

As before, participants were not offered incentives, tokens, or payments as a way to eliminate false or hurried answers from those who answered study questions only for the token. Eventually, surveyparrow.com's website included numerous steps to discourage repeat participation by one person. The IBM SPSS version 22 statistical package was used to synthesize and evaluate the data (IBM Corp., 2015). Outliers and missing data were identified using double data entry, testing of the frequencies and ranges in the descriptive statistics, and testing of the confidence intervals in the descriptive statistics.

In addition to descriptive statistics like mean and standard deviation, frequencies, minimum and maximum scores, and minimum and maximum scores were computed. Calculated for both the first and second study topics were descriptive statistics such as the mean (average), standard deviation (standard deviation), frequencies (frequency distribution), and the minimum and maximum scores. For the third research question, the mean and the sum of the scores for each item were determined for each question. In order to answer the fourth research question, the independent variable (demographic data) was assessed at the nominal level, while the dependent variable (hand washing behaviors) was examined at the ordinal and interquartile range (Likert scale). Multiple Linear Regressions, or ML, were carried out with the overall score of the hand washing commitments as the dependent variables, with the significance threshold set at 0.05 (Khan et al. 2019). The independent factors were age groups, gender, school year level, parents' education, and region of residence. The dependent variables were age groups, gender, and school year level.

RESULTS AND DISCUSSION

Six hundred and ninety-nine respondents were selected from the target population. Demographic variables collected from respondents include gender, age, parents' education, region of residence, and school year level. In terms of gender, it was clear that males participated at a higher rate than females in this study. In terms of age groups and school years, the results indicate that the third year of secondary school is the most frequently represented, with students over the age of 16. However, the results of the parents' education indicate that the majority of participants' parents held bachelor's degrees. The majority of students resided in the Janzen region.

Table 1. Demographics Characteristics of Subjects

Characteristics	Frequency	Percentage
Age		
15 years	2	11.7
16 years	153	21.9
Above 16	464	66.4
Gender		
Male	358	51.2
Female	341	48.8
School Year		
1 st	245	35.1
2 nd	137	19.5
3 rd	317	45.4
Mothers education		
Illiterate	148	21.2
Primary	115	16.2
Secondary	102	14.6
High school	159	22.7
Graduate	175	25.0
Fathers education		
Illiterate	73	10.4
Primary	86	12.3
Secondary	90	12.9
High school	186	26.0
Graduate	268	38.3
Region		
Central region	75	10.7
Western region	94	13.4
Eastern region	32	4.6
Southern region	431	61.7
Northern region	67	9.6

Research Questions 1 and 2: What are the common substances and materials used for handwashing and the reasons for not handwashing by the secondary school students?: Table 1 contains detailed information. Students were given options for handwashing materials such as soap, water, disinfectant, and others. The majority of students chose water and soap. Additionally, the study's findings indicated that the majority of participants forget

to wash their hands. Others stated that they lack the time or opportunity to wash their hands and that the hand washing facilities were not clean (see Table 2).

Students consistently selected "wash your hands before eating," "after eating," "after touching an animal," "after waking up in the morning," "when my hands are dirty," "before preparing meals," "after touching the garbage,"

"after combing my hair," and "after playing." Meanwhile, responses to the remaining ten statements were relatively consistent; respondents chose mostly, occasionally, or rarely. However, respondents almost always selected never for the statements "when I return from school", "after exchanging money", "after sneezing", and "after picking up anything from the ground", indicating that secondary school students are unaware of the dangers of not washing their hands (See Table 3).

Table 2. Reasons for not handwashing and common materials used

Variables	Categories	Frequency	%	Mean (SD)
Material used to wash the hand in last previous months	Water	112	16.0	3.78 (SD= 1.93)
	Water and Soap	401	57.4	
	Disinfectant	9	1.3	
	Others	11	1.6	
	All	166	23.7	
Reasons for not washing the hands	no time	135		2.81 (SD= 1.29)
	no need	19.3		
	Forgetting	113	16.2	
	Hand-washing facilities are not easily accessible	308	44.1	
	Hand-washing facilities are not clean	38	5.4	
		105	15.0	

Table 3A. Reasons to Wash and Not Wash the Hands Response to Each Statement of the Questionnaire" When You Wash Your Hands",

Variables	Never	Rarely	Sometimes	Mostly	Always	μ (SD)
Wash the hands before you eat	93(SD13.3)	12(SD1.7)	42(SD6.0)	126(SD18)	426(SD60.9)	4.12(1.387)
After eating	30(SD4.3)	15(SD2.1)	14(SD2.0)	55(SD7.9)	565 (SD83.7)	4.65(.956)
Wash your hands before going to the toilet	170(SD24.3)	100(SD14.3)	166(SD23.7)	117(SD16.7)	146(SD2 0.9)	2.96(1.456)
Wash your hands after going to the toilet	79(SD22.3)	11(SD2.6)	26(SD3.7)	103(SD14.7)	480(SD68.7)	4.28(1.315)
When I come back from school	184(SD26.3)	64(SD9.2)	99(SD14.2)	110(SD15.7)	242(SD34.6)	3.23(1.624)
After shaking hands with people	128(SD18.3)	180(25.3)	215(SD30.8)	96(SD13.7)	80(SD11.4)	2.74(1.233)
After touching an animal	73(SD10.4)	22(SD3.1)	39(SD5.6)	107(SD15.4)	458(SD65.5)	4.22 (1.315)
After waking up in the morning when my hands are dirty	57(SD8.2)	24(SD3.2)	39(SD5.6)	104(SD14.9)	475(SD68)	4.31 (1.230)

Twenty statements were included in this study, ranging from always (5) to never (1) on a 5-likert scale. The maximum possible score is 100, and the minimum possible score is 20. The response's mean score is 72.8 (SD 12.7). The minimum score was 36, and the maximum score was 100. However, the level of commitment is greater than 70, which is considered to be quite high. Additionally, the results indicate that the majority of respondents always, mostly, or occasionally agree with statements evaluating their commitments to hand washing. Further details about the study group's response to specific situations involving

hand washing are provided in the following sections. For example, Table 4 revealed that less than half (49.5 %) of respondents washed their hands after cleaning their nose and less than three-quarters (37.8 %) of respondents washed their hands after cleaning their ear.

Additionally, Table 5 revealed that over two-thirds (74.4%) of students always washed their hands after combing their hair. There is overwhelming evidence demonstrating the critical role of handwashing in disease prevention, particularly among vulnerable groups such as students.

To this end, a variety of stakeholders contribute to the improvement of adolescents' handwashing commitment. Handwashing is well-documented as a cost-effective method of reducing disease spread. Handwashing with running water and no soap is a better preventative measure than not washing at all (Luby et al. 2011; Mahmud et al. 2015). For children, the practice is even more critical, as

the demography is more susceptible to inhibiting infections and factors affecting personal hygiene. The findings in this study reflect a hot and cold state of affairs in adolescence's commitment to handwashing. The circumstances are the result of a number of factors, as demonstrated by the investigation's findings (Mahmud et al. 2015; Pogrebna and Kharlamov 2020).

Table 3B. Reasons to Wash and Not Wash the Hands Response to Each Statement of the Questionnaire" When You Wash Your Hands",

Variables	Never	Rarely	Sometimes	Mostly	Always	μ (SD)
Before preparing meals	82 (SD11.7)	14(SD2.0)	34(SD4.9)	111(SD15.9)	458(SD65.5)	4.21 (1.340)
After exchange money	133(SD19)	216 (SD30.9)	158(SD22.6)	99(SD14.2)	93(SD13.3)	2.72 (1.291)
After cleaning my ear	147(SD21)	60(SD8.6)	97(SD13.9)	131(SD18.7)	264(SD37.8)	44 (1.561)
After cleaning my nose	121(SD17.3)	42(SD6)	73(10.4)	117(SD16.7)	346(SD49.5)	3.75 (1.529)
After sneezing	175(SD25)	58(SD8.3)	99(SD14.2)	162(SD23.2)	205(SD29.3)	(3.23)1.561)
After rubbing my eyes	167(SD23.9)	142(SD20.3)	156(SD22.3)	107(SD15.3)	127(SD18.2)	(2.84)(1.412)
After touching the garbage	40(SD5.7)	16(SD2.3)	23(SD3.3)	100(SD14.3)	520(SD74,4)	(4.49)(1.068)
After combing my hair	40(SD5.7)	16(SD2.3)	23(SD3.3)	100(SD14.3)	520(SD74,4)	2.91(1.450)

Table 4. Study group according to hand-washing habits after cleaning ear, nose and rubbing eye)

Variables	Never	Rarely	Sometimes	Mostly	Always	μ (SD)
After cleaning my ear	147 (21%)	60(8.6%)	97(13.9%)	131(18.7%)	264(37.8%)	3.44(1.56)
After cleaning my nose	121(17.3%)	42(6%)	73(10.4%)	117(16.7%)	346(49.5%)	3.75(1.1%)
After rubbing my eyes	167(23.9%)	142(20.3%)	156(22.3%)	107(15.3%)	127(18.2%)	2.84 (1.41%)

Table 5. Study population hand-washing habits after combing the hair. n=699

Items	Frequency	Percentage	μ (SD)
Never	40	5.7%	2.91(1.450)
Rarely	16	2.3%	
Sometimes	23	3.3%	
Mostly	100	14.3%	
Always	520	74.4%	

Additionally, there is widespread recognition of the importance of handwashing and its practice among the study population. Adolescents are generally aware of the importance of proper handwashing. It's unsurprising that approximately 50% of participants adhere to acceptable handwashing practices (Wilkinson et al. 2012). There is a high level of commitment to handwashing prior to eating, and more than 60% of respondents wash their hands after these activities. Surprisingly, students routinely washed their hands after sneezing or cleaning their noses. The phenomenon exemplifies the student's awareness of the critical nature of handwashing. Based on a previously published report a high rate of Middle East respiratory syndrome coronavirus (MERS-CoV) transmission via sneezing or nose cleaning.

As such, handwashing after sneezing or washing one's nose is critical for preventing the spread of MERS-CoV. Regrettably, a sizable portion of the population has a low commitment to handwashing. Nevertheless, the importance of consistent and continuous education cannot be overstated (Bolds et al. 2019). Besha et al. studies explain that adolescence's low commitment to handwashing is a result of a number of factors that inhibit or disinterest the group in developing acceptable handwashing practices (Besha et al. 2016; Al-Hazmi et al. 2018; Pogrebna and Kharlamov 2020).

This study increases nursing students' knowledge of the most effective strategies for improving care through infection prevention. The findings of this study will assist nursing administrators in hiring nurses who are knowledgeable about hand hygiene and management. This study provides an opportunity to initiate research into the care practices that nurses might adopt to assist patients with hand hygiene-related illnesses. Schools must ensure that students have adequate access to toilets with sinks and hand hygiene materials, and that there are no barriers to proper washroom use. Recommendations to schedule counseling sessions for students who are ill or at risk of illness, advising them to protect themselves and their peers from pathogen spread through the use of healthy behaviors such as social distance and hand hygiene (Pogrebna and Kharlamov 2020).

Because of the cross-sectional nature of the study and the use of a convenient sampling method in the online survey, the findings of this study cannot be generalized. The knowledge gained from this study indicates that effective handwashing among students can only be achieved through the combination of a number of different factors. Because of the lack of individual rapport between the participants in online questionnaires, the validity of the responses may be reduced as a result. A further external threat exists to the validity of history, given the study's timing, which coincided with the pandemic, and in particular, its section on the importance of proper hand washing. A threat of this nature also has an impact on the generalization of results (Pogrebna and Kharlamov 2020).

Future research on this topic should examine the Saudi government's role in raising civic awareness in schools and other sectors. Students' understanding of hand hygiene must be improved. Students should learn how to wash their hands as a life lesson. To help students learn this vital life skill properly, schools and communities must promote increased knowledge of hand hygiene and infection prevention. Hand washing can be learned and practiced throughout the day. As a result, all secondary schools should have a hand hygiene education program run by school nurses. All secondary school students should be educated by school nurses. The content will cover the importance of handwashing, risk behavior, preventive control measures, and universal precautions (Pogrebna and Kharlamov 2020).

Schools and healthcare facilities can use self-learning, pamphlets, booklets, and exhibits to help students learn more. High school students must be motivated to learn about the most common habits and change their unhealthy attitudes into wellness behaviors. Teachers and school nurses should help students recognize when hand hygiene is required and teach them how to do it correctly. Hand washes with soap and water are preferred in some cases, but waterless hand sanitizers can be used in schools. Hand hygiene should be viewed as a lifestyle change for students. For example, during a pandemic, a better understanding of WASH and hygiene culture is critical (Pogrebna and Kharlamov 2020).

CONCLUSION

The findings of the present study confirm the majority of students prefer water and soap as their primary hand-washing materials at school. Additionally, it indicates that in the Saudi Arabian student population, handwashing behavior improves with age and increased knowledge about the importance of exercise. Additionally, it demonstrates that the Saudi Arabian education sector requires effective hygiene education programs in schools and various sectors of society to raise awareness about the importance of hand hygiene.

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Agricultural Communication

The Need for Production Volumes of Main Agricultural Products as an Integral Part of the Russian Agrarian Policy

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ABSTRACT

The agrarian policy pursued in Russia is primarily aimed at ensuring food security, the main components of which are the economic and physical availability of food to the population. However, the indicators approved in the regulatory legal acts on food security monitoring do not contribute to its objective assessment. They can be used to assess the overall state of food security. A more in-depth analysis is needed for a more objective assessment of the economic availability of food to the population of the Russian Federation, which requires, among other things, changes in the methods of calculating food independence, taking into account the share of household disposable resources used to purchase food. It is also necessary to take into account the energy value of food, the quality of food consumed, etc. To do this, it is necessary to significantly expand the information base on the food consumed, including taking into account its quality and energy value. The paper attempts, based on the available analytical data, to assess the economic availability of food for households depending on their income, as well as in the context of socio-demographic groups, to identify the main factors constraining bringing the level of food consumption to rational norms.

KEY WORDS: ECONOMIC ACCESSIBILITY OF FOOD, FOOD INDEPENDENCE, FOOD SECURITY, THE PURCHASING POWER OF THE POPULATION.

INTRODUCTION

The conducted agri-food policy contributed to an increase in the gross harvest of the main crops. For 2010-2020, gross grain collections increased from 94.2 to 133.5 million tons, sunflower seeds from 5.3 to 13.3 million tons, fruits and berries from 2.1 to 3.7 million tons, vegetables of protected and open ground from 11 to 13.9 million tons. The gross harvest of potatoes and sugar beet are unstable, but at the same time tend to increase. The production of livestock products, which is especially important for ensuring the Doctrine of Food Security, is also increasing at a less dynamic pace. From 2010 to 2020, milk production increased from 31.5 to 32.2 million tons, and the production of livestock and poultry for slaughter (in slaughter weight)

from 7.2 to 11 million tons, marketable eggs from 40.8 to 48.4 billion units, which is still not enough to ensure food security indicators (Rosstat 2021).

MATERIAL AND METHODS

In 2020, farms of all categories produced gross agricultural output of 6.1 trillion rubles, including 3.3 trillion rubles in crop production, 2.8 trillion rubles in animal husbandry (Table 1). The volume of gross output produced in farms of all categories in 2020 increased by 5.3%, including in crop production by 7.2%, in animal husbandry by 3.2%. Even though the volume index of GDP amounted to 97% and has a downward trend, this indicator in agriculture, although unstable, exceeds the average for the economy. The volume index of agricultural production was 1.5% in 2020 (Figure 1). As before, a significant share of agricultural products is produced by large-scale agricultural organizations,

Article Information:*Corresponding Author: 571062@rambler.ru

Received 25/10/2021 Accepted after revision 28/12/2021

Published: 31st December 2021 Pp- 1931-1939

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.82>

which have undoubted advantages in the formation of their production capacity, produced on an industrial scale.

RESULTS AND DISCUSSION

The share of agricultural organizations in the gross output of agriculture in (2020) amounted to 58.3%, households 27.4%, farms 14.3% (Table 2), while agricultural organizations produced only 44.8% gross production in 2010, households 48.0%, farms 7.2%.

Table 1. Agricultural products in farms of all categories of the Russian Federation, billion rubles

	Years							
	2010	2013	2015	2016	2017	2018	2019	2020)
Agricultural products	2,462.2	3,458.3	4,794.6	5,112.3	5,109.5	5,348.8	5,801.4	6,110.8
crop production	1,090.2	1,730.2	2,487.3	2,710.3	2,599.7	2,756.1	3,056.4	3,276.9
animal husbandry	1,372.0	1,728.1	2,307.3	2,402.0	2,509.8	2,592.7	2,745.0	2,833.9

Source: According to Rosstat (2021)

Figure 1: Volume index of Russia's GDP and agriculture

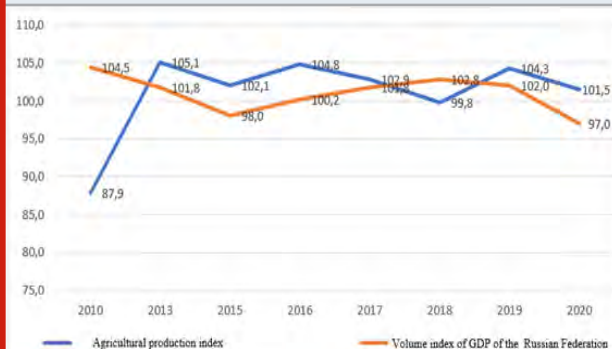


Table 2. Gross agricultural output by category of farms (in actual prices; as a percentage of the total)

	2000	2010	2018	2019	2020
Farms of all categories including:	100	100	100	100	100
agricultural organizations	45.2	44.8	56.5	57.7	58.3
households	51.6	48.0	31.0	28.6	27.4
peasant farm enterprises 1)	3.2	7.2	12.5	13.7	14.3

Source: According to Rosstat (2021)

Table 3. Change in the share of production of the main types of agricultural products by categories of farms for 2000-2020, %

	Households	Agricultural organizations	Peasant farm enterprises
Grain (in weight after completion)	-0.1	-21	21.1
Sugar beet	-0.5	-2.2	2.7
Sunflower seeds	-0.9	-19.6	20.5
Potato	-26	13.4	12.6
Vegetables	-24.6	5.6	19
Fruits and berries	-19.9	11.4	8.5
Cattle and poultry for slaughter (slaughter weight)	-41.8	40.5	1.3
Milk	-15.2	8.2	7
Eggs	-10.8	10	0.8
Wool (in physical weight)	-12.5	-20.2	32.7

Source: Calculated according to Rosstat data (2021)

We believe that at this stage, comprehensive support was needed for farms that can use small fields more efficiently, and in the future will increase the volume

of agricultural production, transforming into industrial agricultural organizations. Therewith, farms and agricultural organizations would replace the production volumes

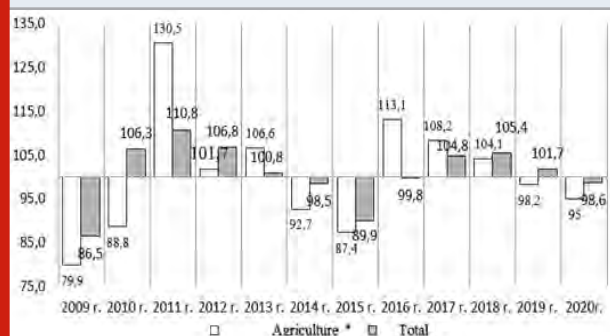
previously produced by households of the population. Such a measure will contribute to increasing the volume of resources used, including labor. The main producers of grain, sugar beet, sunflower seeds, livestock and poultry for slaughter, milk, and eggs are agricultural organizations (Altukhov, Drokin and Zhuravlev 2015).

They are also increasing the production of vegetables. The traditional types of products produced in the households of the population are potatoes, vegetables, fruits and berries, livestock and poultry, milk, and wool. It should be noted that the share and volumes of these types of products in the households of the population are decreasing for several reasons. At the same time, for these types of products, in dynamics, there is an increase in the share of manufactured products in farms and agricultural organizations. The share of potato production in households decreased by 26% in the 2000s. The share of potato production in agricultural organizations increased by 13.4% and by 12.6% in farm enterprises (Table 3) (Altukhov et al 2015).

The share of vegetable production in households decreased by 24.6%, while in agricultural organizations it increased by 5.6%, and in farms by 19%. The share of fruit and berry production in households decreased by 19.9%, while in agricultural organizations it increased by 11.4%, and in farms by 8.5%. An increased in the share of production of basic types of products in agricultural organizations and farms occurred due to a decrease in the share of production in households of the population. As can be seen from the above material, the change in the types of products is uneven.

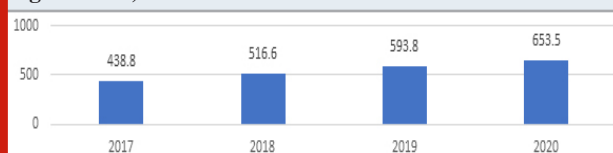
The investment component of the development of agricultural production, which does not contribute to the active increase in the production of agricultural products and food, is of concern (Altukhov et al. 2015). The index of investments in fixed assets of agricultural organizations showed negative trends (Figure 2). By the end of 2020, investments in the industry decreased by 5.0% compared to the previous year, while the Ministry of Agriculture planned an increase of 1.1% following the State Program. This threatens the technological development of agricultural production (Kolesnikov 2019).

Figure 2: Volume index of investments in all types of economic activity, including agriculture for 2009-2020, compared to the previous year, %



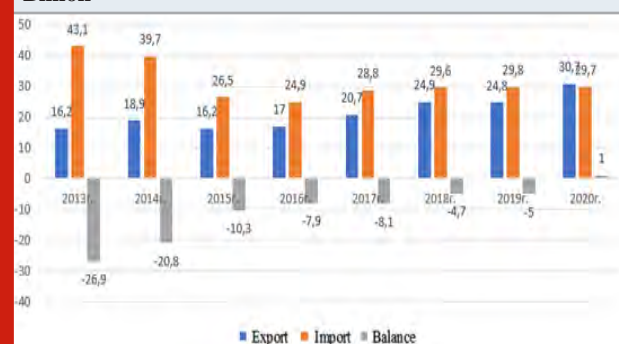
Despite this, positive trends in the development of agricultural production should be noted. The number of high-performance jobs continues to increase. In (2020), there were more than 635 thousand of them (Figure 3), which, according to preliminary estimates, is about 14% of the total number of jobs in agriculture. In this direction, it was necessary to do serious work related to both an increase in the intensity of production and an increase in the incomes of agricultural workers. Together, this would increase the purchasing power of the rural population, and would also contribute to an increase in the level of food independence and economic accessibility of food (Kolesnikov 2019).

Figure 3: The number of high-performance jobs in agriculture, thousand units



According to the Federal Center for the Development of Exports of Agricultural Products of the Ministry of Agriculture of Russia (Agroexport) in 2020, the balance of foreign trade in food products and agricultural raw materials became positive for the first time and amounted to 1 US Dollars Billion (Figure 4). The main buyers of agricultural products were: China – 4.0 US Dollars Billion, the EU – 3.3 US Dollars Billion, Turkey – 3.1 US Dollars Billion, Kazakhstan – 2.1 US Dollars Billion, Egypt – 2.0 US Dollars Billion, the Republic of Korea – 1.8 US Dollars Billion. In the structure of exports, 34% were cereals, 17.0% fish and seafood, 16% fat-and-oil products, 15% food and processing industry products (Kolesnikov 2019).

Figure 4: Dynamics of foreign trade in food products and agricultural raw materials (except textile), US Dollars Billion



It should be understood that the successfully developed market situation in the world food market should not become a reason for abstracting from issues related to increasing the volume of food exports, taking into account the implementation of the indicators of the Food Security Doctrine. Here it was necessary to make efforts to increase the production of agricultural products, as well as to increase the share of the sale of food with high added value. It was also necessary to increase the volume of export products with high conversion rates. Special attention should be

paid to this aspect. This was also due to the adoption by several developed countries of programs to significantly reduce the use of hydrocarbons, the transition to a green economy, which would negatively affect the raw exports of hydrocarbons and reduce the total volume of export revenue.

Table 4. Profit from the results of the activities of agricultural organizations

	2017	2018	2019	2020
Share of profitable organizations, %	81.0	79.7	78.9	80.8
Profit (loss before taxation), billion rubles	270.1	349.8	298.2	542.3

Source: according to Rosstat (2021)

Based on the above, it was necessary to increase the volume of production of agricultural products of high processing, improve its quality, enter new markets for food (Altukhov, Drokin and Zhuravlev 2015; Kolesnikov, Akupiy and Andreeva 2017). Moreover, the profit of agricultural organizations has increased by 2 times over the past 4 years (Table 4). This should contribute to the technical and technological modernization of agricultural production

and increase the volume of agricultural production. However, agricultural organizations are dependent on the supply of imported fixed assets in terms of modernization of production and its innovative development. In 2019, agricultural organizations of Russia spent 242.8 billion rubles on the purchase of imported fixed assets, which is almost comparable to the amount of profit received (Ushachev, Serkov and Maslova 2019).

The consumption of basic foodstuffs by the population does not yet meet rational standards. First of all, this applies to vegetables and melons, fruits and berries, milk and dairy products, for which consumption in 2019 amounted to 77.1%, 62%, and 72% of the recommended rational norms, respectively (Table 5). This problem was not so much agrarian as macroeconomic. The consumption of individual foods remains low. The dynamics of consumption and production of basic foodstuffs are different. The production of meat and meat products increased by almost 2.5 times from 2000 to 2019, while consumption increased by 1.7 times, egg production increased by 33.8%, and consumption by 24.5%, sugar production increased by 17.7%, and consumption by 11.4%, vegetable oil production increased by 4.4 times, and consumption by 41.4%. Thus, an increase in production volumes for these products can meet the growing needs of the population. The situation was different with milk production. Its production volumes decreased by 3.4%, and consumption increased by 8.3% (Ushachev et al 2019).

Table 5. Dynamics of production and consumption of basic foodstuffs in the Russian Federation

	Recommended rational rate of food consumption, kg	Consumption per 1 person, year, kg 2019	Ratio of actual consumption to the rational norm, %	Consumption in 2019 in % to 2000	Production 2020 in % to 2000
Meat and meat products in terms of meat	73	76	104.1	168.9	248.9
Milk and dairy products in terms of milk	325	234	72.0	108.3	96.6
Eggs and egg products – pieces*)	260	285	109.6	124.5	133.8
Sugar	24	39	162.5	111.4	117.7
Vegetable oil	12	14.0	116.7	141.4	434.0
Potato	90	89	98.9	81.7	66.5
Vegetables and food melons	140	108	77.1	136.7	128.1
Fruits and berries	100	62	62.0	193.6	136.1
Bread products	96	116	120.8	99.1	88.1

Source: Calculated according to Rosstat data (2021)

A similar situation was developing for vegetables and fruits: production volumes for vegetables increased by 28.1%, and consumption by 36.7%, and production volumes for fruits increased by 36.1%, and consumption by 93.6%. It is necessary to increase production volumes to meet the needs of the population for these types of products. A non-standard situation is developing for potatoes and bread products. Potato production volumes decreased by

33.5%, and consumption by 18.3%. Production decreased by 11.9% for bread and bread products, and consumption by 0.9%. That is, it is necessary to increase production for these types of products (Ushachev et al 2019).

However, it was provided that the population will have effective demand and trends in the consumption of basic foodstuffs will change for the better, and rational

consumption of basic foodstuffs will not be revised upwards (Kolesnikov 2019). Meanwhile, it is important to understand and take into account the deficit of consumption of basic

foodstuffs in the context of decile groups. The number of products that are consumed according to rational norms increases with a boost in income in the context of decile groups (Table 6).

Table 6. Deficit of consumption of basic foodstuffs by decile groups in 2019, on average per consumer per year, kg

	First	Second	Third	Fourth	Fifth	Sixth	Seventh	Eighth	Ninth	Tenth
Bread and bread products	-7.1	-4.8	-2.8	2.0	4.2	1.2	-1.0	2.0	3.1	-3.8
Potato	-39.7	-36.3	-33.0	-29.7	-30.0	-31.6	-31.6	-28.8	-29.2	-30.5
Vegetables and melons	-72.0	-57.2	-49.5	-39.8	-36.1	-31.0	-27.1	-22.7	-14.5	-14.0
Fruits and berries	-57.1	-45.9	-37.7	-32.0	-27.0	-21.0	-17.0	-7.8	-2.7	0.7
Meat and meat products	-14.1	-1.0	6.8	10.9	18.5	20.7	25.0	32.9	38.7	35.8
Milk and dairy products	-151.9	-116.2	-92.4	-70.8	-35.0	-52.9	-43.3	-20.4	-7.7	-0.7
Eggs, pcs.	-85	-61	-47	-36	-25	-22	-20	-2	17	20
Fish and fish products	-8.2	-5.1	-3.0	-1.5	0.8	0.8	1.2	4.2	5.4	6.0
Sugar and confectionery	0.8	3.7	5.0	7.1	7.8	8.8	8.2	9.4	11.0	7.5
Vegetable oil and other fats	-2.9	-2.3	-1.8	-1.4	-1.0	-1.1	-1.4	-1.1	-0.7	-0.9

Source: Calculated according to Rosstat data (2021)

Table 7. Shortage of funds for the purchase of basic foodstuffs by decile groups per year, rub

	First	Second	Third	Fourth	Fifth	Sixth	Seventh	Eighth	Ninth	Tenth
Bread and bread products	-600.20	-404.28	-231.58	0.00	0.00	0.00	-83.53	0.00	0.00	-318.65
Potato	-1,156.38	-1,057.39	-961.16	-866.69	-875.70	-920.85	-919.89	-840.66	-851.24	-890.04
Vegetables and melons	-1,901.02	-1,509.81	-1,306.03	-1,051.86	-952.93	-819.26	-715.21	-598.40	-382.81	-368.55
Fruits and berries	-6,061.67	-4,880.28	-4,009.45	-3,399.36	-2,869.49	-2,229.44	-1,811.35	-832.15	-290.70	0.00
Meat and meat products	-5,107.63	-357.43	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Milk and dairy products	-9,010.71	-6,895.65	-5,479.98	-4,200.27	-2,077.33	-3,135.48	-2,568.67	-1,212.20	-458.54	-44.07
Eggs, pcs.	-661.99	-476.31	-365.55	-280.23	-197.73	-174.99	-155.87	-14.98	0.00	0.00
Fish and fish products	-1,487.39	-916.08	-545.78	-270.53	0.00	0.00	0.00	0.00	0.00	0.00
Sugar and confectionery	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	375.92
Vegetable oil and other fats	-358.83	-282.11	-218.77	-169.67	-123.57	-140.81	-177.04	-134.06	-81.21	-117.19
Shortage of funds for the purchase of basic foodstuffs, rubles per year	26,345.81	16,779.34	13,118.30	10,237.61	7,096.74	7,420.83	6,431.56	3,632.45	2,064.51	1,362.58

Source: Calculated according to Rosstat data (2021)

Only the consumption of sugar and confectionery is deficient in the first and second decile groups. The consumption of sugar and confectionery, meat and meat products were deficient in the third group. The deficient consumption of bread was added in the fourth group, fish and fish products – in the fifth and sixth groups. The consumption of bread

becomes deficient in the seventh and tenth groups. To meet the needs for basic foodstuffs in the context of decile groups, it was necessary to take into account the shortage of funds, which must be eliminated to meet the needs of the population according to rational nutrition standards (Table 7), (Ushachev et al 2019).

Table 8. Consumption of basic foodstuffs per capita, kg

	Meat and meat products (without by-products of second category and raw fat)	Milk and dairy products	Eggs and egg products	Sugar and confectionery	Vegetable oil	Potato	Vegetables and food melons	Fruits and berries	Bread products
Rational norms									
	73	325	260	24	12	90	140	100	96
WHO nutrition standards									
	78	405	291	47	9.1	117	139	146- 219	117
Actual consumption									
RF	70	234	285	39	14.0	89	108	62	116
CFD	78	219	289	43	15.4	88	102	61	117
NWFD	72	262	298	39	11.9	75	94	62	99
SFD	67	218	308	42	14.7	77	143	77	121
NCFD	58	239	230	42	13.6	89	171	66	124
PFD	65	267	288	37	14.8	98	99	58	114
UFD	66	206	295	37	13.4	92	93	66	118
SFD	66	238	278	34	11.8	93	92	50	122
FEDD	72	199	254	35	12.1	94	95	62	114

Source: Compiled by the author according to Rosstat (2021)

Table 9. Production and consumption of livestock products, thousand tons

	Populati on, million people	Production, 2020.				Consumption according to rational norms			
		Pig meat, thousand tons	Poultry meat, thousand tons	Cattle meat, thousand tons	Milk, thousand tons	Pig meat, thousand tons	Poultry meat, thousand tons	Cattle meat, thousand tons	Milk, thousand tons
RF	146.7	4,281.6	5,016.3	1,633.7	32,225 .5	2,640. 6	4,547.7	2,934	47,677. 5
CFD	39.4	2,209.1	1,857.7	318.2	6,253. 9	709.2	1,221.4	788	12,805
NWFD	14.0	358.9	407.3	49.3	2,000. 8	252	434	280	4550
SFD	16.5	249.8	393.1	262.7	3,774	297	511.5	330	5,362.5
NCFD	9.9	64.7	433.3	154.9	2,743. 9	178.2	306.9	198	3,217.5
PFD	29.3	699.6	1,144.9	459.4	9,987. 6	527.4	908.3	586	9,522.5
UFD	12.4	250	378.6	83.4	1,998. 3	223.2	384.4	248	4,030
SFD	17.1	379.4	366.1	228.6	4,493. 3	307.8	530.1	342	5,557.5
FEDD	8.2	70.1	35.2	77.2	973.6	147.6	254.2	164	2,665

Source: Calculated according to Rosstat data (2021)

In general, actual consumption exceeds rational norms in the Russian Federation for most types of food. The exception is meat and meat products, milk and dairy products, vegetables, fruits, and berries. Russians consume more than the norm for other types of food (Table 8).

It was necessary to pay attention to the increase in the production of livestock products, as well as vegetables, fruits, and berries. Based on the above analytical material, the Russian Federation produces poultry meat and pig meat in sufficient volume, and it approaches rational

consumption standards in terms of the production of cattle meat (Kolesnikov et al., 2017). Milk production was of concern. When consuming 325 kg per year according to rational consumption standards, it is necessary to produce at least 47.7 million tons of milk instead of 32.2 (Table 9). The shortage of food consumption is distributed unevenly across the federal districts of the Russian Federation, which is largely due to different natural and climatic conditions for the production of crops. Based on production and consumption, the deficit of livestock products in federal districts is calculated (Ushachev et al 2019).

Table 10. Shortage of livestock products and the necessary acreage for the production of grain crops

	Pig meat, thousand tons	Poultry meat, thousand	The meat of cattle, thousand	Milk, thousand tons	Additional grain production, thousand	The yield of grain and leguminous crops, c/ha	Additional acreage, thousand hectares
RF	-	-	-1,300.3	-15,452.00	7,717.11	28.6	-2,698.29
CFD	-	-	-469.8	-6,551.10	3,204.56	44.7	-716.904
NWFD	-	-26.70	-230.7	-2,549.20	1,303.69	38.2	-341.28
SFD	-47.20	-118.40	-67.3	-1,588.50	918.915	35.1	-261.799
NCFD	-113.50	-	-43.1	-473.60	489.478	30.2	-162.079
PFD	-	-	-126.6		104.45	24.6	-42.4593
UFD	-	-5.80	-164.6	-2,031.70	1,013.17	13.8	-734.182
SFD	-	-164.00	-113.4	-1,064.20	656.796	17.1	-384.091
FEDD	-77.50	-219.00	-86.8	-1,691.40	1,110.86	21.9	-507.243

Source: Calculated according to Rosstat data (2021)

Table 11. Production and consumption of vegetables, fruits, and berries, thousand tons

	Melon food crops	Vegetables of open and protected ground	Grape plantations	Drupaceous (plum, cherry, sweet cherry, apricot, peach, and other stone fruits)	Pomaceous (apple, pear, quince, etc.)	Berry plantation (wild strawberries, strawberries, raspberries, currants, gooseberries, and other berries)
			Production			
RF	15,843.1	138,639.66	6,819.08	6,017.49	23,416.49	6,952.61
CFD	340.6	25,512.78	56.5	861.78	4,812.73	1,524.8
NWFD	0.14	5,135.96	7.61	176.38	790.87	477.52
SFD	6,871.97	40,717.93	3,703.48	1,673.17	5,911.17	1,123.08
NCFD	2,264.8	22,671.26	2,844.47	1,360.72	6,969.18	188.14
PFD	6,119.97	26,427.58	176.41	1,386.73	3,813.29	1,993.69
UFD	24.3	5,668.54	11.63	251.94	625.38	674.56
SFD	116.39	9,247.23	6.7	238.13	432.46	736.18
FEDD	104.94	3,258.38	12.29	68.63	61.41	234.63
			Consumption			
RF	22,005	183,375	8,802	11,736	85,086	10,269
CFD	5,910	49,250	2,364	3,152	22,852	2,758
NWFD	2,100	17,500	840	1,120	8,120	980
SFD	2,475	20,625	990	1,320	9,570	1,155
NCFD	1,485	12,375	594	792	5,742	693
PFD	4,395	36,625	1,758	2,344	16,994	2,051
UFD	1,860	15,500	744	992	7,192	868
SFD	2,565	21,375	1,026	1,368	9,918	1,197
FEDD	1,230	10,250	492	656	4,756	574

Source: Calculated according to Rosstat data (2021)

Additional volumes of grain production have been calculated. In general, in the Russian Federation – 7.7 million tons, including CFD – 3.2 million tons, NWFD – 1.3

million tons, UFD – 1.0 million tons, FEDD – 1.1 million tons. It was necessary to additionally use about 2.7 million hectares of grain and leguminous crops for the production

of these volumes of grain. Moreover, it was additionally necessary to use the areas under perennial grasses and forage crops. The calculations made assume taking into account the

rational norms of consumption of the main types of meat and milk, without the possibility of exceeding them (Table 10) (Ushachev et al 2019).

Table 12. Deficit of production of vegetables, fruits, and berries, thousand tons

	Melon food crops	Vegetables of open and protected ground	Grape plantations	Drupaceous (plum, cherry, sweet cherry, apricot, peach, and other stone fruits)	Pomaceous (apple, pear, quince, etc.)	Berry plantation (wild strawberries, strawberries, raspberries, currants, gooseberries, and other berries)
RF	-6,161.90	-44,735.34	-1,982.92	-5,718.51	-61,669.51	-3,316.39
CFD	-5,569.40	-23,737.22	-2,307.50	-2,290.22	-18,039.27	-1,233.20
NWFD	-2,099.86	-12,364.04	-832.39	-943.62	-7,329.13	-502.48
SFD	4,396.97	20,092.93	2,713.48	353.17	-3,658.83	-31.92
NCFD	779.80	10,296.26	2,250.47	568.72	1,227.18	-504.86
PFD	1,724.97	-10,197.42	-1,581.59	-957.27	-13,180.71	-57.31
UFD	-1,835.70	-9,831.46	-732.37	-740.06	-6,566.62	-193.44
SFD	-2,448.61	-12,127.77	-1,019.30	-1,129.87	-9,485.54	-460.82
FEDD	-1,125.06	-6,991.62	-479.71	-587.37	-4,694.59	-339.37

Source: Calculated according to Rosstat data (2021)

Table 13. Production of basic foodstuffs for domestic consumption by the population in the Russian Federation in (2036)

	Recommended rational rate of food consumption, kg	Production volumes, million tons, billion pcs.	It is necessary to produce currently, million tons, billion pieces.	It is necessary to produce in 2036 (option), million tons, billion pieces.		
				High 150.1	Average 143.0	Low 134.3
Pig meat	18	4.3	2.6	2.7	2.5	2.4
Poultry meat	31	5.0	4.6	4.7	4.5	4.2
Cattle meat	20	1.6	2.9	3.0	2.8	2.7
Milk	325	32.2	47.7	48.7	46.3	43.9
Eggs and egg products	260	45.1	38.0	39.0	37.2	34.9
Sugar	24	7.3	3.5	3.6	3.4	3.2
Vegetable oil	12	4.6	1.7	1.8	1.7	1.6
Potato	90	19.6	13.1	13.5	12.9	12.1
Melon food crops	15	1.5	2.2	2.2	2.1	2.0
Vegetables of open and protected ground	125	13.9	18.3	18.7	17.8	16.8
Grape	6	0.7	0.9	0.9	0.9	0.8
Drupaceous	8	0.6	1.2	1.2	1.2	1.1
Pomaceous	58	2.3	8.5	8.7	8.3	7.8
Berries	7	0.7	1.0	1.0	1.0	0.9
Bread products	96	7.4	14.0	14.4	13.7	12.9
Grain for domestic consumption	-	78.5	86.2	87.9	83.6	79.3

In addition to the shortage of meat and milk, the population of Russia was experiencing a lack of consumption of vegetables, fruits, and berries. All federal districts, except for the Southern Federal District, the North Caucasian Federal District, and the Privolzhsky Federal District, were experiencing a deficit in melons and gourds. There was no shortage in the production of vegetables, grapes, and stone crops in the Southern Federal District and the North Caucasus Federal District. For the production of seed crops, there is no shortage only in the North Caucasus Federal District (Table 11, 12).

To meet the internal needs of the population for food according to rational norms at the national level, in all variants of the forecast, the production of pig and poultry meat can be left at the current level. Therewith, the demand for pig meat is 2.4-2.7 million tons, and for poultry meat 4.2-4.7 million tons. (Table 13). The production of cattle meat should be increased from 1.6 to 2.7-3.0 million tons. The production of melon food crops should also be increased from 1.5 million tons to 2.0-2.2 million tons (Kolesnikov 2019).

In the future, vegetable growing of protected and open ground will require close attention. The population of the Russian Federation is experiencing a significant shortage in vegetable production, so the production of vegetables on the open and protected ground needs to be increased from 13.9 to 16.8-18.7 million tons. It will also require a slight increase in the production of grapes and stone crops, reserves for the placement of which are available in the North Caucasus Federal District and the Southern Federal District. It will also require an increase in the production of seed crops from 8.5 to 8.7 million tons (Ushachev et al 2019).

In addition, bringing the volume of meat and milk production will contribute to an increase in domestic grain consumption from 78.5 million tons to 79.3-87.9 million tons. Such an increase can be compensated either by the growth of grain production without a decrease in export volumes or without a significant increase in production volumes due to a decrease in export volumes. Increasing the volume of production of basic types of food is necessary to form 100%

of the economic availability of food to the population of the Russian Federation (Ushachev et al. 2019).

CONCLUSION

The findings of the present study suggests that the development of agriculture is characterized by significant, but insufficient success in solving the problem of ensuring food security, therefore, the further development of agricultural production will require significant efforts and state support related to the development of agricultural production in the following areas: increasing the use of domestic scientific and technological potential; an increase in state support for the production of basic foodstuffs; the growth in the volume of exports of agricultural products of high processing; development of rural territories from the standpoint of social and engineering infrastructure, creation of favorable conditions for life in rural areas; stimulating an increase in wages in agricultural organizations in proportion to the growth of labor productivity; the introduction of digital technologies.

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Technological Communication

Universal Robotic Platform for Diagnosis and Spot Spraying of Trees and Shrubs

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ABSTRACT

The number of threats to the plants is constantly increasing. The harmful impact of pests and plant diseases costs billions of dollars to the global economics each year. Chemical protection of plants from diseases and insects became an urgent problem in crop production, forestry and parks. The study aimed to develop an effective mobile robot for treating trees and shrubs from insects and diseases by spraying with chemicals. The work used monographic and general scientific research methods. A review of existing designs and technologies, as well as an analysis of publications in leading scientific journals, led to the conclusion that there is no one generally accepted technology for remote diagnosis and spot spraying of single trees and shrubs. Most of the technical means used have significant technical and environmental limitations. Currently, there is no unambiguous decision about which machine and technology to choose for solving the problems of remote diagnostics and spot spraying of single plant objects with a height of 2 to 4 meters. Taking into account the identified requirements, a robotic platform was developed for diagnosing and spot spraying of ornamental and fruit trees and tall shrubs. The developed robot is equipped with the necessary set of equipment for moving around the territory and spraying objects, has an increased cross-country ability, works according to a given program, has a minimal environmental impact, and can record data for diagnosing problems on plants. The proposed universal robot can work in agricultural, forest and urban areas, in warehouses and production facilities.

KEY WORDS: PLANT PROTECTION, SPOT SPRAYING, UNIVERSAL ROBOT.

INTRODUCTION

Chemical protection of trees and shrubs from insects and diseases in the form of processing them with special preparations is an urgent problem in crop production, forestry and parks. The processed objects in this case are fruit trees and tall shrubs in agricultural areas, deciduous and coniferous trees in forest plantations and protective belts along agricultural fields, decorative objects in the form of freestanding trees and tall shrubs in parks, squares and other areas in the city. Most often used, mounted and trailed sprayers are used for treating trees and tall shrubs with chemicals. In this case, the main limitations and disadvantages are significant height of the processed objects, high consumption of chemicals during continuous processing with fan sprayers, low efficiency in case of continuous processing, since a significant part of the drug falls on the soil, and not on the leaves and fruits,

the obligatory presence of a wide road and a large area for movement and turning of a tractor with a sprayer, the presence of noise and exhaust gases during the operation of the tractor, which is especially undesirable for parks and other recreation areas in cities, damage to lawns from tractor wheels when working in the city, the danger of a moving vehicle for people, especially within the city, the inability to accurately diagnose the degree of damage to a plant object by insects and diseases, especially on the crown of a tree, impossibility to reliably assess the quality of the processing of the object (Ahmad et al. 2020).

Due to the above limitations and technology shortcomings, these machines are usually used in agricultural and forest areas. They are usually unacceptable for the city. One of the options for solving the problem, especially in cities, is modern technologies based on the use of unmanned aerial vehicles. There is a technology for spot processing of plant objects using spray modules installed on unmanned aerial vehicles (Ahmad et al. 2020). However, the use of this technology has significant limitations. The main limitation is the low carrying capacity of the aircraft (no more than 10 kg of the working solution). The second limitation is

Article Information:*Corresponding Author: agro-kem@rambler.ru

Received 14/10/2021 Accepted after revision 14/12/2021

Published: 31st December 2021 Pp- 1940-1945

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.83>

the high cost of aircraft with the specified payload. The third limitation is the short flight time of the apparatus and the need for regular refueling of the reservoir of the spray module. The fourth limitation is the need to engage a professional aircraft operator (Wang et al. 2020).

The qualifications of such an operator should be higher than in conventional photography of territories, since he is required to be able to construct and program with high accuracy a special trajectory of the aircraft's flight. This trajectory should take into account the limited flight time of the aircraft, the need to constantly change the flight altitude due to the different heights of the processed objects, the change in the mass and balance of the aircraft due to the gradual flow of fluid. In addition, it is often necessary to legally issue a flight permit, there is a risk of falling and breakdown of the aircraft, there is a dependence of flights on weather conditions, it is impossible to process the vertical and steep surfaces of objects, etc (Wang et al. 2020).

We see the solution to the problem in the use of a small-sized mobile robot with equipment installed on it for spraying tall plants. We carried out a literary review of scientific publications concerning the possible design options for the robot and the technology for spraying plants with it. Based on the modular principle proposed in studies, it is possible to develop a robotic platform that performs a wide range of plant protection tasks (Levin and Degani 2016). This platform can move through the territory on the basis of operator commands from the control panel or with the help of autonomous navigation, built on modern hardware and software, considered in studies (Oltean 2019; Wang et al. 2020).

The choice of the optimal movement mode depends on the type of territory and the characteristics of the objects being processed. Evaluation of the effectiveness of modes of interaction between the operator and the remotely controlled agricultural robot-sprayer can be carried out on the basis of the methodology described in studies (Adamides et al. 2017). An assessment of the characteristics of spraying greenhouse crops with pesticides by a remote-controlled robot was proposed in studies (Rincon et al. 2020). For us, this article is useful from the point of view of organizing the movement of the robot in a limited area of a small area. The processes of deposition and distribution of the sprayed liquid on the objects to be sprayed and on the ground, depending on the volume of application, the speed of the air flow and the position of the objects, are considered in studies (Musiu et al. 2019; Rincon et al. 2020).

The described technique can be used to assess the quality of plant spraying by a robot under various process parameters. Aerodynamics and analysis of the quality of liquid spraying during spot spraying by a robot from a close distance using compressed air is considered in studies (Malnersic et al. 2016). For our research, this article is of interest from the point of view of optimizing the parameters of pneumatic and hydraulic systems that regulate the intensity and shape of the fluid sprayed by the robot. The work by Oberti et al. (2016) is devoted to spot spraying of bushes in the fight against diseases using a modular agricultural robot. The

results presented in it can be useful in creating hardware and software tools for diagnosing the state of the processed object and making a decision by the robot about spot spraying of the plant surface. The design synthesis and analysis of the working space of a telescopic robot sprayer is considered in studies (Zu-Yun et al. 2019). For us, this article is interesting from the standpoint of developing the design of a vertical telescopic boom for spraying tree crowns. An important factor in increasing the efficiency of the robot sprayer is the development of adequate systems that allow assessing the degree of damage to plants by insects and diseases, as well as systems for automated analysis of the geometric characteristics of the processed object (Rincon et al. 2020).

The method of multifunctional analysis of a tree trunk in a garden using a camera and ultrasonic sensors is described in studies (Chen et al. 2018). Intelligent spraying systems based on various sensors in agriculture are considered in studies (Abbas et al. 2020). An intelligent robotic sprayer based on plant volume is proposed in studies. The information presented in these articles was useful when choosing sensors and cameras in the diagnostic systems of the robotic platform we developed. When operating the robot sprayer, the safety requirements must be observed. The possible effect on the skin of operators using pesticides when spraying crops is discussed in studies (Rincon et al. 2018; Hejazipoor et al. 2021). The issues of safety and ergonomics of interactive agricultural human and robot operations are described in studies (Benos et al. 2020). The materials of these articles were taken into account when developing instructions for the safe operation of the developed robotic platform.

MATERIAL AND METHODS

In the presented article, monographic and general scientific research methods were used. The materials for the study were publications in leading scientific journals.

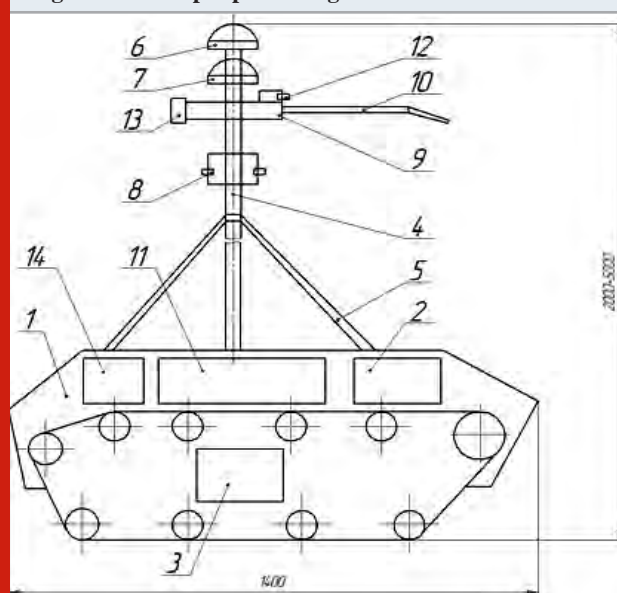
RESULTS AND DISCUSSION

The main requirements for the functioning of the developed robotic platform for diagnosing and spot treatment of trees and tall shrubs were increased cross-country ability, the ability to move from one processing object to another according to a given program or from a remote control panel, the ability to navigate in space, taking into account the boundaries of the cultivated area and the presence of obstacles in the form of shrubs, trees, poles, fences and other objects on the territory, minimal harmful impact on the environment, high safety for the operator and other personnel on the territory, continuity of autonomous operation without recharging the batteries for at least ten hours, the ability to process objects up to 4 m high, the possibility of photographing and video filming of plant objects for their diagnosis and decision-making on the need and intensity of chemical treatment, the minimum duration of the working cycle (moving to the object, lifting the vertical bar to the desired height, monitoring the object, diagnosing a possible problem, processing the object by spraying, recording the results of diagnostics and chemical

processing on an electronic carrier, lowering the vertical bar to its initial position) (Hejazipoor et al. 2021).

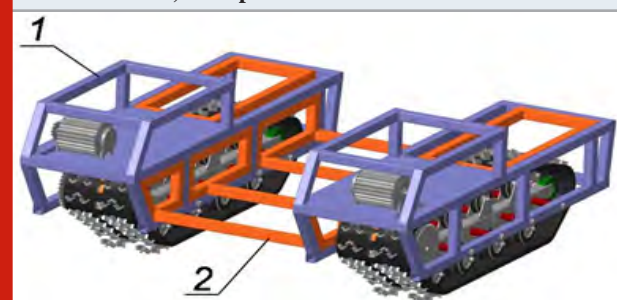
Taking into account the literature review and the above basic requirements, we have designed and manufactured a prototype of a robotic platform for diagnosing and spot spraying trees and tall shrubs. A conceptual diagram of a robotic platform is shown in Figure 1.

Figure 1: Scheme of a robotic platform for high-altitude diagnostics and spot processing:



1 - chassis; 2 - gear motor; 3 - battery pack; 4 - vertical telescopic bar; 5 - diagonal support; 6 - antenna; 7 - additional antenna; 8 - transport video cameras; 9 - working body; 10 - horizontal rod of the working body with nozzles; 11 - hydraulic equipment; 12 - balancer of the working body; 13 - control unit for movement, spraying, vertical boom, video cameras.

Figure 2: Chassis of the robotic platform. 1 - independent tracked mover; 2 - replaceable frame.



All operating actuators, diagnostic equipment and control equipment of the robotic platform were installed on chassis 1. Moving across the territory between the objects of processing was carried out by two onboard caterpillar propellers, each of which was driven by an individual DC electric motor 2. The power of the electric motors 2 was carried out from the battery pack 3. A vertical telescopic rod

4 was installed on the chassis. The height of the stays was remotely changed from 2 to 5 meters thanks to linear drives (electric actuators). The stability of the bar was ensured by four diagonal supports 5, which were rigidly fixed to the bar 4 at a height of 1.5 m (Rincon et al. 2020).

An antenna 6 was installed at the highest point of the stays 4 for receiving and transmitting signals to the operator. An additional antenna 7 was fixed next to it to expand the functionality of the robotic platform. In the upper part of the boom 4 there were several video cameras 8 broadcasting the image to control the trajectory of movement. In addition to video cameras, radars, lidars, GPS-module and other modern equipment were installed to optimize the route. Between the video cameras 8 and the additional antenna 7, a working body 9 was installed for spraying shrubs and trees with a height of 1 to 4 meters. The range and angle of spraying, the shapes of the spray pattern were provided by a remotely controlled horizontal boom 10 with nozzles. The supply of working fluid to the nozzles was carried out by remotely controlled hydraulic equipment 11, including a reservoir with a chemical solution, a hydraulic pump, an electric motor of a hydraulic pump, hydraulic valves.

Monitoring of the state of vegetation and quality control of processing objects was provided by a video camera 12 of the working body. Compensation of the cantilever arrangement of the horizontal rod of the working body was carried out by a symmetrically located balancer 13. The control unit 14 performed the function of processing and fixing information about the position of the robotic platform, the speed of movement, the presence of obstacles on the route and on the horizon. This block, in addition to the motion control function, also controlled the working body 9, the vertical bar 4, and video cameras. The chassis of the robotic platform consisted of two independent tracked propellers 1 connected by a replaceable frame 2 (Figure 2) (Rincon et al. 2020).

This arrangement simplified the design that provided high maneuverability and maneuverability of the machine. This increased its versatility due to the variable track width. A significant influence on the performance of the robotic platform was exerted by the resistance force due to the interaction of its running systems with the soil surface. For the selection of electric motors and batteries, the resistance force to the movement of the developed robotic platform was determined. The main part of the above force was the resistance force arising from the deformation of the soil by tracks, determined by the formula:

$$P_f = \frac{2b\sigma_0^2\eta_\delta}{k} \ln ch \frac{p_c}{\sigma_0\eta_\delta},$$

where b - width of caterpillar, $b = 200$ mm;

k - coefficient of volumetric crushing of the soil, $k = 0,15 \cdot 10^{-7} \text{H/m}^3$;

σ_0 - ultimate strength for uniaxial soil compression, $\sigma_0 = 2,74 \text{MPa}$;

η_{δ} - efficiency of slipping of the caterpillar propeller, $\eta_{\delta} = 0,97$;

p_c - average pressure on the soil, $p_c = \text{kPa}$.

With a uniform distribution of pressure along the length of the support branch of the track, the value of the average pressure was determined by the formula:

$$p_c = \frac{G}{2bL_0},$$

where G – full operational weight of the robotic platform, $G = 3000 \text{ H}$;

L_0 – length of the caterpillar's bearing surface, $L_0 = 800 \text{ mm}$.

$$p_c = \frac{3000}{2 \cdot 200 \cdot 800} = 9,38 \text{ кПа}$$

$$P_f = \frac{2 \cdot 200 \cdot 2,74^2 \cdot 0,97}{0,15 \cdot 10^{-7}} \ln ch \frac{9,38}{2,74 \cdot 0,97} = 686,4 \text{ H}$$

In real conditions, the robotic platform periodically moves uphill. The second part of the resistance force, taking into account the upward movement, was determined by the formula:

$$P_{\alpha} = G \sin \alpha,$$

where α – maximum slope of the agricultural area, $\alpha = 10^\circ$.

$$P_{\alpha} = 3000 \cdot \sin 3^\circ = 520,8 \text{ H}$$

$$P_o = 686,4 + 520,8 = 1207,2 \text{ H}$$

The required total power of the drive motors of the robotic platform was determined by the formula:

$$N_e = P_o \cdot v_{\max},$$

where v_{\max} – the maximum speed of movement, $v_{\max} = 1,94 \text{ m/c}$ (7 km/h).

$$N_e = 1207,2 \cdot 1,94 = 2342 \text{ W}$$

Thus, two 1.5 kW motors were installed on the robotic platform. The required total power of the storage batteries was determined by the formula:

$$N_{ak} = \frac{N_e}{\eta},$$

where η – overall efficiency of a robotic platform powered by electric traction.

$$\eta = \eta_r \eta_{\text{зд}} \eta_k,$$

where η_r - transmission efficiency, $\eta_r = 0,76$;

η_e - efficiency of drive electric motors, $\eta_e = 0,9$;

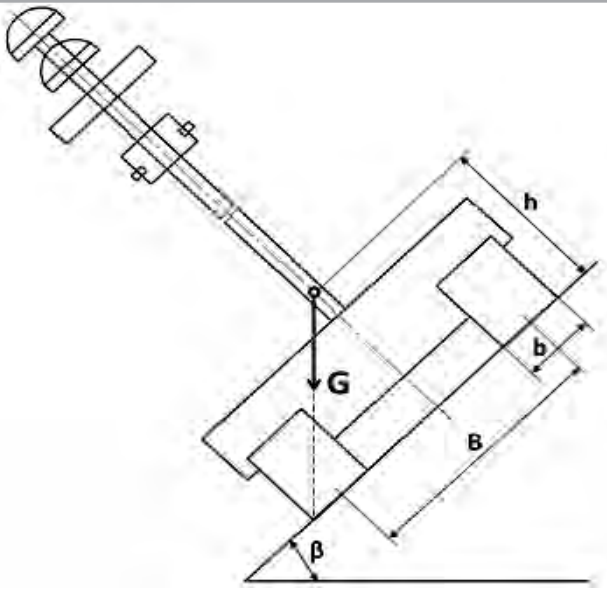
η_k - efficiency of controllers, $\eta_k = 0,95$.

For the accepted operating conditions, the required total capacity of the storage batteries was

$$N_{ak} = \frac{2342}{0,76 \cdot 0,90 \cdot 0,95} = 3604 \text{ Вт}$$

Based on the calculations performed, the choice of specific brands of electric motors and batteries was made. DC motors of the above power were installed on the robotic platform, providing stepless speed control. Taking into account the additional energy consumption (for the electric motor of the sprayer pump, for the operation of video cameras, vertical boom actuators, control equipment, etc.), as well as the fact that the average power consumed by the robotic platform during operation was about 30% of the maximum, accumulators with a total capacity of 300 A·h (Abbas et al. 2020).

Figure 3: Scheme for calculating the lateral stability of a robotic platform



This ensured continuous operation for ten hours. After that, the batteries were charged, which was performed simultaneously with other daily maintenance work on the robotic platform. To achieve high performance of the robotic platform, gel batteries were installed. Their main advantages were: long service life, a large number of charging cycles, no need for urgent charging in the event of a deep discharge, small capacity losses during idle time, safety of operation due to the impermeability of the case. It should be noted that one of the advantages of the robotic platform was the ability to install a replaceable central section. Three sections were manufactured, providing track widths of 700, 900 and 1100 mm. This allowed the track gauge to be changed at

no significant cost to meet new challenges. An essential factor in the safe operation of a robotic platform was its lateral and longitudinal stability when moving over an area with a slope. The lateral stability calculations of the robotic platform were performed for the narrowest center section (Abbas et al. 2020). The design scheme is shown in Figure 3.

Preliminarily, at a special stand, measures were taken to determine the height of the center of gravity of the robotic platform without a vertical boom and equipment for diagnostics and spraying installed on it. At the stand, it was revealed that with a transverse tilt, a robotic platform with a minimum track width of $B = 0.7$ m loses its stability at an angle $\beta_0 = 53^\circ$.

To determine the height of the center of gravity, the formula was used:

$$h_o = \frac{0,5(B + b)}{\text{tg } \beta_o},$$

where B – minimum track width, $B = 0,7$ m.

$$h_o = \frac{0,5(0,7 + 0,2)}{1,324} = 0,34 \text{ m}$$

Calculation of the limiting angle of lateral stability was made according to the formula:

$$\beta = \text{arctg } \frac{0,5(B + b)}{h}$$

Further, the value of the angle β was determined for a robotic platform with vertical rods 2 and 5 m high. Intermediate calculations showed that when equipped with these rods, the height of the center of gravity was, respectively, $h_2 = 0.42$ m, $h_5 = 0.52$ m.

$$\beta_2 = \text{arctg } \frac{0,5(0,7 + 0,2)}{0,42} = 47^\circ$$

$$\beta_5 = \text{arctg } \frac{0,5(0,7 + 0,2)}{0,52} = 41^\circ$$

Since the motors were installed in the front of the robotic platform, the center of gravity was shifted forward along the longitudinal axis. The displacement value was determined on a special stand and amounted to $a = 0.04$ m. The design scheme is shown in Figure 4.

Calculation of the limiting angle of longitudinal stability was carried out according to the formula:

$$\alpha = \text{arctg } \frac{0,5L_o - a}{h}$$

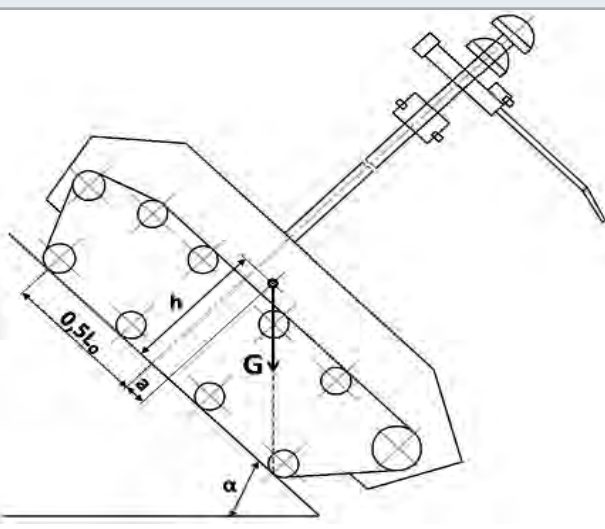
The value of the angle α was determined for the height of the vertical rod of 2 and 5 m.

$$\alpha_2 = \text{arctg } \frac{0,5 \cdot 0,8 - 0,04}{0,42} = 41^\circ$$

$$\alpha_5 = \text{arctg } \frac{0,5 \cdot 0,8 - 0,04}{0,52} = 35^\circ$$

The calculation results showed that the limiting angles of longitudinal stability were less than the angles of lateral stability. Particularly dangerous from the point of view of overturning was the robotic platform movement down the slope. Taking into account that agricultural areas usually have slopes of no more than 10° , and then the calculated critical angle of 35° provided the robotic platform with guaranteed stability even with a maximum height of the vertical telescopic rod of 5 m. The robotic platform had a high degree of versatility. It allowed not only to diagnose problems and to carry out pinpoint processing of plant objects from 1 to 4 meters in height in fields and gardens (Abbas et al. 2020).

Figure 4: Scheme for calculating the longitudinal stability of a robotic platform



This robot diagnosed and spotted treatment with special anti-corrosion and antiseptic solutions, foci of corrosion on the roofs of one-story buildings, foci of corrosion and mold on ventilation and water supply systems, on the walls and ceilings of warehouses, garages, elevators, livestock farms, etc. The treatment of trees and shrubs from insects and diseases by spraying with chemicals was an important factor in maintaining the health and longevity of these plants, as well as consistently obtaining high yields. Most

of the technologies and machines used for this (spraying modules on unmanned aerial vehicles, mounted and trailed sprayers, etc.) were characterized by high economic costs, have technical and environmental restrictions. Therefore, for agriculture, these technologies and machines in a number of cases require justification, and for urban conditions they were often generally unacceptable (Wang et al. 2020).

CONCLUSION

The findings of the present study showed that the developed robot has an increased cross-country ability due to a caterpillar propeller, can move between processing objects according to a given program and from a remote control panel, is able to navigate in space, taking into account the boundaries of the processed area and the presence of obstacles, due to the use of an electric drive, it has a minimal environmental impact, is able to take photographs and video filming of objects to diagnose problems. The developed robot is versatile; it can be used in agriculture, in urban park areas, in warehouses and production facilities.

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Biotechnological Communication

A Computational Approach to Predict the Molecular Drug Targets Against *Candida glabrata*

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ABSTRACT

Non- albicans *Candida* (NAC) species are responsible for 35-65% of all candidaemias in the general population and are associated with a high rate of morbidity and mortality (about 15% to 35%). The availability of few commercially used antifungal drugs against candidiasis and rapid emergences of antibiotic resistance among NAC species has significantly contributed to their increased global outbreak. Green tea is known for its multi-beneficial effects including antimicrobial potential against *Candida*. The present study investigated the molecular drug targets of green tea phytochemicals against inhibition of ergosterol biosynthesis in *Candida glabrata* using *in silico* tools. The molecular interaction was studied between ligands and essential proteins participating in ergosterol biosynthesis in *C. glabrata* using autodockvina software. The protein validation and homology modeling estimation were determined by the SWISS MODEL workspace. The Drug likeness study of all the test ligands was performed using SwissADME, while the toxicity of test compounds was analyzed using the admetSAR 2.0 version. The *in silico* analyses identified Rutin, Chlorogenic acid, Coumaroylquinic acid, Quercetin, Epigallocatechingallate as the potent phytochemicals with significant molecular binding with Erg 6, Erg 27, Erg 8, Erg 7, Erg 24 respectively. The ADMET data suggested an absence of the CYP2 inhibitors indicating the metabolism of all the tested drug candidates in the intestine and liver. The present study highlighted the possible drug targets of green tea phytochemicals against ergosterol biosynthesis protein in *C. glabrata*. It is pertinent that the current study has provided preliminary breakthroughs which could lead to exploring their avenues in potent drug development against NAC species.

KEY WORDS: AUTODOCKVINA, CANDIDA, EGCG, ERGOSTEROL, RUTIN.

INTRODUCTION

Over the last decade, the fungal infections associated with the non-albicans *Candida* species have become a global cause of concern for health professionals (Sardi et al. 2013). Candidiasis is a common fungal infection caused by yeasts from the genus *Candida*. The disease is associated with a diverse range of infections including mucosal, cutaneous, subcutaneous, and systemic mycoses. Candidiasis which was once considered to be majorly caused by *C. albicans*, other non- albicans *Candida* (NAC) species have been recognised to be primary pathogens associated with increase incidences of Candidiasis. Epidemiological studies have shown that NAC species are responsible for 35-65% of all candidaemias in the general population (Presterl et al. 2007; Arendrup, 2014; Ahmed et al. 2020). The most common NAC species include *C. parapsilosis* (20-40% of all *Candida* species), *C. tropicalis* (10-30%), *C. krusei* (10-35%), *C. glabrata* (5-40%), *C. auris* (5%), *C. lusitanae* (2-8%), and

C. guilliermondii (1-5%). Other rare NAC species, are *C. rugosa*, *C. kefyr*, *C. stellatoidea*, *C. norvegensis* and *C. famata* ($\leq 1\%$ of *Candida* infection) (Du et al. 2020).

These NAC species are also identified to be associated with a high rate of morbidity and mortality (about 15% to 35%) particularly in individuals who are immunocompromised like organ transplant recipients, HIV patients, and patients under chemotherapy (Krcmery and Barnes 2002; Bitar 2014; Rudramurthy et al. 2017; Du et al. 2020). A progressive shift in NAC species infection has been linked with indiscriminate use of antibiotics, severe immunosuppressants in immunocompromised such as HIV, cancer or organ transplant patients. The studies have reported that several NAC spp. are inherently resistant to common antifungal drugs and have demonstrated acquired/cross-resistance against antifungals like triazoles (Du et al. 2020).

Indeed, with the availability of a few antifungal drugs commercially used in the treatment of candidiasis and rapid emergences of toxic side effects as well as antibiotic resistance among NAC species, the increased outbreak of NAC associated fungal infection are becoming a global

Article Information:*Corresponding Author: nishantrai1@gmail.com

Received 18/09/2021 Accepted after revision 25/12/2021

Published: 31st December 2021 Pp- 1946-1955

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.84>

threat (Magill et al. 2006; Pfaller and Diekema 2007; Oberoi et al. 2012; Deorukhkar et al. 2014; Chowdhary et al. 2014; Du et al. 2020). Green tea known for its multi-factorial health properties has been scientifically recognised as a possible antifungal drug representative that could along with standard chemotherapeutics enhance the efficacy of the general treatment approach. There is plethora of scientific evidence highlighting the antifungal potential of green tea catechins against *Candida albicans* and identified potent drug candidate's targeting key enzymes participating in ergosterol biosynthesis (Hirasawa and Takada 2004; Anand et al. 2015; Musial et al. 2020; Huang et al. 2020). As an urgent need to explore alternative potent drug candidates for the development of novel medication against NAC infection, the present study attempts to investigate the inhibitory potential of green tea phytochemicals against ergosterol biosynthesis in *C. glabrata* using in silico tools.

MATERIAL AND METHODS

In the present in silico study, we selected 15 phytochemicals present in green tea based on their reported antimicrobial activity against *Candida* spp. These phytochemicals were used as ligands for docking with the ergosterol biosynthesis proteins. Autodockvina(version is 1.1.2) tool was used for assessing the molecular interaction based on binding energies. As a positive control, we used azoles (Fluconazole, Itraconazole, and ketoconazole) and Amphotericin B in molecular docking analysis. The code of SMILES for all the aforementioned green tea phytochemicals and positive controls was procured from the online chemical database PubChem (<https://pubchem.ncbi.nlm.nih.gov/>) (Cheng et al. 2002; Diana et al. 2014).

Table1a. Green tea Phytochemicals used as ligands



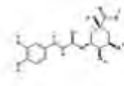


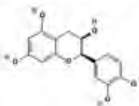
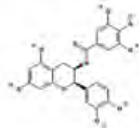
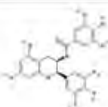
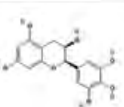
S.No.	Phytochemicals	PUBCHEM ID	SMILES code	2-D Structure
1.	2,5-Dimethyl-4-hydroxy-3-(2H)-furanone	14259114	<chem>OCC1OC(OC2=C(C)OC(C2=O)C)C(C(C1O)O)O</chem>	
2.	B-ionone	26955	<chem>CC(=O)C=CC1=C(C)CCC1(C)C</chem>	
3.	Chlorogenic acid	348159	<chem>O=C(OC1CC(O)(CC(C1O)O)C(=O)O)C=Cc1ccc(c(c1)O)O</chem>	
4.	Coumaroylquinic acid	53420248	<chem>O=C(OC1CC(O)(CC(C1O)O)C(=O)O)C=Cc1ccc(cc1)O</chem>	
5.	Dihydroactinidiolide	27029	<chem>O=C1C=C2C(O1)(C)CCCC2(C)C</chem>	
6.	Epicatechin	1203	<chem>Oc1cc2OC(c3ccc(c(c3)O)O)C(Cc2c(c1)O)O</chem>	
7	Epicatechin gallate	367141	<chem>Oc1cc(O)c2c(c1)OC(C(C2)OC(=O)c1cc(O)c(c(c1)O)O)c1ccc(c(c1)O)O</chem>	
8.	Epigallocatechin gallate	65064	<chem>C1C(C(OC2=CC(=CC(=C2)O)O)C3=CC(=C(C(=C3)O)O)OC(=O)C4=CC(=C(C(=C4)O)O)O</chem>	
9.	Epigallocatechin	1249	<chem>Oc1cc2OC(c3cc(O)c(c(c3)O)O)C(Cc2c(c1)O)O</chem>	

Table 2. Positive controls used in molecular docking study

POTENTIAL DRUG TARGETS AGAINST *CANDIDA GLABRATA* 1948

ERG proteins (Table 3). Among the 15 phytocompounds tested, Rutin and ERG 6 showed the most stable molecular interaction with the least binding energy of -10.80 kcal, compared to the positive control antifungal drugs. It is

considered that at least the docking energy, the higher will be the binding affinity of a compound with the target protein. Hence, Rutin demonstrated the best interaction among all the investigated green tea phytocompounds.

Table 3. Enzymes involved in ergosterol biosynthesis in *Candida glabrata*

S.No.	Gene	Protein	Amino acid Sequence Length
1.	ERG1	Squalene epoxidase (Erg1)	206
2.	ERG2	C-8 sterol isomerase (Erg2)	224
3.	ERG3	C-5 sterol desaturase (Erg3)	364
4.	ERG4	C-24(28) sterol reductase (Erg4)	468
5.	ERG 5	C-22 sterol denaturase (Erg5)	364
6.	ERG6	Sterol 24-C methyltransferase (Erg6)	372
7.	ERG7	Lanosterol synthase (Erg7)	733
8.	ERG8	Phosphomevalonate kinase (Erg8)	445
9.	ERG9	Squalene synthase (Erg 9)	443
10.	ERG 10	Acetyl coA C-acetyltransferase (Erg10)	398
11.	ERG11	Lanosterol 14- α - demethylase (Erg11)	533
12.	ERG12	Mevalonate kinase (Erg12)	430
13.	ERG 13	3-Hydroxy-3-methylglutaryl-coA (HMG-coA) synthase	281
14.	ERG20	Farnesyl pyrophosphate Synthetase (Erg20)	351
15.	ERG 24	Sterol C-14 reductase (Erg24)	437
16.	ERG25	C-4 methyl sterol oxidase (Erg25)	308
17.	ERG26	C-3 Sterol dehydrogenase (Erg26)	350
18.	ERG27	3- keto sterol reductase (Erg27)	348

Table 4. Protein validation and homology modeling estimation by SWISS MODEL workspace.

S.No.	Gene	Template	Sequence identity (%)	Ramachandran favoured regions (%)	Ramachandran outliers (%)	Q-mean score	Mol probity score
1.	ERG1	6c6n.1.A	39.30	94.09	1.48	-2.39	2.04
2.	ERG2	5hk1.1.A	30.54	94.13	1.11	-3.49	1.85
3.	ERG3	6sny.1.A	31.03	100	0.00	0.05	0.50
4.	ERG4	4fgs.1.A	21.49	85.98	4.44	-6.29	2.32
5.	ERG6	3mgg.1.A	18.22	92.52	0.79	-3.87	1.96
6.	ERG7	1w6k.1.A	39.61	93.64	1.11	-2.03	1.76
7.	ERG8	3k17.1.A	20.17	82.49	5.03	-7.5	2.49
8.	ERG 9	3vj9.1.A	48.65	97.37	0.58	-2.37	1.20
9.	ERG 10	5xyj.1.a	81.11	97.72	0.25	0.81	1.17
10.	ERG11	5jlc.1.A	100	96.48	0.20	-0.34	1.24
11.	ERG12	2r3v.1.A	36.67	93.32	1.87	-2.29	2.39
12.	ERG 13	2p8u.1.A	46.77	91.79	0.95	-4.13	2.09
13.	ERG20	4dem.1.A	46.33	96.37	0.29	-0.59	1.49
14.	ERG 24	4quv.2.A	36.74	92.86	2.62	-4.97	2.26
15.	ERG25	1rh5.1.A	26.09	97.44	0.00	-0.09	1.52
16.	ERG26	3lu1.1.A	20.45	90.61	1.88	-3.94	2.43
17.	ERG27	4quv.2.A	31.26	90.40	3.51	-5.63	1.98

Among other ligands, Chlorogenic acid, Coumaroylquinic acid, quercetin, *Epigallocatechin gallate* (EGCG) showed favorable molecular interaction with identified drug targets viz., Erg 6, Erg 27, Erg 8, Erg 7, Erg 24, respectively in ergosterol biosynthesis in *C. glabrata*. These findings are

in agreement with our previous *in silico* investigation in which we highlighted the inhibitory effect of green tea phytochemicals like chlorogenic acid, EGCG, kaempferitrin against *Candida albicans* (Anand et al. 2015; Anand et al. 2021).

Table 5. Binding energies of molecular interaction between ligands and ERG proteins

Green tea Phytochemicals	Ergosterol Biosynthesis (ERG) proteins																	
	Erg 1	Erg 2	Erg 3	Erg 4	Erg 5	Erg 6	Erg 7	Erg 8	Erg 9	Erg 10	Erg 11	Erg 12	Erg 13	Erg 20	Erg 24	Erg 25	Erg 26	Erg 27
Gallic acid	-6.8	-7.8	-5.6	-6.5	-7.9	-6.7	-6.7	-6.6	-6.3	-5.6	-6	-6	-6.4	-6.1	-7.2	-6.4	-6.3	-7.2
Epicatechin	-6.8	-7.9	-6.6	-6.8	-7	-7.4	-7.9	-7.2	-6.9	-5.7	-6.5	-6.8	-7.3	-5.9	-7.5	-6.5	-6.8	-7.8
Dihydroactinidiolide	-6.8	-8.6	-7	-7.6	-7.9	-7.4	-7.9	-7.3	-6.9	-5.7	-6.5	-6.3	-7.4	-5.9	-7.5	-6.1	-6.9	-7.8
Pyrazine	-4.1	-4.5	-3.9	-3.8	-4.5	-4	-3.9	-4.3	-3.6	-3.3	-3.7	-4.1	-3.7	-4.1	-4	-3.8	-3.6	-3.9
Chlorogenic acid	-8.7	-8.9	-8.1	-8.8	-8.9	-9.3	-9.5	-8.6	-7.9	-7.7	-7.8	-7.1	-9	-9.2	-9.3	-7.8	-8.4	-9.4
Coumaroylquinic acid	-8.9	-8.7	-8.5	-7.4	-9.2	-9.2	-9.6	-10.1	-7.8	-7.2	-8.6	-7.4	-8.6	-9.3	-9.7	-7.6	-8.8	-9.6
Quercetin	-7.6	-7.8	-7.8	-8.1	-8.8	-8.6	-9.8	-8.9	-8.9	-7	-7.8	-7.8	-7.8	-8.1	-9	-8.8	-8.7	-7.6
Epigallocatechin	-8.3	-7.9	-7.3	-7.4	-7.8	-8.5	-8.7	-6.9	-7.6	-6.3	-7	-6.2	-7.6	-8.1	-7.9	-6.7	-8.6	-6.9
Epigallocatechin gallate	-8.5	-8.5	-7.5	-8.9	-8.5	-10.5	-4.5	-9.8	-9.1	-8	-10	-7.9	-8.7	-8.6	-10.3	-8.4	-9.1	-8.8
Myricetin	-8.6	-9	-7.6	-7.8	-9.1	-9.1	-9.6	-9.7	-8.8	-7	-8.4	-7.6	-8.7	-7.8	-9.7	-8	-9.3	-8.9
β-ionone	-6.7	-7.2	-6.9	-7.9	-7.2	-7.2	-8.2	-7.2	-7.3	-5.3	-7.1	-6.5	-7.1	-6.3	-7.1	-7.2	-6.7	-8.3
Epicatechingallate	-9.1	-10.1	-9.1	-9.1	-10.1	-10.8	-8.2	-8.2	-9.3	-7.9	-8.7	-9.1	-9.3	-10	-10.9	-8.5	-8.6	-8.2
Kaempferitrin	-11.2	-9.5	-9.5	-10.6	-9.5	-12	-7.7	-9.3	-10.1	-7.9	-9.4	-8.9	-9.9	-8.5	-12	-9.2	-10.7	-9.1
Rutin	-8.2	-8.6	-9.3	-10.1	-8.8	-10.8	-4.4	-8.6	-10.8	-7.6	-10.5	-9.5	-9.1	-8.2	-11.4	-8	-9.2	-8.5
2, 5- Dimethyl-4- hydroxy-3- (2H) – furanone	-5.5	-7	-4.8	-5.5	-7	-6.2	-5.4	-5.5	-5.2	-4.7	-5	-5.4	-5.5	-5.5	-5.8	-5.1	-5.1	-5.3

Table 6. Binding energies of molecular interaction of positive controls and ERG proteins

Inhibitors (antibiotics/drugs)		Targetreceptor	Binding energy
Azoles	Fluconazole	ERG11	-7.62
		ERG 5	-8.24
	Ketoconazole	ERG 5	-11.93
	Itraconazole		-11.69
Amphotericin B		ERG	-12.97

Table 7. Most favorable docking poses for the molecular interaction between green tea ligands and ERG proteins.

S.No.	Most significant molecular bindings	Most favourable docking poses
1	ERG 27 and Chlorogenic acid	ALA 108, PHE 244, ASN 18, SER 17, GLY 14, LYS 48, ARG 44
2	ERG 6 and Rutin	TRY 84, SER 95, GLY 251, LEU 344, ASN 284, TRY 288, PHE 318, ARG 314
3	ERG8 and Coumaroylquinic acid	ALA151, SER 150, LYS 191, ALA 377, GLY 148, SER 149, THR 145, LEU 147, GLY 146, LYS 144, PHE 84, PRO 269
4	ERG 7 and Quercetin	TYR 91, HIS 226, GLY 377, TRP 583, TYR 703, PHE 695
5	ERG 24 and EGCG	ASP 136, GLU 145, TRP 205, TRP 233, MET 230

The Ramachandran plot depicted structural stability and showed confirmation of residues in the favorable region (Figure 1; Table 4). A Ramachandran phi-psi plot for all ERG proteins revealed 82.49% to 100.00% of residues are in the allowed region (light gray), and only 0.00% to 4.44% lay in the disallowed region (white). The above analysis of the predicted structure provides supporting evidence that the predicted 3D structure of ERG protein is of good quality. The protein models of ERG proteins showed local similarity to the crystal structures of target templates (Table 4). The Q mean value of protein models was reliable as depicted in the estimated Figure 2. Based on the binding scores, rutin interacted with ERG 6 at the amino acid residues TRY-84, SER-95, GLY-251, ASN-284, TRY-288, ARG-314, PHE-318, and LEU-344 with the least binding energy of -10.80 kcal (Figure 3, Table 5, Table 6, Table 7) (Gao et al. 2020; Anand et al. 2021).

Figure 1A: Ramachandran plot of Erg3

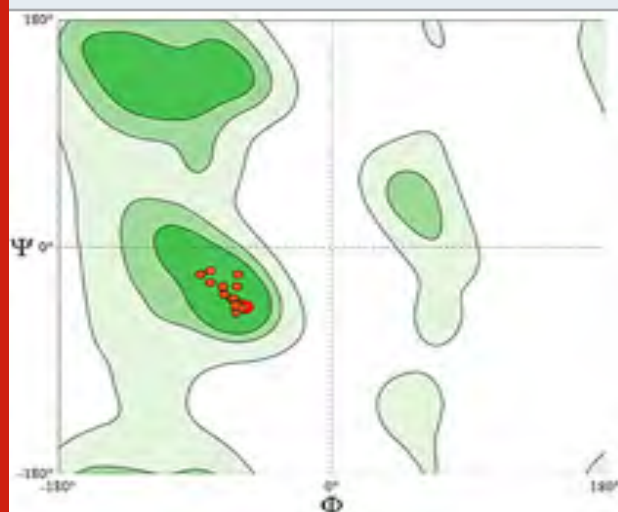


Figure 1B: Ramachandran plot of Erg9

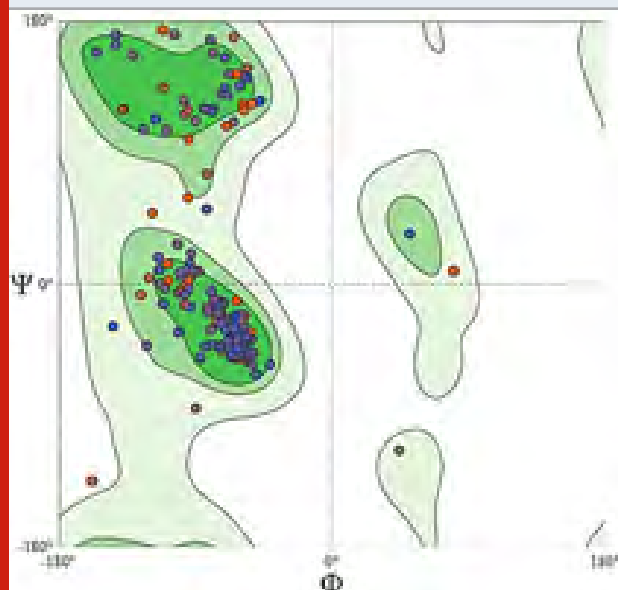


Figure 1C: Ramachandran plot of Erg 25



Figure 1D: Ramachandran plot of Erg 27

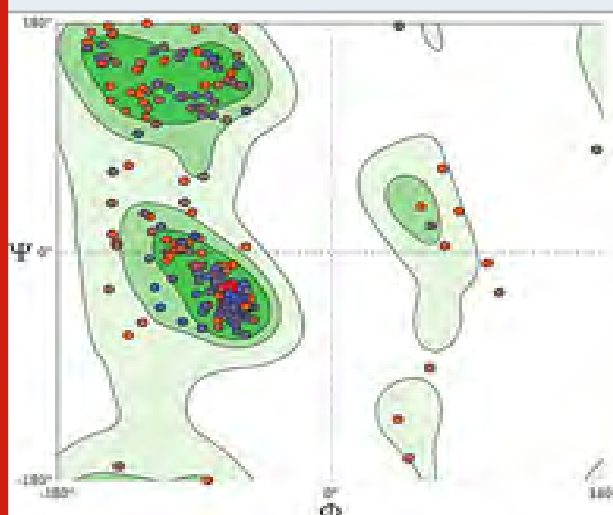
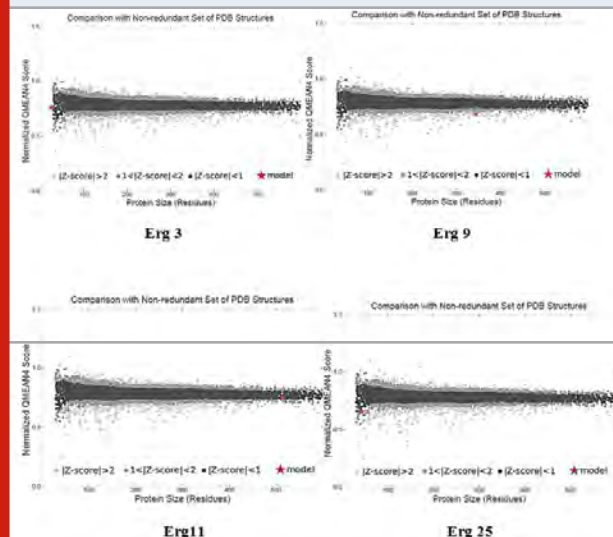


Figure 2: Q mean Z-score value of ERG proteins



Mammalian metabolism and toxicity of all the proposed green tea phytochemicals as potent antifungal drug agents were tested through ADMET analysis. The ADMET analysis depicted that out of 15 ligands, 13 compounds are non-toxic and obeyed Lipinski's rule of five. The drug likeliness report determined that Kaempferitrin, rutin, and EGCG are toxic as they have violated two to three rules from Lipinski's rule of five. Their toxicity level could restrict its application as a potent candidate for antifungal agents; however, before their therapeutic application, the dose regime of these drugs is necessary to be considered and analyzed for the drug toxicity. The solubility, dissolution, and GI permeability of any drug are essential parameters to be determined before their associated medical effect can be induced. These factors are rate-limiting steps for pre-formulation interpretations in drug development (Shekhawat and Pokharkar, 2017; Mitra et al. 2021). In our study, Dihydroactinidiolide and B-ionone, epicatechin, Epigallocatechin, gallic acid, pyrazine, and Quercetin demonstrated high GI absorption, while other ligands were observed to be considerably soluble (Gao et al. 2020; Anand et al. 2021).

Concerning drug potency, the ability of drugs to cross blood-brain barriers (BBB) is estimated. The present study estimated and indicated poor membrane permeation properties of all the tested 13 ligands except Dihydroactinidiolide and B-ionone (Table 8). CYP2 are the family of drug-metabolizing subsets that are involved in the biotransformation of drugs, xenobiotics. The ADMET data suggested an absence of the CYP2 inhibitors indicating metabolism of all drug candidates in the intestine and liver (Table 8) (Tran 2011; Palleri et al. 2013). The Bioavailability score which is an integral part of the pharmacokinetics paradigm was evaluated less than a standard score of 0.55 for chlorogenic acid, EGCG, kaempferitrin, and rutin and thus depicted their poor bioavailability (Martin 2005). The co-existence of CYP3A4 and Pgp at the same site acts synergistically to reduce the bioavailability of the drug (Mohamed and El-Kadi 2012). Recent studies have also reported poor bioavailability of Rutin, EGCG, however novel identified transporter system in human can regulate the increase the bioavailability of EGCG (Ishii et al. 2019; Fatima et al. 2020).

Figure 3: Molecular Docking images of interaction via in silico study.

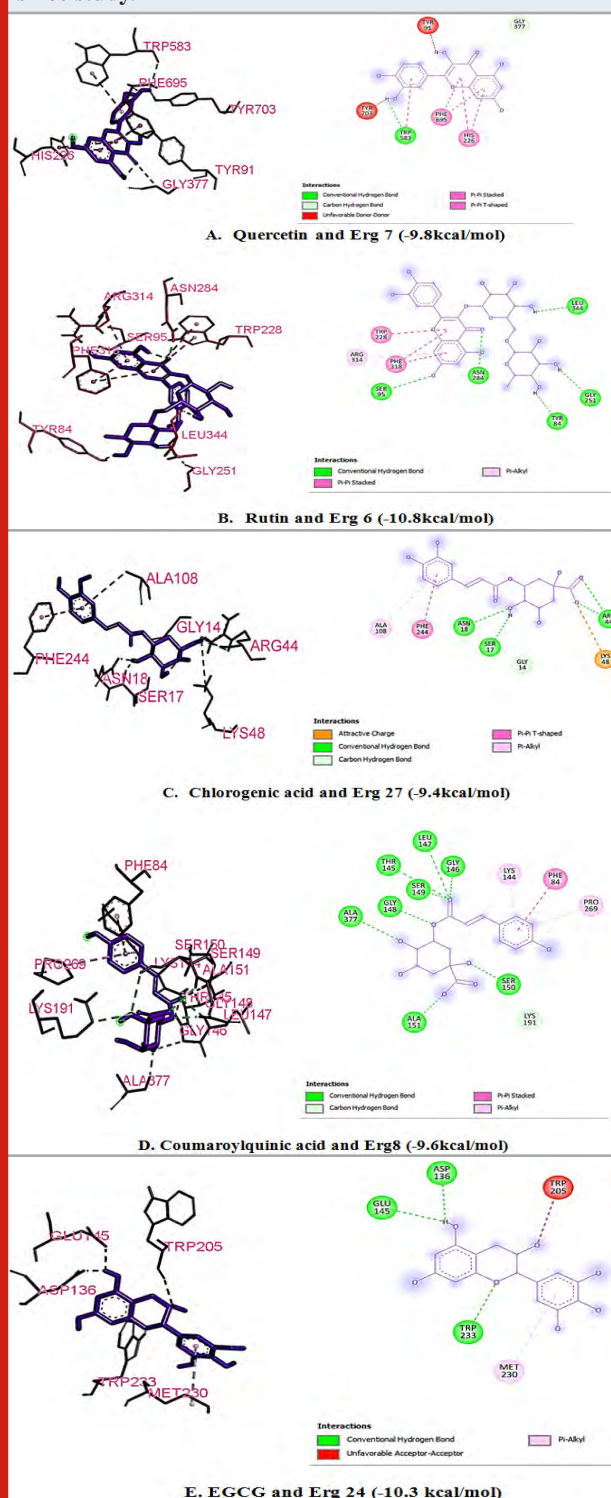


Table 8. Drug likeness report of green tea phytochemicals.

Molecule	ML OGP	ES OL Class	GI absorption	BBB permeant	Pgp substrate	CYP 1A2 inhibitor	CYP 2C19 inhibitor	CYP 2C9 inhibitor	CYP 2D6 inhibitor	CYP 3A4 inhibitor	Lipinski #violations	Gh ose #violations	Ve ber #violations	Egan #violations	Mu egge #violations	Bioa vaila bility Score	Leadlik eness #violations
2,5-Dimethyl-4-hydroxy-3-(2H)-furanone	-2.71	Very soluble	Low	No	Yes	No	No	No	No	No	0	1	0	0	0	0.56	0
B-ionone	2.94	Soluble	High	Yes	No	No	No	No	No	No	0	0	0	0	2	0.55	1
Chlorogenic acid	-1.05	Very soluble	Low	No	No	No	No	No	No	No	1	1	1	1	2	0.11	1
Coumaroylquinic acid	-0.54	Very soluble	Low	No	No	No	No	No	No	No	0	1	1	1	0	0.56	0
Dihydroactinidiolide	2.37	Soluble	High	Yes	No	No	No	No	No	No	0	0	0	0	1	0.55	1
Epicatechin	0.24	Soluble	High	No	Yes	No	No	No	No	No	0	0	0	0	0	0.55	0
Epicatechin gallate	0.05	Soluble	Low	No	No	No	No	No	No	No	1	0	1	1	2	0.55	1
Epigallocatechin gallate	-0.44	Soluble	Low	No	No	No	No	No	No	No	2	0	1	1	3	0.17	1
Epigallocatechin	-0.29	Soluble	High	No	No	No	No	No	No	No	1	0	0	0	1	0.55	0
Gallic acid	-0.16	Very soluble	High	No	No	No	No	No	No	Yes	0	2	0	0	1	0.56	1
Kaempferitrin	-2.69	Soluble	Low	No	Yes	No	No	No	No	No	3	4	1	1	3	0.17	1
Myricetin	-1.08	Soluble	Low	No	No	Yes	No	No	No	Yes	1	0	1	1	2	0.55	0
Pyrazine	-0.92	Very soluble	High	No	No	No	No	No	No	No	0	3	0	0	2	0.55	1
Quercetin	-0.56	Soluble	High	No	No	Yes	No	No	Yes	Yes	0	0	0	0	0	0.55	0
Rutin	-3.89	Soluble	Low	No	Yes	No	No	No	No	No	3	4	1	1	4	0.17	1

CONCLUSION

The findings of the present study highlights the antifungal drug targets of green tea phytochemicals against inhibition of ergosterol biosynthesis in *Candida glabrata*. Rutin, Chlorogenic acid, Coumaroylquinic acid, quercetin, EGCG were screened as the most active green tea phytochemicals with Erg 6, Erg 7, Erg 8, Erg 24, and Erg 27 in ergosterol

biosynthesis pathway as their possible drug targets. The poor bioavailability of rutin, chlorogenic acid, and EGCG is a limiting factor and needs further investigation to enhance their bioavailability. The current study is a preliminary analysis that needs to test *in vitro* to explore the future avenues of potent drug development against NAC species.

ACKNOWLEDGEMENTS

The study was technically supported by Prof. (Dr.) Kamal Ghanshal, Chairman, Graphic Era Deemed to be University, Dehradun.

Conflict of Interests: Authors declare no conflicts of interests to disclose.

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Dental Communication

Awareness and Knowledge of Oral Diseases Associated with Rheumatic Patients Among Dentists in Saudi Arabia

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ABSTRACT

Rheumatic diseases are autoimmune and inflammatory conditions that make the immune system attack the body's joints, muscles, bones, and organs. Oral health is proven to be associated with overall systemic health. Patients with rheumatic diseases present with multiple oral manifestations that might be the first clinical signs and symptoms of a systemic disease. The aim of this study was to assess the awareness and knowledge of dentists of the oral diseases associated with rheumatic patients in Saudi Arabia. A self-administered questionnaire was distributed aimed at the different specialties and degrees of dentistry. The total number of participants in this study was 235. The majority of participants (68.1%) agreed that there is a relationship between oral health and rheumatic disease, but only 31.1% agreed (or strongly agreed) that they have sufficient information about this association. Additionally, 34.9% agreed that there was enough information about medications that can be used in rheumatic patients and their possible side effects on the oral cavity. Almost half of the participants (46.8%) disagreed that rheumatic disease lecture(s) that have been covered at the undergraduate and postgraduate levels, enough to manage rheumatic patients. The majority (64.7%) never attended any form of continuous education lecture(s) or course(s) regarding the management of oral conditions in rheumatic patients. We concluded that most of the participants agreed that there is a relation between oral health and rheumatic diseases. At the same time, they think that they do not have enough information on the association between oral health and rheumatic diseases. This is in addition to information on the medication used in rheumatic patients and their possible side effects in the oral cavity.

KEY WORDS: ORAL HEALTH, RHEUMATIC DISEASES; SYSTEMIC HEALTH.

INTRODUCTION

Rheumatic diseases are autoimmune and inflammatory conditions that cause the immune system to attack the joints, muscles, bones, and organs of the body. Patients with rheumatic diseases present with multiple oral manifestations that might be the first clinical signs and symptoms of a systemic disease. The main oral manifestations of rheumatic diseases are xerostomia, temporomandibular joint disorders, periodontal diseases, and dysphagia (Gualtierotti et al. 2018). The link between oral and systemic diseases is well documented in the dental and medical literature. In (2001),

Mercado (2000) concluded that there is good evidence of an association between rheumatoid arthritis and periodontal diseases (Mercado et al. 2000; Rodríguez-Lozano et al. 2019). Another study demonstrated that periodontitis is associated with an increased risk of developing RA. A study conducted by Chamani (2017) concluded that 51% of rheumatoid arthritis patients have complained of xerostomia. A significant relationship was also found between xerostomia and oral health-related quality of life (Chamani et al. 2017; Choi and Lee 2021).

In response, a better understanding of dentists of this oral and systemic link would help to educate patients to help them improve their oral and general healthcare. In addition, this would also lead to improving the collaboration between dentists and other health care professionals (Chamani

Article Information:*Corresponding Author: hamadnb@hotmail.com

Received 25/08/2021 Accepted after revision 28/12/2021

Published: 31st December 2021 Pp- 1956-1961

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.85>

et al. 2017). Many of the dentists who participated in Nazir's study believed that the patients' awareness of the link between oral and systemic health could increase the number of patients seeking dental care (Nazir et al. 2019). The available literature shows an association between oral diseases and systemic diseases. However, only a few studies have been done on the dentist's awareness of this association, particularly rheumatoid diseases. As mentioned previously, the understanding of this association could be significant for better oral health outcomes among rheumatic patients. A cross-sectional study was conducted by Al Sharrad that assessed the awareness of the relationship between systemic and periodontal diseases among dentists and physicians in the Kingdom of Saudi Arabia and the state of Kuwait. The study sample included a convenient sample of two groups of selected physicians and dentists in Saudi Arabia and Kuwait (Nazir et al. 2019).

Group 1 was comprised of intern physicians, family/internal medicine specialists and other specialists while group 2 consisted of intern dentists, general dental practitioners, periodontists, and other dental specialists. The sample size for the dentists was 484 and for physicians, it was 134. They concluded that the level of awareness is generally low (Sharrad et al. 2019). It has been shown that the attitude and practices of dentists and physicians will determine the advice that they offer to their patients (Merchant et al. 2002). A survey of dentists in the United States found that their awareness of the association between oral and general health can encourage patients to seek out oral care in a dental practice (Song et al. 2013; Sharrad et al. 2019).

A study done by Bhatia showed that almost two thirds of the population in their study were unaware about the oral-systemic diseases link. This emphasized the need for dentists to educate their patients about the oral diseases related to systemic diseases (Hemalatha et al. 2020). This was a cross-sectional study. Another study published in 2001 by Morgan found that the majority of hospital doctors believed that it was important to examine the patient's mouth. However, most of them (77.0%) did not feel that they had sufficient training or enough confidence to examine an oral cavity (Morgan et al. 2001; Hemalatha et al. 2020). The aim of this study is to report on the dentists' awareness and knowledge of the oral diseases associated with rheumatic patients.

MATERIAL AND METHODS

This study's subjects consisted of dentists in Saudi Arabia. Informed consent from the participants was obtained. The participants were informed that their responses and personal information would be kept confidential. A cross-sectional survey was prepared in the English language using Google Forms. The survey was distributed among the selected sample of dentists in Saudi Arabia. The questionnaire was designed based on an established Likert scale to assess agreement. Yes/No questions were also used as part of the assessment. The questionnaire included four questions about the demographic data of the participants: age, gender, specialty, and job title. This was followed by general short questions that were used to assess their agreement or

disagreement regarding the relationship between oral health and rheumatoid diseases. After that, another question was used to assess their knowledge and if they have adequate enough information or not regarding the association between oral health and rheumatoid diseases. This includes whether they can identify the oral signs and symptoms of rheumatoid diseases in their patients and the possible side effects on the oral cavity due to the drugs used to treat rheumatoid diseases.

Furthermore, there were additional questions regarding whether they had experience of cases where they saw an oral manifestation in rheumatic patients, and whether will they consult with a rheumatologist when they suspect oral findings that might be related to rheumatoid diseases. Two general questions were about whether rheumatic patient should be routinely referred to specialized dental clinics for an oral examination or not. The other question assessed their level of agreement or disagreement regarding whether oral diseases, when associated with rheumatic patients, can negatively affect their quality of life. In addition, there was a question about the management of oral conditions.

Another question was on whether the rheumatoid disease lectures at the undergraduate and postgraduate levels are enough to manage rheumatic patients. The last question was about how often dentists attend continuous education regarding the management of oral conditions in rheumatic patients. The survey was prepared using an online form and it was distributed among a sample of dentists in Saudi Arabia. For statistical analysis, the values were presented as numbers and percentage. The Chi square test was used to compare the categories of participants based on gender, age group, and job title. The significance level was set at $p \leq 0.05$. Statistical analysis was performed using SPSS 23.0 (Statistical Package for Scientific Studies, SPSS, Inc., Chicago, IL, USA) for Windows. Microsoft Excel was used to generate the representative graphs.

RESULTS AND DISCUSSION

The total number of participants in this study was 235. More than half of them were male totaling 143 (60.9%) while 92 (39.1%) were females. For the age of the participants, those aged 20 - 30 years totaled 172 (73.2%), 31 - 40 years totaled 43 (18.3%), 41 - 50 years totaled 15 (6.4%), and those above 50 years old totaled 5 (2.1%). More than half of the participants were general dentists totaling 137 (58.3%), followed by oral medicine specialists totaling 23 (9.8%), orthodontists totaling 19 (8.1%), and restorative endodontists totaling 11 (4.7%).

Pedodontists and prosthodontists had the same number of participants totaling 6 (2.6%), while oral surgeons and periodontists also had the same number of participants totaling 3 (1.3%). Other roles totaled 27 (11.5%). The majority of participants (68.1%) agreed (or strongly agreed) that there is a relationship between oral health and rheumatic disease, but only 31.1% agreed (or strongly agreed) that they have sufficient information about this association. Additionally, 34.9% agreed (or strongly agreed) that the sufficient information about the medications that could

be used to treat rheumatic patients and their possible side effects on the oral cavity. Almost half of the participants (48.5%) didn't see any patients with oral manifestations due to rheumatic disease. The majority (77.5%) agreed (or strongly agreed) with the need to consult a rheumatologist when they suspected oral findings in the patients that might be related to rheumatic diseases and 60.4% agreed (or strongly agreed) that patients with rheumatic disease should be routinely referred to a specialized dental clinic.

The majority (66%) agreed (or strongly agreed) that oral manifestations associated with rheumatic patients could negatively affect their quality of life. The majority (88.6%) agreed (or strongly agreed) that the best action to manage oral conditions in rheumatic patients was to refer them to an oral medicine clinic or to treat the patients in the GP clinic as part of a physician's consultation. Almost half of the participants (46.8%) disagreed (or strongly disagreed) that rheumatic disease lecture(s) were covered at the undergraduate and postgraduate levels enough to manage rheumatic patients. The majority (64.7%) had never attended any continuous education lecture(s)/course(s) regarding the management of oral conditions in rheumatic patients.

A significantly higher percentage of females agreed (or strongly agreed) that there is a relationship between oral health and rheumatic disease ($p=0.019$). A significantly higher percentage of females also agreed (or strongly agreed) that they have sufficient information about this association ($p=0.045$) and that they had sufficient information about the medications that can be used to treat rheumatic patients and their possible side effects on the oral cavity ($p=0.020$). A significantly higher percentage of females have seen rheumatic patients with an oral manifestation (31.5%), in comparison to 10.5% of males ($p=0.019$). A significantly higher percentage of females agreed (or strongly agreed) that patients with a rheumatic disease should be routinely referred to a specialized dental clinic for an oral examination. ($p=0.00$). The oral manifestations associated with rheumatic patients could negatively affect their quality of life ($p=0.00$). There was no statistically significant difference found between the participants in the rest of the questionnaire.

A significantly higher percentage of participants in the 41 - 50 year age group agreed (or strongly agreed) that there is a relationship between oral health and rheumatic disease ($p=0.002$). A significantly higher percentage of participants in the same age group (41 - 50 years) also agreed (or strongly agreed) that they have sufficient information about this association ($p=0.00$) and that they had sufficient information about the medications that can be used to treat rheumatic patients and their possible side effects on the oral cavity ($p=0.002$).

A significantly higher percentage of participants in the 41 - 50-year age group have seen rheumatic patients with an oral manifestation (60%), in comparison to 40% and 39.5% of the participants above 50 years or in the 31 - 40-year age group respectively. Only 9.3% of the participants in the 20 - 30-year age group have seen rheumatic patients with an oral manifestation ($p=0.000$). A significantly higher percentage

of participants in the 31 - 40-year-old age group agreed (or strongly agreed) that oral manifestations associated with rheumatic patients could negatively affect their quality of life ($p=0.004$).

A significantly higher percentage of participants (80%) in the 41 - 50-year-old age group considered the best action to manage oral conditions in rheumatic patients to be to refer to an oral medicine clinic. In addition, 50% of participants in the 20 - 30-year-old age group chose to treat patient in the general practitioner clinic following a physician's consultation. There was a statistically significant difference between the age group responses to this question ($p=0.007$). A significantly higher percentage of participants in the 41 - 50 years age group attended 2 to more than 10 continuous education lecture(s)/course(s) regarding the management of oral conditions in rheumatic patients (53.3%), in comparison to 20% and 30.3% of participants above 50 years or in the 31 - 40-year-old age group respectively. Only 17.4% of participants in the 20 - 30-year-old age group had attended 2 or more courses ($p=0.023$). There were no statistically significant differences between the participants in the rest of the questionnaire.

A significantly higher percentage of general dentists disagreed (or strongly disagreed) that there is a relationship between oral health and rheumatic disease ($p=0.00$). A significantly higher percentage (60%) of university professors agreed (or strongly agreed) that they have sufficient information about this association ($p=0.00$), and that they had sufficient information about the medications that can be used by rheumatic patients and their possible side effects on the oral cavity ($p=0.001$). A significantly higher percentage (42.9%) of university professors have seen rheumatic patients with an oral manifestation. This is in comparison to 4.8%, 12.7% and 16.7% of interns, general dentists, and postgraduate students respectively ($p=0.000$). A significantly higher percentage of (91.5%) of university professors agreed (or strongly agreed) that oral manifestations associated with rheumatic patients could negatively affect their quality of life ($p=0.00$).

A significantly higher percentage of university professors (62.9%) considered that the best action to manage oral conditions in rheumatic patients was to refer to an oral medicine clinic, while 60.6% of general dentists chose to treat their patients in the general practitioners' clinic as part of a physician consultation. There was a statistically significant difference in the participants' response to this question ($p=0.003$). A significantly higher percentage of general dentists have never attended or attended only once a continuous education lecture(s)/course(s) regarding the management of oral conditions in rheumatic patients with an oral manifestation (91.6%), in comparison to 76.2%, 72.3% and 65.7% of interns, general dentists and postgraduate students respectively ($p=0.002$). There was no statistically significant difference between the participants in the rest of the questionnaire (Table 1a, 1b, and 1c).

Rheumatoid arthritis is a chronic autoimmune inflammatory disease affecting the synovial membrane of diarthrodial joints. Systemic weight loss, fever, and fatigue may be

among the first presentations of RA. The classic features of this disease are chronic, bilateral, and symmetric polyarthrititis, joint pain, and inflammation that can result in deformity, instability, and the destruction of synovial joints (Abrão et al. 2016). Temporomandibular joint (TMJ) involvement occurs in about 50% of RA patients. This can occur at an early age, and it may result in mandibular

growth disturbance, facial deformity, bilateral and unilateral TMJ ankylosis, a retrognathic mandible, and malocclusion giving a typical bird facies appearance (Voog et al. 2003; Grover et al. 2011). A recent study reported that in patients with RA, the predominant finding was the erosion of the condyle (85%) followed by condylar sclerosis (Voog et al. 2003; Choi and Lee 2021).

Table 1 a. Comparison of knowledge among study participants to various questions according to Job title (Question 5-8)

Questions	Responses	Job title								χ^2	value
		intern		General dentist		postgraduate student		university professor			
		n	%	n	%	n	%	n	%		
Q5-Relationship between oral health &rheumatic disease?	Strongly agree	10	11.9%	12	16.9%	5	27.8%	20	57.1%	42.61	0.00*
	Agree	38	45.2%	36	50.7%	7	38.9%	10	28.6%		
	Neutral	31	36.9%	16	22.5%	5	27.8%	4	11.4%		
	Disagree	5	6.0%	5	7.0%	1	5.6%	1	2.9%		
	Strongly Disagree	0	0.0%	2	2.8%	0	0.0%	0	0.0%		
Q6- Information about the association between oral health & rheumatic diseases?	Strongly agree	3	3.6%	1	1.4%	1	5.6%	14	40.0%	63.34	0.00*
	Agree	23	27.4%	10	14.1%	2	11.1%	7	20.0%		
	Neutral	31	36.9%	29	40.8%	9	50.0%	7	20.0%		
	Disagree	25	29.8%	22	31.0%	4	22.2%	7	20.0%		
	Strongly Disagree	2	2.4%	9	12.7%	2	11.1%	0	0.0%		
Q7- Information about medications used in Rheumatic patients and their side effect on the oral cavity?	Strongly agree	3	3.6%	4	5.6%	0	0.0%	10	28.6%	41.18	0.001*
	Agree	27	32.1%	16	22.5%	3	16.7%	5	14.3%		
	Neutral	27	32.1%	24	33.8%	7	38.9%	13	37.1%		
	Disagree	25	29.8%	19	26.8%	5	27.8%	5	14.3%		
	Strongly Disagree	2	2.4%	8	11.3%	3	16.7%	2	5.7%		
Q8- Have you seen patients with oral manifestations of the rheumatic diseases?	Yes, patients with oral manifest.	4	4.8%	9	12.7%	3	16.7%	15	42.9%	55.81	0.00*
	Yes, patients with no oral manifest	18	21.4%	9	12.7%	1	5.6%	5	14.3%		
	Don't know	10	11.9%	19	26.8%	5	27.8%	2	5.7%		
	No	52	61.9%	33	46.5%	9	50.0%	13	37.1%		
	other	0	0.0%	1	1.4%	0	0.0%	0	0.0%		

Significance level $p \leq 0.05$, *significant, ns=non-significant

Table 1 b. Comparison of knowledge among study participants to various questions according to Job title (Question 9-11)

Questions	Responses	Job title								χ^2	P value
		intern		General dentist		Postgraduate student		university professor			
		n	%	n	%	n	%	n	%		
Q9- Will you consult a rheumatologist when you suspect oral findings in patients that might be related to rheumatic diseases?	Strongly agree	30	35.7%	19	26.8%	7	38.9%	18	51.4%	16.34	0.430 ns
	Agree	40	47.6%	30	42.3%	6	33.3%	12	34.3%		
	Neutral	9	10.7%	13	18.3%	4	22.2%	5	14.3%		
	Disagree	4	4.8%	7	9.9%	1	5.6%	0	0.0%		
	Strongly Disagree	1	1.2%	2	2.8%	0	0.0%	0	0.0%		
Q10- Patients with Rheumatic disease should be routinely referred to specialized dental clinic for oral examination.	Strongly agree	15	17.9%	13	18.3%	3	16.7%	14	40.0%	21.07	0.176 ns
	Agree	32	38.1%	24	33.8%	10	55.6%	14	40.0%		
	Neutral	29	34.5%	22	31.0%	5	27.8%	6	17.1%		
	Disagree	8	9.5%	11	15.5%	0	0.0%	1	2.9%		
	Strongly Disagree	0	0.0%	1	1.4%	0	0.0%	0	0.0%		
Q11- Oral manifestations associated with rheumatic patients could negatively affect their quality of life?	Strongly agree	13	15.5%	9	12.7%	2	11.1%	15	42.9%	42.48	0.00*
	Agree	39	46.4%	30	42.3%	11	61.1%	17	48.6%		
	Neutral	27	32.1%	21	29.6%	5	27.8%	3	8.5%		
	Disagree	4	4.8%	10	14.1%	0	0.0%	0	0.0%		
	Strongly Disagree	1	1.2%	1	1.4%	0	0.0%	0	0.0%		

Significance level $p \leq 0.05$, *significant, ns=non-significant

Table 1 c. Comparison of knowledge among study participants to various questions according to Job title (Question 12-14)

Questions	Responses	Job title								χ^2	P value
		Intern		General dentist		Postgrad. student		university professor			
		n	%	n	%	n	%	n	%		
Q12- What do you think the best action to manage oral condition in rheumatic patients?	Refuse to see patients in GP clinic	4	4.8%	2	2.8%	1	5.6%	0	0.0%	36.04	0.003*
	Refuse to refer patients to specialty clinic	4	4.8%	1	1.4%	0	0.0%	2	5.7%		
	Treat patient in GP clinic under physician consultation	37	44.0%	43	60.6%	8	44.4%	8	22.9%		
	Refer to Oral and Maxillofacial surgery clinic	2	2.4%	5	7.0%	1	5.6%	3	8.6%		
	Refer to Oral Medicine clinic	37	44.0%	20	28.2%	8	44.4%	22	62.9%		
Q13- Do you think rheumatic diseases lecture(s) covered during undergraduate/postgraduate level enough to manage rheumatic patients?	Strongly agree	5	6.0%	5	7.0%	0	0.0%	0	0.0%	20.83	0.185 ns
	Agree	11	13.1%	9	12.7%	2	11.1%	5	14.3%		
	Neutral	36	42.9%	21	29.6%	9	50.0%	11	31.4%		
	Disagree	28	33.3%	27	38.0%	4	22.2%	19	54.3%		
	Strongly Disagree	4	4.8%	9	12.7%	3	16.7%	0	0.0%		
Q14- Did you attend any continuous education lecture(s)/course(s) regarding management of oral conditions in rheumatic patients?	Never	52	61.9%	57	80.3%	10	55.6%	16	45.7%	37.07	0.002*
	Once only	12	14.3%	8	11.3%	3	16.7%	7	20.0%		
	2-4 lectures/courses	17	20.2%	5	7.0%	5	27.8%	9	25.7%		
	5-9 lectures/courses	2	2.4%	1	1.4%	0	0.0%	1	2.9%		
	More than 10 lectures/courses	1	1.2%	0	0.0%	0	0.0%	2	5.7%		

Significance level $p \leq 0.05$, *significant, ns=non-significant

Patients with long-standing active RA may have an increased incidence of periodontal disease, including an increase in pocket depth, furcation involvement, and a loss of the alveolar bone and teeth. It is necessary for the dentist to have an awareness of the patient's current RA medication. It is possible that there may be side effects and interactions with other drugs. Before recommending any additional NSAIDs, the clinician must assess the patient's current medication schedule to avoid renal or gastric toxicity. Gastrointestinal-protective agents such as misoprostol may help to reduce these side effects. Replacement therapy is essential in patients who engage in the long-term use of glucocorticoids to prevent secondary adrenal insufficiency.

In patients with severe RA who have had any of their joints surgically replaced with prosthetic joints, they may require prophylactic antibiotic therapy before undergoing any invasive dental procedures (Grover et al. 2011; Li et al. 2017; Pandey et al. 2018). In a previous qualitative study, the dentists suggested taking care of the oral and general health of patients as primary care providers. Some strongly believed that the patient's education on the oral-systemic link is crucial to the provision of optimal dental care (Song et al. 2013; Nazir et al. 2019).

This study was conducted to assess the dentists' awareness and knowledge of the oral diseases associated with rheumatic patients in Saudi Arabia. In the case of the present study, 160 (68.1%) of participants agreed that in general, there is a relationship between oral health and rheumatic disease. On the other hand, 79 (33.7%) participants disagreed that they have sufficient information about the association between oral health and rheumatic diseases, in addition

to the medications that can be used to treat rheumatic patients and their possible side effects on the oral cavity. This is mostly because the rheumatic disease lectures that have been covered at the undergraduate and postgraduate levels are not enough to manage rheumatic patients. Most of them did not attend any continuous education lectures or courses regarding the management of oral conditions in rheumatic patients.

According to Nazir et al., improving the level of awareness and updating the knowledge of medical professionals can be achieved through a review of the curricula in medical and dental schools in relation to conducting continuous educational programs (Nazir et al. 2019; Choi and Lee 2021). Song et al. (2013) mentioned that when established guidelines do not exist for treating dental patients with additional medical conditions, especially for those with less studied diseases, the dentists in their study took actions based on their clinical experience in some cases.

They sought their colleagues' advice online for others (Song et al. 2013). According to the current study, 117 (48.5%) participants had not seen patients with oral manifestations of rheumatic diseases in their clinic. Additionally, 182 (77.5%) of participants intended to consult a rheumatologist when they suspect oral findings in their patients that might be related to rheumatic diseases. According to Nazir et al., their results confirm the need for effective communication between physicians and dentists by referring patients between the specialties as required (Nazir et al. 2019). In addition, 142 (60.4%) participants agreed that patients with rheumatic diseases should be routinely referred to specialized dental clinics for an oral examination. Additionally, 155 (66%) of

them agreed that the oral manifestations associated with rheumatic patients could negatively affect their quality of life. Referring the patient to an oral medicine clinic is what most of participants agreed on when managing select oral conditions in rheumatic patients. The agreed and strongly agreed responses were combined to describe the results (Choi and Lee 2021).

CONCLUSION

The finding of the present study showed, in the spite of the results, most of the respondents agreed that there is a relation between oral health and rheumatic diseases. At the same time, they think that they do not have sufficient information about the association between oral health and rheumatic diseases, in addition to the medication used in rheumatic patients and their possible side effects in relation to the oral cavity. In general, the awareness and level of knowledge are low, therefore rheumatic disease lectures should be covered well at both the undergraduate and postgraduate levels.

Conflict of Interests: Authors declare no conflict of interests to disclose.

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Biotechnological Communication

Characterization and Genetic Diversity of Taif Chicken Ecotype Using Dense Microsatellites Panel

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ABSTRACT

The current study is the first comprehensive investigation to address the native chicken ecotypes of the Taif region to unravel the genetic diversity using a dense panel of 40 microsatellites (SSR). Blood samples were collected from 25 hens randomly sampled from a village farm at Taif governorate. A total of 147 alleles were detected, with an average of 3.7 alleles per locus. The overall mean of polymorphic information content (PIC) was 0.43. The average observed heterozygosity (H_{obs}) of 0.28 was lower than the expected heterozygosity (H_{exp}) of 0.48. Out of 40 loci only 11 loci showed insignificant deviation from Hardy Weinberg expectation. The ecotypes showed low genetic diversity ($H_s = 0.65$) and a high level of inbreeding ($F_{is} = 0.75$). The high F_{is} is indicative of the endangerment potentiality of this ecotype. Nine SSR showed an inbreeding coefficient of one. The significant estimate of the inbreeding coefficient of the present study calls for an immediate breeding plan to preserve such endangered ecotypes. Results of the present study will provide an initial guide to design further investigations for the development of sustainable genetic improvement and conservation programs for the Taif ecotype genetic resources.

KEY WORDS: ECOTYPE, CHICKEN, MICROSATELLITES, DIVERSITY, HETEROZYGOSITY.

INTRODUCTION

The importance of chicken ecotypes is not only limited to the rural economy and smallholder livelihood but also important in the maintenance and conservation of genetic sources. These ecotypes have been locally adapted for decades. Raising local ecotype/breed, in other words indigenous, is preferred because of their small cost of production and their adaptability to harsh environmental conditions. Also, it has special significance particularly in remote areas where they consider as a source of revenue for underprivileged families. The adaptive privilege they gained over time allowed them to survive harsh local conditions including scarcity of water

and food, and disease epidemics (Muchadeyi et al., 2007, Nxumalo, 2020, Habimana et al., 2020).

Ecotypes represent a valuable genetic resource as they have been used as a framework in diversity studies. Although many definitions are proposed for ecotypes, however, the most common description of ecotypes is the exclusive population that adapted to their local environment and represents a reservoir for genes affecting productive adaptability. For instance, native chicken in Saudi Arabia carries different heat tolerance genes which are crucial in hot climate zones and has the potential in breeding programs. Almost a quarter of local chicken breed populations are globally classified as endangered, hence their genotypes could be lost (Morrison, 2012, Strillacci et al., 2017, Radwan, 2020).

Preservation of these genetic resources comprises a comprehensive knowledge of activities related to the identification, and documentation of native breeds. The

Article Information:*Corresponding Author: saboaba@kau.edu.sa

Received 24/09/2021 Accepted after revision 28/12/2021

Published: 31st December 2021 Pp- 1962-1967

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.86>

importance of ecotypes as evolutionary conservation objectives emerges from their ability to exhibit phenotypic differences in relation to environmental heterogeneity. Many studies have investigated ecotypes in village chicken populations. Indigenous chicken at Taif governorate (1700 m above sea level) are well adapted to the harsh environmental conditions of high altitudes (Muchadeyi et al., 2007, Rudresh et al., 2015, Abdel-Hameed and Bazaid, 2017).

Elevation above sea level is generally known for extreme environmental conditions, including low oxygen content, reduced partial pressure of oxygen, and temperature fluctuations. The genetic makeup of Taif ecotypes is understudied with very limited information available on its adaptability. Advances in molecular markers applications have given a reliable opportunity to evaluate genetic variation at the DNA level. One of the most widely used genetic markers for genetic diversity investigations is microsatellites (SSR). Microsatellites are commonly utilized in genetic diversity assessment since they are codominant, highly polymorphic, abundant, and highly reproducible (Groen et al. 1994, Hillel et al., 2003, Huang et al., 2016; Nxumalo et al., 2020).

Such efficiency has made SSR markers the most common genetic markers for the assessment and characterization of genetic diversity in many commercial and indigenous chicken populations. Numerous SSR markers are accessible in chickens and have been used to construct linkage maps in many populations. The current study is the first to use a dense panel of microsatellites to assess Taif chicken ecotypes genetic diversity in an attempt to highlight the possible effects of inbreeding (Groen et al., 1994, Nones et al., 2005, Seo et al. 2013, Abebe et al., 2015, Nxumalo et al. 2020).

MATERIAL AND METHODS

A total of 25 hens were randomly sampled for DNA extraction during January 2021 from a village chicken farm in Taif (21.28 °N, 40.42 °E), Saudi Arabia, representing the Taif chicken ecotype. Blood samples were collected from hens and prepared for DNA extraction using the procedure described in Hoelzel (1992). Genomic DNA was extracted from the whole blood using a DNA extraction kit (QIAGEN, USA). DNA quality was checked using electrophoresis and further determined using NanoDrop 2000C spectrophotometer (Spectronic Genesys, Thermo Electron Corporation). DNA samples were stored at -20°C until microsatellites genotyping.

Microsatellite selection and genotyping: A total of 40 microsatellite markers were used for sample genotyping (Table 1). These markers were chosen according to the recommendation by Choi et al. (2015), details of SSR markers are provided in Table 1. Polymerase chain reaction (PCR) was carried out according to Seo et al. (2013) and Ibrahim et al. (2015) in a final volume of 25 µL containing 1 µL (20 ng) of genomic DNA, 12.5 µL of Go Taq® Green Master Mix, Promega, USA, 1 µL of each primer (20 pM), deionized distilled water (to a total volume of 25 µL). The

PCR reactions were performed in C1000™ Thermo Cycler Bio-Rad, Germany, using the following conditions; initial denaturation at 94°C for 5 min; 40 cycles of denaturation at 94°C for 30 sec., primer annealing at 52°C for 45sec and primer extension at 72°C for 1 min; final extension at 72°C for 7 min.

Table 1. Marker name, chromosome number (Chrom.), and allele size (bp) of 40 SSR markers used in Taif ecotypes genotyping.

Marker	Chrom.	Allele size	Marker	Chrom.	Allele size
MCW0248	1	205-225	MCW0029	5	139-189
LEI0141	2	220-242	MCW0014	6	164-182
MCW0087	2	267-283	MCW0183	7	296-326
MCW0063	2	132-150	ROS0019	7	119-143
LEI0234	2	217-315	ADL0278	8	114-126
MCW0206	2	226-240	GCT0016	9	108-154
MCW0288	2	108-122	ADL0259	9	106-146
MCW0264	2	224-240	MCW0067	10	175-184
MCW0127	3	227-241	MCW0228	10	221-239
MCW0016	3	134-146	MCW0216	13	139-149
MCW0037	3	152-156	MCW0104	13	189-225
MCW222	3	221-225	MCW0213	13	288-316
LEI0166	3	354-370	MCW0123	14	79-89
MCW098	4	261-265	ADL0293	17	105-119
LEI0094	4	254-280	MCW0330	17	254-286
ADL0317	4	178-204	ADL0304	18	137-159
MCW0295	4	88 -106	MCW0165	23	114-118
ROS0013	5	220-242	LEIO074	26	224-240
MCW0078	5	135-147	MCW0069	26	158-176
ADL0292	5	110-138	LEI0135	28	131-142

To confirm successful amplification, PCR products were separated on 2% agarose/TBE gel and visualized on UV Transilluminator. To determine allele sizes, the genotyping mixture was carried out as follows; 1 µL of diluted PCR products, 10 µL of Hi-Di™ Formamide (Applied Biosystems, USA), and 0.1 µL of GeneScan™-500 LIZ™ size standard marker (Applied Biosystems, USA). Genotyping reaction mixture was denatured for 2min at 95 °C and fragment analysis was performed using capillary array using Genetic analyzer 3130xl (Applied Biosystems, USA). GeneMapper ver.3.7 (Applied Biosystems, USA) was used for allele size estimation.

Genetic diversity analysis: Microsatellite diversity for Taif ecotype was estimated using observed (H_{obs}) and expected (H_{exp}) heterozygosity (Nei, 1987), the fixation index FIS was also calculated. The informativeness of each marker was assessed based on polymorphic information content (PIC), (Botstein et al. 1980). All loci were tested for deviations from the Hardy-Weinberg equilibrium (HWE). Exploratory data analysis and graphical representations of the results were carried out under R statistical environment, R Core Team (2019). Genetic diversity and population genetics

parameters were estimated using the adegenet package (Jombart 2008).

RESULTS AND DISCUSSION

To assess the genetic diversity of the studied ecotype, different genetic diversity estimates were obtained including the number of alleles per locus, polymorphic information content (PIC), and observed (H_{obs}) and expected (H_{exp})

heterozygosity (Table 2). A total of 147 alleles were identified across 40 loci. The number of alleles per locus varied among loci between one and eight with an average of 3.7 allele/locus. The lowest number of alleles per locus was attributed to MCW098 with one allele, while ADL259 had the maximum number of alleles/locus of eight. The monomorphic pattern observed for MCW098 might be associated with the low sample size and/or the presence of null alleles (Choi et al., 2015).

Table 2. Number of alleles (N) polymorphic information content (PIC), observed heterozygosity (H_{obs}), and expected heterozygosity (H_{exp}) for 40 microsatellite markers used in Taif ecotypes genotyping.

Marker	N	PIC	H_{obs}	H_{exp}	Marker	N	PIC	H_{obs}	H_{exp}
MCW248	2	0.14	0.00	0.15	MCW029	7	0.67	0.52	0.71
LEI141	3	0.57	0.44	0.65	MCW014	5	0.25	0.12	0.26
MCW087	3	0.56	0.24	0.64	MCW183	2	0.19	0.16	0.21
MCW063	3	0.50	0.44	0.57	R0S019	7	0.69	0.56	0.74
LEI234	4	0.52	0.20	0.56	ADL278	2	0.33	0.20	0.42
MCW206	3	0.40	0.60	0.46	GCT016	5	0.58	0.00	0.63
MCW288	2	0.19	0.00	0.21	ADL259	8	0.80	0.36	0.82
MCW264	3	0.59	0.60	0.66	MCW067	2	0.32	0.00	0.40
MCW127	2	0.19	0.16	0.21	MCW228	5	0.61	0.36	0.67
MCW016	4	0.55	0.64	0.62	MCW216	2	0.07	0.08	0.08
MCW037	7	0.71	0.60	0.75	MCW104	4	0.45	0.36	0.48
MCW222	2	0.36	0.00	0.48	MCW213	5	0.58	0.20	0.61
LEI166	3	0.34	0.52	0.41	MCW123	5	0.22	0.12	0.22
MCW098	1	0.00	0.00	0.00	ADL293	3	0.54	0.52	0.60
LEI094	6	0.49	0.36	0.52	MCW330	5	0.62	0.40	0.68
ADL317	5	0.70	0.16	0.75	ADL304	4	0.52	0.44	0.58
MCW295	3	0.49	0.00	0.56	MCW165	3	0.14	0.00	0.15
ROS013	4	0.27	0.04	0.28	LEI074	3	0.53	0.64	0.60
MCW078	2	0.37	0.12	0.50	MCW069	3	0.39	0.20	0.44
ADL292	3	0.47	0.64	0.54	LEI135	2	0.19	0.00	0.21

The average number of allele/locus in the current study was lower than its native chicken counterpart reported in South Africa, Zimbabwe, Egypt, and Sweden. The polymorphic information content (PIC) is a common index to infer the allelic polymorphism. PIC ranged between 0.0 and 0.8 with an average of 0.43 (Table 2). Most of the studied loci (45 %) were highly informative ($PIC > 0.50$), while 32.5 % were reasonably informative ($0.5 > PIC > 0.25$), and only 22.5 % were slightly informative ($PIC < 0.50$). The relatively low PIC of the studied markers might be associated with the lower number of alleles per locus where 58% of the studied markers, 23 out of 40 markers, had a maximum of three alleles (Van-Marle-Koster and Nel, 2000, Muchadeyi et al., 2007, Ramadan et al. 2012, Abebe et al., 2015).

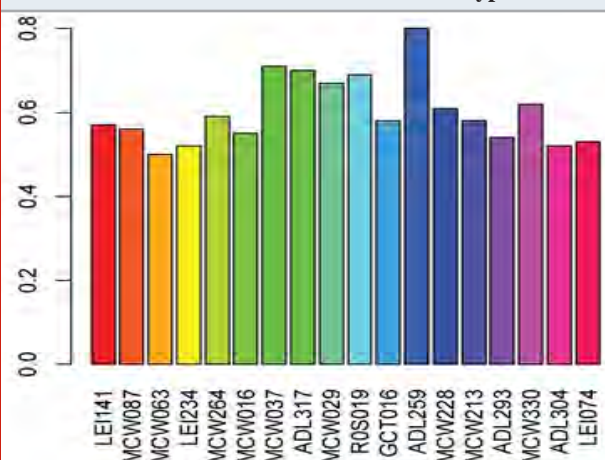
However, the majority of the SSR markers were adequate for the appropriate estimation of the genetic diversity of Taif ecotype populations. The average PIC for SSR in Taif ecotype is lower than that of PIC obtained in different chicken breeds. For instance, Ramadan et al. (2012)

reported an average PIC of 0.65 in Egyptian native breeds, while Seo et al. (2013) estimated the PIC of 0.77 in native Korean lines. Choi et al., (2015) reported a PIC of 0.68 in the commercial Korean breeds. One of the highest PICs reported in recent studies was obtained in Sinai and Norfa Egyptian native chicken breeds ($PIC = 0.84$) as reported by Soltan et al. (2018).

In the present study, about 18 markers had PIC exceeded 0.50 (Figure1) which is indicative of relative informativeness in studying genetic diversity. The observed proportion of heterozygosity (H_{obs}) ranged between 0.0 and 0.64 with an average of 0.28, Table 2. H_{exp} ranged between 0.68 and 0.24 with an average of 0.48. The elevated level of genetic diversity is a crucial factor for species' constant survival and adaptability to a different environment. In the current study, Taif ecotype showed low genetic diversity H_{obs} compared to H_{exp} . The lower H_{obs} as compared to H_{exp} is indicative of the probable effect of random genetic drift and inbreeding

that shaped Taif ecotype heterozygosity (Botstein et al., 1980, Ibrahim et al., 2015).

Figure 1: Polymorphic information content (PIC) for 18 microsatellite loci with PIC > 0.50 of Taif ecotype.



Different evolutionary forces may act differently on various parts of the genome, hence changing the magnitude of heterozygosity. Most of the studied loci showed significant deviation from Hardy Weinberg expectation (Table 3) which might be associated with one or more factors including inbreeding, presence of null alleles, and/or small sample size (Nei, 1978, De La Rua et al., 2001).

Genetic diversity (H_s) and coefficient of inbreeding (F_{IS}) across 40 microsatellite loci of Taif ecotype are presented in Table 4. Genetic diversity represents the magnitude of genetic variability within the population. In the present study, H_s ranged between 0.52 and 0.92 with an average of 0.75. Also, the coefficient of inbreeding (F_{IS}) ranged between 0.10 and 1.00 with an average of 0.65. The F_{IS} in the present study is higher than that reported in other local chicken breeds (e.g., 0.02 in Egyptian local breeds), 0.01 in Korean local breeds, 0.19 in Swedish breeds, and 0.41 in Sinai and Norfa Egyptian chicken breeds (Ramadan et al., 2012, Seo et al., 2013, Abebe et al., 2015, Soltan et al., 2018).

Table 3. Deviation from Hardy Weinberg equilibrium for 40 loci of native Saudi ecotype (loci in HWE are typed in *italics* and P-values marked with NS).

Marker	HWE	P-value	Marker	HWE	P-value
MCW248	0.001	0.00	MCW029	0.041	0.03
LEI141	0.062	0.06NS	MCW014	0.002	0.00
MCW087	0.000	0.00	MCW183	0.267	0.32 ^{NS}
MCW063	0.009	0.01	ROS019	0.000	0.00
LEI234	0.000	0.00	ADL278	0.014	0.02
MCW206	0.282	0.28 ^{NS}	GCT016	0.000	0.00
MCW288	0.000	0.00	ADL259	0.000	0.00
MCW264	0.000	0.00	MCW067	0.000	0.00
MCW127	0.281	0.28 ^{NS}	MCW228	0.000	0.00
MCW016	0.085	0.07 ^{NS}	MCW216	1.000	1.00 ^{NS}
MCW037	0.000	0.00	MCW104	0.001	0.00
MCW222	0.000	0.00	MCW213	0.000	0.00
LEI166	0.096	0.10 ^{NS}	MCW123	0.001	0.00
MCW098	1.000	1.00 ^{NS}	ADL293	0.000	0.00
LEI094	0.013	0.00	MCW330	0.001	0.00
ADL317	0.000	0.00	ADL304	0.124	0.11 ^{NS}
MCW295	0.000	0.00	MCW165	0.000	0.00
ROS013	0.000	0.00	LEI074	0.239	0.25 ^{NS}
MCW078	0.000	0.00	MCW069	0.000	0.00
ADL292	0.338	0.30 ^{NS}	LEI135	0.000	0.00

The coefficient inbreeding (F_{IS}) is indicative of the risk for a particular breed and urge for proper conservation plans (Simon and Buchenauer, 1993). Different thresholds have been set to determine the endangerment status of a specific breed (Ramadan et al., 2012, Soltan et al., 2018). For instance, the breed is considered not at risk when F_{IS} is below 0.05; when F_{IS} falls between 0.05 and 0.15, the

breed is potentially endangered; when F_{IS} is between 0.15 and 0.25, the breed is slightly endangered, and when F_{IS} is between 0.25 and 0.40, the breed is endangered and more than 0.40, the breed is severely endangered. In the current study, a total of 31 (77.5%) of SSR loci had F_{IS} greater than one, indicative of a significantly endangered breed. The significant magnitude of inbreeding of the Taif

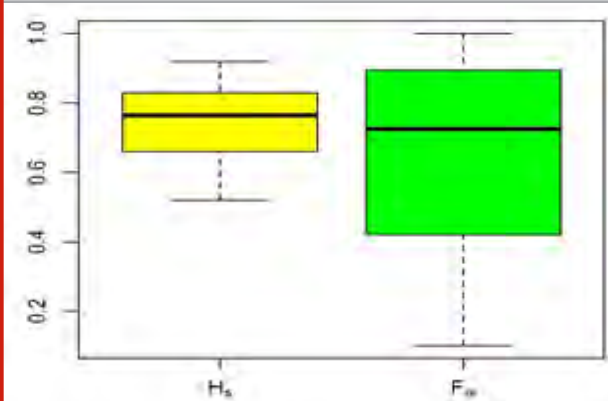
ecotype provides evidence of a severely endangered breed that requires immediate breeding and conservative actions

to be considered in order to preserve such an endangered ecotype.

Table 4. Gene diversity within population (H_s) and coefficient of inbreeding F_{IS} across 40 microsatellite loci of native Taif ecotype.

Marker	H_s	F_{IS}	Marker	H_s	F_{IS}
MCW248	0.60	1.00	MCW029	0.86	0.40
LEI141	0.83	0.47	MCW014	0.65	0.82
MCW087	0.84	0.71	MCW183	0.62	0.74
MCW063	0.77	0.44	ROS019	0.86	0.35
LEI234	0.80	0.75	ADL278	0.73	0.72
MCW206	0.67	0.10	GCT016	0.85	1.00
MCW288	0.63	1.00	ADL259	0.92	0.61
MCW264	0.83	0.28	MCW067	0.73	1.00
MCW127	0.62	0.74	MCW228	0.84	0.57
MCW016	0.76	0.16	MCW216	0.56	0.84
MCW037	0.85	0.29	MCW104	0.74	0.51
MCW222	0.77	1.00	MCW213	0.83	0.76
LEI166	0.67	0.22	MCW123	0.63	0.81
MCW098	0.52	1.00	ADL293	0.76	0.32
LEI094	0.77	0.53	MCW330	0.86	0.53
ADL317	0.90	0.82	ADL304	0.78	0.44
MCW295	0.81	1.00	MCW165	0.60	1.00
ROS013	0.67	0.94	LEI074	0.78	0.18
MCW078	0.77	0.85	MCW069	0.74	0.73
ADL292	0.75	0.15	LEI135	0.63	1.00

Figure 2: Boxplot of gene diversity within a population (H_s) and coefficient of inbreeding (F_{IS}) across 40 microsatellite loci of native Taif ecotype, (lines above and beneath each box refer to max and min values, line within each box refer to median value).



CONCLUSION

In the current study, a panel of 40 microsatellites markers were used to assess the genetic diversity of Taif ecotype chickens. All genetic diversity parameters showed average polymorphism level, yet high level of inbreeding. The

current study highlights the significant magnitude of inbreeding in Taif ecotype that could severely impact trait fixation in the populations. The results of the current study may be used as an initial guideline to design further conservation programs for the development of sustainable genetic improvement plans for Taif ecotype conservation.

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Biotechnological Communication

Anticancer and Gene Expression Analysis of *Piper nigrum* Extract on Colon Cancer Cell Lines

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ABSTRACT

One of the most prevalent malignancies among geriatrics is colorectal cancer (CRC), which starts to develop in the forms of genetic syndromes in young adults. The *Piper nigrum* is one important common spice used in the household having anticancer activities. The current study aims to evaluate *P. nigrum* seed extracts potency as anticancer against CRC cell line (COLO205). The extract is used to elucidate the MTT assay, DNA damage studies (COMET assay), Acridine Orange/Ethidium Bromide dual staining, cell death, cell cycle arrest using Flow cytometry, and regulation of Bcl-2, Bax & P53 gene regulation. To check the cell cytotoxicity by MTT assay methanolic extract was used. To evaluate anticancer activity the sample was extracted in methanol. RT-PCR was used to evaluate gene expression studies of Bcl-2, Bax, and P53. In the dose-dependent mode, the extract inhibited the growth of COLO205 cells and the IC₅₀ value was calculated at 48.2 µg/ml. The DNA fragmentation induced by apoptosis was the primary reason for the cell toxicity as observed by DNA damage studies & AO/EB dual staining technique. The extract concentration ranging from 40 & 80 µg/ml remarkably increased the proportion of cells in the S & G2/M phase. Cells at the late-apoptotic stage were found to be in the range of 22% - 57%. The Bax and P53 were upregulated and Bcl-2 was downregulated when treated with the extract. From this investigation underlying the mechanism of CRC was found to be *P. nigrum* extract caused to induce apoptosis and upregulation of tumor suppressor gene downregulation of apoptosis-suppressing gene bcl-2.

KEY WORDS: AO/EB STAINING, COLORECTAL CANCER, COLO205, COMET ASSAY, PIPER NIGRUM.

INTRODUCTION

In the digestive system of the body colon is an important role in the absorption of food, water & minerals, and aids in the removal of de-nutritional food materials. It is bowel in the large intestine and measures approximately 1.5 mt (Azzouz and Sharma 2019). Cancer is the world's leading cause of mortality with roughly 10 million fatalities recorded in 2020 worldwide (Ferlay et al. 2020). About 2.0 million

deaths related to cancer was reported to be Colorectal cancer (CRC) is prevalent cancer followed by breast and lung cancer. The change in the lifestyle such as consumption of canned foods, low intake of dietary fibers and increase in sedentary activities, smoking, obesity, and aging are leading causes of CRC (Kuipers et al. 2015; Valderrama-Trevino et al. 2017). Furthermore changing in lifestyle has affected the gut microbiota. Normal microflora is disturbed leading to increase in the pathogenic microbiota further aggravating the incidence of occurrence of CRC (Siegel et al. 2020).

The imbalanced gut microbiota leads to a release of toxins which causes DNA damage in turn leading to cell

Article Information:*Corresponding Author: krmaruthi@gmail.com

Received 19/08/2021 Accepted after revision 25/12/2021

Published: 31st December 2021 Pp- 1968-1974

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.87>

transformation (Jahani-Sherafat et al. 2018). Conventional methods of treating CRC are operations, chemotherapy, and radiotherapy, it depends upon the cancer pathology stage. Minimal invasive surgeries like Endoscopic Mucosal Resection, Polypectomy, and Laparoscopic Surgery to excise affected CRC in initial stages. In the advanced phase, fractional colectomy and elimination of lymph nodes are recommended procedures along with immuno-therapy, hormone-therapy, and photodynamic therapy are found to be effective. These methods have an invasive and effect on the quality of lifestyle of affected individuals. Therefore, the new methods of cancer treatment are alternative options to reduce the toxicity of drugs on healthy tissues (Arruebo et al. 2011; Singh and Chaturvedi 2015; Ferlay et al. 2020).

The traditional system of medicine (TSM) Ayurveda, Unani and Amchi system have become alternatives to the conventional method of treatment has shown resistance to cancer, TSM gives better quality of life since they have minimal or no side effects. In TSM utilize ingredients are food & spices as well as having medicinal & therapeutic properties (Greenwell and Rahman 2015). Members of the Piperaceae family are native Indian and many rare species, and have been part of a diet for a long time. Black pepper (*P. nigrum*) contains Piperine, which is an abundant bioactive alkaloid and has a pungent flavor of pepper (Gorgani et al. 2017; Dong et al. 2019). Piperine is a Vaso Modulator & antihypertensive activity, antiplatelet, antioxidant, antitumor, antiasthmatics, antiarthritic and anti-inflammatory, antidepressants, immunomodulatory, anticonvulsant, antimicrobial. In this study the molecular basis of the *P. nigrum* extract as an anticancer agent CRC cell line (COLO205) was investigated (Damanhoury 2014; Zou et al. 2015; Chung 2019; Yu et al. 2020; Haq et al. 2021).

MATERIAL AND METHODS

Freshly harvested *Piper nigrum* seeds were purchased from farmers in Ujire, Dakshina Kannada, India. The seeds were coarsely powdered, passed through a 20 µm stainless steel sieve. The sieved *P. nigrum* seed powder was used for the solvent extraction by decoction method. The extraction was carried out with 400 ml methanol by steam heating at 400 C for 6 hours. (Takooree et al. 2019). The methanolic decoction was filtered using Whatmann filter paper No.42, concentrated using wet heat on a 40° C water bath, and stored in a glass vial at 4° C (Zhang et al. 2018, Patil et al. 2021). Using Dulbecco's Modified Eagle Medium (DMEM) fortified with inactivated fetal bovine serum (FSB - 10%), 100 IU/ml penicillin, 100 µg/ml streptomycin at 37° C in a carbon dioxide incubator (5% CO₂, humidified) cultured CRC cell lines COLO 205 (ATCC® CCL-222™), procured from ATCC (Takashi et al. 2015). The cell dissociation solution containing trypsin (0.2%), EDTA (0.02%), glucose (0.05%) in phosphate basal salt solution. The cells were collected by subjecting to the cell sedimentation at ~1000g (1000 rpm) for a couple of minutes and viability was checked. Additionally, in a 96 well plate, 5 X 10⁴ cells/well were seeded & incubated at 37° C for 24 hours, in a carbon dioxide incubator (5% CO₂).

To investigate cytotoxicity using the MTT test, the cultured cells were trypsinized. The monolayer culture was counted and adjusted to 1 X 10⁵ cells per ml. 100 µl of diluted cell suspension (5 X 10⁴ cells/well) was put to each well of microtiter plates and allowed to develop a partial monolayer (after 24 hours), *P. nigrum* extracts concentrations of 40 & 80 µg/ml of 100 µl each was added and incubated at 37° C for 24 hours. To dissolve the formed Formosan, add 0.1 ml of DMSO to the supernatant and shake.

To calculate the cytotoxicity (IC₅₀ values) microtiter plates were read at 590 nm (Thermo Fisher Scientific, Varioskan™ multimode microplate reader) and cytotoxicity IC₅₀ values calculated by standard protocol (Graph pad, San Diego, CA, USA) (Ref 2021). In a 96-wells plate with 2 ml media, 10⁶ cells were seeded and cultured for 24 hours. The cells were then treated with 40 µg/ml & 80 µg/ml ethanolic extract of *P. nigrum* and incubated for another 24 hours in a 37° C in carbon dioxide incubator with 5%. After the stipulated period of incubation extract-treated cells were aggregated by centrifugation at 3000 rpm for 5 minutes at 4° C. discard the supernatant. The cells were splashed with PBS, air dried, and the pellet was dissolved in 0.5 ml PBS. Following cell isolation, 0.02 ml of cell suspension (1x10⁵ cells/ml), 0.08 ml of 0.5 percent low melting point agarose (LMPA) were blended, layered over the wells, containing Normal Melting Agarose (NMA) in the slide.

In a coplin jar containing lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Trizma Base, 1 percent SDS) place a cell fixed slide for 2 hours at 40. In the electrophoretic buffer (0.3N NaOH and 100mM EDTA) cells were separated by electrophoresis at 0.74 V/cm for 30 minutes. After electrophoresis, the slides were rinsed in chilled distilled water and air-dried followed by a wash with 70% chilled ethanol and air dry. Stain the slides with 80 µl Ethidium Bromide (EtBr) for visualizing. Immediately after staining with EtBr to score the slides, it was observed under a 40X objective lens of the fluorescent microscope and recorded. To estimate the extent of DNA damage 50 to 100 cells are randomly chosen for each analysis. "The cells damage is determined by measuring the length of DNA migration & the percentage of migration of DNA with ImageJ software with the open comet plug-in (www.rsweb.nih.gov/ij/)". The tail moment, the amount of migration per cell, the number of cells with amplified migration, the level of migration among damaged cells, and viability were analyzed using the program (Lan et al. 2014).

Using "5 µl AO-EtBr (Acridine orange & Ethidium Bromide) to a 25 µl (~1x10⁵ cells) of untreated & treated cells stained separate Eppendorf tubes, and after a couple of minutes mixed gently. The AO-EtBr stained cell suspension about 10 µl of was mounted on a glass slide with a coverslip, visualized under a fluorescence microscope with fluorescein filter" (Kasibhatla et al. 2006; Liu et al. 2015). To record apoptosis, "1 X 10⁶ cells per well were placed in a 6-well plate containing the DMEM and incubated". Subsequently floating cells detached by a pipette, make up the volume after 18 hours. Incubated in carbon dioxide incubator for 24 hours at 37° C of 40 & 80 µg/ml *P. nigrum* extract treated samples. "Incubated cell culture medium transferred into

fresh 15 ml centrifuge tubes and centrifuged at 2000 rpm for 5 minutes in 4° C. The cells pellet was rinsed with ice-cold PBS, then suspended in 1 ml 1X binding buffer". After aliquoting 500µL of cell suspension, 10µl of PI and 5µL of Annexin V were added. "The suspension was incubated for 15 minutes at room temperature in dark and was analyzed by FACS Caliber (BD Biosciences, San Jose, CA), as per the guidelines" (Wang et al. 2003).

Two ml of medium were dispensed in a 96-well plate seeded 1×10^6 cells and grown for 24 hours. Subsequently treated with 40 & 80 µg/ml of *P. nigrum* extract, incubate in a carbon dioxide incubator for the next 24 hours at 37° C. Harvesting the cells by centrifuging at lower rpm for a couple of minutes at room temperature. Discard the supernatant with care to retain the cell pellets were retained. "Cell pellets were washed by resuspending them in 2 ml of PBS (1X) and repeating this process twice. Discarded the supernatant and the cell pellets preserved". "Cells were fixed by re-suspending them in 0.3 ml of sheath fluid, then adding 1 ml of chilled 70% ethanol drop by drop with continuous & gentle shaking, repeat the procedure couple of times" (Jackman and O'Connor 2001).

The cells were then stored at a temperature 4° C overnight, centrifuged at lower rpm for a couple of minutes to collect a cells pellet. Then the cell pellets were rinsed in 2 ml of ice-cold 1X PBS and centrifuge. "Re-suspended in 0.45 ml of sheath fluid containing 50 µg/ml PI & 50 µg/ml RNase A & incubated in the dark for 15 minutes. The populations of treated and untreated cells in various phases of the cell cycle were analyzed by FACS Caliber" (BD Biosciences, San Jose, CA) (Magadi et al. 2015; Patil et al. 2020a; Patil et al. 2020b). Using TRizol Reagent (Invitrogen) total RNA was isolated from COLO205. "The isolated RNA was dissolved in 25 µl of DEPC treated water. A semi-quantitative reverse transcriptase-polymerase chain reaction was used to determine the sequence of mRNA of expressed sequence tags of Bax, Bcl-2, P53, and β-Actin (Applied Biosystems, VERITI 96 well thermal cycler). The cDNA was generated by 2 µg of RNA with the Verso cDNA synthesis kit (Thermo Fisher Scientific), with oligo dT primers". The final reaction volume was 20 µl, and cDNA synthesis was carried out for an hour at 42° C and reverse transcriptase inactivated at 85° C for 5 minutes.

RESULTS AND DISCUSSION

Cancer is the second leading cause of death globally, Colorectal cancer (CRC) third most common cancer followed by breast and lung cancer (Siegel et al. 2020; WHO 2018). The conventional approach of cancer therapy is chemo & radiotherapy has side effects, recovery chances shrink with immune compromised patients. Traditional medicine systems have become a promising method of treatment with minimal or no side effects. Plants are the treasure of secondary metabolites such as alkaloids, flavonoids, lignans, saponins, terpenes, and taxanes, synthesized due to biotic or abiotic stress. These phytochemicals are the treasures of therapeutic molecules and it has been used in medicinal practices since ancient times (Upreti 2021).

There are currently many therapeutic approaches that are considered to persuade cytotoxicity, which hinder tumor growth. The goal of any therapeutic approach is to affect the wide number of tumor cells while having no adverse effects on healthy tissue or cells; this can be achieved by inducing apoptosis (Rundqvist et al. 2020). The current research looked at the cytotoxic effect of different concentrations of methanolic extract of *P. nigrum* seeds on Colon cancer cell line COLO205 with increasing dosage (0 – 320 µg/ml), the number of viable cancer cells decreased significantly. In the current study IC_{50} value of *P. nigrum* extract after 24 hours intervals is at 48.2 µg/ml, likewise, crude extract of *P. nigrum* at 13.70 µg/ml was observed in the previous studies (Tedasen et al. 2020) (Figure 1).

Figure 1: CRC COLO205 Cell proliferation inhibition effect of *P. nigrum* extract. Cell viability positively correlated with the maximum concentration of 320 µg/ml extract, using the MTT assay.

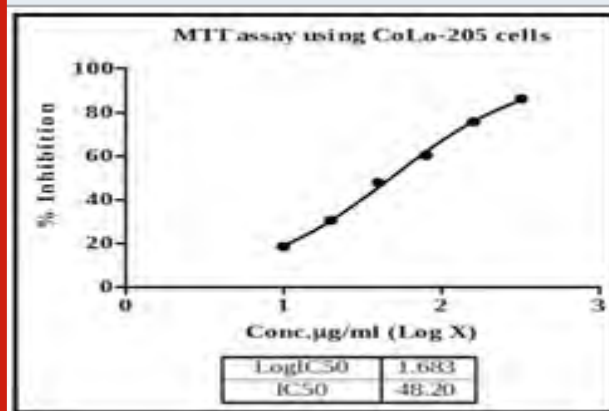


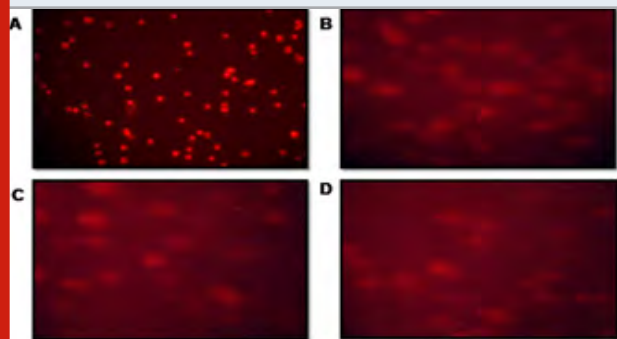
Table 1. The Effect of *P. nigrum* methanolic extract on CRC cell lines showing mean Olive moments against control and standard.

Variables	Olive tail moments Mean ± SD
Positive Control	4.1 ± 11.5
<i>P. nigrum</i> methanolic extract 40 µg/ml	8.8 ± 11.0
<i>P. nigrum</i> methanolic extract 80 µg/ml	11.4 ± 10.3
Negative Control H ₂ O ₂ (150 µM)	12.7 ± 26.2

Bioactive compounds Kusunokinin and piperloguminine isolated from *P. nigrum* were effective against breast cancer, and *P. nigrum* also effectively inhibited the growth of HeLa cells (Priya and Kumari 2017; Sriwariyajan et al. 2017). The Ekowati et al. (2019) combined ginger and pepper extract which triggered apoptotic mechanism due to cytotoxicity in HeLa cell line had IC_{50} at 33.81 µg/ml which is significantly lower than the current study. This study implicates that it is *P. nigrum* has a synergistic effect when combined with other natural food ingredients such as *Citrus limon*, *P. nigrum*, and *Melaleuca alternifolia* (Nikolić et al. 2017). Also, *P. nigrum* in combination with cardamom has a cytotoxic

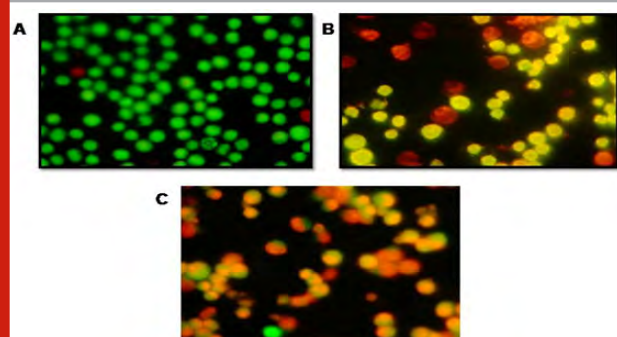
effect against YAC-1 tumor cells (Majdalawieh and Carr 2010; Rundqvist et al. 2020).

Figure 2: The patterns of DNA fragment migration determined by the comet assay were examined using a fluorescence microscope. (A) Control cells, (B) 40 µg/ml extract-treated Cell lines (C) 80 µg/ml extract-treated Cell lines (D) Positive Control: 150µM of H₂O₂ treated Cell lines.



The crude extract potency as a cytotoxic agent was confirmed from the morphological study using COMET assay and AO/EB staining. The extent of DNA damage in CRC cells was measured by DNA migration. Randomly selected cells were analyzed by calculated tail moment. The comet assay images of the Fluorescence microscope were visualized after treatment (Figure 2 and Table 1). The olive movements at 40 & 80 µg/ml were 8.8 ± 11.0 and 11.4 ± 10.3 respectively while the control had 4.1 ± 11.5 was observed. Maximum DNA damage was recorded with 80 µg/ml ($p < 0.05$) of *P. nigrum* extract-treated cells (Figure 2 C). A comparison of the extent of DNA damage with and without oxidant pretreatment was also reported, with the comet assay being declared the best option among all other methods for studying DNA damage (Jahangir et al. 2020).

Figure 3: A fluorescence microscopic image showing CRC Cell lines treated with AO/EB dual staining. (A) Control CRC cells, (B) *P. nigrum* extract (40 µg/ml) treated Cell lines (C) *P. nigrum* extract (80 µg/ml) treated Cell lines.



On observation under fluorescence microscope of AO/EtBr dual stained COLO205 cells treated & untreated cells with *P. nigrum* extract, normal cells had a circular nucleus depicts early-stage apoptotic cells (Figure 3A, 3B), with the nucleus

displaying yellow-green fluorescence due to AO staining & condensed into a semicircle in a corner of the cell. The late apoptotic phase in necrotic condition, evidenced by the EtBr intercalated to the damaged cell's DNA (Figure 3C). Similar results have been reported in *Piper betle* leaf extract and *Syzygium aromaticum* flower extract (Azhagumeena et al. 2021).

Figure 4: AV-FITC/PI dual-staining assay. After treating with methanolic extract of *P. nigrum* cells stained with AV-FITC/PI & analyzed by fluorescence microscopy

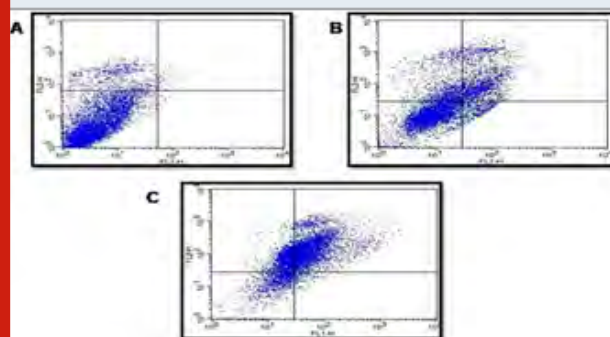
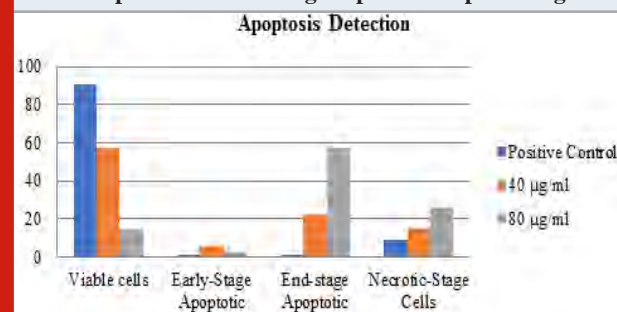


Figure 5: Apoptosis detection: fraction of cells CRC COLO-205 cells present in each stage expressed in percentage.



Staining cells with FITC-Annexin V (green fluorescence) simultaneously with dye exclusion of propidium iodide (negative for red fluorescence) at the same time allows for the differentiation of intact cells, early apoptotic & late apoptotic or necrotic cells. In control G2M was observed to be 8.5%. When treated with 40 & 80 µg/ml of *P. nigrum* extract G2M & S phase arrest was observed to be 2.7%, 16.6% and 17.1%, 21.0% respectively as illustrated in Figure - 6 & 7. When compared to control cells, the 40 & 80 µg/ml treated were induced both early and also late apoptosis in CRC cell line (COLO-205) with 5.7%, 2.2%, & 22.4% (Figure 4 and Figure 5,) apoptotic cells, respectively, and 14.9% and 26.2% necrotic cells, therefore the MTT assay tends to overestimate the efficacy of drugs that alter cell metabolism; it also fails to differentiate cell death from growth inhibition (Gomez-Guitierrez et al. 2021).

Flow cytometric analysis of AV-FITC/PI dual-staining: CRC COLO205 cells left untreated or treated for 24 h 40 & 80 µg/ml extract. A) Untreated, B) After 24 hours treatment with 40 µg/ml of sample, C) After 24-hour treatment with 80 µg/ml of sample. The bottom left quadrants in untreated cells were primarily AV-FITC/PI negative, live cells. The

upper left quadrant shows non-viable cells, undergoing apoptosis (AV-FITC positive and PI negative). The bottom right quadrants indicate the early apoptotic cells. The upper right quadrants indicate that they were in the late apoptosis phase, positive for both AV-FITC and PI.

Figure 6: Data indicating the proportion of cells in each stage of the cell cycle, for the of *P. nigrum* methanolic extract of treated with 40 and 80 µg/ml.

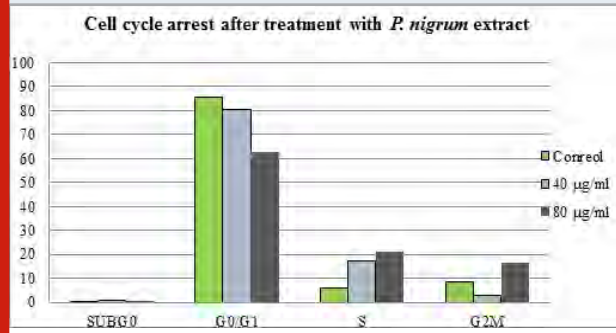


Figure 7: Analysis of CRC COLO205 cell cycle after being treated for 24 hours with 40 & 80 µg/ml extract. A) Positive Control Untreated cells, B) Treated with 40 µg/ml of *P. nigrum* ethanolic extract, C) Treated with 80 µg/ml of *P. nigrum* ethanolic extract.

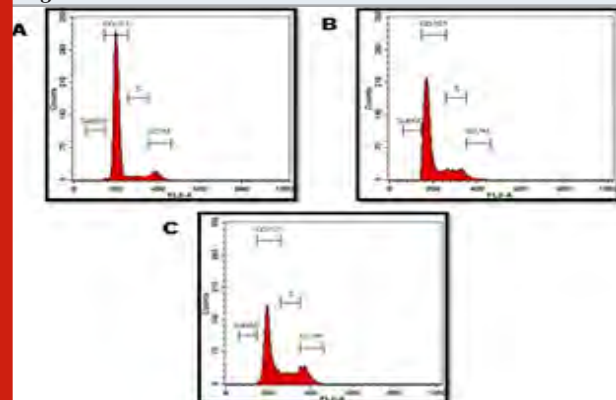


Figure 8: Expression of BAX, Bcl-2 genes. P53-independent on gene expression in DNA damage-induced apoptosis in COLO205 cells.

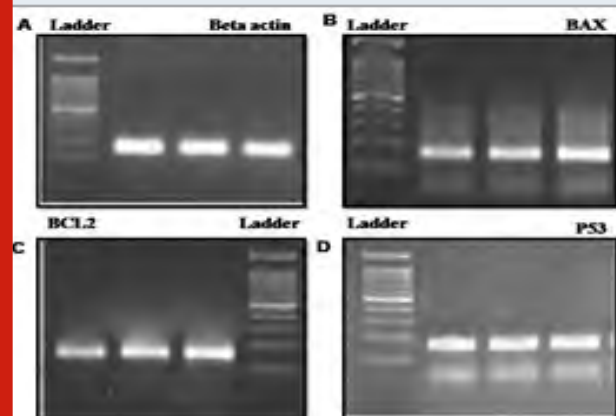
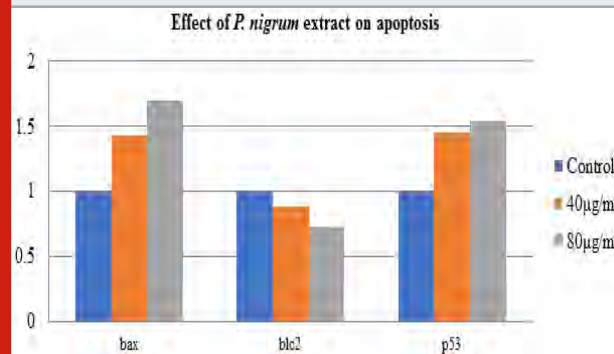


Figure 9: Effect of *P. nigrum* extract on apoptosis-related gene expression: BCL2-associated X protein (BAX), tumor protein P53 & B-cell CLL/lymphoma 2 (bcl2) in COLO 205



Semi-quantitative PCR was used to investigate the effect of *P. nigrum* on gene tumorigenic and oncogene - BAX, Bcl-2, and P53 in COLO205. The housekeeping gene β -Actin was taken as control. The treatment of *P. nigrum* extract led to upregulation of BAX & P53 by 0.4 & 0.5 folds while anti-apoptotic Bcl-2 were down-regulated by 0.7 & 0.5 fold (Figure – 8 & 9). Apoptosis occurs in cells accumulated in the G2/M phase via the BAX-initiated mitochondrial pathway (Celentano et al. 2019).

Our findings are consistent with previous research, indicating that *P. nigrum* is effective on cell death in the colorectal cancer cell line COLO-205 to a significant extent.

CONCLUSION

The results of this study indicate that *Piper nigrum* seed extract is an effective anticancer agent. While awareness on the biological activities of *P. nigrum* has improved, the scientific basis for the conventional use of *P. nigrum* in the treatment of colorectal cancer does not provide adequate information on the efficacy of the drug, the molecular mechanisms. Therefore, the data produced in this study are important to demonstrate evidence of potential pharmacological action. Our results correlate which mainstream research, which suggests that *Piper nigrum* is effective in inducing cell death in colorectal cancer cell line COLO-205 to a considerable extent.

Conflict of Interests: Authors have no conflict of interests to disclose.

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Technological Communication

Big Data Analytics Strategy Framework: A Case of Crowd Management During Hajj

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ABSTRACT

The objective of the present study was an investigation of applications of big data analytics in Hajj and Umrah for pilgrims, who come to Saudi Arabia every year for tourism and observation of religious rites as per the sacred beliefs of Islam. It has now become a necessity to see more applications of big data analytics in these pilgrimages because of the growing number of people every year. Therefore, crowd control, crowd management and conflict management are essential for reduction of stress, troubles, fatalities, accidents, theft and possible deaths during Hajj and Umrah events. Developing a predictive data analytic model for Hajj and Umrah will improve the efficiency, gross domestic product (GDP), surveillance, revenue generation, opportunities and satisfaction for the pilgrimages. In this paper, review of big data tools was presented along with their use in the decision support system and how it can be used for surveillance and crowd management. A robust big data framework applicable for Hajj and Umrah events was also presented in this paper. This was meant to aid seamless adoption and implementation of big data applications across sectors and government parastatals involved in Hajj and Umrah. The presented framework was also included all the relevant use cases related to these pilgrimages.

KEY WORDS: BIG DATA ANALYTICS, HAJJ, UMRAH, MINISTRY OF HAJJ AND UMRAH, CROWD MANAGEMENT, SAUDI ARABIA.

INTRODUCTION

Hajj and Umrah are both important events for Islamic believers. Hajj is a pilgrimage journey to Mecca and is part of the five pillars of Islam; it is required for every Muslim who is financially and physically capable to make this pilgrimage at least once in their lifetime. Performing Hajj successfully, which is one performed without evil commitment, results in erasing sins. Umrah is also linked to erasing of sins as well as a rewarding spiritual experience. With these reasons as the foundation, millions of Muslims from different parts of the world travel to Saudi Arabia for Hajj and Umrah (Ledhem et al., 2020; Luz, 2020).

These annual events attract so many people that it's now considered the world's largest human gathering (Central Department of Statistics and Information, 2014). With the largest gathering, there are aspects that come into play: crowd management and big data frameworks and analysis. Hajj and Umrah are posing big issues due to the fact that the

number is increasing every year. It is estimated that by 2030, there will be more than 5.4 million people in Hajj and around 30 million in Umrah. These huge numbers result in issues related to crowd management and resource control. Big data analytics is a technique that can be used to manage, control, predict and facilitate smooth success of the Hajj and Umrah. Big data refers to a dataset with a large size and complexity. Data mining is an important concept in the big data era. It is the collection, extraction, analysis and predictive use of data which allows the useful and meaningful applications of data. In this paper, we discuss Hajj and Umrah, crowd control and management, big data tools and framework, and design of big data framework application for Hajj and Umrah, to forecast and foster more successful Hajj and Umrah pilgrimages (Wu et al., 2014; Luz, 2020).

We looked at the literature of all the above-mentioned topics and analysed their contribution to the significance of this study. Crowd control and conflict management are a key challenge during Hajj and Umrah. Crowd control is an essential factor to consider in any mass gathering, and Umrah and Hajj are not exceptions. Thus, whatever the reason for mass gathering, there must be provision for eventualities. Hence, security, peaceful processes and

Article Information:*Corresponding Author: haldahawi@kau.edu.sa

Received 21/10/2021 Accepted after revision 25/12/2021

Published: 31st December 2021 Pp- 1975-1984

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.88>

reduction in casualties, fatalities and deaths must be the objectives for the authorities managing Hajj and Umrah. The use of big data analytics to predict the expected behaviours of the crowd helps in pre-incident planning to enable responsibilities, training, organisation, operating procedures and the rules of engagement in case of any eventuality (Felemban et al., 2020).

In fact, for a huge number of people to be gathered in a given place, crowd control and management must come into play, especially when safety and security measures are considered. We can define crowd “as an assembly of persons large enough to produce a sense of considerable mass, casually gathered together without organised discipline or order”. Hence, to have a disciplined and organised crowd requires crowd control and management. Therefore, crowd management is defined as organised and systematically planning the movement as well as assembly of people in an orderly manner as to achieve the desired objective and purpose of the mass gathering effectively. On the other hand, crowd control refers to providing guidelines for people regarding their attitudes and behaviour in a gathering (Brian and Kingshott, 2014).

Data Analysis: The Hajj activities take place for five days and in specific locations. With millions of people carrying out these activities within a limited period of time and in the same places, crowd management has turned out to be an issue the stakeholders of the event, such as the Ministry

of Hajj and Umrah, have had to battle with over the years. In some cases, there have been occurrences of tragedies. According to Alnuaim and Almasry (2012), the table below is a highlight of disastrous accidents that have occurred during Hajj and Umrah due to poor crowd management.

Even though there have been efforts geared towards improving crowd management over the years, there are still numerous challenges that Saudi Arabia faces. The slow turning of the wheel of progress when it comes to crowd management in Hajj and Umrah is linked to issues such as: Infrastructure problems: the pilgrimage activities are limited to a few places in the holy city of Mecca. The infrastructure such as the road networks connecting these places have some problems. Lack of following the schedule: there are schedules rolled out by the Hajj authorities but some of the pilgrims do not fully follow them, thus creating hindrance in the process. Ever increasing number of pilgrims: each year, there is a drastic increase in the number of pilgrims and the event planners can hardly plan adequately, especially when it comes to numbers. Having more people than planned for leads to issues such as long waits to perform the Hajj and Umrah activities, which, in most cases, end up in disaster.

Pilgrims getting lost: according to Amro and Nijem (2012), in 2011, over 30,000 pilgrims went missing as a result of overcrowding at the Hajj holy locations. Children and foreign pilgrims are mostly affected, and the language barriers sometimes make the problem worse.

Table 1. Data Report of Past Hajj Incidences (Alnuaim & Almasry, 2012)

Date	Accidents	Casualties	Place
1975	Fire	Death of 200 pilgrims	Camps for pilgrims near Makkah
1990	Suffocation	Death of 1,426 pilgrims	Inside a pedestrian tunnel
1994	Stampede	Death of 270 pilgrims	Al-Jamarat in Mina
1998		Death of 118 pilgrims	
2001		Death of 35 pilgrims	
2003		Death of 14 pilgrims	
2004		Death of 251 pilgrims	
2006		Death of 346 pilgrims	
2015		Death of 2,411 pilgrims	Mina

Figure 1: Big Data Representation (Adapted from Ezeogu et al., 2019)



Limited guidance at the ritual places: lack of adequate guidance even in direction leads to problems. Research on the areas of crowd management has improved over the years. There are more studies geared towards improving movement and assembly of people. Some of the changes that can be attributed to the continuous growth in research include small cars not being allowed in Mecca to limit congestion. The expansion of the railway network through construction of the monorail Al Mashaaer Al Mugaddassah Metro Line in 2010 to link Mina, Arafat and Muzdalifah has resulted in reducing transportation problems for pilgrims (Reffat, 2012; Still et al., 2020).

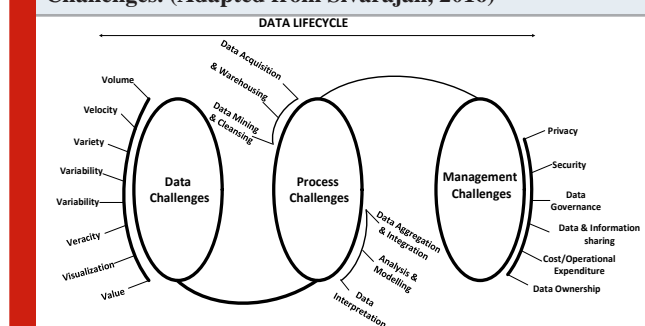
Mahmassani and Sheffi (1981) came up with a one-of-a-kind proposal on the study of the behaviour of people in certain situations. As a result of this proposal, Dr

Felemban's centre is working on the development of a bracelet that would help in tracking pilgrims as well as be a source of guidance for them throughout the various Hajj and Umrah activities. Installation of over 800 surveillance cameras is also a result of research in crowd management. This is slowly helping the stakeholders to improve the safety measures even as the number of pilgrims increases each year. Research on the structural issues of the sacred sites in relation to crowd management problems has also been vital, among other scientists, proposed that the geometry of the sacred sites structures should be refined. This proposal is being implemented, and one of the examples is the reconstruction of the Jamaraat Bridge to help accommodate a larger number of pilgrims performing the Hajj ritual that involves the stoning of the devil, which is carried out on the bridge (Algadhi et al. 2002; Pin et al., 2011).

Furthermore, in 2012, author carried out a study on crowd management of pilgrims with the use of thermography. Through this, they were able to provide a realistic demonstration of behaviour of pilgrims during three Hajj activities. In addition, a study by Maciej et al. (2011) took another approach, by introducing a crowd management system that was based on optical data flow. In this approach, the system was to consider two lines of threads: one being the analysis of behaviour for detection of situations that could be considered dangerous in a crowd, and the second one being the detection of any delays or hold ups in a given area (Khoziun et al., 2012; Still et al., 2020).

Additionally, other studies on crowd management in Hajj and Umrah have been geared towards intelligence-based detection and management of congestion, development of an intelligent computational real-time virtual environment model, a multi-agent approach to the issue of crowd simulation and modelling and use of a wireless sensor network deployment model in monitoring pilgrims in cases such as evacuation (Stefania et al., 2007; Reffat, 2012; Felemban et al, 2020).

Figure 2: Conceptual Classification of Big Data Life Challenges. (Adapted from Sivarajah, 2016)



Big Data Concept: Big data analytics was first developed by (Chen et al., 2012) when they pointed out the relationship between business intelligence and analytics, and the connection they have with mining of data and analysis of statistics. The large number of pilgrims in Hajj and Umrah events bring with it a lot of data, which, if effectively and efficiently captured, stored, managed and analysed, can help the stakeholders involved in planning the Hajj to make

the event safer, more secure and successful. Thus, data, be it audio, video or textual from millions of people, is a lot, especially in recent times of high-volume data consumption; it is considered big data. Therefore, big data is simply defined as a dataset with a size that is more than what a typical software tool can take in terms of capturing, storing, managing and analysing. Big data describes innovative techniques and technologies to capture, store, distribute, manage and analyse larger sized datasets with diverse structures. According to Halevi (2012) and Apollos et al. (2019), in describing big data concept, the five Vs ideology is applied (Manyika et al., 2011; Apollos et al., 2019).

Studies on big data have focused on looking at challenges in the understanding of the whole big data concept. This includes decisions on what data is generated as well as collected, privacy issues and consideration of ethics while mining the data. Tole (2013) indicated that one of the challenges that businesses and other sectors are facing is building a viable solution for big data; therefore, it continues to be a learning process with creation, innovation and implementation of new approaches every now and then. Computing architectures are proposed to replace the von-Neumann traditional computing architecture. The idea behind the new architecture, computation-in-memory using new nanomaterial such as memristor, is to improve storage capacity and speed of computation in the era of big data (Lazer et al., 2009; Boyd & Crawford, 2012; Crawford, 2013; Hargittai, 2015 Ezeogu et al., 2019; Felemban et al, 2020).

Big Data Life Cycle: Today, big data analysis is done using intelligence plans. According to Manyika et al. (2011), big data can contribute positively towards the transparency, performance, creation and innovative decision-making wherever and whenever it is used effectively. According to the author, as opposed to the past when capturing, storing, managing and analysing of big data was difficult, today it is easier thanks to the development of big data systems. With these systems, visualising, detection of trends and development of algorithms to predict occurrence of issues is possible and easier (Mawed et al., 2017; Khalid & Zebaree, 2021).

Computing technologies are used when it comes to capturing, storing managing and analysing big data. However, it is important to note that a lot of challenges come with the big data: data challenges, process challenge and management challenges. These are the stages in a big data life cycle. Akerkar (2014) and Zicari (2014) categorise the challenges of big data into data, process and management challenges, depending on the stage of the data life cycle a set of data is at. The data challenges are the ones that have to do with the data's characteristics including its volume, velocity, variety, veracity, quality, volatility, dogmatism and discovery. Process challenges, on the other hand, are those related to techniques on how the data are captured, integrated and transformed as well as the procedure of selecting the correct analysis model and how to generate results. Lastly, management challenges look at security, privacy, ethical and governance issues of big data. To ensure that decision made by people utilising big data

are evidence-based, choosing efficient data processing or analysing methods is necessary (Gandomi and Haider, 2015; Felemban et al, 2020).

The benefits and potential that comes with using big data is limitless, but the technologies, skills and tools that are available to help with big data analysis can prove to be restrictive. Labrinidis and Jagadish (2012) defined big data analysis as the methods or processes used in the examination and attainment of intellect or viable information from large datasets. Big data analysis, commonly referred to as BDA, is more than just tracing or capturing, categorising, understanding and quoting data (Davenport & Dyché, 2013). They go further to indicate that choosing the right analytical method is crucial, as it also influences the information one can extract from the data. The types of analytics include:

Descriptive Analytics: scrutinises data and information to define the current state of on a situation or organisation, patterns and exceptions, producing ad hoc reports, standard reports and alerts (Joseph & Johnson, 2013). **Inquisitive Analytics:** as the name suggests, this type of analytics focuses on using data to support or reject propositions (Bihani & Patil, 2014). **Predictive Analytics:** here, data is used to forecast and statistically model future possibilities (Waller & Fawcett, 2013). **Prescriptive Analytics:** this is more about optimising and randomly testing data to evaluate how a business or situation is performing (Joseph & Johnson, 2013). **Pre-Emptive Analytics:** this involves using the data to develop the capacity to take precautions that may lead to undesirable occurrences. An example is analysing data to use it to identify possible risks and develop recommendation to avoid or deal with the identified risks (Szongott et al., 2012).

To deal with big data analysis, fields of machine learning (ML) and deep learning (DL) framework were developed and expanded to deal with BDA. ML explores predictive features or patterns in big data, thus allowing prediction of what could happen in future. On the other hand, DL plays a major role in the extraction of data that are useful and meaningful. DL, which was inspired by the human brain, was introduced in the 1940s but had not been determined until 2006 when Hinton and Salakhutdinov (2006) introduced the layer-wise-greedy-learning method as a way of overcoming the deficiency of neural network (NN) method. The NN method worked optimally through trapping into the optima local point that is exacerbated with lack of adequate training data in terms of size.

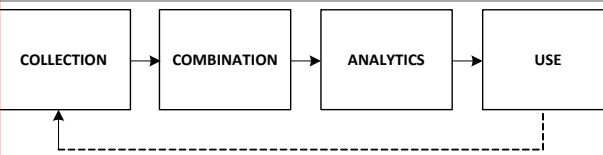
To deal with this challenge, Hinton proposed that unsupervised learning should be used prior to layer-by-layer training. There are four categories of DL algorithms: convolutional neural networks (CNN), auto-encoder, sparse coding and restricted Boltzmann machines. In the case of Hajj and Umrah, there is limited literature on big data and big data analytics even with the large datasets these events come with. There are several technologies being developed that are meant to help the stakeholders to take advantage of this data and use it for better, safer and more organised Hajj and Umrah events (Vanani and Majidian, 2019).

Big Data State, Approaches And Activities: In the literature review, we expounded on the concept of big data. However, in this section, the focus is on big data current state, approaches and activities including possible advantages of using big data in Hajj and Umrah events. Big data poses privacy and reliability requirement as essential in the data management. The task to develop a system that can be scaled to handle data influx and provide sufficient capacity for analysis is a key challenge in big data. This particular challenge includes both hardware resources requirement and architecture, and also processing requirements. Hence, in this section, the current state of big data is briefly discussed along with the approach and activities.

Figure 3: Big Data Approach (Adapted from IBM Student Guide, 2018)



Figure 4: Big Data Activities



Current State of Big Data: Big data has many applications. The intelligent use of big data within the health sector can save over \$300 billion, as seen in precision medicine diagnostics. In addition, as surveyed by Archenaa and Anita (2015), big data analytics is applied in health care and government for patient centric services, detecting spreading diseases earlier, improving treatment method, reducing unemployment rate, providing quality education and addressing needs urgently (McPadden et al., 2018; Keshavarz et al., 2021).

Furthermore, the potentials of big data allow integrating geographical information system and business intelligence, hence building an effective predictive analytic system which can be of very good use and advantage for crowd control

and management. A technology-driven process allows firms to analyse, report and make critical informed decisions. Big data and business analytics market was predicted to increase up to \$203 billion by 2020. It has become the trending practice for organisations to construct and extract valuable information. This is achieved by using open-source applications such as Apache Hadoop, Spark, NoSQL and many others. Hadoop dominates about 60% of the market space (Keshavarz et al., 2021).

With use of big data in business intelligence with ML and AI technologies, we can track of employees, assets, equipment and collect and interpret data to make informed decision, thus resulting in personalisation and better user experience. As reported in McPadden et al. (2018), integrating data-lake and analytic platform used to provide real-time access to health care allows for continuous monitoring of patient in real-time analytics. Big data-lake makes parallelised computation more accessible. Hadoop allows big data storage and batch processing analysis, hence enabling distributed data storage and scalable processing capacity. However, Hadoop has with fewer tools available for streaming data and real-time analysis.

Predictive analytics, intelligent security, internet of things (IoT), edge computing and self-services, in-memory computing and business intelligent application, decision support system and geospatial information system are all different areas and trends on which big data has had a great impact. The current state and trends of big data exploration have various advantages and applications in the industries, government, medical, engineering and social events. Big data is now driving innovations by studying interdependencies between humans, events, institution, processes and then using the insights gained to improve decisions (Keshavarz et al., 2021).

This insight is known as big data visualisation. Visualisation of data is a key purpose of gathering data, which provides insight to the large pool of data. The data visualisation pipeline stages consist of simulating, preparing, mapping, rendering and interpreting. The data flowing from the simulating stage go through preparing, mapping, rendering and interpreting stages, and then return to the simulating stage. The mapping is very essential in the visualisation pipeline in big data analytics. Thus, data visualisation helps to identify inherent patterns and infer correlation and causal relationships (Xuefeng et al., 2020).

The purpose of visualisation is to provide suitable methods and instruments to explore data and information. It enables the extraction of useful information from complex or/and voluminous datasets through the use of interactive graphics and imaging, thus steering the dataset and seeing what may have appeared invisible. Parallel implementation for big data visualisation is used to address scalability issues using many core GPUs, distributed clusters or hybrid architectures (Xuefeng et al., 2020).

Hence, data preparation and rendering during visualisation are achieved using modern GPUs and distributed visualisation frameworks with Hadoop/Spark that

leverages GPUs to compute data aggregates with kernel density estimation. Another example of data visualisation framework is the open-source MapD, which uses GPUs to accelerate the processing of large and complex datasets that can process billions of rows of data in milliseconds. In addition, MAP model and MAP-Vis framework as proposed in Xuefeng et al. (2020) realises millisecond-level multidimensional data querying with good interactive visualisation, and both the MAP model and MAP-Vis framework provide high scalability for processing and online visualisation. They use SPARK as a pre-processing tool and HBase as a distributed storage platform (Perrot et al., 2015; Root & Mostak, 2016; Felemban et al, 2020; Khalid & Zebaree, 2021).

Big Data Approaches and Activities: Big data approaches entail the predictive model design and data mining tools used for big data deployment. Big data processing can be performed by batch processing and stream processing. Batch processing collects and stores data in batches, in order to analyse and generate results, while stream processing is suitable for real-time feedback and requires response time constraints. Stream processing reduces computational time. This big data approach is essential in health care analytics for real-time extraction of needed information from a large amount of patient data, for alerting emergencies and complications; thus, it has been helpful for real-time diagnostics, decision and intervention for urgent care. Figure 3 shows an illustration of big data driving business strategies.

Meanwhile, big data also entails different activities, which includes data collection, combination, analytics and use, as shown in Figure 4 (Klievink et al., 2017). Although, big data is not a technology itself, it is a collection of large datasets that cannot be easily handled by conventional data processing technology due to its great variety, velocity and large volume (Kankanhalli et al., 2016; Klievink et al., 2017). It involves visualisation of data using a more specialised computational tools, storage and analytic technologies.

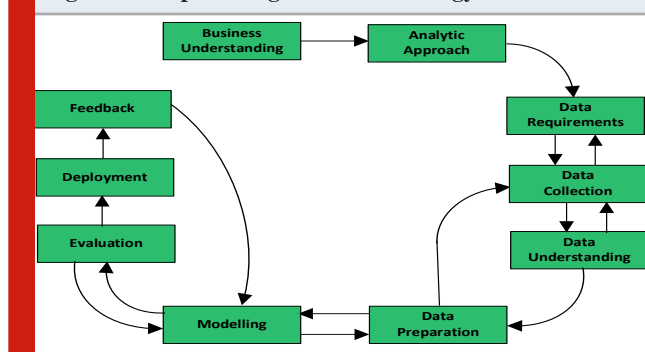
Big Data Advantages in Hajj: There are many advantages of big data in Hajj like: increase perception of risk and more effort in the hospitality management of the visitors, reduce provocation during activities, enhance visitors experience, safety and security, preparation and planning, management and organisation principle and crowd management and response including spontaneous disturbance.

Big Data Methodology: Big data methodology provides methods and analysis of the rules or procedures of inquiry for constructing a model to predict, discover underlying patterns and gain insight in data.

The development methodologies in big data process following a software development life cycle are as follows: feasibility and business understanding; systems analytic approach; data requirement, collection and understanding; data preparation; modelling; evaluation; deployment and feedback. The objectives of a big data project depend on the purpose and must strategically be motivated with strong

executive support, and must meet a business need. Large-scope big data projects may benefit from having managers and a co-project manager, to develop and be executed by project team. If the project will be outsourced, then a process needs to be developed for creating a request for proposals and then evaluating the proposals submitted.

Figure 5: Proposed Big Data Methodology



If the development will occur in-house, development tools and technical issues need to be resolved. The feasibility analysis should have determined if the project could be completed in-house. The project manager should identify tasks that must be completed, resources that are needed and project deliverables. Deliverables are especially important for monitoring the progress of the project. Furthermore, milestones are identified to help non-technical managers monitor a big data project, while the chief information officer (CIO) of the organisation and one or more business managers usually monitor the progress of a large-scope or high visibility big data project. Meanwhile, big data deployment in Hajj objectives are as follows:

1. **Visitors (Pilgrimage) relationship management:** shopping frequency, locational pricing adjustment, identity recognition, performance targeted advertisement campaigns, visited locations, disability care management and many more.
2. **Improved data accuracy and management:** this includes the operational maintenance, support, data analysis and tracking and monitoring of data.

Big data project is an expensive task. It is developed to assist people and the organisation in making effective decisions, predictive analysis, customisation, effective data management and improving the lifestyles and utility of the people. However, the development process and approach may encounter failure. An inadequate development process is one without critical and analytical details of the requirements and feasibility of the big data project. Many factors can contribute to this failure which include the following: Poor and inadequate feasibility and preparation, poor and inadequate documentation and tracking, bad leadership and inexperience project manager, incompetent or lack of technical skills for execution of tasks and projects, inaccurate cost analysis and estimation, ineffective communication at every level of management, choice of big data framework and tools used, organisation culture and Competing priorities (Qi et al, 2020).

Big Data Framework Application In Hajj And Umrah:

The framework for big data application in Hajj and Umrah events is presented in Figure 6. In this section, we describe the various components within this framework.

Big Data Tools: Big data tools are used for designing and implementing big data projects. The characteristics of these tools are as follows: applications are written in high-level language code, work is performed in a cluster of commodity machines, data is distributed in advance that brings computation to the data, data is replicated for increased availability and reliability and scalable and fault tolerant.

Figure 6: Big Data Framework for Hajj

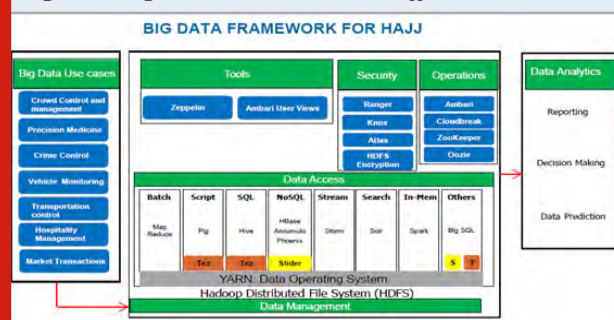


Figure 7: Big Data Tools (Adapted from IBM Student Guide, 2018)



Big data space is a large growing ecosystem. Many tools exist for big data deployment. These tools can be seen in Figure 7. Hadoop is most widely used tool, and it is also an Apache open-source software framework, which is very reliable, scalable and includes distributed computing of massive data. It is developed in Java and consists of three sub projects: MapReduce, Hadoop Distributed File System (HDFS) and Hadoop common. Hadoop is developed in Java and hides the underlying system details and complexity from the user. It is meant for heterogeneous commodity hardware. Its related projects include Hbase, Zookeeper and Avro. Thus, data mining involves using data-intensive and computing-bound algorithms with high processing units to extract information at the required time.

The common use cases for data analytics are extract/transform/load, text mining, index building, pattern recognition, predictive model, risk assessment and collaborative filtering. In the Hajj and Umrah big data

application, it collaborates variety of data and networks to monitor the cases using sensor networks. Thus, sensors are used to monitor people's movements and services, purchase transactions, inventory management, asset tracking, vehicle monitoring and transportation organisation. This design application will require efficient collection, storage and analysis of data. The design framework is an event-driven data processing design that will trigger a certain action when a condition is met. In addition, during the convergence prayer, it will be necessary to carry out stream data processing to monitor events and ensure effective crowd management.

The data access tools for big data design include MapReduce, Pig, Hive, Accumulo, Hbase, Storm, Solr, Spark, Big Sql, Tez and Slider. These are the components that provide data access capabilities (IBM Student Guide, 2018).

MapReduce: It provides frameworks that manage the complexity of parallelisation. Input is split into pieces as HDFS blocks or splits, then the worker nodes process the individual pieces in parallel and store the results in its local file system, where a reducer accesses it. **Spark:** It is a large-scale data processing framework that can be used to write several applications in several languages like Java, Scala, Python and R. The advantage of Spark is that it also allows data to be operated in memory, is easy to use and runs programs faster than MapReduce in memory computation. It can combine SQL, streaming and complex analytics. Furthermore, it runs in variety of environment and also with diverse data access sources such as Hadoop, Mesos, Cloud, Hbase, S3 and Cassandra. **Storm:** Storm is Apache Hadoop stream-processing framework that tracks and determines successful completion of tree of tuples triggered by every spout tuple.

Solr: Apache Solr is a fast open-source enterprise search platform built on Apache Lucene Java search library; it allows full-text indexing search, providing highly reliable, scalable, fault tolerant, centralised configuration and automated failover and recovery. **Hive:** Hive was developed originally by Facebook; it allows abstraction of data on top of non-relational semi-structured data. It provides an SQL-like interface for the user. Hive uses a serialisation/deserialisation interface to read data from table and then write back in any custom format. **Big SQL:** Big SQL builds on Apache Hive foundation, and it uses the native C/C++ MPP engine. It is an SQL-processing engine for Hadoop cluster that provides SQL on Hadoop interface. It requires no new SQL syntax nor propriety storage format. **Pig:** It is a procedural/dataflow open-source programming language originally developed by Yahoo. It is a high-level programming language for data transformation and is better than Hive for unstructured data. Its native language is Pig Latin. The compiler translates it into sequence of MapReduce programs. **HBase:** It is a NoSQL data store and is a distributed and scalable big data store used when random and real time read/write access is needed in big data. Thus, it enables handling of large tables of data running on clusters of commodity hardware. It is modelled after Google's Big Table and provides Big Table-like capabilities on top of Hadoop and HDFS.

Falcon: It is for managing data life cycle in Hadoop clusters for backups, archival of data, feed retention and much more. It is a data governance engine that defines schedules and monitors data management policies. It allows Hadoop admins to centrally define data pipeline, which is used to auto-generate workflows in Oozie. **Ranger:** It is used for data security control over the Hadoop platform. It manages policies for access to files, folders, table, databases and columns. These policies can be set for individual users and groups.

Zookeeper: It provides centralised service for maintain configuration information. It is fast, reliable and ordered. Distributed applications can use it to store and mediate updates to important configuration information.

Design of Big Data Analytics in the Hajj: The big data analytics in the Hajj can be designed for different reasons such as: fraud detection in credit card transactions, credit issuance, increase speed of detection, crowd control and risk management, 360° view of the pilgrimage, planning and monitoring to reduce congestion, real time analysis to weather, identify traffic patterns to reduce transportation cost and save time of arrival and exit, predict trends and prepare for demand for the Hajj attendees and optimise pricing and promotions. Use cases in the Hajj to be tackled: Precision medicine, transportation/congestion control Hospitality management, market transactions, crowd control and conflict management and crime detection and prevention.

Big Data Use Cases: The use cases are the data sources generated by sensors, smart devices, social networks, survey, regulatory agencies and IoT equipment. These use cases are included in the data production phase identified for data management and analysis. In this Hajj framework, the use cases are every identifiable factor that will be involved in the pilgrimage; in addition, people will fall sick, people with disabilities will attend and health care services and monitoring of patients are essential in critical cases. Hence, precision medicine which involves real time monitoring will be used.

Transportation and congestion control to identify best route and traffic congestion control while attending and leaving the prayer centre is very essential. This is best managed and identified using big data. Hospitality management ensures effective accommodation, care and services provided to the visitors during Hajj. Therefore, crowd control and management, conflict resolution and market transactions are all events that must be identified, including credit card frauds during business transactions. An effective crime detection and control is a key identifiable need to ensure vehicle monitoring and safety of lives and properties of the pilgrims.

Big Data Management: This is the next phase; it is where the identified sources are managed. This stage involves the collection, storage and processing of data. The data are explored and processed. Data cleaning, transformation and packaging operation is performed. Big data management in the Hajj design consists of the data access, tools, operations

and security. The data management deals with the entities (objects) in the data model, the relations and granularity of data and the time window available of data (Felemban et al., 2020).

Thus, the mapping of data objects with one another in the existing business process and the supporting infrastructure is achieved. In data management, it is required to address the issue of data requirement and the capacity requirement of the data. The features of the data that must be considered for effective management, which include data availability and the timing and longevity of the data. Data manipulation operations are derived by joining data sources, filtering rows and fields of data sources, aggregating, combining and deriving new features, and appending data sources.

Big Data Analysis: After the processing stage of the data using all the required tools for data management, evaluation of the results for reporting, prediction and decision-making is carried out. The analytic process provides data access for informed decisions, improved and higher security and smooth pilgrimage. A data quality report is necessary; it describes the features of the analytic base table, using standard statistical measures such as central tendency and measures of dispersion. This process is followed by data visualisation using bar charts, graphs, histograms and other convenient data plots methods (Xuefeng et al., 2020).

Consequently, identifying and handling the data quality issues can be addressed here: missing values, outliers and cardinality. Thus, data report is examined here for all the use cases for the Hajj and Umrah events. There are two types of data examinations during the visualisation process: categorical and continuous data. In the categorical data examination, the modes are examined to observe level of domination in the dataset, whereas, in the continuous data examination, the mean and standard deviation are examined to make sense of the central tendency and variations within the dataset.

Big data is the new keyword today that is filled with large pool of data. The need for the use of data is to explore and identify pattern, prediction for informed guesses and infer to quantify what we know. The model for crowd control and management using big data analytics is to first know what is expected and also focus on possible harmful conditions and behaviours that might arise during the pilgrimage. Enhancing visitors experience can be obtained by ensuring public safety, explain changes in the rules, give proper direction and provide guidelines that must be enforced and followed during the Hajj and Umrah pilgrimages. Big data analytics enable the management to target and intervene quickly on danger situations and scenario of disturbance and violence. There must be rules and guidelines that reduce the potentials of misinterpretation of rules.

Additionally, the management and organisers must ensure consistent training and intervention prior and during the pilgrimage. The big data framework for Hajj is designed with three phases: big data uses case, the data management and data analysis. The use cases are the big data production; they are the identifiable sources of data that will be

critically needed and involved during the Hajj and Umrah pilgrimages. As elaborated earlier, Hajj and Umrah involve the largest mass gathering activities in the world (Ledhem et al., 2020).

Thus, there is need to effectively make informed decisions, predictions and innovations in the process to ensure reduction of hazards, risk factors, death and other vices that may meet the visitors. Therefore, big data deployment in Hajj and Umrah will help in minimising all the risk factors and ensure smooth operations and satisfaction of the pilgrimage, thus fostering effective realisation of the goals and objectives of the pilgrimage (Shambour & Gutub, 2021).

This research has also shown that big data implementation starts with feasibility study with formal report or document, providing the business understanding with the risk factors that will affect the potential development and implementation the design. This feasibility study is a major checkpoint with critical information on whether it is possible to develop a system given the project goals and constraints. This is followed by in-house big data development or purchasing the big data software applications and packages. Thus, packaged solutions sometimes may be generalised and may not provide some specialisation need as required by the development team in building the analytical and decision support system for predictive analysis in big data applications. Just as mentioned earlier on big data approaches, it is necessary to consider the nature of the approach, whether it requires stream processing or batch processing. A big data deployment requires a development process which includes methodologies that will be used in the big data development.

CONCLUSION

This paper designed big data framework for Hajj and Umrah Islamic pilgrimage, as it is now very essential in this Islamic pilgrimage that conglomerates an estimated 3 million people in a location. Therefore, because of the growing rate of people participating in the program, there is need for crowd control and management, crime detection and monitoring, congestion and transportation control, market transactions and health care services to deploy big data for the Hajj and Umrah pilgrimage. The framework for Hajj will solve and bring to the barest minimum the existing challenges faced in the pilgrimage. This will improve the efficiency and effective management of the pilgrimage. It will foster reliable surveillance of the process, with increased revenue generation, opportunities and satisfaction for the pilgrims.

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Biomedical Communication

Significance of Addressal of Clinical Investigations of Kidney Functions in Recovery/Mortality of Certain COVID-19 Patients

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ABSTRACT

Clinical management of COVID-19 patients through a robust protocol is key to the good recovery and reduced mortality of patients. Efficient kidney functions during treatment period can contribute for improvised recovery and reduced mortality of patients. Analysis of the kidney function among Recovered and Dead cases of COVID-19 was made to reveal the degree of association of kidney functions with the two categories of patients. 83.4% of recovered patients did not show hyper values of blood urea whereas 72.5% of dead patients showed hyper-urea level in blood. 88.8% of survivors showed non-hyper creatinine level of blood whereas only 40% of dead cases showed hyper creatine level. Strong degree of association of blood urea with recovery/mortality was observed. Sodium levels were seen to be low while potassium and chloride ions were seen to be high in COVID-19 individuals. Our preliminary study suggests that kidney functions especially the value of blood urea and creatinine need to be addressed during COVID-19 patients to ensure the best recovery and reduced mortality. After more number of case studies, the present observation could sensitize consideration for inclusion of addressal and treatment of kidney functions into treatment protocol against COVID-19. It was also interesting to observe that levels of sodium and potassium ions among Survivors and Dead cases have impacted function of the essential ion channels in patient's physiology.

KEY WORDS: COVID-19, ELECTROLYTES, KIDNEY, SARS-COV2, UREA.

INTRODUCTION

Severe Acute Respiratory Syndrome-CoronaVirus-2 (SARS-COV2) has now taken the shape of one of the most rampant human pandemics the world has ever faced. In India, there prevails the second wave of pandemic starting from 11th February 2021 with a total of 178360849 cases and 3869384

as of today as updated on W.H.O's Coronavirus (COVID-19) dashboard (<https://covid19.who.int/>), (Ranjan et al. 2021).

Due to huge number of cases being reported to hospitals every day, the treating clinicians are compelled to focus aggressively only on the anti-viral/anti-bacterial treatments to the patients with parallel attention on normalcy in vital levels of pulmonary oxygen saturation level, cardiac parameters and Blood Pressure levels. However, although many other parameters such as Liver Function Tests, Hematological parameters and Kidney Function Tests etc.

Article Information:*Corresponding Author: vinodjoshidmrc@gmail.com

Received 15/08/2021 Accepted after revision 24/11/2021

Published: 31st December 2021 Pp- 1985-1991

This is an open access article under Creative Commons License,

Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.89>

are being measured and recorded essentially among all the admitted patients, yet kidney function parameters are not paid attention to address them in the corresponding treatment protocols. It is well known that the human cell receptor of SARS-COV2 is Angiotensin-Converting Enzyme-2 (ACE-2) and it is very important enzyme of the RAS pathway (Renin-Angiotensin System) i.e. in regulating the blood pressure homeostasis of the body as well as in maintaining the fluid and salt balance in the body (<https://www.rndsystems.com/resources/articles/ace-2-sars-receptor-identified>), Acc Apr 2021)

This ACE receptor is expressed specifically on the lungs and also on the kidney, gastrointestinal cells, vascular epithelial cells, kidney and Leydig cells. The IFCC (International Federation of Clinical chemistry and Laboratory Medicine) Guidelines on COVID-19 highlights the need of monitoring creatinine levels in critical COVID-19 patients so as to diagnose any injury to kidney at an early stage (IFCC guide on COVID-19, 2020).. The observations reported in the present study are in concurrence with the IFCC guidelines. Previous studies have reported that in the SARS-COV 1, 2003 strain and the Middle East respiratory syndrome (MERS) infection, there were cases with Acute kidney injury (AKI) and subsequent mortality of cases (Elias & Benito, 2018, Cheng et al. 2020; Naicker et al. 2020).

The present study highlights the association between the recovery/mortality of Covid-19 patients with their Kidney Function parameters and reports that surviving patients showed normal and not surviving patients showed abnormal kidney function parameters. Crucial kidney function parameters viz; Blood urea and creatinine level needs addressal and after more number of studies their treatment could be included in treatment protocols of COVID-19 patients. Similarly, the observations reported of Sodium and potassium levels among “survivors” and “dead” sensitized further clinic-basic studies on roles of SARS-COV2 in blocking essential ion channels of human physiological system.

MATERIAL AND METHODS

A study of association between Kidney Function Tests and survival/mortality of COVID-19 patients was undertaken among patients reporting in Noida and Greater Noida, UP, India. Surviving and Dead cases were chosen at random for the study. Few investigations were done in the residential society as per the information of COVID-19 patients obtained from the society notifications/news board. Few samples were also studied among students, staff and faculties in Sharda University who had either been a patient of COVID-19 or had any family members infected with COVID-19 in the past. Some information was collected from the patients records of Sharda hospital, Greater Noida, UP, India after obtaining appropriate permission from the Hospital administration. The contact details of the COVID-19 patients obtained from the above survey was tabulated and patients were contacted telephonically. The aims and objectives of this study were telephonically conveyed to the patients or their family members (who so ever were available on phone for the conversation). After taking their

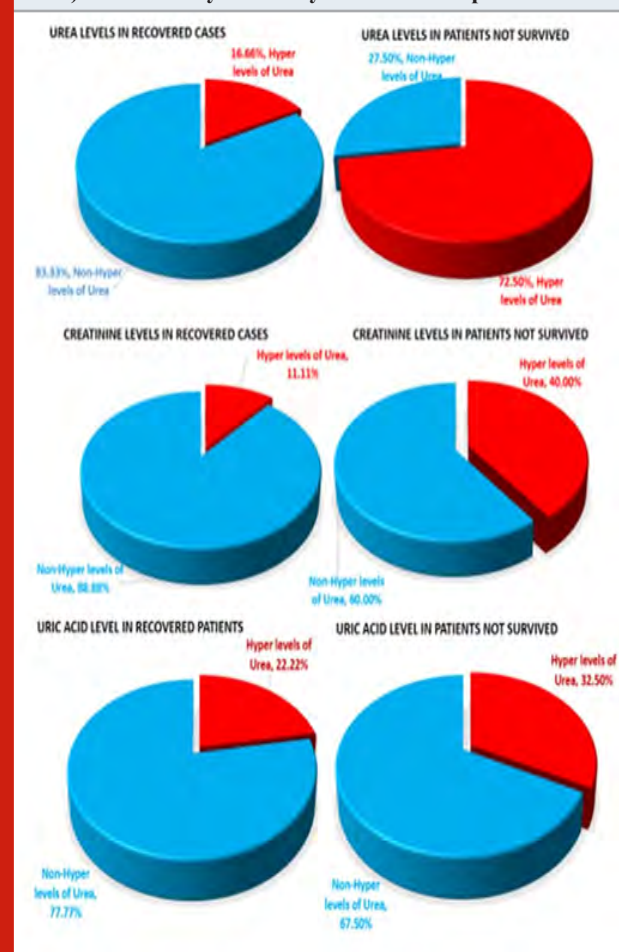
verbal consent for participation in the study, their Kidney function tests parameters were noted down along with other clinical parameters.

RESULTS AND DISCUSSION

A total of 76 patients were included in the study which comprised of 36 “Recovered” patients and 40 “Dead” cases (Table 1 & 2). Association of Kidney Function parameter with the Recovery from COVID-19 infection: Of the 36 study patients, 5.5% (2 patients; 1 Male, 1 Female) were in the age group of 0-20 years; 33.3% (12 patients; 7 Males, 5 Female) were of the age 21-40 years; 27.7 % (10 patients; 5 Males, 5 Females) were of the age 41-60 years and 30.55 % (11 males & 2 Females) were above 61years of age. (Table 1).

Association between Recovery of patients and Blood Urea level: It was observed that of the total 36 “Recovered” patients, 30 patients (83.3%) did not have hyper levels of urea (non-hyper level) in their blood establishing the optimum functioning of kidney with respect to Urea extraction from blood. Only 6 patients (16.6%) who recovered had hyper/higher levels of blood urea. (Fig. 1).

Figure 1: Schematic presentation of degree of association between kidney function values (Urea, Creatinine and Uric Acid) and recovery/mortality of COVID-19 patients

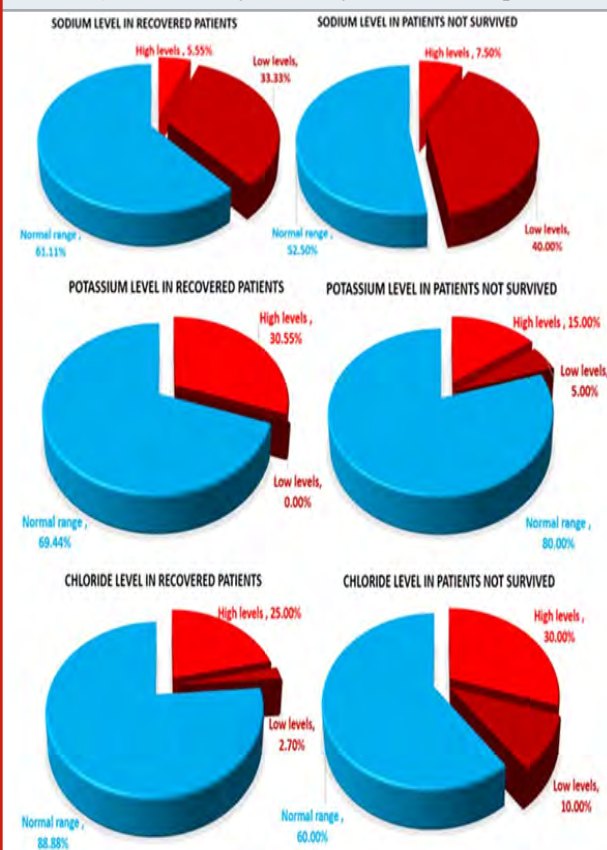


Association between Recovery of patients and Blood Creatinine level: Of the total 36 patients studied who recovered from infection, 32 patients (88.8%) had non-hyper levels of Creatinine in their blood establishing the optimum functioning of kidney with respect to Creatinine extraction from blood. Only 4 patients (11.11%) who recovered had higher levels of blood creatinine. This comprised of 2 females (56& 65years respectively) and 2 males (65& 78 years respectively). (Fig.1).

Association between Recovery of patients and Blood Uric acid level: Out of 36 patients who recovered from COVID-19, 8 (22.2%) patients had hyper levels of Uric acid whereas 28 patients (77.8%) showed non-hyper levels of Uric acid in the blood. The 8 patients included 4 males (between 46-65 years) and 4 females (between 56-75 years) (Fig. 1).

Association between Recovery of patients and Blood Sodium level: The values observed in the recovered patients indicated that 12 (33.3%) patients (8 males and 4 female patients of age 41 to 78 years) had low levels i.e. between 130 to 136mg/Eq/L. Higher values were observed in 2 (5.55%) patients (1 male patient of 46 years and 1 female of age 75years) the value being 148 and 150 mgEq/L respectively i.e. 18 patients (61.11%) had normal sodium levels (Fig. 2).

Figure 2: Schematic presentation of degree of association between Electrolyte values (Sodium, Potassium and Chloride) and recovery/mortality of COVID-19 patients



Association between Recovery of patients and Blood Potassium level: Higher values of Potassium were observed in 11 (30.55%) patients (six female of 23-59 years; 5 males of 23-65 years), the value was found to be between 5.3 to 10.0 mgEq/L. No low values were observed in the recovered patients, the remaining 25 patients had normal levels of potassium account to 69.44%. (Fig. 2).

Association between Recovery of patients and Blood Chloride level: The values observed in the recovered patients indicated that one male patient (2.7%) had low level (age 78 years) of Chloride while Higher values were observed in 9 (25%) patients; six females (age 23-75 years), 3 male patients (age 46-65 years). The remaining 26 (88.88%) had normal chloride levels (Fig. 2).

Association of Kidney Function parameters with the Mortality of patients from COVID-19 infection: Of the total 40 patients who did not survive, their age ranged from 23 to 99 years. 7.5% were of age between 21-40 years (one female & 2 male), 30% were of the age 41-50 years (5 females & 7 males) and 55 % were above 61 years of age (7 females; 15 males) (Table 2).

Association between Mortality of patients and Blood Urea level: Referring to the level of urea to be 20.0-43.0 mg/dl in a healthy person, the values were analyzed among 40 "Dead" patients. It was found that hyper values of blood urea were observed in 29 patients (72.5%) out of the 40 patients (8 females of age 24 to 99 years and 19 males of age 46 to 82 years). The values ranged from 43.2. to as high as 221.8mg/dl. Only 11 (27.5%) "Dead" patients had non-hyper levels of urea (Fig. 1).

Association between Mortality of patients and Blood Creatinine: The values observed for blood creatinine in the "Dead" cases indicated that 16 patients (40%) (3 females and 13 males) had hyper values of creatinine ranging from 1.1 to 7.0mg/dl while 24 patients (60%) had non-hyper levels of creatinine (Fig. 1).

Association between Mortality of patients and Blood Uric acid: The values observed in the dead patients indicated that 13 patients (32.5%) (2 females, 10 males and 1 UnK case) had hyper values. The age group was 24 -63 years in female patients, while in male patients it ranged from 57 to 82 years (within 6.2 to 13.9mg/dl range). However, there were 27 (67.5%) of "Dead" patients who had non-hyper levels of uric acid (Fig. 1).

Association between Mortality of patients and Blood Sodium: The values of blood sodium observed in the patients who did not survive indicated 16 (40%) patients with low Sodium values (5 females, 11males; age group 24-66 years and 53 to 82 years respectively). The values were found to be between 115-136mgEq/L. Higher values were observed in three (7.5%) male patients, of 40, 65 and 67 years with values between 149-153mgEq/L. Remaining 21 (52.5%) had normal levels of sodium (Fig. 2).

Association between Mortality of patients and Blood Potassium: The Potassium levels observed in the patients

who did not survive, indicated that two (5%) male patients of age 71 & 80 years had low levels of potassium (2.6 & 2.8mgEq/L respectively). Higher values were observed

in six (15%) patients; 5 males & 1 UnK of age between 58-82years, the values were between 5.1-6.5 mgEq/L. Remaining 32 (80%) patients showed normal levels (Fig. 2).

Table 1. Values of Kidney function parameters obtained from patients recovered from COVID-19 infection.

S.No	Patient code	Age	Sex	Day of recovery/ discharge after hospitalization	Kidney Function Test			Electrolytes		
					Urea (mg/dl) Ref level: 20.0-43.0 mg/dl	Creatinine (mg/dl) Ref level: 0.52-1.04 mg/dl	Uric acid (mg/dl) Ref level: 2.50-6.20 mg/dl	Sodium (mEq/L) Ref Level: 137.0-145.0 mgEq/L	Potassium (mgEq/L) Ref Level: 3.50-5.10 mgEq/L	Chloride (mgEq/L) Ref level: 98.0-107.0 mgEq/L
1.	SCR 5	41	M	8 th	38.5	0.5	2.2	136	3.7	98
2.	SCR 7	56	F	9 th	99.7	1.1	7.1	138	4.6	103
3.	SCR 9	35	M	10 th	19.2	0.8	5.6	142	4.2	105
4.	SCR 13	62	M	10 th	30.90	0.70	4.0	139	4.4	103
5.	SCR 15	23	F	11 th	19.20	0.4	3.5	140	4.4	105
6.	SCR 16	31	F	10 th	16.2	0.5	1.7	139	4.4	108
7.	SCR 17	25	F	11 th	14.8	0.6	2.9	140	3.8	104
8.	SCR 18	35	F	9 th	22.0	0.5	2.3	139	4.1	104
9.	SCR 19	5	F	6 th	20.5	0.2	3.0	140	4.1	105
10.	SCR 20	65	M	8 th	20.8	0.9	5.2	141	4.2	109
11.	SCR 21	29	M	10 th	25.1	0.7	5.2	145	5.0	107
12.	SCR 22	37	M	3 rd	23.1	0.5	4.4	138	4.8	102
13.	SCR 25	46	M	8 th	24.7	0.9	6.7	148	4.9	109
14.	SCR 26	48	F	5 th	29.7	0.5	1.9	136	5.3	109
15.	SCR 27	65	M	14 th	125.3	3.7	9.6	132	7.7	109
16.	SCR 28	51	M	7 th	23.4	0.8	7.1	141	5.1	110
17.	SCR 31	78	M	13 th	61.4	1.1	5.2	135	4.1	97
18.	SCR 32	58	F	11 th	30.4	0.6	7.3	135	4.0	101
19.	SCR 35	62	M	8 th	25.6	0.9	5.8	140	5.0	102
20.	SCR 37	65	M	11 th	26.5	1.0	7.0	133	5.4	102
21.	SCR 41	23	M	7 th	20.9	0.8	3.9	139	5.8	104
22.	SCR 44	23	F	13 th	17.5	0.6	3.9	145	5.9	110
23.	SCR 45	21	M	13 th	17.9	0.6	5.2	139	10.0	105
24.	SCR 53	59	F	18 th	35.0	0.7	10.0	133	5.8	98
25.	SCR 54	62	M	3 rd	24.3	0.6	5.1	135	5.4	101
26.	SCR 55	74	M	9 th	40.0	0.9	5.1	134	4.3	98
27.	SCR 56	75	F	9 th	41.4	3.0	10.0	150	5.3	112
28.	SCR 60	52	M	7 th	82.8	0.9	5.0	137	4.3	104
29.	SCR 61	-	F	2 nd	21.3	0.6	5.9	139	5.9	106
30.	SCR 62	41	F	8 th	40.1	0.4	5.8	144	5.2	108
31.	SCR 64	35	M	30 th	53.1	0.8	4.5	131	5.1	100
32.	SCR 65	62	M	4 th	34.2	0.7	3.9	133	4.1	100
33.	SCR 67	72	F	4 th	24.5	0.5	5.5	130	3.5	99
34.	SCR 72	14	M	10 th	25.9	0.3	3.6	138	4.4	105
35.	SCR 73	40	M	8 th	22.9	0.7	5.2	139	5.0	103
36.	SCR 74	49	M	12 th	59.4	1.0	5.4	138	4.3	105

Table 2. Values of Kidney function parameters obtained from patients who did not survive the COVID-19 infection.

S.No	Patient code	Age	Sex	Day Till hospitalized	Kidney Function Test			Electrolytes		
					Urea (mg/dl) Ref level: 20.0-43.0 mg/dl	Creatinine (mg/dl) Ref level: 0.52-1.04 mg/dl	Uric acid (mg/dl) Ref level: 2.50-6.20 mg/dl	Sodium (mEq/L) Ref Level: 137.0-145.0 mgEq/L	Potassium (mgEq/L) Ref Level: 3.50-5.10 mgEq/L	Chloride (mgEq/L) Ref level: 98.0-107.0 mgEq/L
1.	SCD 1	60	M	5 th	234.9	7.0	13.3	124	5.6	98
2.	SCD 2	63	F	6 th	52.6	0.8	13.7	142	5.0	99
3.	SCD 3	80	M	5 th	86.6	0.8	4.3	136	3.8	104
4.	SCD 4	70	M	4 th	221.8	4.4	10.0	137	6.2	105
5.	SCD 5	51	F	3 rd	39	0.8	3.5	140	3.6	107
6.	SCD 7	52	F	9 th	114.8	3.4	2.5	141	4.1	106
7.	SCD 8	24	F	4 th	63.5	0.6	6.4	134	3.9	110
8.	SCD 9	80	F	4 th	52.4	0.6	4.8	145	2.8	107
9.	SCD 10	46	M	6 th	123.4	3.3	3.2	143	5.1	116
10.	SCD 11	63	M	5 th	116.3	1.0	3.9	144	4.8	117
11.	SCD 19	23	M	9 th	36.1	0.7	5.5	140	5.0	113
12.	SCD 22	67	M	8 th	29.7	0.8	5.9	137	4.2	103
13.	SCD 23	57	F	7 th	36.3	1.1	5.0	135	4.3	96
14.	SCD 24	68	F	2 nd	47.8	0.5	5.1	140	3.5	98
15.	SCD 26	67	M	5 th	70.3	0.9	6.3	135	3.5	99
16.	SCD 27	70	M	8 th	112.7	10.6	4.8	123	4.1	94
17.	SCD 28	67	M	6 th	87.0	3.0	5.8	149	3.6	115
18.	SCD 29	53	M	3 rd	23.5	0.8	4.5	135	5.0	106
19.	SCD 30	71	M	6 th	55.2	1.5	7.8	141	2.6	97
20.	SCD 31	65	M	10 th	74.1	0.9	3.8	147	3.5	102
21.	SCD 32	58	M	4 th	85.1	0.8	7.2	138	5.7	99
22.	SCD 33	67	M	5 th	35.5	1.1	6.6	133	5.6	97
23.	SCD 34	54	M	5 th	23.2	0.6	3.1	128	4.5	98
24.	SCD 38	57	M	2 nd	43.2	1.5	6.6	137	4.4	95
25.	SCD 42	60	F	3 rd	67	2.2	5.1	134	4.7	110
26.	SCD 45	-	-	5 th	112	1.0	13.9	145	6.5	108
27.	SCD 46	54	M	4 th	36	1.5	4.2	133	4.6	105
28.	SCD 49	61	M	1 st	55.7	0.82	-	133	4.5	101
29.	SCD 52	66	F	8 th	45.2	0.9	5.4	127	3.6	94
30.	SCD 53	66	M	3 rd	75.7	1.5	6.9	139	4.4	104
31.	SCD 55	40	M	6 th	42.3	0.7	2.8	153	4.2	116
32.	SCD 57	78	M	2 nd	91.8	5.7	5.3	139	4.3	104
33.	SCD 58	59	F	2 nd	25.6	0.6	5.1	136	5.0	105
34.	SCD 60	74	F	6 th	86.4	0.8	4.8	145	3.3	114
35.	SCD 61	80	M	5 th	47.7	0.7	3.3	139	3.1	115
36.	SCD 62		M	9 th	44.8	0.7	2.4	137	4.2	98
37.	SCD 64	99	F	3 rd	70.7	0.9	4.8	144	3.9	107
38.	SCD 67	82	M	2 nd	207.7	1.6	7.3	115	5.6	111
39.	SCD 68		M	4 th	54.3	0.6	4.3	133	4.7	99
40.	SCD 69	82	M	4 th	133.9	3.0	7.8	145	4.2	112

Association between Mortality of patients and Blood Chloride: The Chloride values observed in the patients who did not survive indicated low values (94-96mEq/L) in 4 (10%) patients i.e. two females and two male patients (age between 57-66years). Higher values were observed in 12 (30%) patients; one UnK with 108mEq/L; three females (age 24-74 years), had values between 110-114mEq/L. The 6 male patients (age 23-80 years) had values between 114-117mgEq/l. Normal values were seen on 24 (60%) patients (Fig. 2).

Relative association of Blood Urea, Creatinine and Uric acid with survival/mortality of patients: Blood Urea: It was observed that average percentage of the association between hyper blood urea and recovery/mortality of the cases was 77.9%. As 83.4% of "Recovered" patients did not show hyper urea level whereas 72.5% of "Dead" patient showed hyper urea levels.

Blood Creatinine: It was observed that average percentage of the association between hyper blood creatinine and recovery/mortality of the cases was 64.4%. 88.8% of "Recovered" patients did not show hyper creatinine level whereas 40.0% of "Dead" patient showed hyper creatinine levels.

Uric Acid: It was observed that average percentage of the association between hyper blood uric acid and recovery/mortality of the cases was observed to be 55.1%. 77.7% of "Recovered" patients did not show hyper uric acid levels whereas 32.5% of "Dead" patient showed hyper creatinine levels.

Association of Electrolyte levels in survival/mortality in COVID-19 patients: Sodium & Potassium level: An inverse association of Sodium and Potassium levels were observed in study subjects. Of the total number of 36 patients who recovered, it was observed that maximum patients were having Low levels of sodium and high levels of potassium (33.33% and 30.55% respectively) when compared to the high and normal values. Similarly, of the total number of 40 patients who died, maximum patients were reported with Low levels of sodium and high levels of potassium (40% and 15% respectively) when compared with their high and normal values. Chloride level: Chloride levels also had trend like that of potassium levels. Maximum number of patients who survived and who died were comparatively more (25% and 30% respectively).

Present work has been undertaken to study association of kidney function parameters with the survival/mortality of COVID-19 patients. After careful observations and analysis of Kidney Function parameters among the categories of "Recovered" and "Dead" cases, we observed that most crucial of these KFT parameters is the level of Blood Urea followed closely by Blood Creatinine level among the two categories of COVID-19 patients. The degree of association observed between blood urea level versus survival/mortality of patients emerged to be the most significant observation.

Our preliminary study reveals that need for addressal

and/or correction of blood urea level among patients may be considered for possible inclusion into the COVID-19 TREATMENT protocol through appropriate clinical interventions. Fundamentally, when protein is broken down in the body, then urea is made in the liver and is then passed out of the body in the form of urine, which indirectly amounts to the level of nitrogen in the body. So if there is elevated levels of Urea in the blood it is as indication of inefficient kidney functions, which may impact the blood chemistry to lead to cardiac and/or pulmonary problems enhancing mortality of COVID-19 patients.

The second important observation emerged out of present study is a high degree of association of Serum Creatine level with the survival and mortality of patients. The degree of association between creatinine and survival/mortality of patients was computed to be 64.4 which reveals that treatment to keep normal creatinine after studying the observed value of this parameter among patients has to be the part of COVID-19 treatment protocol. Creatine is yet another amino acid which we get through dietary intake. This is also made in the liver and provides energy to the body. It is also present in muscles and when the body utilizes creatine as an energy source, then creatinine is expelled out of the body as waste material (<https://www.medicalnewstoday.com/articles/322380>) (Acc May 2021).

Elevated levels of creatinine in blood may be correlated with decreased excretion and besides this Creatinine is also measured to correlate the Glomerular filtration rate (GFR) in our body (Thomas 2005). The IFFC (International Federation of Clinical chemistry and Laboratory Medicine) Guidelines on COVID-19 highlights the need of monitoring creatinine levels in critical COVID-19 patients so as to diagnose any injury to kidney at an early stage (IFCC guide on COVID-19, 2020).. The observations reported in the present study are in concurrence with the IFFC guidelines. Previous studies have reported that in the SARS-COV 1, 2003 strain and the Middle East respiratory syndrome (MERS) infection, there were cases with Acute kidney injury (AKI) and subsequent mortality of cases (Cheng et al. 2020; Naicker et al. 2020).

Many viruses including SARS-COV2 have been reported to show 'viroporin' activity for which it depends on the ion channels present on the host cells (Charlton et al. 2020; Neiva et al. 2012; Neiva et al. 2015; Royle et al. 2015) as it does for its replication and dissemination activities. As it causes infection and complication when it enters specific cell or tissue, similarly it may also cause impaired ion-channels and may contribute to internal imbalance or disturbances. Of the whole array of 300 ions channels known in humans (Yu et al. 2005), there are many channels which have been studied for their involvement either in viral entry or in replication or in dissemination and also in the involvement in dysfunctions generalized as 'Channelopathies' and hence causing chaos in the ion channels associated with the nervous, musculoskeletal, cardiovascular, immune systems, (Kim, 2014; Elias & Benito, 2018; Vaeth & Feske, 2018, Charlton et al. 2020).

It has also been known that the E protein of the SARS-COV2 virus has more affinity for sodium ions than potassium ions (Kai et al. 2016; Melton et al. 2002), while the 3a protein have a tendency to form ion channel and thus aid in virus dissemination (Lu et al. 2006). The low levels of sodium as seen in the patients who did not survive may indicate utility of ion channels by the virus and thus leading to decrease in the sodium channels availability for the human system to perform its normal functioning such as disturbance in the RAS/homeostasis pathway.

The fact that low levels of sodium had been observed in recovered patients also can be supported by the fact that may be use of ion channel blockers are hypertensive medication may have contributed as a modulator of this viral activity. This necessitate further studies of adding ion channel modulators in the treatment protocol (only after careful analysis of KFT test reports) in complicated cases. The Dysregulation of sodium channels specifically of the respiratory tract has been observed in Human Respiratory Syncytial virus (Chen et al. 2009). This observation also focuses studies to be extended on understanding role of SARS-COV2 with chloride and potassium levels too.

CONCLUSION

All the parameters i.e. urea, creatinine, uric acid and electrolytes are very important and speak for their individual existence and persistence within the human body, but the fact needs to be considered whether these are symptoms prior to viral infection or whether these are the post effects of COVID-19 infection. The findings of the present study thus highlight an important observation which needs to be considered to improvise renal treatment regime of the COVID-19 patients. Preliminary observations reported in the present paper sensitizes to further understand and confirm the reported association of kidney function and recovery/mortality of patients and if proved the treatment protocol of COVID-19 may be considered for possible modification.

Disclosure: The authors declare that there are no conflicts of interest in this work.

ACKNOWLEDGEMENTS

We thank Dr Ashutosh Niranjana, Medical Superintendent, Sharda Hospital for kindly providing us with data for analysis of this work and also to the staff and students who have worked in providing us the data.

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Biotechnological Communication

Isolation and Molecular Identification of Phenol Degrading Bacterium from Industrial Wastes, Collected from Jeddah Saudi Arabia

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ABSTRACT

In the past two decades, phenolic compounds have had different applications, however their use in densification has increased considerably due to Covid 19. Discharge of these dangerous materials is highly toxic and causes risk and severe problems to the environment and health of human and animals, in addition to it being harmful to the aquatic life. Phenol degradation is very important due to high toxicity and stability. The aim of this study is to isolate phenol-degrading aerobic bacteria from hydrocarbon contaminated soil or wastewater, collected from the industrial area of Jeddah. Minimal medium containing phenol as carbon source was used to isolate different bacteria. About 30 actinomycete isolates were obtained, purified and preserved on Starch nitrate. Out of 30 isolates, eight isolates (27%) grow well in medium containing 0.1% phenol. After growing in broth medium, isolate BA4 and isolate BA8 were very active in phenol degradation. Growth and phenol degradation was measured in liquid medium for the two isolates. Morphological and physiological characters of these isolates were detected using different methods. Using molecular methods, they were belonging to a genus of actinomycetes. They were identified as *Streptomyces flavabus* BA4 and *Streptomyces* sp. BA8. The effects of some growth factors on growth and phenol degradation were determined. Growth was measured by dry weight (mg/l) while phenol degradation was detected by assaying the residual phenol concentration. The presence of electron donors such as glucose, starch, glycine, peptone, and Na acetate affect both growth and phenol degradation. It was clear that addition of 1 g/l peptone enhanced both growth and phenol degradation. The isolate use phenol and its derivatives m-cresol and o-cresol as carbon sources and addition of vitamin B complex increased the bacterial growth. In conclusion, phenol degradation was detected by actinobacteria and was affected by some physical and biochemical factors. It was noticed that optimization of growth conditions enhanced both growth and phenol degradation by the two selected *Streptomyces* isolate. Degradation process by isolate BA4 could be a promising solution for removal of phenol from wastewater.

KEY WORDS: PHENOL, STREPTOMYCES, DEGRADATION, 16SRRNA, WASTEWATER.

INTRODUCTION

Increasing population and industrialization has critical effects on human beings and the environment. Many studies have proved that these industrial effluents are the main source of many kinds of pollution of natural water. In the effluents of major industries, phenolic materials are present as dangerous pollutants especially in oil refineries, petrochemical plants and industrial effluents of paper mills. Paper industries produce a huge amount contaminated water with organic and inorganic pollutants in addition to coloring materials which

destroy soil and growing plants in these soils (IARC, 1989, Mörsen and Rehm, 1990, Baruah et al. 1996, Gerginova et al., 2007, Hussain et al., 2008, 2009, Hussain et al., 2010, Alhazmi et al., 2018, Xu et al., 2021).

Several studies have been conducted to examine the effluent constituents of textile, dyes, coal processing and plastics and pharmaceutical industries in addition to the effluents of pulp and paper, oil refineries, polymeric resins, insecticides, pesticides and steel plants. They have reported that these effluents contained phenolic compounds and phenol is the main pollutant which destroys the skin, cause vomiting, paralysis, lung failure and cardiac arrest, modify the water taste and odor. LD50 of these dangerous materials to fishes and human beings have been found to be 5–25 and

Article Information:*Corresponding Author: balaidaros@kau.edu.sa

Received 18/09/2021 Accepted after revision 15/12/2021

Published: 31st December 2021 Pp- 1992-2001

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.90>

10–24 mg/l, respectively being potentially carcinogenic at these concentrations, (Pazarlioglu and Telefoncu, 2005, Shazryenna et al 2015, Xu et al., 2021).

Due to the high toxicity of phenol and its derivatives, several regulatory laws all over the world such as MOEF, GOI, EPA and USEPA have reported them as the main dangerous pollutants with low permission limits in water and before discharge contaminated waste water into any natural sources, removal of phenol is prominent and urgent for developing green and sustainable environments. Many chemical methods like adsorption, extraction, ion exchange, oxidation, polymerization and coagulation are applied for removal of phenol up to the limit of WHO recommendation for drinking water but these methods were not efficient or effective. Biological methods are the solution for wastewater treatment and reducing the poisonous organic compounds (Sonawane and Koreke 2016, Mallick et al., 2021).

Several microorganisms can be used to treat phenol contaminated effluents by mainly techniques including phenol degradation enzymes like peroxidase and laccase and biosorption on live or dead cells. Untreated effluents used to be pumped to the Red sea cause a negative impacts on marine environments. Studies must be planned for isolation and screening of phenol-degrading bacteria from contaminated water effluent to decrease pollution (Park et al., 2007, Malaviya and Rathore, 2007, Dubey and Hussain 2014, Xu et al., 2021).

Saudi Arabia is a big country, located in the arid area and its area is about 2.15 million km². Its problem is water, where about 80% of the used water is from shallow grounds which can be contaminated by the phenolic materials or heavy metals. Therefore, it is expected that the situation of groundwater contamination may become more alarming in the coming years, mostly because of the rapid industrialization and urbanization in this country, (Al-sefry and Sen, 2006, Mallick et al., 2021).

In the effluents of different petrochemical, pharmaceutical and paint industries, phenolic products are found which are classified as highly hazardous chemicals that cause dangerous problems to man, animals and aquatic and terrestrial environments. In animals, gastrointestinal irritation, diarrhea, weight loss and liver and kidney toxicity have been noted after exposure to phenol. Contamination with the priority pollutant, phenol in waste water must be limited for good natural system-functions and sustainable environment. All International regulatory bodies have reported that phenol level must be brought to the required levels not exceeding 9–25 mg/l, which is the toxic level for living cells (APHA, 2005, Mallick et al., 2021).

It was also observed that chemical contamination is limited to shallow aquifers only while the deeper aquifers are safe. Besides, it is important to emphasize the presence of phenolic compounds in groundwater. Therefore, there is a need to carry out a systematic and exhaustive study of the toxic materials in the groundwater of the country. The efforts are made to describe the groundwater in KSA, toxicities of the metal ions in the groundwater, sources of

metal ions contamination and the future challenges and the remediation measures needed to protect groundwater resources. Certainly, studies about phenol degradation will be important for environmental and the regulatory authorities. Bacteria, fungi and actinomycetes have the ability of using phenol as carbon source and metabolized it to CO₂ and water. Phenol-degrading microorganisms which successfully completely removed phenol from waste water are still needed to be discovered (Xu et al., 2021). Considering the above-mentioned facts, this study was aimed to carry out the isolation and molecular identification of promising potential actinobacteria from contaminated soil for phenol degradation and optimization the degradation conditions for maximum activities in treating phenolic wastewater.

MATERIAL AND METHODS

Contaminated soil (10) and wastewater (10) samples were collected from Wastewater treatment plant, Jeddah industrial city, Jeddah, Saudi Arabia (Figure 1). They were collected either in sterile plastic bags or sterile bottles. Soil samples were air dried and sieved. The bacteria from contaminated soil or wastewater samples were obtained on starch-nitrate agar medium which was adjusted to pH 7.0 and contained (g/l): starch, 20; KNO₃, 2; K₂HPO₄, 1; K₂HPO₄, 0.7; MgSO₄·7H₂O, 0.7; agar, 20 after 7 days of incubation at 37 °C. All the obtained bacterial isolates were purified and preserved on the same medium on slants at 4 °C until used (Arifuzzaman et al., 2010, Tork et al., 2018, Aly and Tork, 2018, Khalel et al., 2021).

Different bacterial isolates (30) were screened on mineral salt agar medium containing Phenol as carbon source (100 mg/l) for 7 days at 37 °C and the most active isolates that showed the highest growth (8 isolates) were selected and screened in liquid broth medium and Phenol degradation was determined (Ali et al. 1998). The eight bacterial isolates that showed growth in the presence of phenol were screened in 250 ml Erlenmeyer flasks containing 50 ml of the basal Mineral salt medium broth medium supplemented with phenol (0.1 g/l) as a sole carbon source for 7 days. The medium pH was adjusted to pH 7. Each flask was inoculated with 2 ml of fresh prepared bacterial suspension, containing 6 x 10⁶ cfu/ml and incubated in shaking incubator at 37 °C and 120 rpm. At the end of the growth period, the growth was determined as g/l. Cells were harvested by centrifugation at 5,000 rpm for 5 min, washed dried at 60 °C for 3 days and weighted. Phenol degradation was measured quantitatively in the culture filtrate by the increase in the absorbance using spectrophotometric method. All experiments were made in triplicate and averages were calculated.

In any contaminated area, the amount of phenol was detected using 4-aminoantipyrine reagent and UV–Vis spectrophotometer (Systronics UV–Vis spectrophotometer 118) as described by APHA (2005), Sachan et al. (2019). All the chemicals used in present study were obtained from Hi-Media Laboratories Pvt. Ltd, Mumbai, India. The isolates BA4 and BA8 were grown on MSA medium containing different concentrations of Phenol (100–1300 mg/ml). The plate was inoculated with 1 ml of the bacterial

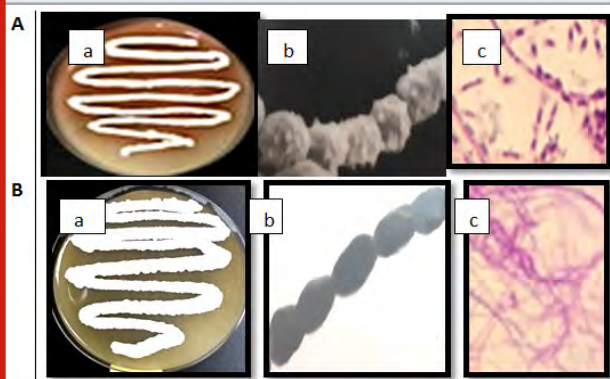
Table 1. The isolated Gram positive bacteria, color of aerial and substrate mycelia, growth and diffusible pigment production

Bacterial isolate	Source of isolation	Colony color	Growth	Diffusible pigment	Melanin pigment
BA1	Wastewater	White	++	-ve	+ve
BA 4	Wastewater	Pink	++++	+ve	+ve
BA 6	Wastewater	Gray	++	-ve	+ve
BA 8	Wastewater	Gray	++++	+ve	+ve
BA 15	Wastewater	Gray	++	-ve	+ve
BA 17	Soil	Gray	++	-ve	+ve
BA 19	Soil	Gray	++	+ve	+ve
BA25	Soil	Gray	++	+ve	+ve

++++: high growth, ++: moderate growth, +: poor growth, +ve: pigment present ,
-ve: pigment absent

Table 2. Growth and ph enol degradation by the selected actinomycete isolates

Phenol concentration Bacterial isolate	500 mg/l		1000 mg/l	
	Growth (Dry weight, mg/ l)	A ₂₃₅	Growth (Dry weight, mg/ l)	A ₂₃₅
BA1	0.21±3.04	0.10 ±0.06	0.ND	ND
BA 4	0.89 ±5.06	0.16 ±0.08	0.48 ±5.06	0.16 ±0.08
BA 6	0.19 ±6.07	0.11 ±0.07	0. ND	ND
BA 8	0.82 ±6.07	0.21 ±0.9	0.32 ±6.07	0.11 ±0.07
BA 15	0. 29±2.09	0.10 ±0.01	ND	ND
BA 17	0. 25±2.09	0.15 ±0.04	ND	ND
BA 19	0. 33±8.02	0.14 ±0.08	ND	ND
BA25	0. 39±3.07	0.14 ±0.03	ND	ND

Figure 2: A: The isolate BA4 and B: the isolate BA8, a: on starch nitrate agar, b: under electron microscope, c: under light microscope.

Moreover, Table 5 showed the growth of the two bacterial isolates BA4 and BA8 on different carbon and nitrogen sources. Also, the antimicrobial activities (diameter of the

inhibition zone, mm) of the two tested bacterial isolates against different bacterial pathogens and compared o control were shown in Table 6. From the previous results, the two isolates were belong to genus *Streptomyces*. Identification of the two tolerant isolates was confirmed using molecular methods and they were belonging to the same genus which was belonging to actinomycete genera. They were identified as *Streptomyces flavabus* BA4 and *Streptomyces* sp. BA8. Phylogenetic tree based on 16s rRNA of the two selected *Streptomyces* isolates was detected (Figure 3).

Tolerance of the two isolates to phenol was recorded on starch nitrate agar containing increasing concentration of phenol. It was found that increasing phenol concentration decreased the growth up to 1200 mg/l where there is no growth (Table 7). The growth of the selected isolate BA4 with increased concentrations of phenol (0.1- 1.2%) was shown in Figure 4. The isolate BA4 was grown in MS broth medium with phenol as carbon source. It was noted that at low phenol concentration the growth was maximum and increasing phenol decreased the growth. No growth was

recorded at 1300 mg/ml phenol. The quantity of phenol present in the solution increased with increasing the used phenol concentration (Figure 5).

Table 3. Morphological characteristics of the two selected isolates

Characters	Isolate BA4	Isolate BA8
Source	Waste water	Waste water
Gram stain	Gram-positive	Gram-positive
Color	Pink	Yellow
Motility	Absent	Absent
Respiration	Aerobic	Aerobic
Substrate Mycelium	Branched	Branched
Spore chain	Positive	Positive
Motile spores	Absent	Absent
Aerial and substrate mycelia	Present	Present
Optimum temperature	30 °C	37 °C
Optimum pH range	6.5 - 7.0	6.5 - 7.5
Catalase	Positive	Positive
Penicillin	Sensitive	Sensitive
Cephalosporin	Resistance	Resistance

The effects of some growth factors on growth and phenol degradation were determined. Growth was measured by dry weight (mg/l) while phenol degradation was detected by assaying the residual phenol concentration. The addition of some electron donors such as glucose, starch, glycine, peptone, and Na acetate on growth and Phenol degradation was determined. It was clear that addition of these materials significantly enhanced both growth and phenol degradation except starch which showed no significant differences compared to control (Figure 6). The best results were obtained with peptone. It was clear that addition of 1 g/l peptone enhanced both growth and phenol degradation. Increasing peptone concentration decreased phenol degradation by the tested bacterium BA4 (Figure 7). The isolate BA4 can use phenol and its derivatives m-cresol and o-cresol as carbon sources and addition of vitamin B complex increased the bacterial growth and phenol degradation (Table 8).

The currently methods for removing and degradation of toxic wastes and chemicals that harm human and animal health by bacteria or fungi are effectively used. Industrial effluents are mainly contained phenol and/ or phenolic compounds that must be safely bio- removed or biodegraded. In wastewater, presence phenol cause severe problems during treatment process, thus phenol biodegradation is a necessary process in the wastewater treatment process.

Table 4. Growth of isolate BA4 and isolate BA8 on different growth media for 10 days at 30 °C.

Media	Isolate BA4		Isolate BA8	
	Growth	Color of aerial mycelia	Growth	Color of aerial mycelium
Starch Nitrate agar	Heavy	Pale Pink	Heavy	Light yellow
Yeast extract-malt extract agar (ISP-2)	Moderate	Pink	Heavy	Light brown
In-organic salt-starch iron agar (ISP4)	Heavy	Dark brown	Heavy	Creamy
Glycerol asparagine agar (ISP-5)	Moderate	White	Moderate	Yellow
Tyrosine agar (ISP-7)	Heavy	Light brown	Heavy	Pale yellow
E-Medium (ISP-9)	Moderate	Yellow	Moderate	Creamy

Table 5. Growth of bacterial isolates BA4 and BA8 on different carbon and nitrogen sources

Carbon source	isolate BA4	Isolate BA8	Nitrogen source	isolate BA4	Isolate BA8
Glucose	+++	+++	Ammonium sulfate	±	±
Sucrose	+	+	Ammonium chloride	+	+
Starch	+++	++	Potassium nitrate	+	+
Lactose	+	+	Glycine	++	++
Vanillin	±	±	Dextrose	+	+
Peptone	+++	+++	Maltose	+	+

+++ : high utilization, ++ : moderate utilization, + : weak utilization, ± : very weak utilization

Wastewater is unique environment due to the extreme conditions that is preventing easy growth. In the contaminated

area, phenol bioremediation and studying the intermediate compounds are needed (Borghei and Hosseini, 2004; Abd

El-Zaher et al., 2011, Xu et al., 2021). Nakagawa et al. (1963) isolated and characterized catechol oxygenase from *Brevibacterium fuscum* for biodegradation of phenol. Isolation of bacteria for biodegradation of phenolic compounds from wastewater or polluted soil has been reported by Tallur et al. (2006) and Atsushi et al. (2006). Khleifat and Kaled, (2007) isolated *Actinobacillus* species that degraded phenol. Similarly, Nagamani et al. (2009) isolated and identified *Xanthobacter flavus* for removal of phenolic materials while Nair (2007, 2008) tried to purify paper factory effluent using a phenol degrading *Alcaligenes* sp. Also, Nilotpala and Ingle (2007) mineralized phenol by *Serratia plymuthica* strain GC isolated from sludge sample while Hasan and Jabeenb (2015) isolated *Pseudomonas* sp. and *Bacillus subtilis* from malathion and phenol contaminated soil.

Table 6. The antimicrobial activities (diameter of the inhibition zone, mm) of the two tested bacterial isolates against different bacterial pathogens and compared o control.

Tested pathogen	Isolate BA4	Isolate BA8	Amoxicillin
<i>Staphylococcus aureus</i>	19±3.1	17±3.1	21±4.11
<i>Bacillus cereus</i>	23±3.1	20±3.1	29±2.10
<i>E. coli</i> (MTCC 443)	23±3.1	20±3.1	29±3.19
<i>Pseudomonas aeruginosa</i>	17±3.1	16±3.1	21±3.11

Figure 3: Phylogenetic tree based on 16s rRNA of the two selected Streptomyces isolates.

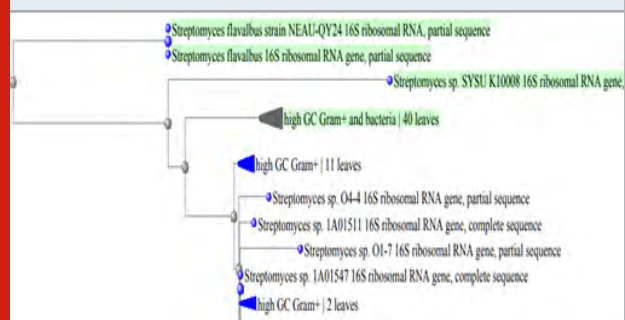


Table 7. Tolerance of the two selected isolates to different Phenol concentrations.

Phenol concentration (mg/l)	100	200	400	800	1000	1200	1300
Isolate BA4	+++	+++	+++	++	++	+	-
Isolate BA8	+++	+++	++	++	++	-	-

+++ : high utilization, ++ : moderate utilization, + : weak utilization, - : no utilization

In this study, waste water and soil were used as source of phenol degraded bacteria. Similarly, Mohite et al. (2010) used contaminated soil sample for isolation of Streptococcus epidermis which use was isolated on medium containing phenol as carbon and energy source.

Figure 4: The growth of the selected isolate BA4 grow on starch nitrate medium with increased concentrations of phenol (0.1- 1.2%).



Figure 5: Growth and Phenol degradation by the isolate BA4 grown in different concentration of phenol

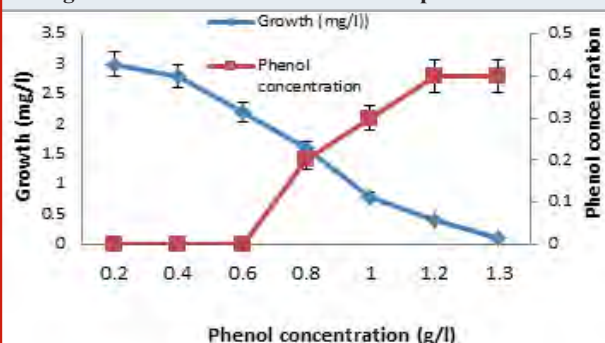


Figure 6: Growth and Phenol degradation by the isolate BA4 grown in medium subdifferent concentration of phenol

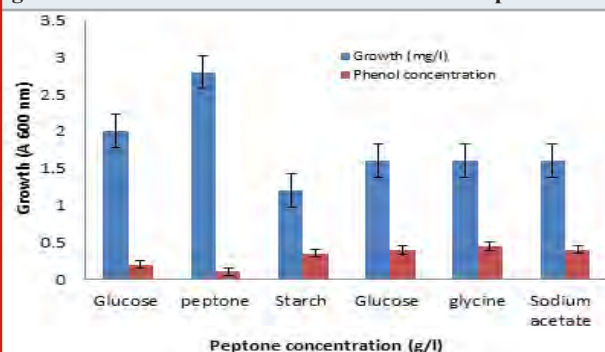
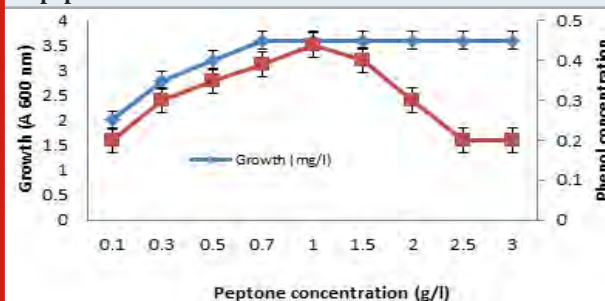


Figure 7: Growth and Phenol degradation by the Actinomycete isolate BA4 grown in different concentrations of peptone



Recently Sachan et al., (2019) have reported removal of phenol from waste-water by two bacterial isolates up to 1800 mg/L. Starch nitrate agar was used for growing and maintained the two selected isolates. The previous medium was used by many authors to isolate and grow Actinobacteria (El-Naggar et al., 2014, Aly et al., 2018). On MSA medium containing 0.1% phenol, eight isolates (27%) showed growth and all the eight isolates were melanin pigment producers. Melanin pigments are natural biopolymers which have special biological activities and protect organisms from difficult environmental conditions. During the past decade, melanins have attracted increasing attention for their use in drug delivery, photoprotection and environmental bioremediation (Tran-Ly et al., 2020).

Table 8. Effect of some activators on growth of BA4 on starch nitrate medium containing phenol or its derivatives.

Tested material (0.1 g/100 ml)	Diameter of colony (mm)		
	Phenol	m-cresol	o-cresol
Control	30.0±5.0	17.9±5.3	17.0±5.0
B1	37.9±3.1*	17.4±5.3	17.9±5.0
B6	33.9±2.5	17.5±5.1	17.6±5.0
B12	33.3±5.0	23.2±5.0	16.9±3.0
B1+B6+B12	39.3±5.0*	29.2±5.0*	19.9±1.3
Indole acetic acid	30.5±1.3	17.3±3.1	12.4±2.0*
nicotinic acid	27.0.9±1.4	17.0±2.0	17.5±1.5

*: significance $P \leq 5\%$

Using enrichment technique, two bacterial isolates BA4 and BA8 were selected for their maximum ability to degrade phenol. Similarly, isolate ABO11 used phenol (0.8 g/l) as carbon and energy source (Aburas, 2016) while lower degradation was obtained by Mohite et al. (2010), who recorded degradation of 200 mg/l phenol. Phenol removal was generally by oxidation using phenol hydroxylase to catechol. The two active bacterial isolates BA4 and BA8 were belonging to Gram-positive Actinobacteria which was worldwide in a variety of natural habitats. Actinobacteria are rich with a high guanine plus cytosine (55-70 %) content and they were extremely diverse group of microorganisms. Their members differed in the chemical structure of the cell wall, cell morphology, and physiological characters (Singh et al., 2016, Tran-Ly et al., 2020).

In this study, the two isolates had hyphae that branch, generating aerial mycelium with lengthy chains of spores. They were identified according to phenotype and genotype characterization. Sequencing of the 16S rDNA gene shared 93 and 95% identity with that of genus *Streptomyces*, respectively. They were identified as *Streptomyces flavabub* BA4 and *Streptomyces* sp. BA8. Phenol treatment was applied for isolation of rare Actinomycetes from soil samples which was suspended in 1.5 % (w/v) phenol solution at 30°C for 30 minutes they boated 61 isolates, and most of them were not *Streptomyces* (only 24.6%), whereas other genera such as *Micromonospora*, *Actinomadura*, *Microbispora* and *Polymorphospora* were isolated with ratios of 49.2%,

13.1%, 9.8%, and 3.3%, respectively (Istianto et al., 2012). The results of Azadi and Shojaei (2020) showed that a range of *Nocardia* species that belonging to actinobacteria have not received much attention but have great potential for bioremediation purposes.

Phenol biodegradation with least two bacterial enzymes, involved in the process, is widely used through definite methods. Also, *Candida tropicalis* was used by Mohd and Piakong (2006) for phenol biodegradation while Shawabkeh et al., (2007) reported that within 72 hr *Klebsiella oxytoca* degraded 75% of 100 ppm of phenol. Adjusting growth conditions improved phenol degradation process. In this study, addition of 1% peptone enhanced both growth and phenol degradation using Succinic acid and glycine as carbon and nitrogen source. Capacity of *Pseudomonas aeruginosa* for phenol biodegradation was enhanced of addition of different organic nitrogen sources (Aspartic acid, Beef extract, Peptone, Tryptone and Yeast extract. They added that maximum phenol degradation was obtained using yeast extract and peptone addition to the used medium while moderate degradation was obtained with the other nitrogen sources.

Wastewater had unpleasant smell and brown color due to the presence of high concentration of phenol and its derivatives (Madan et al. 2018) which have direct inhibitory effects on some bacteria. These materials are not easily biodegraded and cause increase the COD, suspended and dissolved and total dissolved solids in such type of wastewaters. The discharge of waste water into water bodies may cause a drop or increase in their pH values due to the size and activities of microbial population. High biochemical oxygen demand and chemical oxygen demand indicated the presence of high concentration of organic matter, thus treatment of wastewater is required before discharging. Biological treatment using useful microfora especially bacteria is the only choice for treatment (Marihal and Jagadeesh 2013). Phenol-degrading enzymes are broadly distributed in different microorganisms that play an important role in the degradation of phenol. (Marihal and Jagadeesh, 2013, Haritash and Kaushik, 2009). Bacterial isolation by enrichment method was used to obtain specific bacteria that degrade phenol (Sachan et al., 2019).

They added that many phenol tolerant bacteria and fungi were isolated from phenol contaminated area and they identified 16 bacterial isolates from paper effluent sample that were grow on MSM broth medium with phenol. At higher concentration of phenol (2000 mg/l), negligible growth was recorded which was due to bacteria sensitivity to higher concentration of phenol. Some isolates required acclimatization on different concentrations of phenol (Abd-El-Haleem et al., 2002). Gradually, bacteria adapted themselves to degrade wastes (Arutchelvan et al., 2005, Sun et al., 2012). After acclimatization, bacteria showed surprising ability in fast reproduction and phenol removal. Annadurai et al., (2000a, b) reported that absorption and biodegradation of phenol was increased by using Chitosan immobilized *Pseudomonas putida*. Screening by growth studies on medium with phenol lead to isolation of species with high capability of phenol removal that can

be utilized to purify wastewaters, containing high phenol concentrations.

Similar to our results, Yang and Lee (2007) reported that phenol has a potentially inhibitory effect on cell growth. Two bacterial isolates SP-4 and SP-8 showed good growth minimal salt medium with phenol in the presence of 1% glucose (w/v), whereas no growth has been observed in the absence of glucose. Both the strains showed fast and luxuriant growth at phenol concentration of 0–1000 mg/l. Isolate SP-4 is tolerate phenol up to 1600 mg/L while isolate SP-8 can tolerate the phenol up to 1800 mg/l and no growth has been observed for the two bacterial isolates at 2000 mg/l phenol.

Information about degradation potentials of bacteria, isolated from polluted places, is essential in designing stable bioremediation methods (Azadi and Shojaei, 2020). Majority of studies has deals with isolation of actinomycetes from normal habitats and determine their biological activities while actinomycetes that live in unusual environments was not studied well and are unexplored (Singh et al., 2016, Aly et al., 2020, Bahamdain et al., 2020).. Isolation of actinobacteria for cleanup soil of pesticides, metals, and mixed pollution had been reported. Due to their high catabolic ability and durability in harsh conditions and polluted area, screening new regions for unexplored actinomycetes discovered novel isolates with excellent and significant bioremediation abilities (Azadi and Shojaei, 2020). The xenobiotic material p-Nitrophenol is dangerous and highly toxic to soil microflora, released into soil after pesticides degradation organophosphate. Two actinomycete isolates A1 and A5 were found to be promising PNP bio- degraders.

Isolate A5 was identified as *Streptomyces coeruleorubidus* and optimization growth conditions can improve the biodegrading abilities of the selected actinomycete isolate (Jaweria et al., 2013). For removal of toxic chemical and pesticides from contaminated soil and wastewater effective system is needed. Inorganic compounds are difficult to be removed while organic compounds were completely degraded to less toxic materials. Using some bacterial genera in bioremediation process are used to solve these problems. Actinobacteria are excellent choice due to their high presence in soil and water and they previously maintained ecological balance between soil flora. Several species of Actinobacteria have been found to use phenol and pesticides as carbon sources, completely degrading them to nontoxic compounds (Fuentes et al., 2010).

For example, several strains of *Streptomyces* (including *Streptomyces spinosus*) have been found to produce tyrosinase enzymes, which are helpful and more effective than that obtained from other bacteria in the removal of phenols, a component of many pesticides that polluted water and soils. In addition, these bacteria produced secondary products that can be used for some pollutants removal, thus they are excellent candidates for organic pollutants removal without damage the environment (Myronovskiy et al., 2020). Finally, we can conclude that some Actinobacteria,

isolated from contaminated region can be used in cleaning the environment and waste water from phenol pollution.

CONCLUSION

Phenol degradation was detected by actinobacteria and was affected by some physical and biochemical factors. It was noticed that optimization of growth conditions enhanced both growth and phenol degradation by the two selected *Streptomyces* isolate. Degradation process by isolate BA4 could be a promising solution for removal of phenol from wastewater. Its potential for use in biological treatment of phenol in industrial wastewater must be studied to improving the quality of the final wastewater. These results provided useful information about use of the phenol-degrading bacteria for cleaning of industrial wastewater and the quality of the resulting wastewater will be improved. In occlusion, the degradation of phenol helps to overcome some of the pollution problems associated with the use of detergent.

ACKNOWLEDGEMENTS

This Project was funded by the Deanship of Scientific Research (DSR) at King Abdulaziz University, Jeddah, Saudi Arabia, (Grant number J 32- 247-1439). The author therefore acknowledges DSR for technical and the financial support.

Conflict of Interests: The author declares no conflict of interest exist.

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Biomedical Communication

On the Body Image and Standard Score Scale for Ideal Body of Women in Ho Chi Minh, Vietnam

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ABSTRACT

Developing the shape of human bodies is both one of the important tasks of the sports industry in each country in the world and the need of each individual, especially women. So, what body image is considered standard? We have researched and initially built a rating scale for each of the basic body standards indicators of women including 7/9 body standards indicators. At the same time, it also developed a standard to evaluate the body image of women's body shape through the ratio between waist measurement and indicators such as standing height, bust measurement, and hips measurement. During this study, we used common methods such as reference methods related to research objectives; expert interviews; anthropometric; Statistical mathematics. The study has developed a scale to evaluate the indicators of body beautiful image for each criterion of ideal body standards. This is the basis for them to be able to calculate the measurements of each fitness criterion to exercise in proportion to their height so that they have an ideal body standard, as well as a source of reference for other athletes, trainers, body image trainers, physical education teachers, or researchers on women's health in Vietnam. From there, it helps the practitioner know the correct rings needed have to work out based on his height. This is the basis for them to be able to calculate the measurements of each fitness criterion to exercise in proportion to their height so that they have an ideal body standard.

KEY WORDS: BODY IMAGE; BUST, HIPS MEASUREMENT, HEIGHT, WAIST.

INTRODUCTION

The measurement of body standards in humans, especially the ideal body standards of women today, we need the field of anthropometry, it is the science of measuring the human body, from which it can be compared, and determine the size and body image characteristics in different poses. Size and body image are important in many applications, such as clothing design, machinery design, transportation sector, medical/healthcare sector, aircraft cockpit design, spacesuit design for astronauts, safety, biometrics, criminology, interface design for household/industrial products, etc. (Godil and Ressler 2008). Along with health promotion, balanced and harmonious development is both the goal and the main motivation of women and men when participating in physical training and sports, (Pirkko 1995; Hartmann et al. 2018).

So each person needs to train their body to have an ideal body standard. In addition to healthy eating during sports

and activities that require low body fat or low body weight to improve performance body standards are the goals participants want to achieve, so participants in physical training need to understand and control their weight (Goldfield 2009). The majority of the population in Western countries would benefit from regular exercise as part of a healthier lifestyle, but a small percentage of individuals engage in regular physical activities, obsessions and that can be harmful physiologically, psychologically, and socially. The thinness scale of unhealthy eating, and the desire to be a lean and toned body, with men expressing their opinion in recent years (Garner 1991; Thompson et al. 1999; Homan 2010; Hartmann et al. 2018).

According to researchers, some people develop muscular disorders, consider themselves too skinny, and may feel pressure to gain muscle size and/or strength even though they have The body does not have beautiful muscles (Tod and Lavalley 2010). Manifestations of muscular disorders include disfigurement/dissatisfaction with body image, inappropriate diet, pharmacological support, supplements old body dependence, concealment of stature, and low self-esteem (Muscle Dysmorphia Inventory) (Rhea, Lantz and Cornelius 2004; Hartmann et al. 2018). These components,

Article Information:*Corresponding Author: thuchus@gmail.com

Received 20/09/2021 Accepted after revision 13/12/2021

Published: 31st December 2021 Pp- 2002-2006

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.91>

exercise dependence, have been defined as “a craving for leisure time, a focus on physical fitness that leads to uncontrollable excessive exercise behavior and manifests in physiological symptoms (eg, tolerance, withdrawal) and/or psychological symptoms (eg, anxiety, depression)” (Hausenblas and Downs 2002). It has also been measured using the Vibration Scale (EDS) (Downs, Hausenblas and Nigg 2004; Hartmann et al. 2018).

The above studies show that having a beautiful body image is desirable for both men and women across the globe. However, ideal body standards for men and women are different. The ideal body standards have become slim in Western societies, although body standards vary considerably by gender and country (Peter 1997). Several national groups emerged where, depending on their location, such as Slovakia, Bulgaria, and France, body slimness was valued more in women than in men. This group mainly includes European countries, along with Israel and the Philippines. France takes the number one spot: while men don't appreciate body slimness (37%), women (53%) do so much more. It is the country with the largest relative disparity between men's and women's ideals, a finding that may be related to the large gender disparity in body standards, (Robineau et al. 2013; Hartmann et al. 2018).

Countries (Austria, Mexico, and Uruguay) have the opposite situation, where more respondents say body slimness is ideal for men than women. In contrast to the countries in the first group, the respondents here rated body slimness more highly in men than in women, and the body beautiful

image of women as larger. On the one hand, countries like Ireland, where body beautiful images are preferred for both men and women, and on the other hand, countries like South Korea, where body slimness is appreciated for both sexes (Robineau et al. 2013; Hartmann et al. 2018). In Vietnam, how body image is considered a body standard, this issue has also been studied by many domestic and foreign authors and made their assessment. In the scope of this research, we want to understand the ideal body standards of women through the height index, to determine the value of bust, waist, hips measurement of women so that it is reasonably balanced.

MATERIAL AND METHODS

During this study, we used common methods such as reference methods related to research objectives; expert interviews; anthropometric; Statistical mathematics, we use Microsoft Excel and SPSS 20.0 software to analyze research data. Research object: 70 women 25 to 35 years old who are participating in regular aerobic exercises at gym clubs in Ho Chi Minh City (HCMC), Vietnam.

RESULTS AND DISCUSSION

The reality of body image of 25-35-year-old female HCMC: To select tests to evaluate the reality of body image of 25-35-year-old females, we conducted interviews with 72 female trainers and trainees participating in exercise at gym clubs aerobics in Ho Chi Minh City, Vietnam. About the body standards that women were interested in practicing to improve. The results are presented in Table 1.

Table 1. Body standards improve when exercising

level of concern	height		weight		Bust measurement		Waist measurement		Hips measurement		belly fat layer		subcutaneous fat of arm		subcutaneous fat of back	
	S	%	S	%	SL	%	SL	%	SL	%	S	%	SL	%	SL	%
Concern	8	11.11	4	66.67	40	55.56	60	83.33	40	55.56	3	44.44	24	33.33	40	55.56
Less interested	3	44.44	2	33.33	16	22.22	12	16.67	24	33.33	2	33.33	40	55.56	24	33.33
Do not care	3	44.44	0	-	16	22.22	0	-	8	11.11	1	22.22	8	11.11	8	11.11

Thus, according to the interview results in Table 1, most of the interviewed women were interested in waist measurement (reaching 83.33%). This was also a result consistent with the ideal body standards needs of women from the past to the present. Along with this interview result, we added BMI into 9 basic indicators used to assess body image, including height, weight, BMI, bust, waist, hips measurement, belly fat layer, subcutaneous fat of

arm, subcutaneous fat of back. With these 9 indicators, we tested 70 women, 25-35 years old who were participating in aerobic exercises at gym clubs in Ho Chi Minh City, Vietnam, obtained the results as shown in Table 2:

The results in Table 2, we saw that: The body image indicators of HCMC women developed unevenly in such indicators as weight, BMI and belly fat layer, subcutaneous

fat of arm, subcutaneous fat of back. Bust, waist, hips measurements were relatively uniform ($v\% < 10\%$). However, most of the body standards are reliable ($p < 0.05$), except for the weight and belly fat layer, subcutaneous fat of arm, subcutaneous fat of back criteria. Women 25-35 years old in HCMC, all have a beautiful body image: BMI is in the range of 18 to 24.99 – in the normal range. (WHO published index for Asians, 2020).

Developing standards for assessing women's body standards in HCMC: In this study, we only build a scale for 7/9 of the body image indicators that have been shown (Table 3). Because normally researchers do not build a 10-point scale for BMI and weight. In this scale, there were waist measurement and belly fat layer, subcutaneous fat of arm, subcutaneous fat of back inverse scale.

Table 2. The reality of body image of 25-35-year-old female HCMC.

No.	Tests	M	SD	V%	p
1	Height (cm)	153.8	5.76	3.74	0.01
2	Weight (kg)	49.3	7.71	15.6	0.06
3	BMI	20.7	2.48	11.9	0.04
4	bust measurement (cm)	81.1	4.94	6.1	0.02
5	waist measurement (cm)	68.1	4.78	7.03	0.03
6	hips measurement (cm)	89.6	6.2	6.92	0.03
7	belly fat layer (mm)	27.9	6.46	23.1	0.09
8	subcutaneous fat of arm (mm)	16.6	3.11	18.7	0.07
9	subcutaneous fat of back (mm)	21.03	4.15	19.7	0.07

Table 3. The rating scale for body beautiful image indicators of women

Standard score	Height (cm)	Bust measurement (cm)	Waist measurement (cm)	Hips measurement (cm)	belly fat layer (mm)	subcutaneous fat of arm (mm)	subcutaneous fat of back (mm)	Values
	153.80	81.10	68.10	89.60	27.90	16.60	21.03	Means
	5.76	4.94	4.78	6.20	6.46	3.11	4.15	Standard
1	142.28	71.22	77.66	77.20	40.82	22.82	29.33	deviation
2	145.16	73.69	75.27	80.30	37.59	21.27	27.26	
3	148.04	76.16	72.88	83.40	34.36	19.71	25.18	
4	150.92	78.63	70.49	86.50	31.13	18.16	23.11	
5	153.80	81.10	68.10	89.60	27.90	16.60	21.03	
6	156.68	83.57	65.71	92.70	24.67	15.05	18.96	
7	159.56	86.04	63.32	95.80	21.44	13.49	16.88	
8	162.44	88.51	60.93	98.90	18.21	11.94	14.81	
9	165.32	90.98	58.54	102.00	14.98	10.38	12.73	
10	168.20	93.45	56.15	105.10	11.75	8.83	10.66	

Our grandfather once had the sentence in Vietnam, "The best body image, the second skin", reflects Asian views on the beauty of women and asserts in "The body image" that waist measurement makes sense extremely important, reflected in the Vietnamese folk song "Those who wear the bottom of the bee's waist, both skillfully pamper their husbands, and raise children well". Therefore, to supplement the scale according to each body image criterion, we build a scale according to the ratio between waist measurement/height and Bust, hips measurement, see table 4:

With the results of Table 4, when a woman with a height of 160 cm wants to score 10 body beautiful image, her waist measurement is: $\text{Waist measurement} = \text{height} \times 39.13/100$
 $\Rightarrow \text{Waist measurement} = 160 \times 39.13/100 = 62.61 \text{ cm}$

From there, we can find bust measurement and hips measurement of women with waist measurement of 62.61 cm who want to score 10, that is: $\text{Bust measurement} = \text{Waist} \times 100/75.40 = 62.61 \times 100/75.40 = 83.04 \text{ cm}$, $\text{Hips measurement} = \text{Waist} \times 100/69.13 = 62.61 \times 100/69.13 = 90.57 \text{ cm}$

Body beautiful image rating scale is based on the ratio between waist measurement and indicators such as Height, bust measurement, and hips measurement. From there to help women physical exercise know the standards bust, waist, hips measurement required to exercise based on their height. It should also be added: The lucky waist measurement is one of the few indicators that its owner has both health and beauty but even so is not without risk.

Many people who rapidly reduce their waist measurement through unhealthy diets have caused serious physical harm. To improve waist measurement, regular and proper physical exercise is a method that brings many real benefits instead of the rush, non-scientific, which is both unmaintained, expensive, and harmful to health (Hartmann et al. 2018).

Overall, with bodybuilders having spent more years, they possess ideal body standards, time in the gym, and work out harder than body image practitioners. This finding is similar to the differences in exercise frequency reported

between bodybuilders and Gymers (Hale et al. 2010). But Hausenblas and Symons (2002) reported exercise behavior alone, and history is not an adequate predictor. But these studies have not provided standard indicators of body beautiful image in women. Besides gymer's finding when typical bodybuilder training is moderate-high versus light-moderate intensity. It is similar to the recent finding by Cook, Hausenblas and Rossi (2013), who reported that Gymers who wanted to gain weight had significantly higher amounts of strenuous exercise than women who wanted to lose weight supported (Cook, Hausenblas and Rossi 2013; Hartmann et al. 2018).

Table 4. Rating scale according to the proportion of body standards of women

Standard score	Percentage			Values
	Waist measurement / Height	Waist / Bust measurement	Waist / Hips measurement	
1	44.28	83.97	76.00	Mean
	2.06	3.43	2.75	Standard deviation
	48.40	90.83	81.50	
2	47.37	89.12	80.13	
3	46.34	87.40	78.75	
4	45.31	85.69	77.38	
5	44.28	83.97	76.00	
6	43.25	82.26	74.63	
7	42.22	80.54	73.25	
8	41.19	78.83	71.88	
9	40.16	77.11	70.50	
10	39.13	75.40	69.13	

Therefore, it is necessary to have standard indicators for women to have an ideal body standard, based on which they can exercise easily, and control their weight. The current study has proven it with women in HCMC, Vietnam. For USA teenagers, for every 10,000 teenagers living across the United States, 8% of girls and 12% of boys said they use products to improve appearance, muscle mass, or strength. Girls and boys reported frequently thinking about wanting to improve their body shape (Field et al. 2005; Cook, Hausenblas and Rossi 2013; Hartmann et al. 2018). Previous research results have not found any specific standards of ideal body standards for a person, especially focusing on body beautiful image for Vietnamese women, as well as men. Therefore, this study has found the standard indicators of ideal body standards of women in HCMC, Vietnam. From there, build a standard scale, for HCMC women to practice a beautiful body image for them based on that.

CONCLUSION

The findings of the present study has developed a scale to evaluate the indicators of body beautiful image for each criterion of ideal body standards. On that basis, the authors have built a rating scale according to the ratio between the body standards indicators of women in Ho Chi Minh City and Vietnam. This is the basis for them to be able to calculate

the measurements of each fitness criterion to exercise in proportion to their height so that they have an ideal body standard, as well as a source of reference for other athletes, trainers, body image trainers, physical education teachers, or researchers on women's health in Vietnam.

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Biotechnological Communication

Improved Cleansing Process of Stuck Soil Brush from Cleaners during the Sugar Beet Harvest

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ABSTRACT

Purpose of the present research is to improve the quality of cleaning sugar beetroot crops with a brush cleaner by developing a device for removing stuck soil. The article defines the dependence of the impact velocity on the distance to the place of impact, which changes linearly, increasing with increasing distance to the place of impact. Analysis of the dependence shows that the speed can be most significantly influenced by the speed of the conveyor and the radius of the trajectory of the end of the bending lint, which directly depends on the diameter of the cylindrical brush. Nomogram was obtained to determine the most optimal parameters of the impact site on the lint. When conducting studies with heavy loamy chernozem soils with a moisture content of $\approx 28\%$, it revealed that the minimum required brush lint impact velocity to separate soil from the coils of the spring is about ≈ 2.5 m/s. The analysis of the dependence shows that the most effective cleaning of the brush lint from the stuck soil (90-98%) occurs when a blow is applied at a distance of 40 to 78 mm from the place of attachment of the lint with a lint length of 100 mm. The impact velocity of the brush lint should be large enough to separate the soil of maximum stickiness from the coil of the spring, however, it should not be greater than the speed causing lint cutting, i.e. the speed at which the brush lint are destroyed.

KEY WORDS: ROOT CROPS, STUCK SOIL, SUGAR BEETROOT.

INTRODUCTION

The purpose of harvesting sugar beet is to collect root crops, which provide the highest yield of sugar per hectare and minimal resource costs. The harvest and the quality of beets depend on the soil moisture in the second half of the growing season, its duration, and the quality of harvesting operations. For cereals, the harvest period can be easily set by the achieved ripeness phase. Sugar beet does not have such indicators. Beets can be considered ripe when root crops spend more energy reserves on the respiration for several days forming new reserve constituents by assimilation. But for healthy beets, this period comes in late autumn (Kuznetsov et al. 2020).

Sugar hybrids achieve an acceptable yield and maximum collection (yield) earlier than yielding hybrids. Harvesting machines must ensure the following agrotechnical and technological requirements: completeness of beetroot extraction from the soil (at least 98%); minimal losses (no more than 5% of sugar beetroots mass, and no more than

18% of tops); minimal contamination of beetroot (no more than 10% of beetroots, and 0.5% of tops). Losses under optimal conditions of mechanized harvesting amount to 5%. Under unfavorable weather and soil conditions, they can reach 20% or more. When the soil is wet, the contamination of root crops and tops is higher, and the screening of soil impurities worsens. High quality of work during harvesting should provide the optimal level of cutting the tops, a minimum of damage to the surface of root crops, a low proportion of soil stuck on root crops, and the least beet losses and destruction of the soil structure (Scott and Cooke 2012; Kleuker and Hoffmann 2021).

The costs of beet crops transportation to/from the plant depends on the weight of the soil stuck on root crops. Besides, the removal of the soil results in the worsening of its fertility (Tuğrula et al. 2012; Rajaeifar et al. 2019). For example, in Germany in 1991, the contamination of root crops amounted to 15%, the soil removal per hectare averaged seven tons. With an area under sugar beet in the amount of 570 thousand hectares, four million tons of soil were lost per year. For economic and environmental reasons, it is necessary to reduce the percentage of soil stuck on root crops by applying the following measures: selecting hybrids; conducting liming; ensuring high field germination; harvesting under optimal weather and soil conditions;

Article Information:*Corresponding Author: pank-77@mail.ru

Received 04/10/2021 Accepted after revision 25/12/2021

Published: 31st December 2021 Pp- 2007-2012

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.92>

choosing the optimal speed of the harvesting machines; providing additional cleaning sprockets, cleaning drums, and drive stages; adjusting the drum sugar beets cleaner to the average diameter of root crops (Haggar 2010; Tuğrula et al. 2012; Rajaeifar et al. 2019; Rajaeifar et al. 2019). The present work aims to improve the quality of cleaning sugar beetroot crops using a brush cleaner by developing a device for removing stuck soil from it when harvesting in conditions of high soil moisture.

MATERIAL AND METHODS

To calculate the theoretical speed, according to the impact theory and taking into account the elastic properties of the brush lint, one can use the formula

$$V_y^T = V_y^S + V_y^E,$$

where V_y^T is the speed of impact at the moment of touching brush lint with the coil of the spring, used for cleaning, is calculated by the formula $V_y^S = \omega \times R$, m/s, where $\omega = \text{const}$ is the angular velocity of brush rotation ($\omega = 2 \times 3.14 \times 548 / 60 = 57.39$ rad/s) R is the distance from the rotation axis of the brush drum to the point of impact, m; V_y^E is the speed that occurs when the lint is straightened after escaping the root crop surface due to its elastic properties, m/s (Figure 2) (Solovyov and Kuznetsov 2016).

Figure 1: Receiving rod conveyor of the ROPA Euro Tiger combine with installed brush drums



RESULTS AND DISCUSSION

In the design of mechanisms designed to extract root crops from the soil, digging plowshares and disk diggers were used. Given that the excavated root crops carry a large amount of soil and impurities, they undergo repeated cleaning on the separation devices of root harvesters. Combinations of different types of separating devices were used (cleaning screws, rod conveyors, rotating turbines, etc.). It was established that the cost of transporting root crops to sugar factories was 25-30% of the beet production cost. The offered working tools for cleaning root crops (Figure 1) allowed continuing harvesting under complex conditions (at increasing soil moisture by up to 32%). Field trials showed a significant reduction of irreversible losses of topsoil when using upgraded working tools in comparison

with the regularly used serial tools, as well as preserving their operational capacity at the highest soil moisture (up to 32%). Such conditions take up to a third of the harvesting time (Gil et al. 2013; Zavrazhnov et al. 2019).

Considering the lint as a physical pendulum, it is possible to determine the period of its small oscillations T , 1/s according to the formula:

$$T = 2\pi \sqrt{\frac{I}{mgl}} \quad (2)$$

where I is the pendulum (lint) inertia moment relative to the rotation axis, $\text{kg} \cdot \text{m}^2$, m is the mass of the pendulum (lint), kg, l is the distance from the rotation axis to the center of mass, m. The length of the lint's end travel path when cleansing the root crop can be represented as a sum of paths, traveled with the relative (with speed $\omega = 57.39$ rad/s) and transfer velocities (with velocity v) of the brush drum, on which the lint is fixed, and transported root crop (Figure 3).

Figure 2: Schematic diagram for determining the impact velocity: 1 - The center of impact of the lint on the spring coil; 2 - Extension spring; 3 - The trajectory of the lint point which impacts the surface; 4 - A lint bunch; 5 - Brush drum; 6 - The distance from the rotation axis of the brush drum to the center of impact

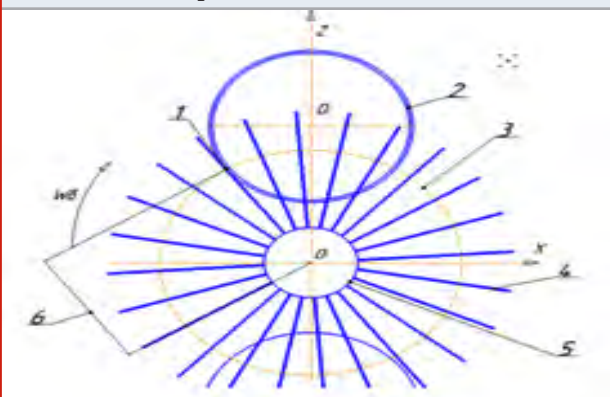
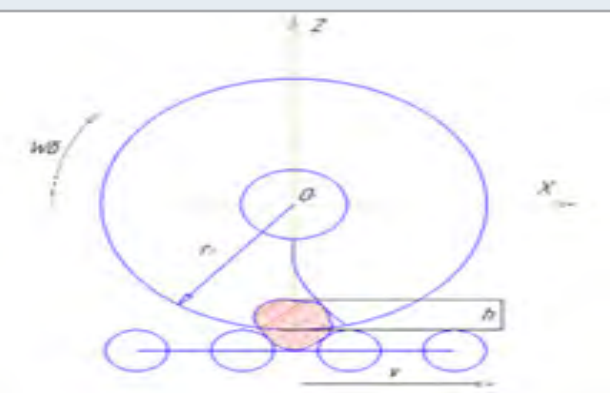


Figure 3: The schematic diagram for determining the length of a lint trace



Considering these movements as independent, and summing up their results, we determine the length L of the lint

trajectory during its contact with the root crop.

$$L = 2 \left(r + \frac{v}{\omega} \right) \arcsin \frac{\sqrt{r(h-r)}}{r} \quad (3)$$

where v is the transfer velocity of the conveyor, m/s; r is the radius of the trajectory of the bending lint end, m; h is the maximum depth of immersion of the lint into the contamination array, m.

Based on formulas (2) and (3), the velocity V_y^E can be determined as the ratio of the path to the time during which the lint is straightened:

$$V_y^E = \frac{L}{t} = \frac{2 \left(r + \frac{v}{\omega} \right) \arcsin \frac{\sqrt{r(h-r)}}{r}}{2\pi \sqrt{\frac{I}{mgl}}} \quad (4)$$

Thus, it is possible to calculate the velocity at which the brush lint impacts the coil of the spring, using formulas (1) and (4).

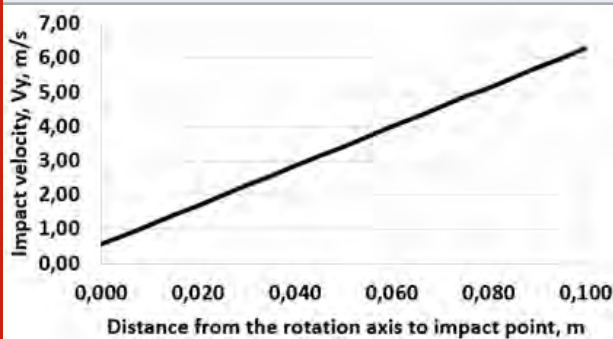
$$V_y^T = \omega \times R + \frac{2 \left(r + \frac{v}{\omega} \right) \arcsin \frac{\sqrt{r(h-r)}}{r}}{2\pi \sqrt{\frac{I}{mgl}}} \quad (5)$$

Finally, after some algebraic transformations, the theoretical impact velocity can be defined as

$$V_y^T = \omega \times R + \frac{\left(r + \frac{v}{\omega} \right) \arcsin \frac{\sqrt{r(h-r)}}{r}}{\pi \sqrt{\frac{I}{mgl}}} \quad (6)$$

As a result, one can get the impact velocity depending on the distance to the impact point. Figure 4 shows that this velocity varies linearly, increasing as the distance to the impact point increases. The analysis of the dependence shows that this velocity can be most significantly influenced by the velocity of the conveyor, as well as the radius of the trajectory of the bending lint end, which directly depends on the diameter of the cylindrical brush (Kuznetsov et al. 2020; Kleuker and Hoffmann 2021).

Figure 4: The dependence of the impact velocity on the distance to the impact point



To determine the necessary impact velocity on the lint to clear it from the soil, it is necessary to know and take into account certain technological properties of the soil. In this case, these include the stickiness and frictional properties of the soil. At certain soil moisture, adhesion, and friction act together. When the soil slides over the surface

of the working tool both processes manifest themselves simultaneously in the form of resistance to its sliding. Then the slip resistance is defined as

$$T_{\text{total}} = F_{\text{fr}} + F_{\text{ad}} = fN + p_0 \times S + p \times N \times S \quad (7)$$

where F_{fr} is the friction force of the soil on the surface of the working tool, N; F_{ad} is the adhesion force of soil to the surface material of the working tool, N. V_y^T 6 determined earlier must be equal or greater than theoretical impact velocity on the lint $V_y^{T_{th}}$ necessary to clean it from the soil, i.e.

$$V_y^T \geq V_y^{T_{th}} \quad (8)$$

The maximum value of the friction force F_{fr} is achieved when sliding. The values of F_{fr} are within the range from 0.25 to 0.90, $\varphi = 14...42$. Besides, friction is significantly affected by soil moisture. At decreased moisture from 0 to 8-10%, moist soil does not stick to the surface. It is the case of true friction when the friction coefficient does not depend on humidity. The increase in F_{fr} is explained by the appearance of the molecular attraction forces of soil particles to the surface. When the moisture increases to 50-80%, it plays the role of a lubricant, decreasing F_{fr} . The friction force F_{fr} is influenced by the mechanical composition of the soil, i.e. the content of physical clay (particles less than 0.1 mm in diameter). To determine the stickiness, one can also use the well-known equation:

$$G_A = \frac{P_{\text{det}}}{S}, \quad (9)$$

where P_{det} is the force required to detach from the soil, N; S is the area of the contact surface, m². This force can manifest itself in two ways. - as a resistance when the soil slides on the surface of the working tools. - as a resistance corresponding to the separation of solids in contact with working tools. Slip resistance from sticking is determined by the formula, (10):

$$F_{\text{sl}} = p_0 \times S + p \times N \times S, \quad (10)$$

where p_0 is the coefficient of specific adhesion in the absence of normal pressure, Pa; p is the coefficient of specific adhesion caused by normal pressure, Pa; S is the visible contact area, m²; N is the normal pressure force, N (Kuznetsov et al. 2020).

Comparing $G_A = \frac{P_{\text{det}}}{S}$ and $F_{\text{sl}} = p_0 \times S + p \times N \times S$, it is clear that the friction and adhesion laws have significant differences. Adhesion, unlike friction, depends not only on the normal pressure and the properties of the working surface of the material, but also on the contact area, and is manifested even in the absence of normal pressure N . The stickiness of the soil depends on the mechanical composition (dispersion), moisture content, the material of the surface of the working tool, and the specific pressure. As the dispersion increases, the stickiness increases, therefore clay soils are more sticky than sandy ones. Structureless soils are more

sticky than structural ones. Stickiness manifests itself only at certain moisture:

- for structureless soils – at a relative humidity of 40-50%;
- for structural soils – 60-70%.

With increasing moisture, the stickiness first increases, and then decreases. The lint of the brush drum is made of nylon or dacron, which reduces the stickiness. The soil will stick to the working surface of the lint if the adhesion and friction forces between the soil particles or aggregates of the moving layer are less than the friction and adhesion forces between the soil and the working surface, i.e.

$$(C_0 + \sigma \times \tan \varphi) \times S < fN + p_0 \times S + p \times N \times S, \quad (11)$$

where C_0 is the soil adhesion coefficient, (for chernozem $C_0 = 6 \dots 9 \text{ N/m}^2$); σ is the normal pressure, ($\sigma = \frac{N}{S}$), Pa. Since the force is nothing but the pulse velocity or the rate of change in momentum mv , for further calculations we will express the impact velocity through the force. First, we write down the equation for determining the period of free vibrations for the lint fixed in the drum

$$T = 2\pi \frac{l_l^2}{3.56} \sqrt{\frac{\delta}{EIg}}, \quad (12)$$

where l_l^2 is the length of the lint, m; δ is the specific weight of the lint per unit length N/m^4 ; E is the elastic modulus of the lint, Pa; I is the second moment of the lint, m^4 ; g is the acceleration of gravity ($g = 9.81 \text{ m/s}^2$) (Kuznetsov et al. 2020; Kleuker and Hoffmann 2022). To make lint lagging behind the coil of the spring at the impact, the period of lint's own vibration $\frac{2\pi}{\omega}$ should be greater than that of the coil against which the impact occurs. Therefore, the period of free oscillation of the lint will be

$$T = 2\pi \frac{l_l^2}{3.56} \sqrt{\frac{\delta}{EIg}} < \frac{2\pi}{\omega}, \quad (13)$$

hence

$$\omega < \frac{3.56}{l_l^2} \sqrt{\frac{EIg}{\delta}}. \quad (14)$$

Now it is possible to determine the necessary and sufficient force impulse to clean the lint surface from the soil. We substitute the values of force and time, defined above (the period of free oscillation) into the following formula:

$$P = F \times t,$$

where $t = T$ and F will take the values $(C_0 + \sigma \cdot \tan \varphi) \cdot S$, or $fN + p_0 \times S + p \times N \times S$, discussed above. Then the impulse offeree will be equal to

$$P = (fN + p_0 \times S + p \times N \times S) \cdot 2\pi \frac{l_l^2}{3.56} \sqrt{\frac{\delta}{EIg}} \quad (15)$$

while the impulse offeree, necessary and sufficient for cleaning the surface of the lint from the soil must be greater than this value, namely:

$$P_{io} > P = (fN + p_0 \times S + p \times N \times S) \cdot 2\pi \frac{l_l^2}{3.56} \sqrt{\frac{\delta}{EIg}}. \quad (16)$$

Based on the inequality (11), it is possible to determine the theoretical impact velocity on the brush lint, necessary for cleaning it from the soil

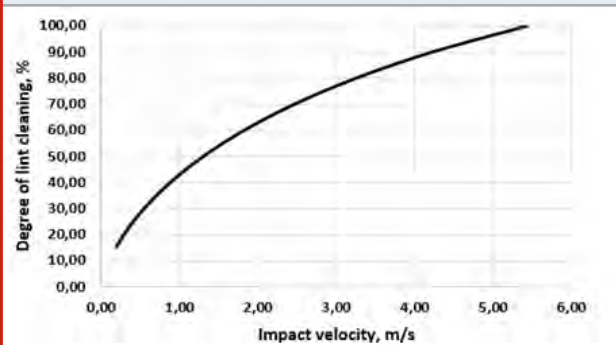
$$V_{yth}^T > \frac{P}{m} = \frac{(fN + p_0 \times S + p \times N \times S) \times 2\pi \frac{l_l^2}{3.56} \sqrt{\frac{\delta}{EIg}}}{m}. \quad (17)$$

Substituting expressions (6) and (17) into formula (8), we get

$$V_y^T = \omega \cdot R + \frac{\left(\frac{r+v}{\omega}\right) \arccos \frac{\sqrt{r(r-v)}}{\pi}}{\pi \sqrt{\frac{l}{mg}}} > \frac{(fN + p_0 \times S + p \times N \times S) \times 2\pi \frac{l_l^2}{3.56} \sqrt{\frac{\delta}{EIg}}}{m}. \quad (18)$$

Based on the obtained formula, we construct a graph (Figure 5).

Figure 5: Dependence of the degree of lint cleaning on the impact velocity



To determine the diameter of the wire for the spring, it is required to calculate the contact area between the lint and the coil of the spring. Based on the basic formulas of the contact interaction mechanics, and taking into account that a contact spot is fairly small, we make the following assumption: the lint and the spring wire are considered as the contact of two cylinders (Figure 6) crossing at an angle $\pi/4$, and due to the small contact spot, we assume that the spot is circular. To determine the friction force (7), it is necessary to determine the normal force and the friction coefficient (Kuznetsov et al. 2020; Kleuker and Hoffmann 2022). The normal force is determined by

$$N = F = \frac{4}{3} E^* \tilde{R}^{1/2} d^{3/2}, \quad (19)$$

where $N(F)$ is the normal force, N; E^* is the reduced modulus of elasticity, Pa; $\tilde{R} = \sqrt{R_1 R_2}$ is the Gaussian

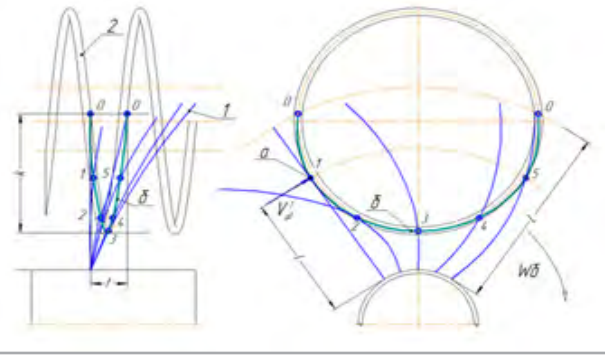
radius of curvature (R_1 and R_2 are the radii of the cross-section of the lint and the coil of the spring, respectively, m); d is the penetration depth, m;

The reduced modulus of elasticity is defined as:

$$\frac{1}{E^*} = \frac{1-v_1^2}{E_1} + \frac{1-v_2^2}{E_2}, \quad (20)$$

where E_1 and E_2 are the elastic moduli, Pa; and ν_1 and ν_2 are the Poisson coefficients of the bodies.

Figure 6: The schematic diagram of the interaction of the lint with the coil of the spring: 1 – lint, 2 – coil of the spring; a – the contact area; b – the trajectory of the lint motion in a contact with the coil of the spring, k – the contact spot of the lint when moving along the coil of the spring



Let's express E^* by transforming the formula (20)

$$E^* = \frac{1}{\frac{(1-\nu_1^2) \times E_2 + (1-\nu_2^2) \times E_1}{E_1 \times E_2}} = \frac{E_1 \times E_2}{(1-\nu_1^2) \times E_2 + (1-\nu_2^2) \times E_1} \quad (21)$$

The contact spot a is defined according to $a = \sqrt{R \times d}$.

Since we previously assumed that the contact area (spot) is a circle, the contact area will be $S = \pi \times a^2$ m². After the transformation, the contact area between the lint and the coil of the spring can be calculated using the formula $S = \pi \times R \times d$, m². The penetration depth will then be defined as

$$d = \frac{S}{\pi \times R} \quad (22)$$

Substituting the expressions (21), (22) in the formula (19) we get

$$\begin{aligned} N = F &= \frac{4}{3} \times \frac{E_1 \times E_2}{(1-\nu_1^2) \times E_2 + (1-\nu_2^2) \times E_1} \times \sqrt{R_1 R_2} \times \sqrt{\left(\frac{S}{\pi \times R}\right)^3} \\ &= \frac{4}{3} \times \frac{E_1 \times E_2}{(1-\nu_1^2) \times E_2 + (1-\nu_2^2) \times E_1} \times \sqrt{R_1 R_2} \times \sqrt{\left(\frac{S}{\pi \times \sqrt{R_1 R_2}}\right)^3} = \frac{4}{3} \\ &\times \frac{E_1 \times E_2}{(1-\nu_1^2) \times E_2 + (1-\nu_2^2) \times E_1} \times \frac{R_1 R_2 \times S^3}{\pi^3 \times (\sqrt{R_1 R_2})^3} \end{aligned} \quad (23)$$

Finally, we get:

$$N = F = \frac{4}{3} \times \frac{E_1 \times E_2}{(1-\nu_1^2) \times E_2 + (1-\nu_2^2) \times E_1} \times \frac{S^3}{\pi^3 \times \sqrt{R_1 R_2}} \quad (24)$$

The friction force at the contact interaction between the lint and the coil of the spring will be

$$F_{fr} = fN = f \times \frac{4}{3} \times \frac{E_1 \times E_2}{(1-\nu_1^2) \times E_2 + (1-\nu_2^2) \times E_1} \times \frac{S^3}{\pi^3 \times \sqrt{R_1 R_2}} \quad (25)$$

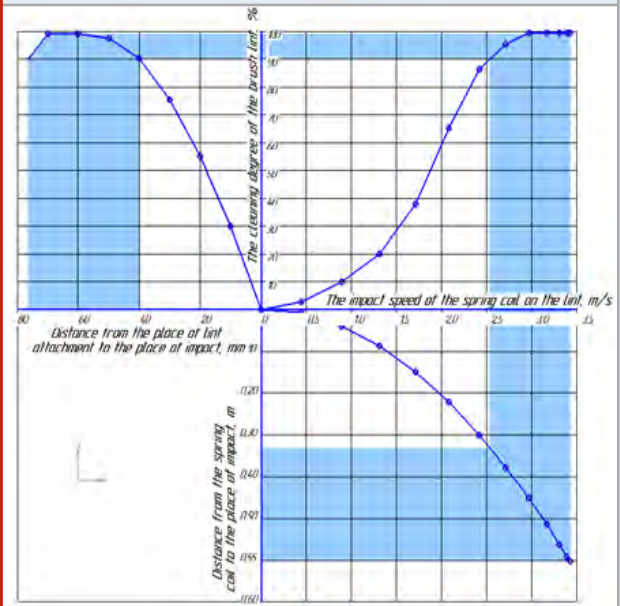
Let us express from the formula (25) the radius of the wire R_2 for making the spring

$$R_2 = \frac{S^5 \times (4 \times f \times E_1 \times E_2)^4}{\pi^5 \times [3F_{fr}(1-\nu_1^2) \times E_2 + (1-\nu_2^2) \times E_1]^4 \times R_1} \quad (26)$$

The diameter of the wire will be equal to the double radius, respectively $d = 2R_2$. Next, we determine the main design dimensions of the cleaning static spring: $d = 4$ mm. According to the design dimensions of the brush and the condition of the static spring, we take the spring diameter $D = 130$ mm. The spring stiffness will be $c_1 = 130/4 = 32.5 \ll 450$, which corresponds to the condition of self-cleaning of the static spring. The dependence of the cleaning degree of the lint on the impact velocity of the coil of the spring, as well as on a distance (height) of the coil to the point of impact, and the distance from the lint attachment point to the point of impact is presented in the form of nomogram (Figure 7) (Rajaeifar et al. 2019; Rajaeifar et al. 2019).

When conducting studies with heavy loamy chernozem soils with a moisture content of $\approx 28\%$, (which corresponds to the greatest stickiness) it is revealed that the minimum required brush lint impact velocity to separate soil from the coils of the spring is about ≈ 2.5 m/s. At the same time, the impact velocity of the brush lint should be large enough to separate the soil of maximum stickiness from the coil of the spring, however, it should not be greater than the velocity causing lint cutting, i.e., the velocity at which the brush lint is destroyed (Rajaeifar et al. 2019; Rajaeifar et al. 2019).

Figure 7: Nomogram for determining the optimal parameters of the impact point location of the lint to the coil of the spiral (the highlighted area corresponds to cleaning degree equal to 90-98%)



CONCLUSION

The findings of the present study obtained dependencies allowed revealing that with an increase in the height of the pendulum's center of mass, the impact velocity on the

lint also increases. The analysis of the dependence of the cleaning degree of the lint on the impact velocity shows that with an increase in the impact velocity on the lint, the cleaning degree increases. At velocities ranged from 0 to 1.7 m/s, the cleaning degree increases to 40%. With an increase in the velocity of more than 1.7 m/s, the cleaning degree increases up to 65% and higher. The velocities exceeding 2.5 m/s provide the most complete cleaning of the brush lint from stuck soil.

Conflict of Interests: Authors declare no conflicts of interests to disclose.

ACKNOWLEDGEMENTS

This study was financially supported by the Ministry of Science and Higher Education of the Russian Federation and JSC Millerovoselmash. The research results presented in the article were obtained as part of the implementation of the Agreement No. 075-11-2019-041 of November 22, 2019, between the Ministry of Science and Higher Education of the Russian Federation and JSC Millerovoselmash for conducting R&D on the topic "Creating high-tech production of multifunctional complexes for sowing and cultivating row crops and vegetable crops in the system of accurate and zero arable farming based on intelligent mechatronic modules". The research is performed in the organization of the General Contractor (Federal State Budgetary Educational Institution of Further Professional Education Michurinsk State Agrarian University).

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Technical Communication

Data Mining and Knowledge Discovery in Big Data for Decision Making in Higher Education

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ABSTRACT

Artificial intelligence and data mining plays a fundamental role in improving the intelligence of education through special standards for improving teaching quality, better learning experience, predictive teaching, assessment method, effective decision-making, and improved data analysis. BD (Big Data) are also used to assess, detect, and anticipate decision-making, failure risk, and consequences to improve decision-making and maintain high-quality standards. According to the findings of this study, certain universities and governments have adopted BD to help students transition from traditional to smart digital education. Many obstacles remain in the way of complete adoption, including security, privacy, ethics, a scarcity of qualified specialists, data processing, storage, and interoperability. Learning today is getting smarter, thanks to the rapid development of the use of data and knowledge for big data analysis. Besides delivering real-world knowledge discovery applications, specialized data mining methodologies, and obstacles have real-world applications. Therefore, this article aims to explain the current concept of an intelligent learning environment in higher education. It explores the main criteria, and presents evaluation methods through the use of the proposed model.

KEY WORDS: ARTIFICIAL INTELLIGENCE, DATA MINING, KNOWLEDGE DISCOVERY, BIG DATA, AND E-LEARNING.

INTRODUCTION

Data-driven decision-making is rapidly evolving in education, it has quickly evolved into the far more sophisticated idea of big data, which is based on software techniques known as predictive analytics. Big data and analytics for educational applications are still in their infancy and will take a few years to mature, but they are already making an impact and should not be overlooked. While big data and analytics aren't a silver bullet for all of the problems and decisions that higher education administrators confront, they can be integrated into many educational periodicals' embedded solutions. The goal of this research was to expand Saudi higher education's use of big data and analytics. It looks at the nature of these notions, present basic definitions, evaluate potential applications, and, last but not least, highlight issues with their implementation and expansion (Alsheikh2019; Aljahdaliet al., 2020; Alkhalil, 2021).

Data mining is a method for identifying previously undiscovered associations in large amounts of data. Data mining and this technique is used as a useful approach for many areas of application in educational settings, particularly in higher education, but has not been generally accepted by users of the educational industry in general and the database community in particular. The nature of data mining jobs is primarily responsible for this. Data mining is a challenging and time-consuming task that requires a lot of practice to succeed, (Kaiser et al 2013).

Data mining usually involves selecting techniques, creating models, and tuning parameters to support analytical activities. Data mining technology helps companies make use of their existing data more effectively and gain insightful information that gives them a competitive advantage. Data mining is the process of extracting hidden predictive information from massive databases. It's a strong tool that may assist higher education institutions to focus on the most significant data in their data repositories while making decisions. Data mining techniques enable all higher learning institutions to make proactive, knowledge-based decisions by predicting

Article Information:*Corresponding Author: Hanaa.e@mu.edu.sa

Received 10/09/2021 Accepted after revision 30/12/2021

Published: 31st December 2021 Pp- 2013-2019

This is an open access article under Creative Commons License,

Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.93>

trends and behaviors. Automated and prospective analyzes of data mining go beyond analyzes of past events presented by potential tools used in decision support systems, (Kaiser et al., 2013; Gandomi et al., 2015).

Data mining techniques also enable to quickly answer problems that previously took a long time. They create databases to search for hidden patterns and prognostic information that experts may overlook since it is outside of their expectations. The proposed use of association rules, simple decision tree, and Logistic Regression Algorithms as means for data mining is a useful technique for many risks that can face higher education systems. Existing studies related to intelligence analysis have focused on news analysis or information analysis forums, but unfortunately, few studies use data mining and knowledge discovery techniques from big information in education (Picciano 2012; Muktharet al., 2017; Baiget al., 2020). This paper consists of 3 sections: The first section includes a brief overview of data mining and knowledge discovery, and the second section includes the data mining methodology, decision tree model, and logistic regression algorithms. Section 3 includes the conclusion and future work.

Data Mining and Knowledge Discovery in “big data”: There are many applications for data mining and knowledge discovery. Many conferences on Artificial Intelligence, Intelligent Systems, Databases, and Statistics have discussed data mining and knowledge discovery. The process of detecting validated, novel, and plausible patterns is referred to as knowledge discovery. Data mining, or discovering information from big data, is the process of applying the discovery process to large databases or data sets. Creating the target dataset, building an understanding of the application domain, etc. are all stages in the discovery process.; finding useful features for data representation; data cleaning and preprocessing; It uses data mining to look for patterns of interest; Interpretation and standardization of the detected patterns ((Muktharet al., 2017; Aljahdali et al., 2020; Alkhalil, 2021).

From an obvious point of view, many algorithms seem to be completely different, but from the point of view of reality, they have common components that make them similar. To decode and understand data mining and model extrapolation contribute to decision making, it is necessary to delve into the level of these components. This, in turn, will lead to an understanding of the overall contribution and good application of the KDD process as shown in Figure 1.

Big Data Analytics' Importance in Higher Education: Big Data Analytics (BDA) is the first phase in the knowledge discovery process for Higher Education, and it involves the algorithms that are used to extract potentially significant patterns, connections, trends, sequences, and dependencies from data, as shown in Figure 2.

1. Determination: Getting to know the application domain:
2. Preparation: Cleaning and preprocessing of data:
3. Data reduction and projection (transformation):
4. Data Mining: Applying Data Mining Functions and

Algorithms: Rules of association, classifying,

5. and grouping
6. Validation and Verification of Patterns: Validate and verify identified patterns:
7. Applying everything you've discovered:
 - A. Data cleaning (a) (to remove noise and inconsistent data)
 - B. Data fusion (where multiple data sources may be combined)
 - C. Data selection (data from the database that is relevant to the analytical activity)
 - D. Data transformation (conversion or aggregation of data into acceptable forms for a certain purpose).
 - E. Data mining is the process of finding and extracting meaningful patterns from massive volumes of data (Data visualization).
 - G. Results verification and assessment; formulating conclusions

Figure 1: The Process of Knowledge Discovery (Aljahdali et al., 2020).

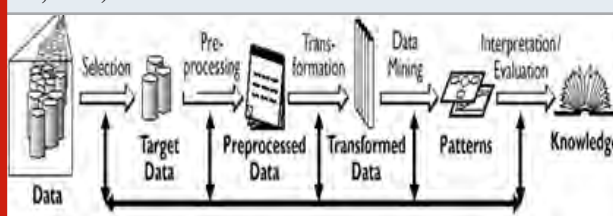
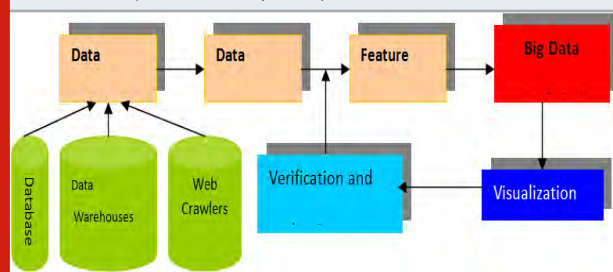


Figure2: Big Data Analytics' Importance in Higher Education (Boulila et al., 2018).



This section describes the main aims of data mining, particularly in the higher education sector, as well as the approaches utilized to attain these goals and the data mining algorithms employed in these procedures. Are the first, third, fourth, and fifth numbers. Knowledge discovery goals can be described in terms of the system's intended usage. Two fundamental aims can be distinguished (Boulila et al., 2018; Verma et al., 2018; Alkhalil et al., 2021). 1) Validation, 2) Investigation.

Challenges: Big data information systems, especially electronic higher education systems, are vulnerable to a variety of threats. Since information is exposed to a variety of risks and attacks through the collection, processing, and retrieval stages - whether reading, printing, or downloading - as well as the transmission, exchange, and storage stage, these risks, and attacks differ according to these. Operations,

each stage has its risks and means of protection (Aljahdaliet al.,2020; Aseeriet al., 2020).

Figure 3: Fields of DM Combination



System penetration: This occurs when an unauthorized individual gains access to a computer system and engages in unlawful actions such as changing application software, stealing private data, damaging files, software, or the system, or simply for illicit purposes. Or by exploiting system flaws, such as circumventing control and protection mechanisms, or by using knowledge gathered by the hacker from material or moral sources. These are the most notable threats and assaults (Menget al., 2014). Cultivation of flaws: Typically, this threat arises as a result of an illegal entry or a legitimate user beyond the scope of the authorization provided to him., so that the individual creates an opening for eventual penetration (Segooaet al., 2018).

Usage of the right of authorization: This occurs when a person who is authorized to use the system for one purpose uses it for another without first getting permission. External threats are mitigated by exploiting a flaw in the system to gain access to it through a valid method or a legitimate element and then engaging in criminal actions (Ndaet al., 2019). Monitoring communications: By merely monitoring communications from one of the victims' computers, the offender can get sensitive information, which is typically information that helps further infiltration of the system, (Baiget al., 2020).

Concept of Data Mining: Data mining is a logical technique for searching through enormous volumes of data to identify essential information, that shown in figure 4. The purpose of this method is to discover previously undiscovered patterns. You may utilize these patterns to address a variety of issues after you've discovered them. Data mining (knowledge discovery) is the process of uncovering data from many angles and presenting it in a form of value that can be used to enhance its value, reduce costs, or both (Al-Medlej, 1997; Segooaet al., 2018).

Data mining software is one of the many analytical methods available for data analysis, especially big data. Higher education groups enable the study of data from a variety of viewpoints, their classification, and the description of the links discovered in them to make the appropriate decision. Data mining is the process of identifying patterns or links between hundreds of variables in large databases. Data

mining is a strong tool since it may supply businesses with pertinent information that can be used to their advantage. When businesses have the appropriate knowledge, all they have to do is use it in the right way to get the desired outcome (Janssenet al., 2017).

Nowadays, obtaining information is pretty simple. However, obtaining relevant information that might assist you in achieving the desired objective is not always simple. This is when data mining comes into play. This is where data mining becomes an important technique to master. It has the capability of predicting certain system behavior (Menget al., 2014).

1. Model Validation and Evaluation in Statistics
2. Pattern recognition and machine learning: Symbolic neural network learning
3. Data warehousing and databases
4. The Algorithm (online analytical processing)
5. Knowledge representation in artificial intelligence
6. Report production using data visualization
7. Recognizing Patterns

Figure 4: Knowledge Discovery and Data Mining (Mukthar,2017)



Data mining techniques may be divided into two categories: tasks and methods (Hashim et al., 2010).

Methodology: The proposed use of association rules, simple decision trees, and Logistic Regression Algorithms as data mining tools is a useful technical method for assessing data security, classifying and analyzing data, and ensuring that it is not transmitted to provide strategic information for the various services provided, as well as finding important management points. Effective educational services, what kind of data should be utilized, what type of data was communicated properly, what are the conditions or needs of the user in the data, knowledge organization and arrangement in terms of time, priority, and so on.

Classification of activities, or in terms of its importance or the moment of its discovery Has there been a breach of information, the underlying structure for sharing knowledge, which leads to the addition to the network based on the numerous efforts made to the mechanism of this process to explore, analyze data and test its integrity and within the framework of prediction, based on the criteria used, which help in choosing between many alternatives? The challenges

and risks that higher education systems face. Algorithms for mining spatial, textual, and other complex data.

1. Methods of incremental discovery and re-use of previously acquired information.
2. Methods of discovery are combined.
3. Data mining data structures and query evaluation techniques
4. Data mining approaches that are parallel and distributed.
5. Problems and obstacles in working with large or small data sets
6. Statistics, databases, optimization, and information processing are fundamental topics as they apply to the difficulties of extracting patterns and models from data.

The practical fundamental goals of data mining may be summed into two basic goals: "prediction" and "description."

1. Data mining prediction entails using certain database variables to forecast future values of other variables of interest.
2. The search for human-interpretable patterns that can characterize the data is the focus of the description process. To put it another way, descriptive modeling (clustering) is the grouping of related data into a single group.

Verification and testing of availability Using rules of association: The use of the term "availability" in communications as a factor to measure and describe the degree to which cyberspace remains functional. In most cases, it indicates a fraction, such as 9998 for the available simple. A, and in the case of subtracting from it the downtime (the time taken), (given the percentage of the expected work value & performance).

$$A = \frac{E[UPtim]}{E[Downtim]} + \frac{E[UPtim]}{E[UPtim]}$$

If you define a function, it will be $x(t)$. At Tim t , Status 1 = sys Function

$$X(T) = \begin{cases} 0 & \text{otherwise} \\ 1 & \end{cases}$$

Savings possibilities include the following:

$$|x(t) = 1|A(t) = Pr[E|x(t)] = X.Prx(t) = 1t > 0 |E|x(t)] = X.Prx(t) = 1$$

As a static random, you'll also need to know the average on a real-time basis availability. The following is how the availability average is calculated:

$$A_{C=\frac{1}{c}} = \frac{1}{c} \int_0^c A(t) dt, \quad c > 0$$

As a symbol denoting limited availability.

$$A = \lim_{t \rightarrow \infty} A(t)$$

The availability average on a real-time basis timeline is taken into account, along with field and randomization. As a result, the availability average can be represented as follows:

$$A_{\infty} = \lim_{C \rightarrow \infty} A_C = \lim_{C \rightarrow \infty} \frac{1}{C} \int_0^C A(t) dt \quad c > 0$$

Protective monitoring of big data as periodic monitoring is critical to ensuring that cyberspace remains effectively protected, as we can eliminate vulnerabilities by identifying weak points and taking into account protection arrangements. The above equations have been applied to a sample of data. Since we can set availability at 96 percent and timeliness at 94 percent, systems can detect and prevent eavesdropping vulnerabilities, as well as control and respond appropriately.

Use a simple decision tree model for safety and security testing and verification: The basic decision tree model (String algorithm) is used to classify data, including input fields and variables shown in table no 1. The result of using this model to classify the higher education large data set. This template provides constrained reporting of database branches, such as classification or rejection decisions, as well as the scope for automatic error detection in big data.

Table 1. Description of Field name

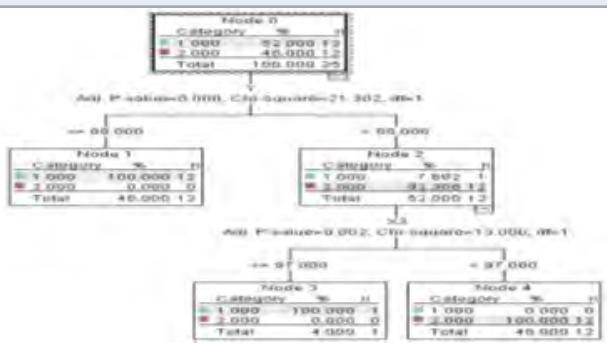
Parameter	Description,
Input variable	x_1, x_2, x_3, x_4, x_5 & Y
Security rating	Security rating: 0= attack 1= security
Data risk	NO of test range of security 1=< 88.00 , 0 >88.00

The data input is coding correct(0, 1)&that dependant[x1-x2-x3-x4-x5-y].

The parse is located at the top of the first node of the tree shown in the image as infigure 5. It compiles data collection. We find in the typical dataset that is the safe protected data, with a rate of 54%, we can detect a rate of 4 percent and 12 degrees. It makes up 15% of the known risk and

vulnerability ratings for unprotected data. Since the initial stage in the study is to improve protection performance and security, let's see if each tree might provide any evidence of problematic components. The first split, as can be seen, depends on the level of the input data. As a result, it will be able to allocate or provide marks based on the maximum revenue of the class (2 knots). This rating has the highest percentage of unprotected data, indicating that data in this category is at risk and requires protection. As a result, the data in this category has a rate of 54 percent, indicating a risk, if not apparent.

Figure 5: Model of Decision Tree



Although the prediction model cannot respond in practice, we must ensure that the data is sufficiently fair enough to allow us to anticipate future risks and respond to potential threats for each degree based on the available data, just as if we analyzed the data referenced by Node 2. As can be seen, the vast majority (92308%) agree. It appears unprotected, which poses a risk and necessitates the implementation of a new security mechanism. As a result, we discovered that each score is an indicator of this model, and we may enhance security requirements in this data set to reduce risk. By allocating a specific node, we will be able to detect weak places.

Table 2. Descriptions of variables and parameters

Through this stated, the following notation	
N	The total number of instances observed
P	The number of parameters
Y	the jth case's the observed value of the dichotomous dependent variable
X	parameter n x 1 vector
B	Likelihood Function
W	log – likelihood function
L	The total number of occurrences found.
L	The number of parameters is represented
I	the observed value

Preliminary Verifiability Control Calculations Using Logistic Regression Algorithms: The class A index is shown, followed by the Class B index X2 and the reference class IS C. Each score h passed through a logistic regression lateral model, the scores belonging to this category equally x1, x2, model set 0,0 which prospects for the threat that

each score h passed through a logistic regression lateral model, where each score h passes through A model prepared for logistic regression, the following formula is used to calculate the expected value as well as the degree of reliability: “most likely z = 1 value of scores and computation” is the expected value.

Table 2 shows that Possibility of ["flexible value feedback" & "Objectivity" & "accuracy verifiability"].

$$\hat{\pi}_i = \text{Exp}(\hat{\eta}_i) \left[\frac{1}{1 + \text{Exp}(\hat{\eta}_i)} \right]$$

where $\hat{\eta}_i$ as $(X_i' \hat{\beta})$

If $\hat{\pi} > 0.5$,

In this case, the projected value is 1; otherwise, it is 0.

Confidence: The confidence value is (1-) for records having a projected value of y = 1, whereas it is (1+) for records with a predicted value of y = 0.

$$(1-) \cdot \sum_{i=1}^n w_i (y_i - \pi_i) x_{ij} = 0, \text{ for the parameter}$$

Where $x_{i0=1}$ for $i = 1, \dots, n$.

Table 3. Model fitting information

Model Fitting Information				
Model	Model Fitting Criteria	Likelihood Ratio Tests		
	-2 Log Likelihood	Chi-Square	df	Sig.
Intercept Only	34.617			
Final	.000	34.617	8	.000

Pseudo R-Square	
Cox and Snell	.750
Nagelkerke	1.000
McFadden	1.000

Is the usage of kind Newton Rafsson the Verifiability Algorithm? We can't rely on convergence, so we use Newton Rafsson-type algorithms to obtain the verifiability. We can't rely on closeness, so we use Newton Rafsson-type methods. • The estimated absolute difference between the frequencies. • The probability difference in percent. • Intervals between successive frequencies, the maximum number of frequencies specified to determine the validity of the data (verifiability) according to the frequency which is the lowest in all circumstances, and ranges from 10 to 8. The probability of redundancy occurring is very near to nil. The message's frequency will be off, and all values will either be 1-0 or be released. Getting inverse of the first information matrix is the upper bound of the probability estimates and the variance matrix is the proxy estimated since the

previous. As well as Note: This example demonstrates how to use the total % as a design guide for the form. It might be a matter of precision. It might be the detective in certain circumstances. In general, the original; zero models were 72.6 percent accurate, Although the final model and expectation had a whole accuracy of 79.1%, as we saw, the real individuality of separate classes on a broad scale was among the forecasts' accuracy.

Table 4. expected values

Parameter Estimates									
Worla)		B	Std. Error	Wald	df	Sig.	Exp(B)	95% Confidence Interval for Exp(B)	
								Lower Bound	Upper Bound
No	Intercept	-174.349	256116.563	.000	1	.999			
	Y	1.503	2034.026	.000	1	.999	4.494	.000	(b)
	X1	.303	625.467	.000	1	1.000	1.354	.000	(b)
	X2	-.406	1117.323	.000	1	1.000	.667	.000	(b)
	X3	1.373	4523.229	.000	1	1.000	3.948	.000	(b)
	X4	-1.175	2069.738	.000	1	1.000	.309	.000	(b)
	X5	.026	1861.721	.000	1	1.000	1.026	.000	(b)
	[Gra=1.000]	14.383	35793.597	.000	1	1.000	1764094.730	.000	(b)
	[Gra=2.000]	33.400	54032.791	.000	1	1.000	320059904887103.000	.000	(b)
	[Gra=3.000]	0(c)		0					

a. The reference category is: Y.
b. Floating point overflow occurred while computing this statistic. Its value is therefore set to system missing.
c. This parameter is set to zero because it is redundant.

The output class with the highest value is the expected value for the scores. Opportunities with a logarithmic scale.

$$r_{ij} = \log \left(\frac{\pi_{ij}}{\pi_{ij'}} \right) = x_i' \beta_j$$

for $j = 1, \dots, j-1$. The log it for reference categoryj, r, l, is 1.0.

$$\hat{\pi}_{ij} = \frac{\exp(r_{ij'})}{1 + \sum_{k=1}^{j-1} \exp(r_{ik'})} = \frac{\exp(x_i' \beta_j)}{1 + \sum_{k=1}^{j-1} \exp(x_i' \beta_k)}$$

Table No. 3 shows that there are unlikely to be any changes for this classification when calculating the probability of occurrence of any of the previous criteria by calculating (exp), as we can see that "1-the lowest probability of occurrence is 0.390, which we find in the x4 variable, and the highest probability of occurrence is 4.494, which we find in the variable y 2- This model appears to be criteria. The outcomes of linking, feasible alternatives, and evaluating the likelihood of all j classes given comparable data are shown in Table 4.

CONCLUSION

Various educational bodies faced numerous challenges and obstacles, including network crimes, a lack of information technology governing security data, and the ability for education to create positive work climates by simplifying the processes for making necessary decisions with companies and reducing management steps to comply with regulatory obligations. Through the employment of a data mining model for the needed educational departments, artificial intelligence plays a significant role in monitoring the information analysis of e-learning systems.

It is recommended to analyze the data and look for hidden information in it. This technique, which uses association rules, basic decision trees, and Logistic Regression Algorithms as data mining tools, is a beneficial solution for a variety of threats that higher education institutions may face. maybe generalized; the suggested control strategy is quite thorough and lowers information security risks that impact senior management in educational institutions' strategic decisions. The findings of this study are highly valuable for developing a plan to assess the efficiency of learning performance to enhance the educational system's effective management, as well as the sort of data that will be employed, i.e. Finally, via real analysis discovered in dialogues, research, and blogging, this article may become an essential tool for learners in decision-making and monitoring of large data.

ACKNOWLEDGMENTS

The authors extend their appreciation to the deputyship for Research & Innovation, Ministry of Education in Saudi Arabia for funding this research work through the project number (R-2021-275).

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Agricultural Communication

Insecticidal Efficacy of Medicines During Cat Flea Infestations in Tyumen

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ABSTRACT

From time immemorial people began to domesticate wild animals and throughout many generations, they are kept by people genetically isolated from external pathogens. In the prevailing era, every pet owner is trying to breed and protect his animal from contagious and noncontagious diseases. Having said that, lack and inadequacy of knowledge and mass media on simple preventive measures results in the widespread occurrence of infectious and invasive diseases, including flea, *Ctenocephalides felis* infestation among dogs as well as cats. The present paper primarily attempts to present the results of therapeutic efficiency of Komfortis, Foresto, and Fitoelita medical agents used during flea infestation among domestic cats. In order to meet the purpose of the study, research was carried out within the framework of study and analysis of the epizootic state of invasive diseases of agricultural and unproductive animals, bees, and birds. Studies of changes in the species composition, and bioecological patterns of the development cycle of parasites under shifting boundaries of their ranges were also done. For the study, the animals were divided into three groups of 10 animals each. Standard clinical and hematological research methods were used during the study. Blood sampling included three stages: before insecticide treatment, the intermediate interval of 15 days, and the final stage. Based on the results obtained, it was found that Komfortis and Foresto have 100% therapeutic efficacy at all stages of flea development and possess a long-lasting action during flea infestation. The morphological blood analysis showed that the proposed medical agents have no toxic side effects.

KEY WORDS: APHANIPTERA, CATS, *CTENOCEPHALIDES FELIS*, FLEA INFESTATIONS, INSECTICIDES.

INTRODUCTION

Flea, *Ctenocephalides felis* infestation of domestic cats is one of the urgent problems which is being studied by modern veterinary science, but despite the achieved success of parasitic pharmacy in their elimination, fleas are widely spread and represent a danger to cats, dogs, and other domestic carnivorous animals kept in close contact with human beings, the owners and those contacting them, (Arkhipov et al., 2001; Roslavtseva, 2011; Berezina, 2015; Nikonov et al., 2017; Stolbova et al., 2018; Stolbova, 2018; Stolbova & Skosyrskikh, 2020). To prevent the distribution of flea infestations it is necessary to account for ensuring continuous epizootic control over timely medical and preventive measures to eliminate the focus of disease, (Glazunov, 2014; Berezina, 2015; Domatsky, 2016; Cooper et al., 2020).

Today the veterinary science employs various anti-parasitic agents that are different in their forms, chemical composition, mode of administration, and price. However, despite the variety of insecticidal agents created to fight against flea infestations, some current flea species are resistant to some classes of insecticides containing two and more active drug substances, (Lyutikova and Arkhipov, 2008; Stepanov et al., 2014; Glazunova and Tkachyova, 2017; Stolbova, 2019; Packianathan et al., 2020).

The resistance level may be high to exclude the possibility of any further use of either medical agent. There are known cases when the resistance of parasitic arthropods is developed even at the stage of testing a new medicine prior to its industrial output, (Udavliyev et al., 2012; Gerke, 2013; Kruglov and Stolbova, 2017; Stolbova et al., 2018; Stolbova, 2018; Stolbova, 2020; Tieleman et al., 2021). In this regard, the efforts of experts are aimed not only at the creation of new medicinal compounds with a different mode of action but also at the search of such insecticide strategy, which could prevent the formation of resistant types of

Article Information: *Corresponding Author: russia@prescopus.com

Received 15/10/2021 Accepted after revision 29/12/2021

Published: 31st December 2021 Pp- 2020-2024

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.94>

2020

parasitic arthropods. Thus, it is critical to develop new medical agents, mainly complex ones containing several active drug substances. This combination is advantageous since various active substances cause a synergistic impact on all stages of flea development.

MATERIAL AND METHODS

The study was performed during 2014-2020 at a vivarium of the Institute of Biotechnology and Veterinary Medicine of the Northern Trans-Ural State Agricultural University Russia. The experimental part was conducted at the laboratories of the All-Russian Research Institute of Veterinary Entomology and Arachnology – branch of Tyumen Research Center, SB

RAS, and the veterinary clinics of Tyumen. This research was conducted within the framework of the topic № 296-2021-0018 -Thirty animals were picked for the study (different breed, age, nutritional state) thus forming three equal groups (n=10). Komfortis was applied to the first test group, Foresto – to the second, and Fitoelita shampoo – to the third control group. Komfortis is an insecticide released as pill for oral use, it contains spinosad – 53.33%, is active towards fleas such as *Ctenocephides felis* parasitizing dogs and cats. The mechanism of action involves the activation of n-cholinergic receptors of parasitic arthropods, causing nervous strain, muscle cramps, tremors, and paralysis leading to the death of insects (Instruction for medical use of Komfortis).

Table 1. Insecticide efficacy during cats FLEA Infestation in Tyumen

Assessment period	First test group Komfortis agent (n=10)		Second test group Foresto collar (n=10)		Control group Fitoelita shampoo (n=10)	
	Number of detected fleas	%	Number of detected fleas	%	Number of detected fleas	%
1st day	144	31.44 ± 0.67	121	25.69 ± 0.71	157	15.31 ± 0.76
5th day	157	34.28 ± 0.58	138	29.30 ± 0.69	104	10.15 ± 0.54
10th day	112	24.45 ± 0.52	124	26.33 ± 0.52	161	15.71 ± 0.48
15th day	29	8.52 ± 0.42	68	12.31 ± 0.37	130	12.68 ± 0.65
20th day	6	1.31 ± 0.34	39	8.28 ± 0.29	187	18.24 ± 0.44
25th day	0	0 ± 0.25	11	2.34 ± 0.21	133	12.97 ± 0.76
30th day	0	0 ± 0.28	0	0 ± 0.17	153	14.93 ± 0.51
Total	458		471		1025	
Infection intensity	45.8		47.1		102.5	

RESULTS AND DISCUSSION

During the study, it was noted that Komfortis and Foresto have high insecticidal effects reaching 98-100%. The insecticide efficacy was considered within a month, every 5 days from the moment of applying for the medicines. It shall be noted that the number of dead images was sharply increasing from the first to the tenth day of the study in test groups. The maximum mortality rate was on the 5th day of observations, where for the first group this made – 34.28%, and for the second – 29.30%. During the following observation, the number of fleas in test groups was reduced. The therapeutic efficacy of applied medical agents made 100% in the first group on the 25th day of observation and in the second group on the 30th day. The smallest number of parasites per one infected specimen was observed with Komfortis – 45.8. The analysis of the insecticidal activity of the control group showed that the efficacy of the medical agent was indicated only at the time of drug administration, during the interim periods the number of fleas was minimum, the maximum mortality rate was observed

on the 20th day, and made 18.24%. The highest invasiveness per one specimen of the control group made 102.5, which indicates the progressive growth of fleas at all control points. All results are shown in Table 1.

The study of the therapeutic efficacy of Komfortis, Foresto, and Fitoelita during flea infestation of cats did not reveal any negative impact on their organism, there were no toxicoses and gastrointestinal upsets. The hematologic study showed that during the primary blood sampling a minor increase of white blood cells was noted among all groups of animals (Stolbova, 2018; Stolbova & Skosyrskikh, 2020). After the use of insecticidal agents, the follow-up tests were performed at interim intervals. The first and the second groups are characterized by the decrease of indicators to the minimum, while the control group – by the increase in leukocytes to $7.98 \pm 0.41\%$, eosinophils to $2.68 \pm 0.46\%$, other indicators remained unchanged.

At the final stage of blood sampling from the groups exposed to Komfortis and Foresto the animals were fully recovered,

the blood values fell within the physiological range, the indicators of the control group did not change drastically from primary tests at the beginning of therapy. All results are shown in Table 2. The hematologic blood changes of cats with flea infestations demonstrate that at different invasiveness the values do not change considerably from physiological range. Thus, the hematologic study made it possible to confirm that the treatment with such medical agents does not exert a pathogenic impact on blood values, which fall within the physiological range. Foresto is an insect-acaricidal collar, it contains imidacloprid – 1.25 g and

flumethrin – 0.56 g. The mechanism of imidacloprid action is based on the interaction with acetylcholine receptors of ectoparasites. It causes neurotransmission disorder resulting in complete paralysis leading to the death of ectoparasites. Flumethrin is a pyrethroid insecticide affecting voltage-sensitive sodium channels, it blocks nerve conduction resulting in spasm causing motor reflexes of arthropods leading to death (Instruction for medical use of Foresto). Fitoelita is an insecticidal shampoo, it contains permethrin – 0.3%, blocks neurotransmission of peripheral nerves ganglion leading to death through paralysis of flea adult and larval phases (Instruction for medical use of Fitoelita).

Table 2. The main hematologic changes of blood during cat's flea infestation in Tyumen

Indicator	Leucocytes, 10 ⁹ /l	Eosinocytes, %	Band neutrophils, %	Segmented neutrophils, %	Lymphocytes, %	Monocytes, %
Blood test at the beginning of study						
First group Komfortis	7.61 ± 0.41	2.38 ± 0.45	2.41 ± 0.38	37.68 ± 1.26	31.87 ± 0.29	2.13 ± 0.33
Second group Foresto	7.64 ± 0.44	2.42 ± 0.38	2.40 ± 0.21	37.62 ± 1.62	31.81 ± 0.25	2.15 ± 0.39
Control group Fitoelita	7.55 ± 0.39	2.44 ± 0.42	2.40 ± 0.21	37.65 ± 1.64	31.86 ± 0.28	2.12 ± 0.38
Interim blood test on the 15th day						
First group Komfortis	6.81 ± 0.42	1.86 ± 0.52	1.38 ± 0.33	36.22 ± 1.26	27.45 ± 0.34	1.61 ± 0.35
Second group Foresto	6.93 ± 0.38	1.97 ± 0.49	1.42 ± 0.36	36.28 ± 1.28	28.16 ± 0.32	1.89 ± 0.29
Control group Fitoelita	7.98 ± 0.41	2.68 ± 0.46	2.14 ± 0.31	37.15 ± 1.12	31.64 ± 1.12	1.64 ± 0.32
Final blood test on the 30th day						
First group Komfortis	6.24 ± 0.51	0.82 ± 0.34	0.28 ± 0.12	35.46 ± 0.66	26.14 ± 0.29	1.25 ± 0.31
Second group Foresto	6.28 ± 0.49	0.86 ± 0.42	0.34 ± 0.15	35.51 ± 0.69	26.17 ± 0.26	1.54 ± 0.28
Control group Fitoelita	8.14 ± 0.53	2.74 ± 0.38	2.15 ± 0.22	37.02 ± 1.16	31.42 ± 1.24	2.49 ± 0.29

Figure 1: Collection of fleas and eggs during combing

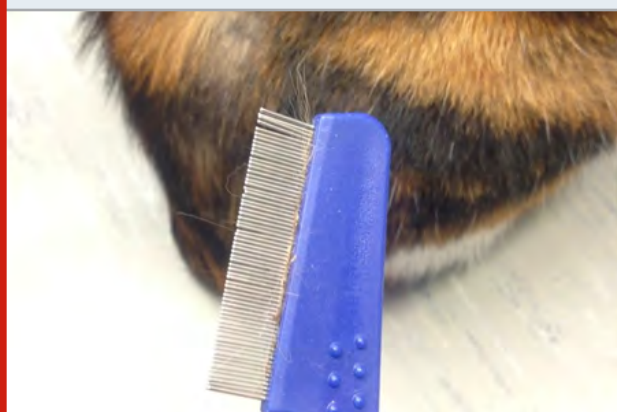


Figure 2: Ctenocephalides felis in Professional Dawn solution after combing

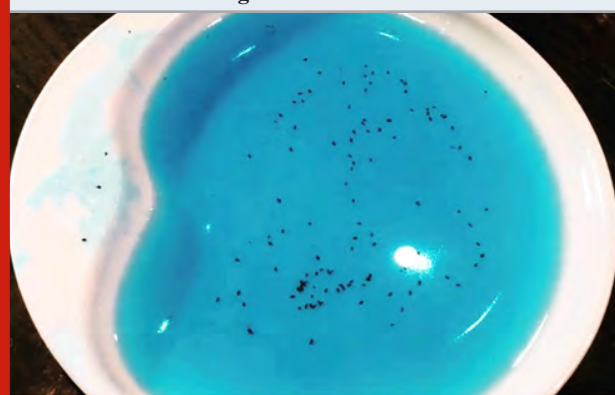


Figure 3: Ctenocephalides felis under microscopic vision**Figure 4: Hair print with Ctenocephalides felis**

The following special methods were used: hair combing (to define the number of alive and dead fleas) (Fig. 1), flea counting in Dawn Professional Dawn solution (Fig. 2), microscopic study (to define flea viability) (Fig. 3), hair print using acetate bands (to detect fleas and products of their activity) (Fig. 4). The obtained results were processed statistically via Statistica Ultimate Academic Bundle (Stolbova, 2018; Stolbova, 2020; Tielemans et al., 2021). The dosage of Komfortis pills was selected individually taking into account animal weight prior to treatment. Komfortis was applied at a dose of 50 mg/kg. The insect-acaricidal Foresto collar was used as per instructions for cats (38 cm). The medical agents were applied once only for test groups. The insecticidal Fitoelita shampoo was used at the rate of 1 ml/kg, its application was repeated after 10 days as per the manufacture's recommendations. The efficiency of applied medical agents during flea infestations of domestic animals was assessed following the results of clinical, hematologic, and special trials, and estimation of infestation intensity.

The blood tests were analyzed via the Abacus Junior 5 (Vet), sampling was done on the first day, further periods were broken into interim stages of 15 days. The flea infestation was validated using the special study of hair and skin cover

of all groups of animals before treatment and after the application of insecticidal medicines

CONCLUSION

The efficacy of using such insecticidal medical agents as Komfortis and Foresto collars as the therapy of cat flea infestations makes 100% effective. The application of such insecticides allows facilitating and reducing the length of treatment of pets. Further observation of patients after treatment did not reveal any reinfestation cases, which indicates its long-term action and serves as an excellent preventive method.

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Biomedical Communication

Antimicrobial Activities and Molecular Signature of Endophytic Fungi of *Opuntia ficus-Indica* Cacti and the Cactus-Like Plant *Aloe vera*

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ABSTRACT

Endophytes are fungi that colonize the internal tissues of plants without causing immediate adverse effects. Saudi Arabia (SA) is rich in *Opuntia ficus-indica* cacti and the cactus-like plant *Aloe vera*, which grow in the southern and western areas of SA. This study aimed to isolate and identify endophytic fungi from cacti and cactus-like plants in the Jeddah, Taif, and Al Baha regions KSA and then determine their effects on pathogenic fungal and bacterial growth. The isolates were grouped into 16 distinct operational taxonomic units based on the sequence of the internal transcribed spacer in the rDNA gene with the primers ITS1 and ITS4. *Mucor circinelloides* was the endophytic fungus found most frequently, with a relative frequency of 20.43%, followed by *Talaromyces funiculosus*, with a relative frequency of 16.12% when isolated from *Opuntia ficus-indica* and *Aloe vera*. Nine out of sixteen endophytic fungi exhibited strong antifungal activity against all the tested pathogens. *P. funiculosus*, *Aspergillus versicolor*, *Penicillium janthinellum*, and *Fusarium oxysporum* showed vigorous antimicrobial activities against the human pathogenic bacteria *Escherichia coli*, *Shigella* sp., and *Salmonella typhimurium*.

KEY WORDS: ALOE VERA; OPUNTIA FICUS-INDICA; ENDOPHYTIC FUNGI; ANTIMICROBIAL ACTIVITIES; PATHOGENIC BACTERIA.

INTRODUCTION

Endophytes are fungi that colonize the internal tissues of plants without causing immediate adverse effects (Khiralla et al. 2017). They are considered a promising source of new natural drug leads with great potential for medicinal and agricultural applications. For instance, many of the products currently used for human or animal therapy are produced by microbial products or derived from them. Furthermore, with the increasing incidence of drug resistance in human, animal, and plant pathogenic bacteria, which are among the major causes of death worldwide, endophytic fungi are considered important biotechnological tools because of the many secondary metabolites that they produce (Bara et al., 2013). Research on endophytic fungi has demonstrated that they constitute a promising source of biocontrol agents. Fungal endophytes enhance the resistance of their hosts against abiotic stress, disease, insects, and mammalian herbivores

by producing a broad range of biologically active fungal metabolites. Indeed, several of the interesting metabolites isolated from endophytic fungi belong to diverse chemical classes, including alkaloids, steroids, flavonoids, terpenoids, quinones, and phenols (Khiralla et al. 2017).

According to Suryanarayanan et al. (2005), various studies have shown that some endophytic fungi are neither artificially residents nor normally latent pathogens of plant hosts. They may protect the plant from insect pests, fungal pathogens, or increase host fitness in harsh environments in addition to possibly playing a role in litter degradation. However, very few plants growing in extreme or harsh habitats have been screened for fungal endophytes. Cacti are a good source of endophytic fungi (Wani and Lone, 2016). Medicinal plants have provided a rich source of novel antimicrobial agents throughout human history, with many infectious diseases traditionally being treated using herbal medicines. A wide range of medicinal plant parts are used to extract raw drugs that possess different medicinal properties, (Suryawanshi et al., 2016). Cactus-like plants are an important food source for wild animals; they are also used in the medicine, food,

Article Information:*Corresponding Author: waalsheri@uj.edu.sa

Received 20/10/2021 Accepted after revision 28/12/2021

Published: 31st December 2021 Pp- 2025-2031

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Published by Society for Science & Nature, Bhopal India.

Available at: <https://bbrc.in/> DOI: <http://dx.doi.org/10.21786/bbrc/14.4.95>

2025

chemical, spinning, and cosmetic industries; furthermore, they are a cheap source of readily available raw materials (Suryanarayanan et al., 2005; Mauseth, 2021).

The antimicrobial agents of *A. vera* gel have been reported to effectively eliminate or greatly reduce the growth of a range of wild pathogens (Lawrence et al., 2009; Bashir et al., 2011; Stanley et al., 2014 and Gharibi et al., 2016). *A. vera* and other cacti plant species have other uses, such as for bactericidal, antibiotic, fungicidal, anti-inflammatory purposes as well as moisturizing tissues and relieving pain associated with joints and muscles (Roşca-Casian et al., 2007, Surjushe et al., 2008, Lawrence et al., 2009, Silva-Hughes et al., 2015 and Ríos and Recio et al., 2005). Several studies have reported the isolation of 44 endophytic fungi species colonizing *O. ficus-indica* cacti plants in Brazil (Bezerra et al., 2013, 2017). In India, more endophytes were isolated from *A. vera* and other cacti such as *O. ficus-indica* (Yadav et al. 2015, Gangurade et al. 2019, and Vyawahare et al. 2019). In the USA, approximately 108 endophytic fungal isolates corresponding to 17 different taxa were obtained and identified as the species most frequently associated with *O. humifuse* cacti through use of molecular methods (Silva-Hughes et al. 2015). In Arizona, 21 cactus

species occurring in various localities were screened for the presence of fungal endophytes (Suryanarayanan et al. 2005). The southern and western areas of Saudi Arabia are rich in *A. vera*. Endophytic fungi species were previously isolated from *A. vera* collected from the Asir Desert (Ameen et al., 2021).

This study aimed to isolate and identify and characterize the endophytic fungi from cactus-like plants in Jeddah, KSA, toward defining the endophytic mycobiota of cacti in addition to evaluating the antimicrobial activities of the isolated endophytic fungi and plant extracts on pathogenic microbes. We hypothesized that the endophytic fungi isolated from cactus-like plants have a wide range of therapeutic applications against several diseases.

MATERIAL AND METHODS

Collection of Plant Samples: The cacti and cactus-like plants used in this study were fresh, naturally grown stems, leaves, and roots of *Aloe vera* and *Opuntia ficus-indica* harvested from the Jeddah, Taif, and Al Baha regions in SA in September 2019 and January 2020. The selected plants, which belonged to different families, are listed in Table 1.

Table 1. List of Plants Utilized in This Study.

NO	Scientific Name	Family	Common Name	Part of Plant Used	Collection Site
1	<i>Aloe vera</i>	Asphodalaceae	<i>Aloe vera</i>	Roots and Leaves	Jeddah
2	<i>Opuntia ficus-indica</i>	Cactaceae	<i>Opuntia</i> Teen Shouki Barshoumi	Roots and Stems	Albaha and Taif

Isolation of Fungal Endophytes: Following Schulz et al., (1993), with modifications, samples of *Aloe vera* and *Opuntia ficus-indica* plants were selected and washed with running tap water to remove soil particles. The samples were cut into small 1 cm pieces and immersed in 70% ethanol for 2 minutes and 5% sodium hypochlorite solution for 5 minutes for surface sterilization. The samples were washed with distilled water several times and then transferred to a dry sterilized surface. The sterilized segments were placed in Petri dishes containing potato dextrose agar (PDA) medium (HiMedia, Mumbai, India); these were sealed with parafilm and incubated at $28 \pm 1^\circ\text{C}$ for two weeks. Fungi growing out of the plant segments were isolated using the method described in Domsch et al., (1980) and identified based on morphological characteristics with reference to fungi identification manuals (kirk et al., 2011).

DNA Extraction, Amplification, and Sequencing: A 2 μL aliquot of potato dextrose broth (PDB) (HiMedia, Mumbai, India) was poured into PDA tubes and vortexed to disperse the spores. The spore–PDB mixtures were then added into flasks containing 100 mL of PDB. The flasks were kept undisturbed at room temperature for two to three days. The mycelium was harvested by filtration, frozen at -80°C for 30 minutes, lyophilized, and stored at -80°C .

The mycelium was ground in liquid nitrogen with a sterile mortar to obtain mycelium powder. DNA was extracted from 20 mg of mycelium powder using a DNeasy Plant Mini Kit. The DNA quantity and quality were checked by electrophoresis on 0.8% agarose gel and visualized with ethidium bromide under UV transillumination.

The internal transcribed spacer (ITS) region of ribosomal DNA was amplified by PCR with the ITS1-F (5'-CTTGGTCATT TAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers (White et al., 1990; Gardes and Bruns 1993). PCR amplifications were carried out in a final volume of 50 μL , containing 2 μL of DNA, 0.5 mM of each primer, 150 mM of dNTP, 1 U of Taq DNA polymerase (Promega), and PCR reaction buffer. Amplification was carried out in a thermal cycler with an initial denaturation of 3 mins at 94°C , followed by 35 cycles of 1 min at 94°C , 1 min at 50°C , 1 min at 72°C , and a final extension of 10 min at 72°C . The amplified products were checked by electrophoresis on 1% agarose gel and visualized with ethidium bromide under UV transillumination based on the manufacturer's instructions. The PCR products were purified using an Exo SAPIT kit (USB Corporation, Amersham Place, UK, under license from GE Healthcare). The purified products

were sequenced in an automated DNA sequencer (ABI PRISM 3700) using the Big Dye Deoxy Terminator cycle-sequencing kit (Applied Biosystems, Darmstadt, Germany). Sequences were submitted to GenBank, NCBI (<http://www.ncbi.nlm.nih.gov>). Sequences obtained in this study were compared with the previously deposited sequences in the GenBank database, using BLAST, on the NCBI website (<http://www.ncbi.nlm.nih.gov/BLAST/>).

ITS Sequence and Phylogenetic Analysis: DNA sequences were initially aligned with Clustal Omega (Sievers et al. 2014). TREECON (Van de Peer and Wachter, 1994) for Windows (version 1.3b, 1998) was used to construct a neighbor-joining tree using the Jukes–Cantor model (Jukes and Cantor, 1969).

Antimicrobial Activity of Endophytic Fungi: Three pathogenic fungi, *Fusarium oxysporum*, *Aspergillus terreus*, and *Penicillium funiculosus*, and three human pathogenic bacteria, *Escherichia coli*, *Salmonella typhimurium*, and *Shigella* sp., obtained from King Fahad Researcher Centre in Jeddah, were used as target fungal and bacterial pathogens in this study. Following Balouiri et al. (2016), the cross-streak method was used to detect the antagonistic activity of fungi strains against endophytic fungal strains. The widths of the inhibition zones between the pathogen and the endophytes were grouped as follows: strong inhibition (+++), moderate inhibition (++) , weak inhibition (+), and no activity determined (–) (Paul et al., 2007).

Statistical Analysis: The colonization frequency (%CF) and the percentage of the dominant endophytic fungi were calculated (Gherbawy et al., 2014):

$$CF = \frac{\text{Number of segments colonized by endophyte}}{\text{Total number of segments analyzed}} \times 100$$

RESULTS AND DISCUSSION

Isolation of Fungal Endophytes: A total of 92 pure isolates of endophytic fungi were obtained from 132 cacti and cactus-like plant samples (leaves, stems, and roots) and were screened for the presence of endophytic fungi. Samples of 16 species and 8 genera were obtained from the leaves, stems, and roots segments of *A. vera* and *O. ficus-indica*. *O. ficus-indica* was found to have a higher endophytic diversity (relative frequency 54.83%) than *A. vera* (45.16%). The isolates were identified as follows: 4 species of *Aspergillus* from 14 isolates, 2 species of *Curvularia* from 2 isolates, 1 species of *Epicoccum* from 1 isolate, 3 species of *Fusarium* from 18 isolates, 3 species *Penicillium* from 15 isolates, 1 species of *Talaromyces* from 15 isolates, 1 species of *Rhizopus* from 8 isolates, and 1 species of *Mucor* from 19 isolates. The most commonly isolated species were *Mucor circinelloides*, with an overall colonization frequency of 20.43%, and *Talaromyces funiculosus*, with an overall colonization frequency of 16.12% (Table 2).

Table 2. Colonization Frequency of Endophytic Fungi Isolated from Leaves, Stems and Roots on PDA Medium at 28 ± 1°.

No	Fungal Endophyte	Isolate Number	CF ^a	Dominant Fungi ^a
1	<i>Aspergillus chevalieri</i>	2	2.15	1.51
2	<i>Aspergillus niger</i>	1	1.07	0.75
3	<i>Aspergillus terreus</i>	4	4.3	3.03
4	<i>Aspergillus versicolor</i>	7	7.52	5.30
5	<i>Curvularia khuzestanica</i>	1	1.07	0.75
6	<i>Curvularia</i> sp. MR-2019o strain LC12021	1	1.07	0.75
7	<i>Epicoccum sorghinum</i>	1	1.07	0.75
8	<i>Fusarium falciforme</i>	8	8.6	6.06
9	<i>Fusarium oxysporum</i>	4	4.3	3.03
10	<i>Fusarium redolens</i>	6	6.45	4.54
11	<i>Mucor circinelloides</i>	19	20.43	14.39
12	<i>Penicillium funiculosus</i>	3	3.22	2.27
13	<i>Penicillium janthinellum</i>	9	9.67	6.81
14	<i>Penicillium minioluteum</i>	3	3.22	2.27
15	<i>Rhizopus oryzae</i>	8	8.6	6.06
16	<i>Talaromyces funiculosus</i>	15	16.12	11.36
	Total	3	NA	NA

ITS Sequence and Phylogenetic Analysis: The molecular analysis of fungal rDNA sequences is a powerful technique for assessing fungal diversity at the genus level. The ITS

sequences of the isolated species provided by Macrogen in Korea were compared with the sequences previously deposited in GenBank using BLAST. The isolates used for

sequencing analysis along with their codes and GenBank accession numbers are listed in Table 3. The sequence results

were corroborated by the morphological identification of the isolated fungal endophytes. Most of the isolates were of the Ascomycota (87%) and Mucoromycota (13%) phyla.

Table 3. Identified endophytes related to the species and the identity percentage found in the CBS.

NO	Isolate Code	Accession Number	The Closet Genebank Taxa	Similarity %
1	Fung1_ITS1	MT510010.1	<i>Penicillium janthinellum</i>	99.39
2	Fung2_ITS1	MT579855.1	<i>Fusarium oxysporum</i>	100
3	Fung4_ITS1	MT279285.1	<i>Mucor circinelloides</i>	99.46
4	Fung5_ITS1	MK762588.1	<i>Epicoccum sorghinum</i>	100
5	Fung6_ITS1	MT563399.1	<i>Fusarium redolens</i>	100
6	Fung7_ITS1	MG437415.1	<i>Rhizopus oryzae</i>	95.27
7	Fung9_ITS1	MH688044.1	<i>Curvularia khuzestanica</i>	99.27
8	Fung10_ITS1	MT487830.1	<i>Aspergillus chevalieri</i>	99.79
9	Fung12_ITS1	KX262973.1	<i>Talaromyces funiculosus</i>	99.80
10	Fung13_ITS1	MT558939.1	<i>Aspergillus terreus</i>	100
11	Fung14_ITS1	MN215703.1	<i>Curvularia</i> sp. MR-2019o strain LC12021	100
12	Fung15_ITS1	JX500735.1	<i>Penicillium funiculosum</i>	99.80
13	Fung16_ITS1	MN555417.1	<i>Fusarium falciforme</i>	99.60
14	Fung17_ITS1	JN620402.1	<i>Penicillium minioluteum</i>	99.62
15	Fung19_ITS1	MT497452.1	<i>Aspergillus versicolor</i>	98.88
16	Fung20_ITS1	MT628904.1	<i>Aspergillus niger</i>	99.24

Table 4. Antimicrobial Spectra of Endophytic Fungi.

No	Isolate Code	Fungal Endophyte	Pathogenic Fungi			Pathogenic Bacteria		
			F. <i>oxysporum</i>	A. <i>terreus</i>	P. <i>funiculosum</i>	<i>Shiglla</i>	<i>E.coli</i>	<i>S. tyhpimurium</i>
1	Fung1_ITS1	<i>Penicillium janthinellum</i>	++	+	+++	++	++	++
2	Fung2_ITS1	<i>Fusarium oxysporum</i>	-	+	+++	++	++	++
3	Fung4_ITS1	<i>Mucor circinelloides</i>	+	-	++	-	-	-
4	Fung5_ITS1	<i>Epicoccum sorghinum</i>	-	-	-	-	-	-
5	Fung6_ITS1	<i>Fusarium redolens</i>	+	+	+++	+	++	++
6	Fung7_ITS1	<i>Rhizopus oryzae</i>	++	-	+	-	+	+
7	Fung9_ITS1	<i>Curvularia khuzestanica</i>	++	-	++	+	++	++++
8	Fung10_ITS1	<i>Aspergillus chevalieri</i>	++	-	++++	+++	+	+
9	Fung12_ITS1	<i>Talaromyces funiculosus</i>	++	+	++++	-	+++	-
10	Fung13_ITS1	<i>Aspergillus terreus</i>	++	-	-	-	-	-
11	Fung14_ITS1	<i>Curvularia</i> sp. MR-2019o strain LC12021	+	++	+	-	+	+
12	Fung15_ITS1	<i>Penicillium funiculosum</i>	+	++	-	-	-	++
13	Fung16_ITS1	<i>Fusarium falciforme</i>	+	-	+	+	++	+
14	Fung17_ITS1	<i>Penicillium minioluteum</i>	+	-	+++	-	-	-
15	Fung19_ITS1	<i>Aspergillus versicolor</i>	+	-	+++	+++	++	+++
16	Fung20_ITS1	<i>Aspergillus niger</i>	+	-	-	-	-	-

Antimicrobial Activity of Endophytic Fungi: Most endophytic fungi exhibit significant inhibition against

a wide range of pathogenic plant fungi and pathogenic human bacteria. The *P. janthinellum* (Fung1_ITS1), *F.*

redolens (Fung6 ITS1), *T. funiculosus* (Fung12 ITS1), and *Curvularia* sp. MR-2019o strain LC12021 (Fung14 ITS1) isolates showed strong inhibition toward pathogenic plant fungi. Ten isolates in this work exhibited promising growth-inhibitory activity against at least one of the pathogenic test microbes. Seven endophytic fungi exhibited antimicrobial activity against all three pathogenic bacteria, and four endophytic fungi exhibited antimicrobial activity against all three pathogenic fungi.

The number of fungal isolates displaying antimicrobial activity against *F. oxysporum*, *A. terreus*, and *P. funiculosus* were 1, 5, 9, and 11, respectively. The *P. janthinellum* (Fung1 ITS1), *F. oxysporum* (Fung2 ITS1), *F. redolens* (Fung6 ITS1), *C. khuzestanica* (Fung9 ITS1), *A. chevalieri* (Fung10 ITS1), *F. falciforme* (Fung16 ITS1), and *A. versicolor* (Fung19 ITS1) isolates displayed the highest level of inhibition against the pathogenic human bacteria *Shigella*, *E. coli*, and *S. typhimurium*. The *P. janthinellum* (Fung1 ITS1) and *F. redolens* (Fung6 ITS1) isolates displayed good activity against all pathogenic microbes. However, isolate numbers 1, 7, 8, and 15 displayed strong activity against all pathogenic microbes (Table 4).

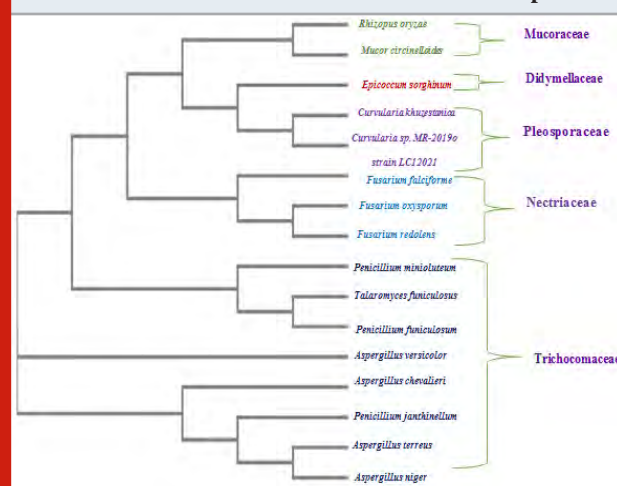
The present study aimed to isolate and identify endophytic fungi in *O. ficus-indica* and *A. vera* plants collected from Saudi Arabia, classified at the species level. A total of 93 isolates, representing 16 species and 8 genera, were recovered from plant leaves, stems, and root segments. Most of the fungal genera obtained as endophytes of *O. ficus-indica* and *A. vera* were described as endophytes and the eight genera were *Aspergillus*, *Curvularia*, *Fusarium*, *Epicoecium*, *Penicillium*, *Rhizopus*, *Mucor*, and *Talaromyces*. *M. circinelloides* was the species most frequently isolated, with a colonization frequency of 20.43%, followed by *T. funiculosus*, with a colonization frequency of 16.12%.

The species isolated with the lowest frequency were *Curvularia* sp., *A. niger*, and *E. sorghinum*. In a study conducted by Gangurde et al. 2019 in Sri Lanka, the highest colonization frequency of endophytic *Penicillium* sp. in *A. vera* found in India was 60%, followed by *Aspergillus* sp. at 50%, *Nigrospora* sp. at 33%, *Fusarium* sp. at 20%, and *Alternaria alternata* at 8%. The findings of Ratnaweera et al., 2015 support *A. niger* as the species showing the highest colonization in the cladodes of *O. dillenii*. Bezerra et al., 2013, isolated forty-seven species of endophytic fungi from *O. ficus-indica* from Brazil, and the most commonly isolated species was *F. oxysporum*. Among all of the endophytic fungi that have been isolated and identified from cacti and cactus-like plants, our study is the first report of these species isolated specifically from *O. ficus-indica* and *A. vera* in SA. An explanation for the overall low rate for frequency of colonization noted in this study could be the harsh environmental conditions and dryness in the areas in which the cacti grow.

The molecular analysis of fungal rDNA demonstrated that most of the fungal isolates described in this study belong to *Ascomycota* (87%) and *Mucoromycota* (13%). The ITS sequences of the isolated species were compared with the sequences previously deposited in GenBank. In this study, compared with the sequences on GenBank, more fungal isolates were found that belonged to the *Ascomycota* taxon, which confirms Vyawahare's findings of 93% of fungal isolates being represented by four endophytic fungal groups, namely Deuteromycetes, Ascomycetes, Zygomycetes, and Basidiomycetes, each with different isolation frequencies (Vyawahare et al., 2019).

Moreover, similar results were also obtained by Mane et al., (2018), who observed Deuteromycetes (55–72%) with high isolation frequencies and Ascomycetes (10–35%) with low isolation frequencies in *A. vera* and other medicinal plants. According to Silva-Hughes et al., 2015, in the USA, Tremellomycetes and Basidiomycota represent the first reported endophytes associated with Cactaceae. Basidiomycota are rarely isolated as endophytes and are associated with only eight species of cacti (Chlebicki, 2009). Fisher et al., 1994, studied 600 fragments of cacti from Australia and isolated 617 endophytic fungi across 23 taxa within *Ascomycota*. Suryanarayanan et al., 2005, used 1050 fragments of cacti from Arizona (USA) to isolate 900 endophytes belonging to 22 fungal species (*Ascomycota*), and Bezerra et al. (2012) used 45 fragments of forage cacti from Brazil to obtain 44 isolates of endophytic fungi belonging to 13 species (*Ascomycota*).

Figure 1: Phylogenetic tree based on neighbor-joining analysis of the rDNAITS sequences of the endophytic fungal isolates obtained from various tissues of two cacti plants.



Some of the fungi in this study (*F. oxysporum*, *A. terreus*, and *P. funiculosus*) are well-known plant pathogens. An endophyte in one plant may act as a pathogen in another plant depending on the balance between the pathogenicity and endophytes of the microorganism in different hosts. The fungi isolated in this study have previously been isolated

as endophytes from a different host, such as *O. ficus-indica* and *A. vera*. The endophytic fungi were tested for antifungal and antibacterial activity, and the results show that the endophytic fungi have stronger inhibition against plant pathogenic fungi and human pathogenic bacteria. This could be due to the natural existence of the endophytic and plant pathogenic fungi in the same habitat. Ramirez-moreno et al. (2017) tested the antimicrobial activity of the *O. ficus-indica* seed oil against *C. albicans*, *E. coli*, *S. aureus*, *L. monocytogenes*, *P. aeruginosa*, *S. cerevisiae*, and *S. typhimurium*. Their results show that the oil extract had high antimicrobial activity against Gram-positive and Gram-negative bacteria. In our study, the endophytic fungi isolated from *A. vera* constitute the first endophytic fungi apart from *A. niger*, *A. terreus*, and *F. oxysporum* to be identified and tested for antimicrobial activity. *O. ficus-indica* is a medicinal plant that has been used traditionally for controlling many different pathogenic bacterial infections (Jean et al., 2006). The discovery of novel antimicrobial metabolites in medicinal plants such as *O. ficus-indica* and *A. vera* provides an important alternative to conventional drugs and may help to overcome the increasing levels of drug resistance by human pathogens.

CONCLUSION

The assessment of molecular genetics using 18S rDNA gene sequencing is a fast and reliable technique for characterizing fungal taxonomy. The obtained data reveal that Saudi Arabian cacti and cactus-like plants possess an enormous diversity of endophytic fungi. A phylogenetic tree was constructed for each isolated endophytic fungus, and the results confirm that the obtained fingerprints indicate differences within the endophytic fungal community in cacti and cactus-like plants, alongside verifying that the reproducibility of 18S rDNA PCR amplification and its usefulness in such analyses.

Conflict of Interest: None to declare

Authors' contribution: All authors have made substantial, direct, and intellectual contribution to the work and approve it for publication.

Funding: None

Data availability: All datasets generated or analyzed during this study are included in this manuscript.

Ethical approval: This article does not contain any studies with human participants, or animals performed by any of the authors.

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2. Periodicity of its Publication : Six Monthly

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For Science & Nature

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Bhopal-462001, India

4. Publisher's Name : Ayesha S. Ali on Behalf of Society
For Science & Nature

(Whether Citizen of India) : Yes

Address : H. No. C-52, H.B. Colony, Kohefiza
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Bioscience Biotechnology Research Communications

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Bioscience Biotechnology Research Communications

P-ISSN: 0974-6455 E-ISSN: 2321-4007

CODEN (USA): BBRCBA

Publishers: Society for Science and Nature, Bhopal India

Journal Unique Identifier: Cross Ref DOI: <http://dx.doi.org/10.21786>

Periodicity: Jan-Feb-March, April-May-June, July-Aug-Sep and Oct-Nov-Dec

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Bioscience Biotechnology Research Communications

An Open Access International Journal www.bbrc.in

Post Box 01, GPO, Bhopal 462001 India

P-ISSN: 0974-6455 O-ISSN: 2321-4007

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Author 3 Name.....Orcid Id.....Signature:.....

Address.....Email.....

Use Extra Space if required.