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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.

Bioscience Biotechnology Research Communications is an open-access international platform for publication of original research articles, exciting meta-reviews, case histories, novel perspectives and opinions in applied areas of biomedical sciences. It aims to promote global scientific research and development, via interactive and productive communications in these areas.

The journal in a short span of time, has become a favorite among biologists and biomedical experts in the Asia-Pacific region and wider international scientific community, because of its standard and timely schedule of publication. It has been able to help scholars to present their cherished fruits of research grown on toiled and tilled trees of hard work in life sciences. Being the single publication of a non-profit Society for Science and Nature, Bhopal India, since 2008, Biosc Biotech Res Comm strongly believes in maintaining high standards of ethical and quality publication. The journal strictly adheres to the guidelines described in the Principles of Transparency and Best Practice in Scholarly Publishing.

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
Editor-in-Chief
Bioscience Biotechnology
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Biomedical
Communication

On the Severity of COVID-19 in Intensive Care and the Role of Invasive Ventilation: A Proportion Meta-Analysis

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ABSTRACT

Coronavirus Disease 2019 (COVID-19) is an emerging infectious pandemic,which has led to a worldwide public health emergency. The clinical spectrum of COVID-19 is varied, and has been explored in many studies. there is still a need to quantify the extent of the risk of developing the severe clinical manifestations of COVID-19 that require admission to intensive care unit (ICU) and mechanical ventilation initiation. The present study aims to assess ICU admission among COVID-19 confirmed cases and those who required invasive mechanical ventilation. MEDLINE, Web of Science, and SCOPUS electronic databases were searched for epidemiological studies on confirmed cases of COVID-19 atthe end of April 2020. Eligible articles that reported on admission to ICUs and mechanical ventilation were included. A random-effects model was used to pool results. A total of 23 articles reported on a total of 6124 confirmed COVID-19 cases. The majority of included articles were from China. The proportion of all hospitalized patients with confirmed COVID-19 who required ICU admission was between 0.01% to 53%, with the pooled proportion of 18% (95%CI 22,73%, I2 = 97.2%, p<0.001). The pooled proportion of ICU patients who had required invasive mechanical ventilation ranged from 4% to 94%, with the pooled estimate at 34%(95%CI 24 to 44%, I2 = 99%,p<0.001) Around a fifth of patients with confirmed COVID-19 diagnoses required admission to the ICU, and at least a third of those cases needed invasive mechanical ventilation. Still, there is a need for additional research with careful study design to identify the predictors and pathogenesis of severe cases.

KEY WORDS: COVID-19, INTENSIVE CARE, INVASIVE VENTILATION, META-ANALYSIS.

INTRODUCTION

Coronavirus Disease 2019 (COVID-19) is a novel coronavirus outbreak which first appeared in December 2019 in China, and has now become an international public health emergency, (The Lancet, 2020; Zhu et al., 2020). The causative pathogen for this pandemic is a positive-strand RNA virus named as the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). Although the original source of SARS-CoV-2 transmission has not yet been identified, by the end of April 2020, there were over 2 million confirmed cases of COVID worldwide (WHO, 2020). In most cases, this pathogen results in a syndrome that leadsto a respiratory condition that requires specialized management at intensive care units (ICU) and the usage of mechanical ventilation (Yang et al., 2020; Rodriguez-Morales et al., 2020; Sun et al., 2020).

The clinical spectrum of COVID-19 is varied, and has been explored in many studies (Yang et al., 2020; Rodriguez-Morales et al., 2020). However, it is important to accurately quantify the precise severity of COVID-19 in order to properly understand the clinical burden of

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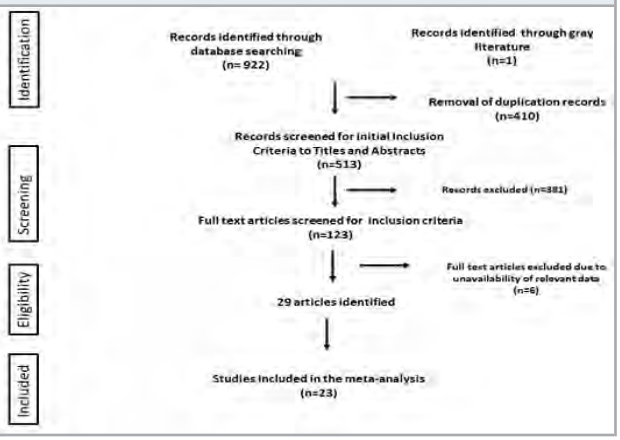
this emerging illness. Mortality is one risk that can be used to measure the extent of the COVID-19's severity. In addition, admission to the ICU and the need for mechanical ventilation could also be used to estimate the likelihood of developing severe COVID-19 complications. Current research has indicated that older adults, mainly those who had underlying health conditions, were at a higher risk for severe COVID-19 illness(Lian et al., 2020; Wei-jie et al., 2020). In the USA, a study reported that 53% of confirmed cases required ICU admission(Bialek et al., 2020). Recently published reports from meta-analyses and systematic reviews have described the symptoms and comorbidity predictors for severe COVID-19 associated illnesses and complications(J. Yang et al., 2020; Rodriguez-Morales et al., 2020).

However, there is still a need to quantify the extent of the risk of developing the severe clinical manifestations of COVID-19 that require admission to ICU and mechanical ventilation initiation, as this was not explored in those previous reviews. This will help in understanding the epidemiological determinants of those risks, which would allow us to correctly assess the clinical burden of COVID-19. Therefore, this study aims to estimate the proportion of ICU admission among confirmed COVID-19 cases.

Figure 1: Flowchart of the included studies



MATERIAL AND METHODS

The present study is a meta-analysis that has been conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement (Moher et al., 2009). The protocol of this study has been approved by King Abdullah International Research Center Riyadh KSA, (protocol numberRC20/164/R). The main objective of this study is to assess the proportion of ICU admission among confirmed COVID-19 cases and those who required invasive mechanical ventilation.

Search strategy and eligibility criteria: Published studies that referred to the clinical description and prognosis of COVID-19 cases were retrieved from MEDLINE (PubMed),Web of Science, and SCOPUS electronic databases until 31 April 2020. The following search terms were used in defining relevant articles:"SARS-CoV-2", "Wuhan pneumonia", "Wuhan coronavirus","2019 nCoV", "severe Acute Respiratory Syndrome Coronavirus 2", "coronavirus 2019", "novel coronavirus", and"COVID-19",in combination with "hospitalization","intensive care" and"ICU". Moreover, we searched for additional articles using the reference list and grey literature. Eligible study designs were case series, case-control studies, cohort studies, and case reports (only ones with a sample size of more than 5 were eligible). Review articles, editorial articles, and surveillance reports which did not present original data were excluded. Language restriction was applied. Thus, we only included articles that have been published in English.

Study selection and data extraction: The articles that resulted from the initial search strategy were first screened based on the information obtained from the title and abstract, and afterwards, were independently reviewed by two authors (FO and TI). The full texts of the potentially relevant articles were assessed for inclusion according to the following outcome: studies that focused on patients with confirmed cases of COVID-19, and documented primary data on the proportion of patients admitted to the ICU and patients undergoing mechanical ventilation. Secondary data on mortality were also collected from those studies. Studies reporting cases with incomplete information were excluded. When more than one article reported on information from the same hospital within the same period of time, the data was obtained from the article with the more recent publication date. Data extraction forms were filled for each study, including information on the type of publication, the period of collection data, country and area, month of publication, the number of confirmed cases, number of cases at ICU, number of cases who required invasive ventilation, proportion of patients who die, and demographic information. The primary data were the proportion of patients admitted to the ICU and patients who required invasive ventilation.

Assessment of risk of bias in included studies: The risk of bias was assessed using a scoring system for the evaluation. We used the quality assessment tool published by the National Institutes of Health (NIH) to determine the methodological quality of the included studies (National Institutes of Health, Study Quality Assessment Tools, no date). The assessment tool has 9 items, of which we used either 0 pointsor 1 point to score each item, and then calculated the sum of the scores for all items to generate an overall quality score that ranged between0 and 9. The criteria and each article were reviewed by two independent reviewers, the results were compared, and any conflicts were resolved by the third reviewer.

Statistical approach: The meta-analysis was performed using Stata 15 software system (StataCorp LP, College Station, TX). For study outcomes, we dealt with them as dichotomous variables. The pooled prevalence and their 95% confidence interval (CI) were used to summarize the weighted effect size for each study using

the binary random effect model. We adapted the metan command, which is specific to binomial data(Nyaga, Arbyn and Aerts, 2014). This allowed the computation of proportions using the exact binomial method, as well as allowing the within-study variability to be modelled using the binomial distribution. The heterogeneity among identified studies was statistically assessed using the I2 statistic. A forest plot was used to illustrate the distribution of the outcome and the effect size obtained from each published study. We performed subgroup analysis of the type of population included in the studies (hospitalized or ICU patients).

Table 1. Data extracted from each included study.

Study Author	Country	Study Frame	Population (n)	ICU cases (%)	patients used NIV (%)	Mortality (%)	quality score	mean age (SD)	gender (female (%))
Guan et al (Wei-jie et al., 2020)	China	11 Dec to 31 Jan	1590	99(6.2)	50(3.1)	50(3.1)	9	48.9(16)	674(42)
Huang et al (Huang et al., 2020)	China	16 Dec to 2 Jan	41	16(39%)	4(10%)	6(15%)	9	49(12)	11 (27)
Yang et al (X. Yang et al., 2020)	China	24 Dec to 26 Jan	52	52(100%)	33 (63.5%)	32 (62%)	7	59.7(13)	17 (33)
Du et al (Du et al., 2020)	China	25 Dec to 15 Feb	109	51 (46.8%)	33(30)	100	7	70.7(10)	35 (32)
Zhou et al (Zhou et al., 2020)	China	29 Dec to 31 Jan	191	50(26%)	_	54	8	56(15)	72 (38)
Chen N et al (N. Chen et al., 2020)	China	1 Jan to 20 Jan	99	23(23%)	4	11(11%)	8	55.5(13)	32(32)
Chen TL et al (T. L. Chen et al., 2020)	China	1 Jan to 1 Feb	203	107(52)	39(19.2)	26(12.8)	8	54.7 (20)	46(22)
Wang D et al (D. Wang et al., 2020)	China	1 Jan to 3 Feb	138	36(26)	17 (12.32)	6 (4.3)	8	56.5(20)	63 (45.
Lei et al(Lei et al., 2020)	China	1 Jan to 12 Feb	20	1 (5.0%)	2(10)	0	7	43.2(14)	10 (50.
Mo et al(Mo et al., 2020)	China	1 Jan to 5 Feb	155	55(35)	36(23)	22(14)	7	54(34)	69(44)
Cao et al(Cao et al., 2020)	China	3 Jan to 15 Feb	104	18(17.6)	14(13.7)	17(16.3%)	8	54(22)	49(48)
Xu et al(Xu et al., 2020)	China	10 Jan to 26 Jan	62	1(1.6)	--	0	9	41(14)	27(44)
Zhang et al (Zhang et al., 2020)	China	13 Jan to 26 Feb	28	6(21.4)	10(35.7)	8(28.6)	7	65(28)	10(39)
Lian et al (Lian et al., 2020)(A)	China	17 Jan to 12 Feb	652	9(1.38)	5(0.77)	0	8	41.11)	303(46)
Lian et al (Lian et al., 2020)(B)	China	17 Jan to 12 Feb	136	13(9.56)	6(4.41)	0	8	68(7)	8(5)
Chen J et al (J. Chen et al., 2020)	China	20 Jan to 6 Feb 6	249	22(8.8)	__	2(0.8%)	8	51(20)	123(49)
Wang R et al (R. Wang et al., 2020)	China	20 jan 9 Feb	125	19(15.2%)	4(21)	0	8	38 (13)	54(43)
Young et al (Young et al., 2020)	Singapore	23 Jan to 3 Feb	18	2(11%)	1(6%)	0	8	47(31)	9(50)
Wang Y et al (Y. Wang et al., 2020)	China	25 Jan to 25 Feb	344	344(100)	100 (29.1)	133(38.6)	7	64(7)	165(47)
Grasselli et al (Grasselli et al., 2020)	Italy	20 Feb to18 Mar	1591	1591(100%)	1150 (88)	405 (26)	8	63(10)	287 (18)
Arentz et al (Arentz et al., 2020)	USA	20 Feb to 5 Mar	21	21(100)	15 (71%)	11 (52.4)	6	70(14)	10(48)
Bhatraju, et al (Bhatraju et al., 2020)	USA	24 Feb to 9 Mar	24	24(100%)	18(75%)	12(50)	8	64(18)	9(38)
Barrasa et al (Barrasa et al., 2020)	Spain	4Mar to 31 Mar	48	48(100)	45(94%)	6(13)	7	63(12)	21 (43)
Simonnet, et al (Simonnet et al., 2020)	France	27 Mar to 5 Apr	124	124(100)	85 (68.6%)	18 (15%)	7	60 (14)	34(27)

RESULTS AND DISCUSSION

Description of included articles: The flow chart of the search process and study selection is shown in figure 1. The initial search in the electronic databases produced

922 results. After removing duplication, a total of 513 potential articles were screened for eligibility from title and abstract. This yielded 123 full-text articles that had been assessed for eligibility by applying the eligibility criteria. A total of 23 articles were selected as eligible articles, and were all subject to meta-analysis(Arentz et al., 2020; Barrasa et al., 2020; Lian et al., 2020; Mo et al., 2020; N. Chen et al., 2020; R. Wang et al., 2020; Simonnet et al., 2020; T. L. Chen et al., 2020; Wei-jie et al., 2020; X. Yang et al., 2020; Xu et al., 2020; Y. Wang et al., 2020; Bhatraju et al., 2020; Young et al., 2020; Zhang et al., 2020; Zhou et al., 2020; Cao et al., 2020; D. Wang et al., 2020; Du et al., 2020; Grasselli et al., 2020; Huang et al., 2020; J. Chen et al., 2020; Lei et al., 2020). The majority of excluded articles did not include the proportion of patients who had been admitted to the ICU. Two articles were published from the same institution during the same period; therefore, we only included the more recently published one.

Figure 2: Forest-plot of the prevalence of ICU admission of confirmed cases

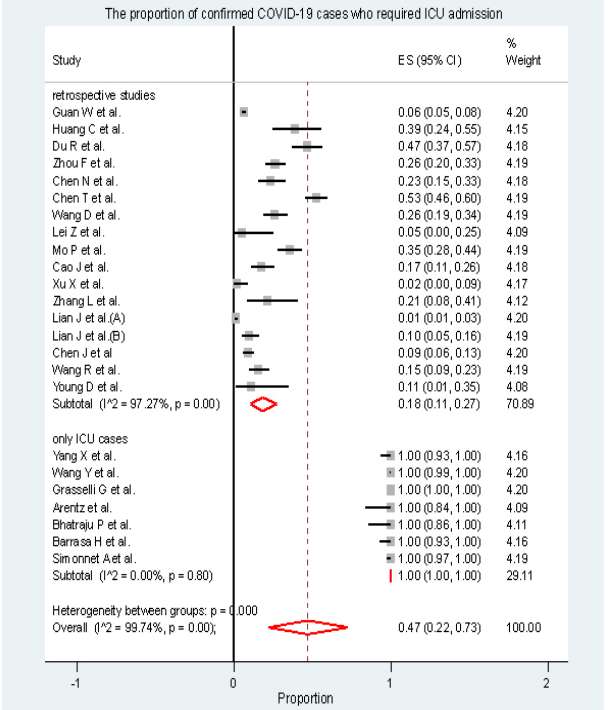
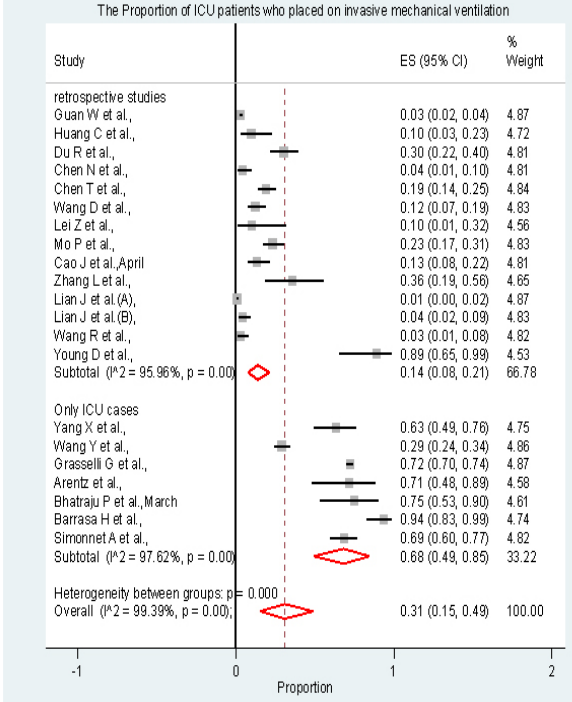


Table 1 demonstrates the details of all included studies. The 23 articles reported on a total of 6124confirmed COVID-19 cases. The majority of the studies were retrospective case series studies in design, and the majority were reported from China (Cao et al., 2020; Wang et al., 2020; Chen et al., 2020; Wei-jie et al., 2020; X. Yang et al., 2020; Xu et al., 2020; Y. Wang et al., 2020; Zhang et al., 2020; Zhou et al., 2020; Du et al., 2020; Huang et al., 2020; J. Chen et al., 2020; Lei et al., 2020; Lian et al., 2020; Mo et al., 2020; N. Chen et al., 2020; R. Wang et al., 2020).Seven studies only included ICU admitted patients(Arentz et al., 2020; Barrasa et al., 2020; Bhatraju et al., 2020; Grasselli et al., 2020; Simonnet et al., 2020; Y. Wang et al., 2020). The proportions of invasive mechanical ventilation among ICU admitted cases were available in 21 articles(Arentz et al., 2020; Barrasa et al., 2020; Mo et al., 2020; N. Chen et al., 2020; R. Wang et al., 2020; Simonnet et al., 2020; T. L. Chen et al., 2020; Wei-jie et al., 2020; X. Yang et al., 2020; Y. Wang et al., 2020; Young et al., 2020; Zhang et al., 2020; Bhatraju et al., 2020; Cao et al., 2020; D. Wang et al., 2020; Du et al., 2020; Grasselli et al., 2020; Huang et al., 2020; Lei et al., 2020; Lian et al., 2020).

Figure 3: Forest-plot of the prevalence of using mechanical ventilation among ICU admission cases

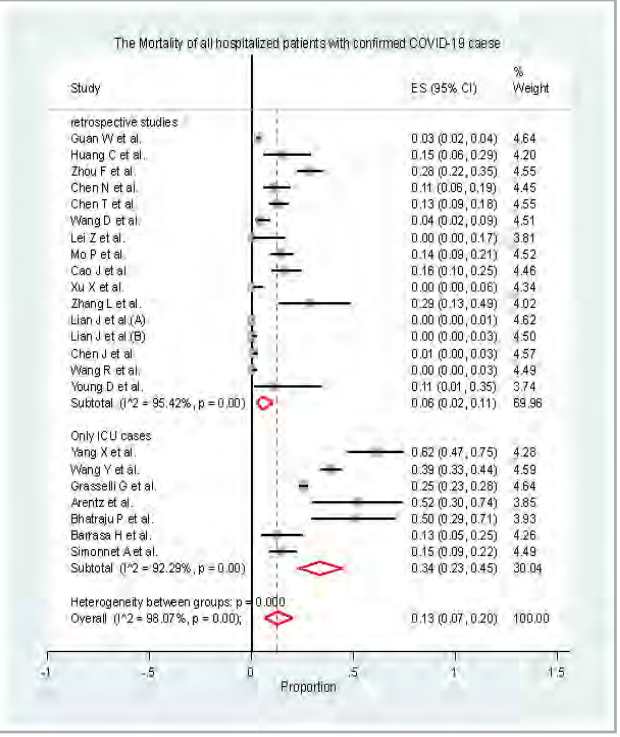


Quality of Included Studies: All of the included studies were case series analyses and were critically appraised using the quality assessment tool published by NIH. Each article was assigned an overall quality score based on each item in the assessment tool. Although the majority of articles have an overall quality score between 6 to 9, there was some inconsistency between the studies in defining the critical cases that required ICU admission. The studies from China used the WHO-China Joint Mission on COVID-19(Report of the WHO-China Joint Mission on Coronavirus Disease 2019 (COVID-19), 2020)) to define the more severe cases that required ICU admission while other studies used different criteria. In this meta-analysis, we identified ICU cases as those cases that required ICU admission or used invasive mechanical ventilation.

Pooled analysis of patients who were admitted to

ICU: Figure 2 demonstrates the pooled ICU admission prevalence. The proportion of all hospitalized patients with confirmed COVID-19 who required ICU admission was between 0.01% to 53%, with the pooled proportion of 18%(95%CI 22,73%, I2 = 97.2%, p<0.001). The pooled proportion of ICU patients who were placed on invasive mechanical ventilation ranged between4% and94%, with the pooled estimate at 34%(95%CI 24 to 44%, I2 = 99%,p<0.001) (figure3).

Figure 4: Forest-plot of the mortality among COVID-19 patients



The mortality of all hospitalized patients with confirmed COVID-19 cases is demonstrated in figure 4. Among the cases who required ICU admission, the mortality rate was 34% (95%CI 23-45). In comparison, the overall mortality rate for all hospitalized patients was 13%(95% CI 7-20%). Age and gender distributions in relation to the ICU admission of those studies which only include ICU populations are shown in figure5. The overall mean age of the patients with confirmed COVID-19 was 57 years (95%CI 52.9-62.8),and 37% (95% CI 31-43%) were female patients.

The COVID-19 pandemic has affected the healthcare sector in all nations around the world. Countries are still facing this epidemic disease and great efforts are still needed to understand its epidemiology, clinical presentation, pathological manifestation, and the appropriate techniques needed for the management of the infected cases. One critical aspect that also needs to be addressed is the severity of the COVID-19 infection associated with the evolution of this emerging epidemic. This can be examined by estimating the proportion of patients who needed ICU admission and initiation of mechanical ventilation, which is the core of this present meta-analysis.

Figure 5: Forest-plot of the propotion of female COVID-19 patients

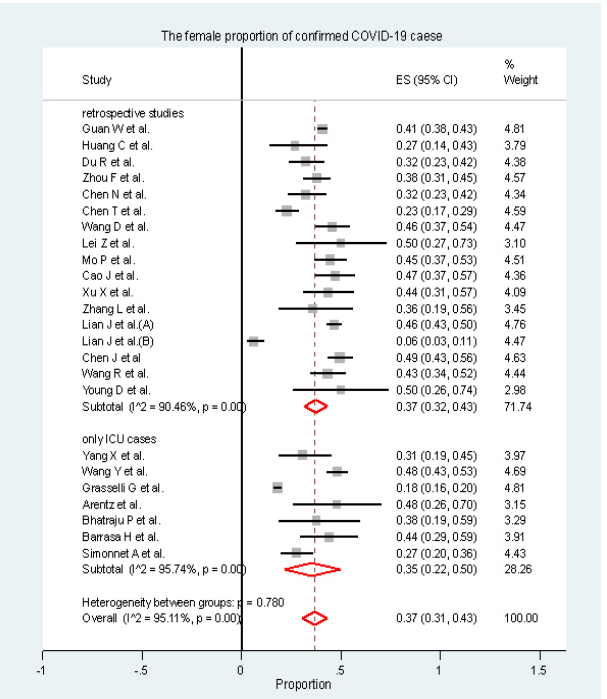
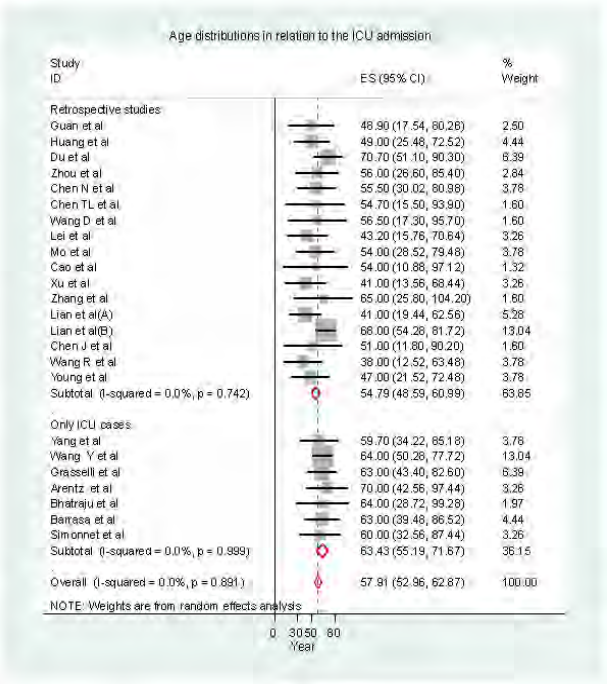


Figure 6: Forest-plot of the age distrubution of COVID-19 patients



In this study, we tried to initially summarize the clinical data on confirmed COVID-19 cases during the first four months of the outbreak. In this study, we found that

18% of hospitalized subjects with confirmed COVID-19 required ICU admission. The results also indicated that COVID-19 cases that required the use of invasive mechanical ventilation were 14% of all hospitalized subjects with confirmed COVID-19 and 67% of all ICU admissions. The overall mortality among COVID-19 confirmed cases was low (6% and 13% hospital and ICU mortality, respectively).

For patients admitted to the ICU, our findings were comparable to previous studies. These previous studies identified that between 18%and 20% of their study population required ICU admission (Rodriguez-Morales et al., 2020; Sun et al., 2020). This is explained by the similarity in the included studies in these meta-analyses. However, the proportion of patient who were admitted to the ICU and required invasive mechanical ventilation have not been examined in previous studies.

Thus, in this study, we examined the number of cases which required mechanical ventilation. The proportion of those patients appeared to vary between the studies. From acritical care perspective, an estimation of the number of patients who required mechanical ventilation is crucial. This can help healthcare authorities in predicting the number of expected severe cases that will require invasive ventilation in terms of allocating the resources. However, there is still a need for more studies that include a cohort follow up of those ICU admitted patients in order to determine the clinical outcome in terms of invasive ventilation requirement.

Strength and limitations: This meta-analysis has some limitations. First, the individual identification information of the patients who had been involved in the published studies was absent. Thus, we could have overestimated the total number of cases in our pooled analysis by referring to the same patients more than once. This risk is especially heightened for most of the earliest studies published from Wuhan hospitals, which collected information during the same period. Although we excluded one study that potentially had the same study population as another, we could not identify this potential bias in other studies.

Second, the majority of the included studies in this meta-analysis were from China, whereas many regions around the world are affected by COVID-19 and have not yet published their clinical data. Although we included data from Spain, Italy, and USA in this analysis, more studies from other countries are still needed in order to expand the growing volume of available data. In addition, the epidemiological understanding of the severity of COVID-19 would be improved through the inclusion of more detailed patient information regarding the ICU course and the mode of invasive mechanical ventilation. It is advisable to set joint case registration across geographical regions as well as allocated identification numbers for each case to allow the epidemiological researcher to better identify risk factors of ICU admission and associated outcomes. On the other hand, this meta-analysis provides useful information regarding the risk of ICU admission and the usage of invasive ventilation in severe COVID-19 case.

CONCLUSION

Around a fifth of patients who are infected with COVID-19 require admission to ICU, and at leasta third of those cases need invasive mechanical ventilation. Still, there is a need for additional research with careful study design to identify the predictors and pathogenesis of severe cases.

Author contributions: FO proposed the original idea for the study, planned the study design, provided research materials, performed the statistical analyses, and writing the first draft of the paper. TI helped in planning the study design, extract the data, revising the drafts of the paper. AM contributed to study design and concept, analysis planning, and interpretation of results, as well as to revising the drafts of the paper. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

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Dental
Communication

Surgical Exposure and Orthodontic Management of Unilateral Impacted Maxillary Central incisor: A Case Report

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ABSTRACT

Even though the impaction of maxillary central incisor during the mixed dentition stage is uncommon, it poses esthetic and phonetic problems that need early detection and management. This case report aims to present a common finding of an 11-year old boy with impacted permanent maxillary left central incisor, affecting the facial aesthetic and the patient's smile. This impaction was managed by an interceptive orthodontic approach that consisted of surgical removal of the obstruction, and regaining of space for the impacted tooth. This was followed by surgical exposure, orthodontic traction, and proper alignment of the impacted tooth 21. This case report illuminates the importance of early diagnosis of the impacted central incisor and the effectiveness of interceptive orthodontic management in insuring successful long-term aesthetic outcomes.

KEY WORDS: IMPACTED TOOTH, ORTHODONTIC TRACTION, ORTHODONTIC MANAGEMENT, SURGICAL EXPOSURE.

INTRODUCTION

Impaction is an impeded tooth eruption due to several local and systemic causes leading to a delayed or even failure of eruption of the tooth. Impaction of permanent maxillary incisors usually occurs because of displacement of the tooth bud or pathological obstruction, such as the presence of supernumerary teeth that comprise about 56-60% of permanent incisors impaction (Al-Zoubi et al., 2017; Syriac et al., 2017; Khandelwal et al., 2018). The pathological obstruction could also happen as a result of odontomas, cysts, as well as root dilaceration following a trauma to the primary incisor tooth (Chokron et al., 2010; Jayam et al., 2014; Narsapur and Choudhari, 2015). In addition, crowding, early loss, and/or over retained (ankylosed) deciduous tooth are considered among local causes of permanent central incisors impaction (American Academy of Pediatric Dentistry, 2020).

Cleidocranial dysplasia, amelogenesis imperfect, and endocrine deficiencies are the most common syndromes of systemic factors for tooth impaction (Choukroune, 2017; Puranik and Gandhi, 2019). Even though the impaction of maxillary central incisor is uncommon, with an impaction prevalence rate of less than 1%, compared to third molars or canines with 1-4% of incidence rate, it poses esthetic and phonetic problems that require early detection and management (Alhammadi et al., 2018; Yemitan, 2018; Alyami et al., 2020).

Interceptive orthodontic treatment is needed to correct any eruption disturbances and to eliminate any functional and/or skeletal interferences (Keerthana et al.,2020; American Academy of Pediatric Dentistry, 2020). Hence, the aim of this article is to present a detailed and informative report on a clinical case with unilateral impaction of permanent maxillary central incisor, in mixed dentition stage, caused by the presence of supernumerary tooth (mesiodens), and managed with a combined surgical-orthodontic approach.

Case Report: An 11-year-old boy was brought by his mother and was admitted into a pediatric clinic, with a chief complaint of delayed eruption of permanent maxillary central incisors at the time of that visit. The pedodontist removed the retained deciduous central incisors to allow for a spontaneous eruption of permanent successors. Following the eruption of the right maxillary

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central incisor, the pediatric dentist referred the patient to orthodontic clinic for evaluation of delayed eruption of the left maxillary central incisor (tooth # 21) at the Department of Orthodontics, College of Dentistry, King Saud University, Riyadh, Saudi Arabia. The patient was medically fit and had no history of dental trauma. He was presented with skeletal Class I malocclusion and balanced facial proportions. Intraoral examination revealed upper midline shift to the left side due to missing maxillary permanent left central incisor (tooth 21) that was bulging labially at the mucogingival junction. In addition, an Angle's Class I molar relationship was observed in the early mixed dentition phase (Figure 1).

Figure 1: pretreatment extraoral and intraoral photographs showing unerupted tooth 21 with midline shift.

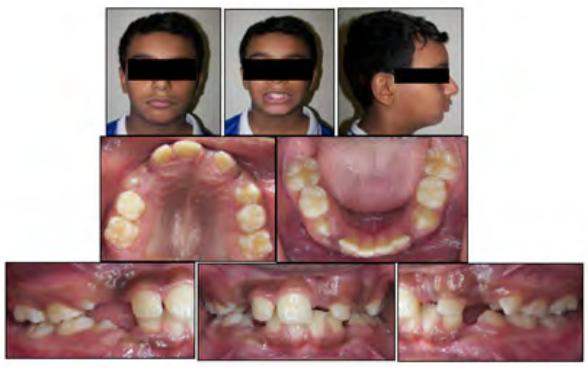
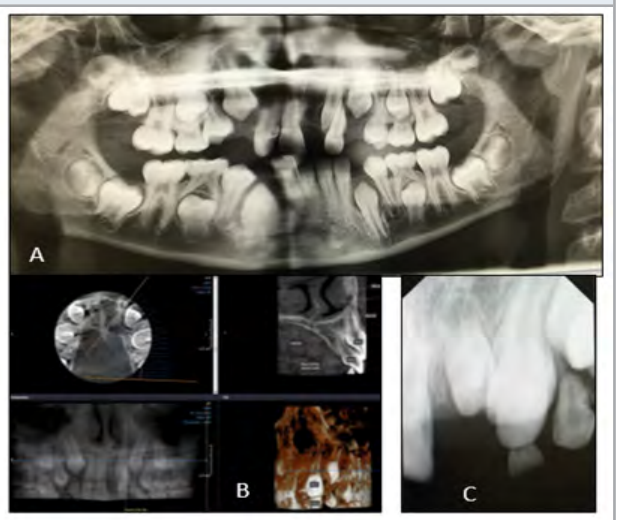


Figure 2: Pretreatment radiographs. A) Panoramic; B) CBCT images; C) intraoral periapical radiographs showing supernumerary tooth, and an impacted permanent left central incisor overlapping with the permanent left lateral incisor.



Several radiographs were taken for the patient as part of the routine pre-treatment records including, a panoramic (OPG), lateral cephalometric radiographs, and intraoral periapical (PA) radiographs of upper anterior region. The radiographs revealed the presence of supernumerary tooth (mesiodens) in the pre-maxillary region and an impaction of tooth 21 (Figure 2). Furthermore, a cone-beam computed tomography (CBCT) scan was performed to assess the position and the morphology of the impacted tooth 21. CBCT images showed that tooth 21 is located buccal to tooth 22 and is in direct contact with it. The maxillary left central incisor is positioned superiorly where the tip of the root seems to be close to the nasal cavity (Figure 2).

Therefore, an extra caution was required to avoid any injury to the adjacent lateral incisor or to the nasal cavity. The main objectives of the interceptive treatment were the following. First, to extract the retained deciduous lateral incisor, and remove the supernumerary tooth surgically. In addition, the treatment aimed at regaining space for tooth 21 followed by surgical exposure of the maxillary left central incisor. The final objective was to perform orthodontic traction and proper alignment in to the arch. The entire treatment procedure was explained to the patient and his mother. Written consent forms for both treatment and publication were signed before starting the interceptive orthodontic treatment. The author ensured the identity of the patient remains anonymous and the data was classified to ensure confidentiality and the privacy of information.

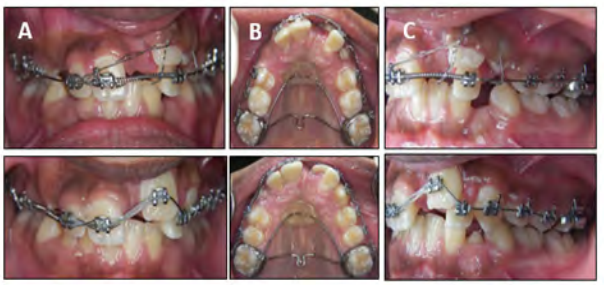
The patient was scheduled to be fitted with fixed orthodontic appliance following the surgical extraction of the mesiodens with a mucoperiosteal flab. Transpalatal arch with Nance button was fabricated and cemented on the maxillary permanent first molars, as an anchorage device, during the orthodontic traction of tooth 21 and maxillary canines. Furthermore, for teeth leveling and alignment, an orthodontic 0.022″ preadjusted brackets were bonded on the labial surfaces of all maxillary existing teeth, and a 0.016-inch nickel–titanium (NiTi) arch wire was fitted on the maxillary arch of the patient. In a period of four months, an adequate space for tooth 21 was regained by frequent activation of Ni Ti open coil spring between tooth 11 and tooth 22, and by anchoring a heavy stainless steel arch wire (0.017X0.025-inch). A closed eruption surgical technique was achieved with raised flab, little bone removal with preservation of the mucoperiosteum and gingival tissues, bonding an attachment to tooth 21, and lastly repositioning of the flab to its former position.

Two weeks following the surgical exposure of the impacted tooth, maxillary labial frenectomy was performed in order to correct the inferior position of the maxillary labial fraenum attachment, and before starting the orthodontic traction of the impacted tooth. Following another period of four months, the impacted tooth 21 was erupted into the oral cavity. Repositioning of the attachments on teeth 21 and 23 and a light flexible arch wire (0.016-inch copper NiTi) was used to fully engage these teeth (Figure 3). Five months later, the impacted maxillary left permanent central incisor was successfully positioned in and was properly aligned with the rest of the upper teeth and with good periodontal condition. At the same time, the lower arch was scheduled for bracket bonding and aligning of the lower teeth to be in a proper occlusion with the upper arch. Permanent

fixed retainers were placed in both arches at the end of the treatment. Successful interceptive orthodontic treatment was completed with ideal alignment of the impacted tooth 21, ideal over jet, ideal over bite, and proper interdigitation.

The clinical examination of the orthodontically treated incisor tooth 21 showed normal vitality test with acceptable gingival margins and attached gingiva (Figure 4). Furthermore, the final radiographs presented intact roots with proper root parallelism and good inclination of the treated teeth (Figure 5). The patient and his mother were satisfied with the results and a three months follow up appointment was scheduled to reevaluate the gingival contour of tooth 21 to assess if further esthetic gingival recontouring is needed.

Figure 3: Intraoral photographs of the orthodontic traction stages of tooth 21. A) Frontal view; B) Occlusal view; and C) Left side view.



RESULTS AND DISCUSSION

The treatment of an impacted permanent central incisor depends on the severity of the impaction, type of malocclusion, and the degree of root completion and dilacerations (Chaushu et al.,2015; American Academy of Pediatric Dentistry, 2020). The literature shows two alternative treatment options for treating such cases that need interdisciplinary approach. The first option includes extraction of the impacted central incisor followed by either closing of the space or prosthetic replacement. The second treatment option includes surgical exposure of the impaction with orthodontic traction followed by an alignment of the impacted central incisor (Singh et al., 2107; Khera et al., 2017; Noorollahian and Shirban, 2018).

Early correction of the impacted central incisor in the present clinical case was recommended by eliminating the cause of the problem, which was the prolonged retention of the deciduous central incisor, as well as, the presence of the supernumerary tooth (mesiodens) in the premaxillary region as confirmed by the two-dimensional (OPG, PA) and three-dimensional (CBCT) imaging. Therefore, early diagnosis is required for an accurate visualization and evaluation of the impacted tooth, and hence formulating an ideal treatment plan (Singh et al., 2107; American Academy of Pediatric Dentistry, 2020). In this case, the second treatment option, mentioned above was chosen. This option consisted of the following procedures. First, removal of the eruption obstacles (retained deciduous incisor and mesiodens). Second, regaining space for the impacted tooth. Lastly, surgical exposure and orthodontic traction to align the impacted central incisor since spontaneous eruption had not occurred, as recommended in the literatures (Khera et al., 2017; Singh et al., 2107; Noorollahian and Shirban, 2018; American Academy of Pediatric Dentistry, 2020).

Figure 4: Posttreatment extraoral and intraoral photographs showing the proper alignment of the impacted tooth 21, ideal over jet, ideal over bite, and proper interdigitation.

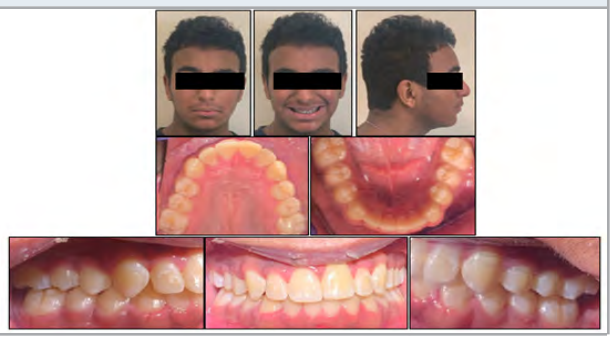
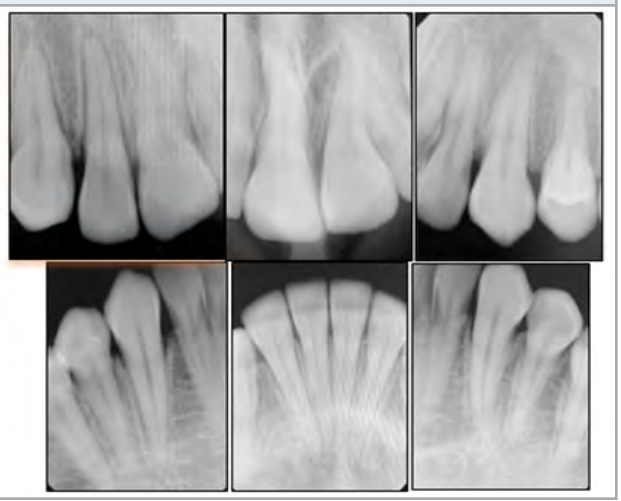


Figure 5: Post treatment periapical radiographs showing intact roots with proper root parallelism and good inclination of the treated teeth.



The extraction of the impacted tooth in the present case was not considered as a plan of treatment in order to reduce the risk of alveolar bone loss and to preserve the edentulous alveolar ridge of the impacted tooth 21. In addition, no root resorption was found on tooth 21, and on palpation of the labial sulcus, and there was bulging of the impacted tooth at the mucogingival junction (Sun et al., 2014; Spuntarelli et al., 2015).

Moreover, the surgical exposure of the impacted permanent left central incisor was carried out by using a closed eruption technique as suggested by Becker et al. (Becker et al., 2002). This technique is the most common surgical procedure, and it is ideal for high impaction

located above the mucogingival junction, which was taken into account for this case in order to obtain an adequate keratinized tissue and a better periodontal condition around the erupting tooth (Shi et al., 2015; Noorollahian and Shirban, 2018; Henner et al., 2108). The removal of the maxillary labial fraenum by frenectomy is another surgical procedure that was carried out in this case along with the orthodontic traction of tooth 21 to eliminate the inferiorly positioned fraenum that encroaches on the gingival margins, and hence facilitate orthodontic closure of the space between the maxillary central incisors (Naini and Gill, 2018; Kadkhodazadeh et al, 2018).

CONCLUSION

This case report presents a common finding of an 11-year old boy with impacted permanent maxillary left central incisor, affecting the facial aesthetic and the patient's smile. This impaction was managed by an interceptive orthodontic approach that consists of surgical removal of the obstruction, regaining of space for the impacted tooth. Followed by surgical exposure, orthodontic traction, and proper alignment of the impacted tooth 21. This case report highlights the importance of early diagnosis and the effectiveness of interceptive orthodontic management to ensure a successful long-term aesthetic outcomes.

Data Availability: Data are available from the corresponding author on request.

Case Report (Human Studies) Ethical Clearance Statement: Thank you for submitting your proposal to the King Saud University Institutional Review Board (IRB). Your proposal was evaluated in light of the KSU IRB policy and national regulations that govern the protection of human subjects in research, and concludes that your research project and its procedure does not pose 'more than minimal risk to the human participants'. The KSU IRB has determined that your proposed study is 'Exempt' from further IRB review. Please note that this approval is for the research ethics perspective only. You still need to at least notify or get approval from the department head or unit of KSU/KSUMC or external institution prior to collecting data.

Please note that even though your project is given exemption from IRB review, the research must be conducted according to the ethical guidelines and KSU IRB policies. In case of any significant change(s) to the approved protocol, you must submit a revised protocol along with a request for amendment for IRB approval/ favorable opinion. Any changes to the research protocol may prevent the research from qualifying for exempt review and require to be halted until approval of amendment by the IRB is granted.

Conflicts of Interest: The author declares that there is no conflict of interest regarding the publication of this paper.

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Environmental Communication

An Updated Review on the Bioremediation of Marine Plastic Pollution

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ABSTRACT

Almost 300 million tonnes of plastic waste are generated annually. Although this issue could be overcome by switching to biodegradable polymers, the existing detrimental effects of synthetic plastic wastes must be dealt with. Various modes of plastic degradation have been tried so far which include the Physical, Thermal, and Chemical means of degradation. Recently biodegradation of synthetic polymers has caught the eyes of researchers and a wide range of microorganisms have been found as potential degraders of these plastics. A concern to protect the environment and human safety led us to explore and research to fill this knowledge gap. Microorganisms have been found to have the capability to adapt themselves to the environment and alter their catabolic pathways in such a way that they either directly utilize these plastic wastes as a carbon source or produce by-products that target the polymer structures. This review paper deals with plastic pollution in the marine environment and how biodegradation could be a solution to it. It shows the various issues faced by marine wildlife and draws a focus on the microplastics that act as a pelagic habitat for the microorganisms. It also talks about the potential microbes from marine sources that can degrade plastics and potential enzymes produced by some of them. These findings pave the way to further enhance the development of environment-friendly degradation processes and products by protein engineering of these enzymes, strain engineering, understanding the genomics and proteomics of the enzymes, and generating an enzyme-based product for large-scale plastic waste management.

KEY WORDS: BIOREMEDIATION, ENZYMES, MARINE POLLUTION, MICROORGANISMS, PLASTIC DEGRADATION.

INTRODUCTION

Since the early 1950's synthetic plastics have gained huge importance for their astonishing physical and chemical properties. Now they have become a crucial part of our lives. Tonnes of plastics are produced every year and about 50% of them are designed for single use. Over the years their use has been exploited by mankind and now plastic wastes have become omnipresent. Almost 300 million tonnes of plastic waste are generated annually. According to marine researchers, the plastic debris could serve as a geological indicator of the Anthropocene epoch. Although this issue could be overcome by switching to biodegradable polymers, the existing detrimental effects of synthetic plastic wastes must be dealt with (Peng et al., 2020).

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A concern to protect the environment and human safety led us to explore and research to fill this knowledge gap. Various modes of plastic degradation have been tried so far which include the Physical, Thermal, and Chemical means of degradation. Recently biodegradation of synthetic polymers has caught the eyes of researchers and a wide range of microorganisms have been found as potential degraders of these plastics. Microorganisms have been found to have the capability to adapt themselves to the environment and alter their catabolic pathways in such a way that they either directly utilize these plastic wastes as a carbon source or produce by-products that target the polymer structures. This review paper focuses on plastic pollution in the marine environment and how biodegradation could be a solution to it (Miraj et al., 2019, Peng et al., 2020).

Plastics and the Marine Environment: In the past 70 years, a major concern regarding the marine environment is the marine pollution that ranges from the surface till the deepest of waters. Chemicals such as persistent organic

pollutants (POPs), polycyclic aromatic hydrocarbons (PAHs) and plastic polymers bio-accumulate and harm the marine life According to Reddy et al., (2006), on an average, for every 1 kg of intertidal sediments about 81mg of small plastics fragments were collected. Upon examining under Fourier Transform Infra Red Spectroscopy (FT-IR) and scanning electron microscope (SEM) they were found to be polyurethane, nylon, polystyrene, polyester and glass wool (Reddy et al., 2006, Miraj et al., 2019, Catania et al. 2020). The accumulation of these small fragments is not yet completely understood. This problem is pervasive throughout the world and is evident in the terrestrial environments, the oceans, on the shores and even in freshwater ecosystems (Barnes et al., 2009).

Figure 1: FT-IR spectra of small plastic fragments in the sediments of Alang- Sosiya ship-breaking yard. (a) Thermocol (polyurethane), (b) styrofoam (polyurethane), (c) nylon, (d) transparent plastic (polystyrene), (e) colored plastic (polyester), (f) glass wool (Reddy et al. 2006)

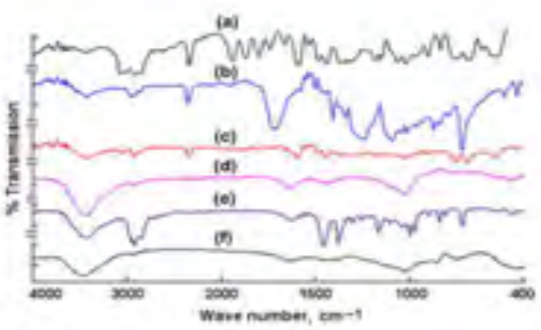
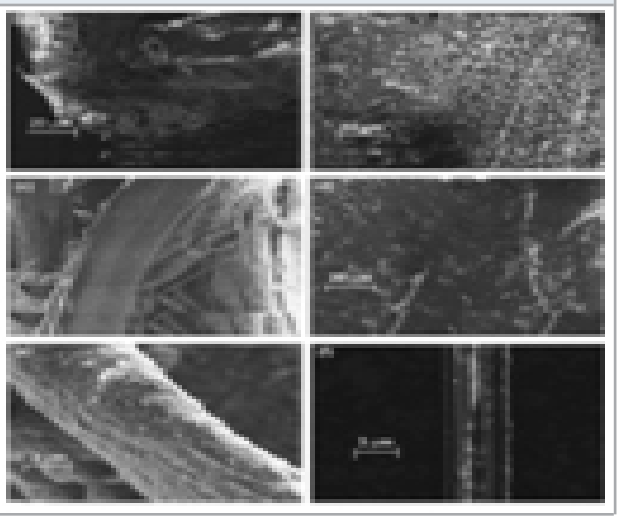
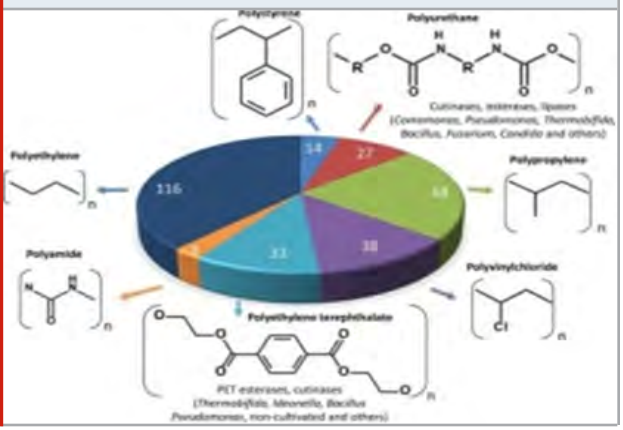


Figure 2: Scanning electronic microscopic (SEM) images of small plastic fragments. (a) Thermocol (polyurethane), (b) Styrofoam (polyurethane), (c) nylon, (d) poly- styrene, (e) polyester, (f) glass wool present in sediments of Alang-Sosiya ship-breaking yard. (Reddy et al. 2006)



Dangers to the Marine Wildlife: Ingestion, entanglement and chemical contamination effects of the plastic waste are the major problems faced by the marine wildlife due to the persistent forms of marine debris. The study was focussed on three major marine taxa, viz. seabirds, sea turtles and marine mammals. Used buoys, traps, pots, fishing nets, monofilaments, plastic bags, some plastic utensils, balloons, food packaging and other EPS packaging posed a huge risk of entanglement of the marine wildlife. The ingestible debris included the plastic utensils, plastic bags, butts, caps, balloons, monofilaments, food packaging and other EPS packaging. Chemical contamination, a secondary consequence of ingestion, was found to be mainly caused by hard plastic containers, plastic bags, butts, plastic utensils and other EPS packaging materials. Straws, stirrers, takeout containers, plastic lids, beverage bottles, cups, plates and cans were other sources of marine debris which posed minor threats to ingestion and chemical contamination. Around 8 million tons of plastic debris is dumped into the oceans each year. A majority of this is due to the intentional disposal of plastics into the sea/oceans. In this crucial phase, policy-based changes as well as consumer driven changes are essential in order to protect our marine wildlife. (Wilcox et al., 2016).

Figure 3: Main synthetic polymers globally produced in 2016. Numbers in the chart indicate the global annual production (millions of tons) of the specified synthetic polymer.Indicated are the names of bacterial genera producing verified enzymes with available protein sequences that are known to be involved in the breakdown of the high-molecular-weight polymers. (Danso.et al. 2019)



Issues with microplastics: The large sized plastics that once were a huge threat now seem negotiable in front of the microplastics. Unlike the mega- or macroplastics that remain floating in the waters, these microplastics can travel to considerable distances deep into the ocean (Barnes et al., 2009). The fragmentation of the large plastics is related to the chemical, thermal and photo and biological degradation. These involve the processes such as UV induced degradation, chemical leaching, ingestion by animals and birds (Barnes et al., 2009). According to Jayasiri et al., (2013), in comparison with the meso, macro and mega plastics, the microplastic litter were found in abundance along the coasts of recreational beaches in Mumbai. The Juhu beach showed the highest number of about 55.33 % of microplastics.

This poses a high risk to the marine beings as there is a huge possibility of ingestion. It is also reported that the beaches are more contaminated by smaller fragments of plastic than by virgin plastic pellets. Upon investigation it was revealed that land-based sources are responsible for the plastic pollution in these beaches (Jayasiri et al., 2013 Miraj et al., 2019).

Table 1. Microorganisms isolated from Marine sources that are capable of degrading different types of plastic wastes:

Microorganism	Source of the Microorganism	Type of Plastic	Reference
Bacillus cereus, *Bacillus sphericus*	Shallow Marine water from Indian Ocean	Low and High Density Polyethylene (LDPE and HDPE)	Sudhakaret al. 2008
Bacillus sp.	Coastal Marine Water	Polyvinylchloride (PVC), LDPE, and HDPE	Kumariet al. 2019
Pseudomonas, *Alcanivorax*, *Tenacibaculum*	Deep sea water	Aliphatic polyesters poly(ε-caprolactone) [PCL], poly(β-hydroxybutyrate /valerate) [PHB/V], and poly (butyrene succinate) [PBS]	Sekiguchiet al. 2011
Brevibacillus borstelensis	Marine water, soil spilled marine water	HDPE sediment and oil	Mohanrasu et al., 2018
Lysini bacillus, *Salini bacterium*	Marine water from Coastal sites in Northern Crete;	Linear Low Density Polyethylene (LLDPE)	Syranidou et al.,2017
Alcanivorax borkumensis	Agios Onoufrios Marine sediments and water-sediment interface	LDPE	Delacuvellerie et al.2019
Kocuria palustris, *Bacillus pumilus*,	Pelagic Waters, Arabian Sea	LDPE	Harshvardhan and Jha 2013
Bacillus subtilis *Pseudomonas spp*, *Streptococcus spp*, *Staphylococcus spp*, *Micrococcus* spp and *Moraxella spp*, *Bacillus subtilis*,	Choked Sewer Line	LDPE and Starch Blend	Prabhat et al., 2013
Bacillus amylolyticus, *Arthobacter defluvii* *Vibrio alginolyticus*,	Benthic zone sediments of	LDPE, Blends	Raghul et al., 2014
V.parahaemolyticus *Bacillus sp.*,	various marine environments Mangrove sediments	of PVA- LLDPE Microplastics of	Auta et al., 2018
Rhodococcus sp. *Muricauda sp.*,	Marine Water	polypropylene (PP) Polyethylene	Debroas et al. 2017
and *Thalassospira sp.* *Alphaproteobacteria*, *Gammaproteobacteria*, *Bacteroidetes*, *Planctomycetes*, *Erythrobacter*,	Seawater in the area of Cape Tonkiy	terephthalate (PET) Polystyrene (PS)	Tourova et al., 2020
Maribacter, and *Mycobacterium* *Alpha proteobacteria*, *Gamma proteobacteria*, *Bacteroidetes*, *Pseudomonas*,	Industrial Water	PS	Tourova et al. 2020

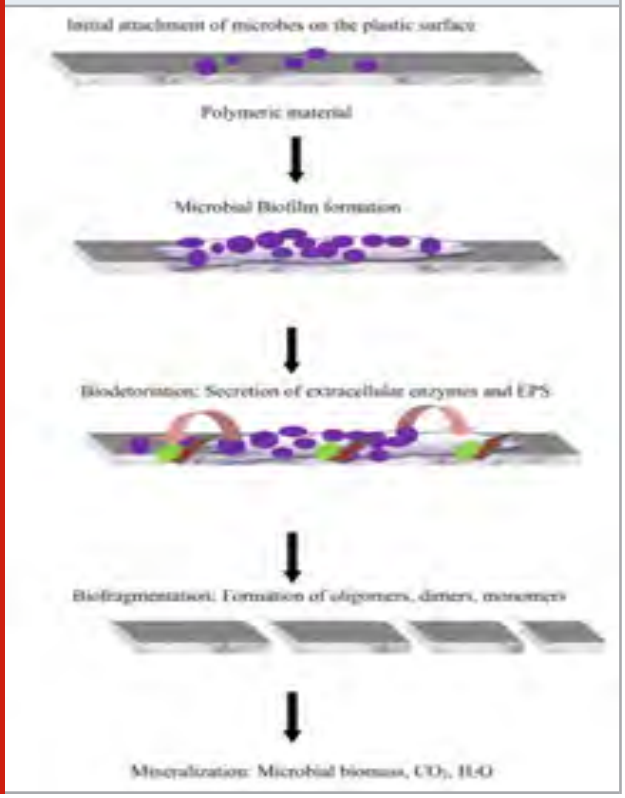
Arenimonas, Acidovorax, and *Mycobacterium*			
Thalassospira	Marine Waters	Polyvinyl-alcohol (PVA)	Nogi et al. 2014
povalilytica sp. nov.			
II. Fungi			
Aspergillus niger,	Choked Sewer Line	LDPE and Starch Blend	Prabhat et al. 2013
Aspergillus glaucus			
Aspergillu stubingensis,	Marine Coastal	HDPE	Devi et al. 2015
Aspergillus flavus	Dumpyard		
III. Algae			
Alariaesculenta,	Benthic Marine Water	Nylon, PP, Polyethylene (PE)	Welden and Cowie 2017
Palmariapalmata			
Diatoms: Amphora,	Marine Surface Waters	Microplastics of PS, PE, PP	Reisser et al. 2014
Achananthes,			
Cocconeis,			
Cymbella,			
Grammatophora,			
Haslea, Licmophora,			
Mastogloia, Nitzschia,			
Microtabella, Minidiscus,			
Thalassionema, Thalassiosira			
Coccolithophores:	Marine Surface Waters	Microplastics of PS, PE, PP	Reisser et al. 2014
Calcidiscus,			
Emiliania,			
Gephyrocapsa,			
Umbellosphaera,			
Umbilicosphaera,			
Coccolithus,			
Calciosolenia			
IV. Barnacles			
Lepas	Marine Surface Waters	PS, PP, PE	Reisser et al. 2014

Microplastics are plastics that are less than 5mm in size. These are most abundant in the surface sea waters and are known to be supporting the lives of many microbes and small invertebrates. Upon observing under the scanning electron microscope, around 14 genera of diatoms, 7 genera of Coccolithophores, Bryozoans, Barnacles, a Dinoflagellate, an Isopod, a marine worm, marine insect eggs, as well as bacteria, Cyanobacteria, and fungi were found to be present on the surface of these microplastics. The surface also had a textured appearance which indicated that these microbes enhanced their degradation. In big picture, since these microplastics are found in floating water, they are believed to be game changers in the ecological niche, organism scattering and ocean productivity. (Reisser et al., 2014). According to Peng et al.,. (2020), microplastics cause malnutrition, inflammation, chemical poisoning, growth thwarting, decrease of fecundity and death in marine life due to destruction in the internal organs/tissues. Also, research shows that nanoplastics have the potential to cross biological barriers which results in their bioaccumulation in the important organs of the marine animals, (Peng et al., 2020).

International Policies to mitigate plastic use and wastage: Globally, the governments have made policies for reducing the use of plastics by banning plastic bags, making them taxable for those who sell them, etc., While some countries, such as North America, Australia and the United Kingdom, have imposed partial bans few other countries in Europe have imposed fees per bag. Several other countries in Africa and Asia have imposed complete ban on the usage of plastic bags. Microbeads are another type of single use plastics. Various governments have also imposed policies against the use of these microbeads, but no strict ban had been imposed as of in 2017 (Xanthos and Walker, 2017). India banned the plastic bags sized less than 20µm, in 2002, in order to arrest the clogging of municipal drainage systems and put a stop of mortality of cows due to ingestion of plastics. But this was enforced only in 2005, with a ban of bags sized less than 50 µm. In 2016, Karnataka imposed a complete ban on the use of plastic bags. Also, India is committed to ban all single use plastics by 2022. But although a lot of policies have been imposed against the use of plastics, many countries fall short of execution approaches. The short- and long-term impacts of these measures must be researched and various campaigns for the public could bring awareness among the public (Xanthos and Walker, 2017 Miraj et al., 2019).

How far are degradable plastics really degradable?: These days the focus of research has shifted to research in bio-based polymers as they have similar properties and are environmentally friendly. This is believed to be a sustainable solution in managing the growing plastic use and wastage. (Catania et al., 2020). According to O'Brine et al., (2010), compostable plastic bags tend to degrade faster when compared to oxo-biodegradable plastic bags and conventional plastic bags. This was observed by comparing the decrease in tensile strength. The compostable plastic bags were completely degraded between 16 and 24 weeks whereas about 98% of the other plastic bags remained even after 40 weeks. This reveals that the so called degradable or biodegradable plastic bags usually last longer (approx. 18 months) than they are thought of and hence they must be reused and recycled rather than being used for a single application. Hence even though the degradable plastics seem convincing, there are certain limitations to its degradability which might affect their applications. (O' Brine et al., 2010).

Figure 4: Schematic illustration of plastic biodegradation by microorganisms. (Kumar Aet et al., 2020)



Degradation of the Plastic Wastes: The plastic wastes in the marine environment undergo weathering and degradation due to their exposure to the sunlight, oxidants and physical stress. Such abiotic degradation is usually followed by biological degradation mechanisms. Hence the pathways of degradation and their products must be analysed from an environmental chemist point of view in order to evaluate their properties and potential risks to the environment. Plastics such as polypropylene (PP), polyethylene (PE), polystyrene (PS) and polyvinyl chloride (PVC) have a carbon backbone whereas polyethylene terephthalate (PET) and polyurethane (PU) have carbon and hetero atoms in their backbone (Gewert et al., 2015). The plastics having a carbon backbone at first undergo the photo-initiated oxidative degradation. This breaks the polymers into smaller fragments that can easily pass through the microbial cell membrane and undergo biodegradation. Biodegradation causes the polymers to break into monomers and the monomers undergo mineralization. The degree and rate of degradation depends on the amounts of additives present in the plastic as additives tend to inhibit degradation. PET and PU on the other hand have an increased thermal stability and undergo hydrolytic cleavage at their ester or amide groups. This is followed by biodegradation (Gewert et al., 2015 Miraj et al., 2019).

Biodegradation: A solution to the issue: Biodegradation seems to be a promising solution as it is eco-friendly and affordable. The plastic wastes span the marine sources right from the surface till the ocean bed. The microbes present in each of these niches are capable of easily adapting to the plastic wastes and are likely to form biofilms on the surface of the plastic debris. Various factors play key roles in the biodegradation mechanisms, of which, the polymer characteristics and environmental conditions are the most important ones (Kumar et al., 2020).

The marine debris is broken down by microbes in one of the two ways

- The microbes utilize these chemicals as their carbon source with the help of certain key catabolic enzymes.
- The microbes produce by- products that attack the polymer structure

Immobilized enzymes offer a greater potential for treating wastewaters polluted with recalcitrant materials (Catania et al., 2020).

The degradation mechanism by the microbial enzymes involves the following steps:

- Formation of microbial Biofilm: Initial attachment and formation of plastisphere.
- Biodeterioration: Action of microbial exoenzymes on the mechanical, chemical and physical properties of the plastics.
- Biofragmentation: Enzymatic depolymerization into oligomers, dimers or monomers
- Assimilation: Plastic is converted into Carbon-dioxide, water, methane and biomass (Lucas et al., 2008; Kumar et al., 2020).

Products formed after biodegradation of plastics: Plastics upon biodegradation initially form smaller subunits which further get degraded into small inorganic molecules such as carbon dioxide and water (Andrady, 1998). According to Lucas et al., (2008) and

Restrepo-Flórez et al., (2014), "Once the molecular size of the synthetic polymers has been reduced to a range of 10–50 carbon atoms, the degradation products can be taken up into the cell for further metabolization" (Wei and Zimmermann, 2017).

Table 2. Potential Microbial enzymes those are capable of degrading different types of plastic wastes:

Microorganisms	Enzymes	Plastic	References
Aspergillus clavatus			Ishii et al., 2007
Alcaligenes faecalis	PHB depolymerise	PHB and PHB valerate (PHBV)	Kita et al., 1995 Mabrouk and Sabry, 2001
Streptomyces sp. SNG9			
Candida antarctica	Lipase B	Polyurethane (PUR)	Shibasaki et al. 2009
Ideonella sakaiensis *Paraglaciecola agarilytica,*	PETase	PET	Palm et al., 2019
Marinobacterium litorale *Penicillium* sp.,	Styrene monooxygenases Oxidase, Hydrolase	Styrene	Pu et al., 2018
Geotrichum fermentans	and Dehydrogenase	PVA	Kawai and Hu, 2009
Pestalotiopsismicrospora	Serine hydrolase	Polyester	Jonathan et al., 2011
Pseudomonas chlororaphis	Polyurethanases	PUR	Howard et al., 2007
Pseudomonas protegens	Lipase	PUR	Hung et al., 2016
Sphingomonas terrae	PEG-Dehydrogenase	Polyethylene glycol (PEG)	Sugimoto et al., 2001
Thermobifida fusca	Hydrolase	PET	Muller et al., 2005
B. cereus, B.sphericus	Peroxidase	HDPE and LDPE	Sudhakar et al., 2008
Nocardia	Esterase	PET	Sharon et al., 2012

Table citation: (Kumar A et al. 2020)

CONCLUSION

This review paper analyses marine plastic pollution, the various factors that cause it, and how it can be treated using biodegradation by microorganisms. It shows the problems faced by marine wildlife and draws a focus on the microplastics that act as a pelagic habitat for the microorganisms. It also talks about the potential microbes from marine sources that can degrade plastics and potential enzymes produced by some of them. These findings pave the way to enhance the development of environment-friendly degradation processes and products by protein engineering, strain engineering, understanding the genomics and proteomics, and generating an enzyme-based product for large-scale plastic waste management.

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Medical
Communication

Is *Helicobacter pylori* Association Suspicious of Gastric Cancer? A Descriptive Retrospective Study

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ABSTRACT

Stomach cancer is the second leading cause of cancer-related mortality worldwide. The present study aimed to answer whether *Helicobacter pylori* associated is suspicious of gastric cancer as a cause for gastric biopsy over-diagnosis. In this descriptive retrospective study, we retrieved data referring to gastric biopsies for one year back from the Department of Pathology at King Salman Hospital, Hai'l, Northern Saudi Arabia. The study included data referring to gastric biopsies patients diagnosed during the period from October 2019 to November 2020. The most identified histopathological finding was chronic inflammation 179/192(93.2%) included 84/88(95.5%) males and 95/104(91.3%) females. Acute inflammation was identified in 6/192(3%) patients, included 2/88(2.3%) males and 4/104(3.8%) females. Benign lesions were diagnosed in 3/192(1.6%) cases, 1/88(1.13%) male and 2/104(1.92%) females. Carcinoma of the stomach was diagnosed in 3/192(1.6%) patients, included 1/88(1.13%) male and 2/104(1.92%). Females. There is an overuse of gastric endoscopic diagnosis. *H. pylor*i infection is prevalent in Northern Saudi Arabia. It frequently makes confusion for gastric carcinoma. The incidence of gastric cancer and other benign stomach tumors is very low in Northern Saudi Arabia. Strict guidelines for gastric endoscopy is deemed necessary in Northern Saudi Arabia.

KEY WORDS: STOMACH CANCER, GASTRIC CANCER, H. PYLORI, SAUDI ARABIA, GASTRIC BIOPSY, ENDOSCOPY.

INTRODUCTION

Gastric carcinoma is the second leading cause of cancer-related mortality worldwide. However, the epidemiology has been changed in the past few years due to increased awareness toward gastric cancer risk factors (Sitarz et al., 2018). Notably, gastric cancer's geographical epidemiology had changed in recent years with progressive eradication of *Helicobacter pylori* (*H. pylori*) and improved hygiene (Fock, 2014; Marghalani et al., 2020). Many risk factors have been implicated in stomach cancer's etiology, such as *H. pylori*, tobacco smoking, alcohol consumption, obesity/overweight, and eating red meat, etc. (Poorolajal et al., 2020a; Poorolajal et a. 2020b). However, *H. pylori* infection

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represents the primary cause of gastric cancer (Holleczek et al., 2020), usually associated with chronic gastritis (Chen et al., 2020).

The available literature from Saudi Arabia reported an incidence of 2.7% of gastric cancer in 2013 (Abuderman, 2019); however, later report has shown that gastric cancer increased 3-fold (Althubiti and Nour Eldein, 2018). A study from Saudi Arabia has reported a prevalence of 58.1% for *H. pylori* infection, which is relatively higher than the global reports (Akeel et al., 2018).Several laboratory tests exist to diagnose *H. pylori* infection (Nakamura, 2001) other than the invasive endoscopic biopsy. However, overdiagnosis of gastric tumors may be encountered with *H. pylori* infection. Therefore, the present study aimed to answer whether *H. Pylori*-associated suspicious of gastric cancer a cause for gastric biopsy over-diagnosis.

MATERIAL AND METHODS

In this descriptive retrospective study, we retrieved data referring to gastric biopsies for one year back from the Department of Pathology at King Salman Hospital, Hai'l, Northern Saudi Arabia. The study included data referring to gastric biopsies patients who were diagnosed during the period from October 2019 to November 2020. The diagnosis of gastric tissue biopsies was confirmed by conventional histopathology. The re-evaluation of the histopathological diagnosis of the tissue samples was completed to verify the prior diagnosis and categorize the lesion's classification into benign and malignant types or other varieties.

Statistical analysis: Data were analyzed using computer software; Statistical Package for Social Sciences (SPSS version 16; SPSS Inc, Chicago, IL). Chi squire test was employed to statistical significance ($P < 0.05$ was considered significant).

Ethical consent: The protocol of this study was established agreeing with the 2013 Declaration of Helsinki. Additionally, the study's protocol was approved by the ethics committee of the College of Medicine, University of Hail, Saudi Arabia. Ethical Committee approval number: EC00069.

RESULTS AND DISCUSSION

This study investigated 192 patients denoted for gastric biopsy, 88(45.8%) were males, and 104(54.2%) were females. Their ages ranged between 14 to 84 years, with a mean age of 38 years. Most patients were aged < 25 years & 25-34 years, constituting 45/192(23.4%) each, followed by 35-44 years and 45-54 years representing 39/192(20.3%), and 32/192(17.7%), respectively. Most males were at age range < 25 years 21/88(23.9%); hence, most females were at the age group 25-34 years 25/104(24%), as indicated in Table 1, Fig 1.

The most identified histopathological finding was chronic inflammation 179/192(93.2%) included 84/88(95.5%) males and 95/104(91.3%) females. Acute inflammation was identified in 6/192(3%) patients, included 2/88(2.3%) males and 4/104(3.8%) females. Benign lesions were diagnosed in 3/192(1.6%) cases, 1/88(1.13%) male and 2/104(1.92%) females. Carcinoma of the stomach was diagnosed in 3/192(1.6%) patients, included 1/88(1.13%) male and 2/104(1.92%). Females, as indicated in Table 2, Fig 2, as shown in Image 1.

Table 1. Distribution of the study subjects by sex and age

Age	Males	Females	Total
<25 years	21	24	45
25-34	20	25	45
35-44	20	19	39
45-54	11	21	32
55-64	11	12	23
65+	5	3	8
Total	88	104	192

Figure 1: Description of the study subjects by age and sex

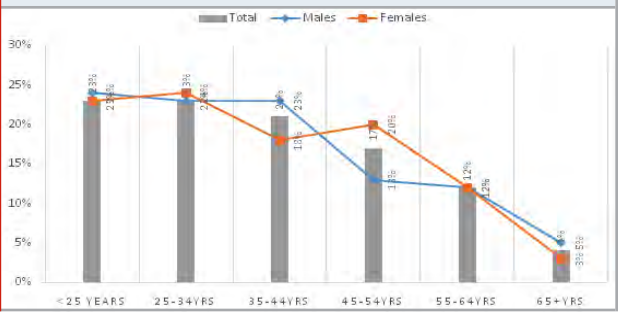


Table 2. Distribution of the study subjects by sex and histopathological diagnosis

Diagnosis	Males	Females	Total
Histopathology			
Acute inflammation	2	4	6
Chronic inflammation	84	95	179
Benign lesion	1	2	3
Gastric Carcinoma	1	2	1
Total	88	104	192
H. pylori			
Positive	55	67	122
Negative	33	37	70
Total	88	104	192

H. pylori infection was diagnosed in 122/192(63.5%), included 55/88(62.5%) males and 67/104(64.4%) females. The relative risk (RR) of association between *H. pylori* infection and females sex, and the 95% confidence interval (95%CI); RR (9%CI) =1.0308 (0.8307 to 1.2791), P = 0.7832, z statistics = 0.275, shown in Table 2, Fig 2.

Microphotographic 1. Gastric Carcinoma (H&E) x400

Table 3, Fig 3, summarized the distribution of the study subjects by sex and histopathological diagnosis. Chronic inflammation was more frequent among the younger population <44 years. Acute inflammation was common between 35 to 54 years. Benign lesions were observed between 35 years and 64 years. Gastric carcinoma was distributed in younger age, middle age, and older age. Positive *H. pylori* were more frequent in the younger age ranges. However, when calculating the percentages within each age group, variable proportions can be observed, as shown in Fig 3 and Image2.

Microphotographic 1. Gastric Carcinoma (H&E) x100

Image 1

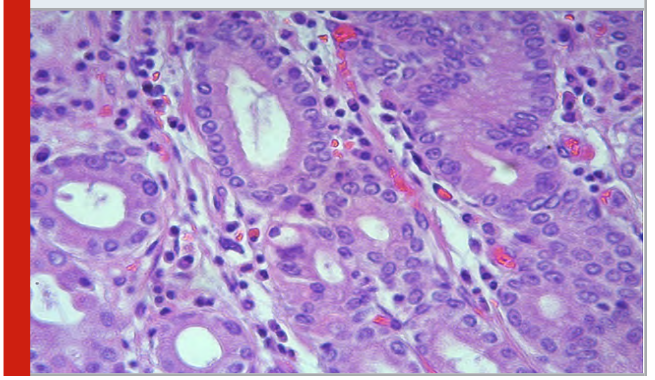


Figure 2: The study subjects by sex and histopathological diagnosis

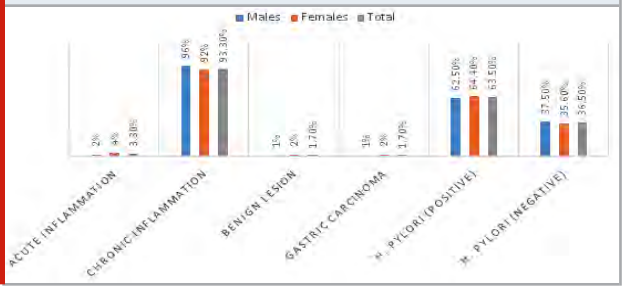


Table 3. Distribution of the study subjects by age and histopathological diagnosis

Diagnosis	<25years	25-34	35-44	45-54	55-64	65+	Total
Histopathology							
Acute inflammation	2	0	2	2	0	0	6
Chronic inflammation	43	44	36	28	21	7	181
Benign lesions	0	0	1	1	1	0	3
Gastric Carcinoma	0	1	0	1	0	1	2
Total	45	45	39	32	23	8	192
H. Pylori							
Positive	27	28	23	24	15	5	122
Negative	18	17	16	8	8	3	70
Total	45	45	39	32	23	8	192

As cancer is growing to be a concern for many people, and with a greasing level of awareness programs in addition to widespread of cancer early detection tests, the number of over-diagnosis is increasing with the application of invasive diagnostic methods. With the increased prevalence of *H. pylori* infection, several patients with the infection are subjected to gastric endoscopic biopsies. In the present study, the incidence of gastric cancer was 1.5%, and the incidence of benign conditions was 1.5%. The remaining 97% biopsies were performed for inflammatory diseases, some of which were associated with *H. pylori* infection. This incidence of gastric cancer was much lower than the previously reported incidence of 2.7% (Abuderman, 2019).

The incidence of *H. pylori* infection in the current study was 63.5%. This incidence may predict a high prevalence rate in the country. *H. pylori* infection is the primary cause of chronic active gastritis, peptic ulcer, and gastric cancer. *H. pylori* infect around 50% of the world population, making it the most widespread infection worldwide (Akeel et al., 2018). Studies from Saudi Arabia report high prevalence rates of *H. pylori* infection. A study from Saudi Arabia reported *H. pylori* infection 67-87% of the children diagnosed with peptic ulcer (El Mouzan and Abdullah, 2004).

Another study from Saudi Arabia investigated school children with chronic recurrent abdominal pain; 73% were detected as positive for *H. pylori* infection, including 62.9% and 82.1% positive infection in an intermediate and secondary school in that order (Telmesani, 2020). Another study from Saudi Arabia has found a relatively lower prevalence of *H. pylori* infection, 49.8% in the investigation included 303 children (Hasosah, 2019). In a random analysis of 3551 healthy children, seropositive for *H. pylori* infection was detected in 40% (Al-Hussaini et al., 2019).

pylori infection and other gastric problems were more apparent among females. With the lack of evidence showing the close association between *H. pylori* infection and female gender, the relationship between female sex and increased risk of gastric cancer has been documented

(Choi and Kim, 2016).The association between gastric conditions mentioned in the current study did not show any significant indications, and similarly, the connection with *H. pylori* infection. Although the present study has some limitations, including its retrospective setting, it provides valuable guidance for physicians and health managers for suitable guidelines for more accurate management of patients selected for gastric endoscopy.

Figure 3: The proportions of the study subjects by age and histopathological diagnosis within the entire age group

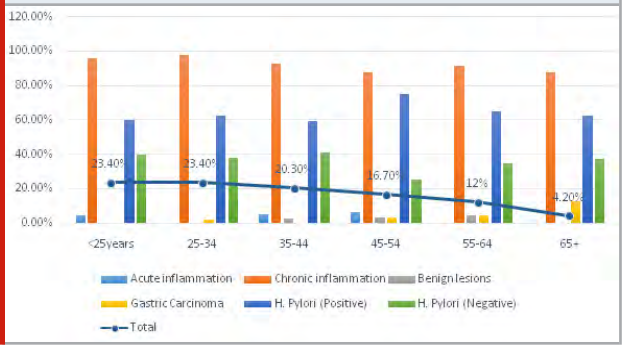
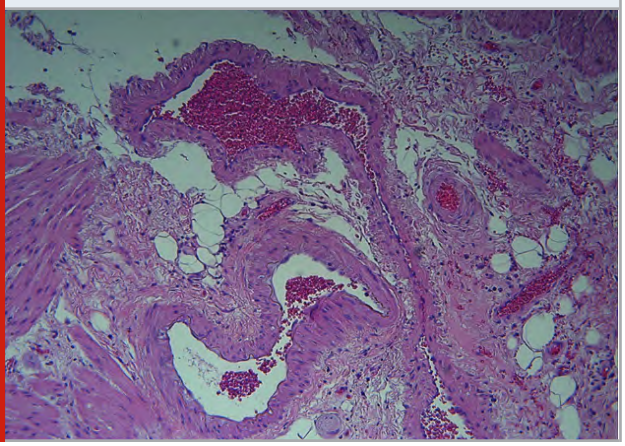


Image 2



CONCLUSION

There is an overuse of gastric endoscopic diagnosis. *H. pylori* infection is prevalent in Northern Saudi Arabia and frequently creates confusion for gastric carcinoma. The incidence of gastric cancer and other benign stomach tumors is very low in Northern Saudi Arabia. Implementing strict guidelines for gastric endoscopy is deemed necessary in Northern Saudi Arabia.

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Genetical
Communication

Analysing the Impact of Greenhouse Gases on Genetic and Human Health

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ABSTRACT

The state of the Earth's surrounding atmosphere, especially recently due to Covid-19, worries many people because it affects their health. It is proven that the leading greenhouse gases: carbon dioxide, water vapor, and ozone,influence genetic activity and affect humans. It is also known that because of this effect, water vapor in the Finnish sauna and Russian bath also has a positive impact on a person. In this paper, a comparative analysis of this phenomenon is carried out based on the obtained experimental data. In the first case, the total radiation power (absorption) was calculated in the dipole approximation, taking into account the dipole moment and the resonant frequency of the DNA molecule—the number of all DNA in the cell and all human cells. The emission power of greenhouse gases was determined, taking into account the radiation power in its model spectrum and the coefficient corresponding to the ratio of the integral intensities of the observed spectrum and the spectrum according to the model representations. In the second case, a calculated model of the greenhouse effect was used to estimate the impact on humans of the optical power of radiation in the I.R. region in the band of deformation vibrations of water vapor in the Finnish sauna and Russian bath. This power was calculated using the classical formula for dipole radiation in quantum mechanical analysis. Consequently, the power was determined by the energy of the considered oscillation and the probability of spontaneous transition to the main oscillatory level of water vapor, taking into account humidity and temperature in the Russian bath Finnish sauna. It turned out that the two powers at these given parameters coincide, and they are much larger than the corresponding optical powers for greenhouse (atmospheric) gases falling on a person.

KEY WORDS: GREENHOUSE, GREENHOUSE GASES, GENETIC ACTIVITY, ABSORPTION SPECTRUM, RADIATION POWER.

INTRODUCTION

Most people, especially recently due to Covid-19, are concerned about the state of the Earth's surrounding atmosphere, as it is related to their health. As you know, mitochondrial and nuclear deoxyribonucleic acid (DNA) have different linear dimensions, and therefore different resonance frequencies (Chirkova, 1992), to which the genome reacts. It is important to note that the frequency of 1660 cm^{-1} of the first overtone of nuclear DNA and the fundamental tone of 1061 cm^{-1} of mitochondrial DNA, calculated from model representations and lying in the infrared (I.R.) region, coincide within the error with the frequencies of the corresponding intensity maxima in the DNA absorption spectrum (Tymchenko & Polyanichko, 2017; Sheikhshoaie et al., 2018; Asgari2021). (see table 1, corresponding values are given in parentheses). The same coincidence is observed for the fundamental tone of the frequency 830 cm^{-1} of nuclear DNA. Theoretical and experimental research in this area has long been of great interest, for example, work (Oktyabrskiy and Ryazanceva, 2020).

In this paper, as in the first mentioned above, we are talking about the particle-wave dualism of the genome, which can be beneficially affected in vivo in the IR, visible and ultraviolet regions with the help of external

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optical radiation sources, including lasers. In addition, it is known (Asgari 2021) that, first, the main atmospheric (greenhouse) gases: water vapor, ozone and carbon dioxide; due to the greenhouse effect, affecting the human genetic activity in the IR region, have a beneficial effect on his health. By genetic activity we will understand such a state of the genome when the frequency of incident radiation coincides with the frequency of its absorption, and the power reaches the activation threshold, that is, in the case of resonance: "setting the receiver to the frequency". Secondly, because of this greenhouse effect, water vapor in the Russian bath and Finnish sauna also has a positive effect on humans.Therefore, the question arises of a comparative analysis of the experimental data obtained for these two effects due to the greenhouse effect. This study is devoted to the answer to this question (Asgharzadeh and Manda, 2021).

Since a sufficiently strong absorption of solar radiation by overtones and composite frequencies of water vapor is observed in the visible and near-IR regions, the "greenhouse glass" model, which corresponds to the classical interpretation of the greenhouse effect, does not pass in the case of the atmosphere. Still, using this traditional terminology, we will mean that in our IR range there is radiation from greenhouse gases due to the absorption of thermal radiation from the Earth. In the case of the Finnish sauna and the Russian bath, the role of the Earth is played by the furnace, and the impact on the person is due to the radiation of water vapor, which in turn absorbs the thermal radiation of the furnace, that is, under the influence of the same greenhouse effect (Sajjadi and Moosavi, 2019; Ilina et al., 2020). Overall, in this study, it is attempted to analyze of impact of greenhouse gases on genetic and human health considering the acquired experimental data, especially in the era of COVID-19. Firstly, the total radiation power (absorption) is calculated in the dipole approximation, taking into account the dipole moment and the resonant frequency of the DNA molecule. Secondly, a calculated model of the greenhouse effect is used to estimate the effect on humans of the optical power of radiation in the IR region in the band of deformation vibrations of water vapor in the Finnish sauna and Russian bath.

MATERIAL AND METHODS

To satisfy the aim of the study, a comparative analysis of is conducted based on the obtained experimental data. Initially, the total radiation power (absorption) was calculated in the dipole approximation, considering the dipole moment and the resonant frequency of the DNA molecule, as well as the number of all DNA in the cell and all human cells. Second, a calculated model of the greenhouse effect was applied to estimate the effect on humans of the optical power of radiation in the IR region in the band of deformation vibrations of water vapor in the Finnish sauna and Russian bath. This power was calculated using the classical formula for dipole radiation in quantum mechanical analysis.

RESULTS AND DISCUSSION

Water molecules, like ozone, belong to the point group of symmetry, C_2v, that is, the main operation is rotation by 180° (axis of rotation C_2). Having two components of the dipole moment (relative to two coordinates in the plane of the molecule), which also change during its vibrations, water and ozone molecules have a vibrational-rotational absorption spectrum (radiation). The main tones of vibrations of interest to us (Table 1) these molecules are located in the mid-IR region. Their activity in the radiation (absorption) spectrum is determined based on the theory of point symmetry groups. The oscillation frequency is active only when the amplitude of the matrix element of the dipole moment differs from zero. This means a situation in which the product of the wave functions of the initial and final states, as well as the corresponding component of the dipole moment in the basic symmetry operation of this group (C_2) does not change sign. This is exactly what is observed in our case.

As for carbon dioxide, it is a linear molecule belonging to the symmetry group D∞h. Therefore, it has a center of symmetry (inversion). Although the molecule does not have a dipole moment in the equilibrium position, however, it appears in valence asymmetric and doubly degenerate deformation vibrations. The last frequency (010), which is 667 cm^{-1}, is interesting because within the error range (Table 1) it coincides with the basic tone of nuclear DNA calculated from model representations (Chirkova, 1992). In addition, in one vibrational band there are hundreds of rotational bands, that is, we have a so-called vibrational-rotational band. Depending on the change in the rotational quantum number J (takes the values: -1, 0, +1) during the transition from the lower vibrational level to the upper one, in general, we have, respectively, branches P, Q and R with symmetry E_u, which corresponds to a perpendicular band: the dipole moment is directed perpendicular to the axis of the molecule.

In the first case, the optical power of radiation for the corresponding DNA was calculated per unit area (the effective area of a person is approximately 2 m^2), unit frequency interval (in wave numbers) and unit solid angle. The calculation was performed in the dipole approximation (Elyashevich, 2014), using the classical formula, which includes the dipole moment (second degree) equal to 1015 D (Oktyabrskiy and Ryazanceva, 2020), as well as the resonant frequency of the DNA molecule (fourth degree). Taking into account the number of all DNA in the cell and all human cells (or only the liver), the total radiation power was determined. Optical power emission of greenhouse gases was determined based on the radiation power spectrum and its coefficient corresponding to the ratio of the integral intensities of the observed spectrum and the model spectrum by ideas from (Borisov, 2011; Ilina et al., 2020). Human thermal radiation was also calculated (as for a black body) at the frequency of greenhouse gases and the corresponding DNA.

In the second case (see above) numerical value of the power per unit area (2 m2), a unit frequency interval (for half-width of bands in the spectrum of absorption around 250 cm^{-1}(Borisov, 201) and unit solid angle was calculated from the ratio of the dipole approximation (Elyashevich, 2014), obtained from well-known classical formula for the dipole radiation in the quantum-mechanical consideration. As a result, the power was determined by the product of the probability of spontaneous transition, taking into account its dependence on the frequency and dipole moment of water molecules equal to 1.84 D, the population at the main level of water vapor (010) at a given humidity, respectively, in a Russian bath and a Finnish sauna, the pressure of saturated water vapor in this volume, equal to approximately 1 m^3, temperature and frequency energy (010) (Ilina et al., 2020). The thermal radiation of the furnace was also calculated as the radiation for a completely black body (see above).

As a result, (Table 1), it was found that within the error (Δp) calculated by the formula of indirect measurements (Asgari 2021), the optical power of radiation (P) of water vapor for the Finnish sauna and the Russian bath is the same, although the temperature in both cases differed by 30 K, and the humidity – by 8 times. Large errors, in turn, are associated with a large half-width of the band of non-degenerate deformation vibrations (010) of water vapor (Borisov, 2011). These optical powers were approximately an order of magnitude greater than the corresponding thermal powers calculated for the Finnish sauna and Russian bath furnaces, and three orders of magnitude greater than in the case of the greenhouse effect for water vapor in the Earth's atmosphere (Jlina et al., 2020) and more than three orders of magnitude greater than human thermal radiation at the frequency of greenhouse gases and the corresponding DNA.

Table 1. The frequency of greenhouse gases (GG), DNA radiation and their respective radiation powers, including the Russian bath and the Finnish sauna.

	GG, DNA	F, cm^{-1}	ΔF, cm^{-1}	P, w/ (m^2 cm^{-1}sr)	ΔP, w/(m^2cm^{-1}sr)
I	GO Mitochondrial DNA from liver cell	 1042 1061 (1070)	 25 53 (27)	 0.02 0.16-0.33	 0.01 0.03-0.06
II	GDC Nuclear DNA of human cell	667 830 (837)	144 42 (27)	0.30 0.16	0.10 0.03
III	GWV Nuclear DNA of human cell, 1-st overtone	 1595 (1678)	 250 (87)	 0.02 0.03	 0.01 0.01
IV	Russian bath (333 K, humidity 40%)	1595	250	37	17
V	Finnish sauna (363 K, humidity 5%)	1595	250	30	14

CONCLUSION

Thus, as a result of the analysis of experimental data obtained in two cases in accordance with the two proposed models, the following conclusions can be drawn:As in the case of greenhouse (atmospheric) gases, water vapor in the Finnish sauna and Russian bath due to the greenhouse effect influence the genetic activity of a person and has a beneficial effect on his health.Due to the significant predominance of the optical power of water vapor falling on a person in both baths over the power emitted by the greenhouse gases of the Earth's atmosphere, the impact on a person in the first case is much greater.Direct human exposure in both cases can be carried out through the membranes of certain skin cells.

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Conflict of Interests: the authors declare that there is no conflict of interest in this study.

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Pharmacological Communication

Pharmacoeconomic Evaluation of Anti-Hypertensive Therapy

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ABSTRACT

This study aimed to analyse the price variation between various prescribed brands of anti-hypertensives and the direct medical costs involved in the treatment of hypertension. A retrospective study was conducted at a super specialty hospital over a period of eight months from September 2019–April 2020. Ethical approval was obtained from the institutional ethics committee before initiating the study. A total of 400 hypertensive patients of either gender, aged above 18 years, prescribed with at least one antihypertensive agent were enrolled in the study. All relevant information was collected from the concerned patient treatment charts and patient hospital bills. Patient details such as age, gender, occupation, body mass index, domiciliary status, social habits (smoking and alcohol), family history of hypertension, co-morbid conditions and duration of hypertension were collected. All the data obtained were then analysed using SPSS (version 20.0). The majority of the patients were males, n=234 (58.5%) and n=166(45.5%) were females. A total of n=285 (71.25) were prescribed with more than one antihypertensive agent and only n=115(28.75%) patients were on monotherapy. In monotherapy, prazosin 25 mg was found to have the maximum price variation of 7.76, followed by spironolactone 50 mg with a price variation of 7.73. The least variation was observed with telmisartan 80 mg. In case of multiple drug therapy, maximum variation was seen with metoprolol 50 mg. Lab charges, being the highest median medical cost has resulted in the maximum burden for the patients. The average lab charges were found to be 4997.33 INR (64.46USD). The least median direct medical cost was accounted to antihypertensives. The cost of antihypertensives was found to be 134.48 INR (1.96 USD).

KEY WORDS: ANTI-HYPERTENSIVES, CLINICAL PHARMACIST, METOPROLOL, PRAZOSIN, PRICE VARIATION.

INTRODUCTION

Hypertension is associated with an increased morbidity, mortality and economic impact on both the individuals and the society. It is surprising to know that 1 billion individuals around the globe have already been diagnosed with hypertension and 7.1 million deaths per year account to this cardiovascular condition (Bakare et al., 2016; Mishra et al., 2017). High healthcare costs associated with the management of uncontrolled hypertension has imposed a heavy economic burden on the society. The cost of medication and diagnosis has increased proportionately with the increase in comorbidities associated with hypertension (Dipiro et al., 2017; Kostova et al., 2020).

Pharmacoeconomic studies provide us with insights on the economic burden of this disease, associated comorbidities, arising the need for chronic medications (Paul et al., 2020; Sunny et al., 2020). Various existing studies has highlighted the utilization and prescription pattern to help us understanding the clinical trends of this disease. Enabling us to design the most effective, safe, and economical therapy which clinical pharmacists are competent enough to carry out such studies (Al-Jabri et al., 2019; Mohammed 2020; Voora et al., 2020). Keeping this existing need in mind, this study was carried out with the objective of analyzing the price variation between different brands of antihypertensives prescribed and the direct medical costs involved in the treatment of hypertension.

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Table 1. Distribution of antihypertensive agents prescribed

S. No	Class of drug	Antihypertensive drugs	Number of drug prescribed (n)
#Monotherapy			
1	Calcium channel blockers	1.Amlodipine	19
		2.Diltiazem	8
		3.Verapamil	1
		4.Nifedipine	7
		5.Clinidipine	10
2	ACE Inhibitors	1. Enalapril	8
		2. Ramipril	6
3	Angiotensin receptor blocker	1. Losartan	7
		2. Telmisartan	6
4	α-adrenergic blockers	1. Prazosin	3
5	Central sympatholytic	1. Clonidine	2
6	β-adrenergic blockers	1. Propranolol	8
		2. Metoprolol	7
		3. Atenolol	3
		4. Nebivolol	3
7	Diuretics	1. Hydrochlorothiazide	3
		2. Furosemide	3
		3.Torsemide	11
Total			115
#Multidrug therapy			
1	Calcium channel blockers	1. Amlodipine	166
		2. Diltiazem	36
		3. Verapamil	6
		4. Nifedipine	34
		5. Clinidipine	73
2	ACE Inhibitors	1. Enalapril	46
		2. Ramipril	6
3	Angiotensin receptor blocker	1. Losartan	26
		2. Telmisartan	35
4	α-adrenergic blockers	1. Prazosin	21
5	Central sympatholytic	1. Clonidine	10
6	α+β adrenergic blockers	1. Labetalol	5
		2. Carvedilol	1
7	β-adrenergic blockers	1. Propranolol	50
		2. Metoprolol	25
		3. Atenolol	18
		4. Nebivolol	4
8	Diuretics	1. Hydrochlorothiazide	27
		2. Furosemide (IV)	36
		3. Spironolactone	1
		4. Amiloride	3
		5. Torsemide	63
		6. Mannitol (IV)	24
Total			716

MATERIAL AND METHODS

A retrospective study of eight months was conducted in a super specialty teaching hospital located in Dakshina Kannada, South India. The ethical approval was obtained from the institutional ethics committee before initiating the study. This study included inpatients of both genders, aging more than 18 years who were diagnosed with hypertension, prescribed with at least one antihypertensive agent and admitted to the hospital

for at least two days. The patient data collection form was designed as per the need of the study. All relevant information was collected from the concerned patient treatment charts and patient hospital bills. Patient details such as age, gender, occupation, body mass index, domiciliary status, social habits (smoking and alcohol), family history of hypertension, co-morbid conditions and duration of hypertension were collected. Characteristics of drug therapy like generic and brand name of the drugs, dosage form, frequency, route of administration and number of drugs per prescription were recorded in the data collection form. All costs in Indian rupees were converted into U.S. dollars and all the values were represented in median with inter-quartile range (IQR) (Sunny et al. 2020). Descriptive statistics was applied for analysing the collected data using Statistical Package for Social Science (SPSS) 20.0 for windows (Kim et al., 2021).

RESULTS AND DISCUSSION

Demographic characteristics of the study population: Out of 400 patients enrolled in the study, the majority were n=234(58.5%) males and the remaining n=166(41.5%) were females. A majority of the patients were found in the age group of 60-79 years, n=222(55.5%), followed by the age group of 40-59 years, n=153(38.25%) and a smaller number of patients belonged to the age group of 20-39 years, n=25(6.25%). The mean age of the study population was 58.93±11.14 years.

Table 2. Analysis of direct medical cost among the hypertensive patients

Cost category	Hypertension with and without comorbidity(INR)		Hypertension with and without comorbidity(USD)	
	Median	IQR(Q3-Q1)	Median	IQR(Q3-Q1)
Medication cost	136.48	307.06-72.65	1.96	4.40-1.04
Comorbidity medication cost	1781.50	3000.50-953.00	25.53	43-13.66
Laboratory cost	4497.33	7122.5-2540.0	64.46	102.08-36.41
Consultation charge	657.68	1270.57-356.88	9.43	18.21-5.12
Nursing charge	331.12	580.12-185.34	4.75	8.31-2.66
Treatment charge	300.00	500-200	4.3	7.17-2.66
Surgical charge	738.465	1022.25-538.50	10.58	7.17-2.66
Hospital charge	2000.00	3430-1100	28.66	14.65-7.72
Miscellaneous charges	397.77	616.89-212.99	5.70	8.84-3.05
Total	10840.345	16560.18-6574.53	155.37	237.35-94.23

Distribution based on number of antihypertensive drugs per prescription: A total of 831 antihypertensives were prescribed to 400 patients, with an average of 2.07±1.01 antihypertensives per prescription. Out of them, monotherapy was noted among n=115(28.75%) patients, and in multidrug therapy, the majority were prescribed with 2 drugs, n=141(35.25%), followed by 3 drugs n=103(25.75%). The remaining, n=41(10.25) were prescribed with more than 3 anti-hypertensive drugs.

Distribution of anti-hypertensive agents prescribed: In monotherapy, the majority of antihypertensive drugs prescribed were from the category of calcium channel blockers, n=45. This was followed by β-adrenergic blockers, n=20 and diuretics, n=18. Similarly, in multiple drug therapy, the category of drugs prescribed the highest was found to be calcium channel blockers, n=315, followed by diuretics n=154, and β- adrenergic blockers n=97. The details are shown in Table: 1.

Price variation: Among the various agents prescribed to the patients as monotherapy, prazosin 25 mg was found to have the maximum price variation. There were six brands of prazosin which showed a price variation of 7.76. This was followed by spironolactone 50 mg, which had three brands with a price variation of 7.73. The least variation was observed with telmisartan 80 mg (0.05), which had only two brands. Within the multidrug therapy section, maximum price variation was found with metoprolol 50 mg. Metoprolol had four brands and it showed a price variation of 10.69 which was followed by prazosin 25 mg, having six brands and a price variation of 7.76. The least variation in prize was shown with propranolol 10 mg, (0.04) which had only two brands.

Analysis of direct cost: The total cost of illness (COI) includes consultation, laboratory, medication, nursing and hospital charges. Table 2 summarizes the annual median cost spent by hypertensive patients with and without complications. The highest median direct medical cost was found to be of laboratory charges (INR 4497), followed by hospital (INR 200) and comorbidity medication charges (INR 1781.50). The least median medical cost (INR136.48) was for the treatment associated with hypertension.

A total of 400 patients were evaluated during the study period. In the current study, males (58.5%) were more than the females (41.5%). Similar results were

obtained in a study conducted by (Malpani et al. 2018). Based on the prescription pattern, most of the patients were prescribed with calcium channel blockers and among patients who received monotherapy as well as multiple drug therapy, amlodipine was prescribed the maximum. This was consistent with the study conducted by (Forouzanfar et al., 2017). Prazosin 10 mg showed maximum price variation among various antihypertensive drugs prescribed as monotherapy. The funds spent on laboratory tests accounted for Rs. 4497.33. This, when converted to U.S. dollars equals to 64.46 USD. The median total annual direct cost was Rs 10840.35 which in U.S. dollars amounts to 155.37 USD. These results were in contradiction to a similar study conducted in 2019 (Oyando et al., 2019). The cost was less than the study conducted in 2021 (Bryant et al., 2021).

CONCLUSION

In direct medical cost, laboratory charges were found as the most prominent factor contributing to the increased burden on the patients. Along with the hospital stay charges, medication cost may further increase the burden. Significant price variation was also noted in each drug used for the management of hypertension. So, there is always a possibility to prescribe the drugs which are at low costs to reduce the overall healthcare expenditure and the economic burden on the patients.

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Biotechnological Communication

Microbial Naringinase and its Applications in Debittering Technology –A Mini Review

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ABSTRACT

Naringinase is an omnipotent enzyme found in animals, plants, and microorganisms. Subsequently, naringinase is increasingly getting recognition in industrial application, particularly, in debittering technology of citrus fruit juices. The other industrial sectors considered a potential user of naringinase are antibiotic preparation, wine enhancement, preparation of rhamnose, hydrolysis of glycoside, and biotransformation. Biochemically, naringinase is a multi-complex enzyme, in general, is an enzyme that catalyzes the hydrolysis of α-rhamnose and β-glucose. In addition to this, naringinase can also outnumber other natural glycosides. This natural compound is receiving increasing attention because it carries major potential for application in food and pharmaceutical industries. However, in near future, the growing multifold potential of naringinase application is expected to trigger its share to reach a leading position. The microbial naringinase getting popularity among researchers for its availability, easy handling, multifold property, and abundant supply. In nature, naringinase is obtained from different origins promoting variation in reaction specificity, thus making it a versatile and interesting element of study. The existence of industrially important microbial strains with diverse genetic resources cannot be ruled out. Isolation and screening of microbial strains found from the diverse ecological niches may lead to the isolation of novel naringinase-producing strains. Research has been conducted worldwide on the isolation of novel naringinase primarily through two different strategies namely; (1) screening of natural resources for isolating microbial strains bearing novel naringinase activity (2) applications of molecular biology tools to develop desired properties from the existing naringinase. The industrial demands of naringinase having high catalytic specificities continue to stimulate the search for new enzyme sources. Hence, an effort has been made through this review to discuss the isolation and characterization of naringinase enzyme along with its applications from different microbial origins in nature.

KEY WORDS: BITTERNESS, DEBITTERING, NARINGINASE, MICROORGANISMS.

INTRODUCTION

Excessive bitterness is the main problem in the citrus juice industry as it leads to reduced quality and profitable value of the final juice products in the market (Purewal and Sandhu 2021). The processing of citrus fruit juices face forbidding problem of "bitterness" thereby directly affecting consumer acceptability (Lafuente et al., 2021). Without a suitable debittering technology method, the commercial citrus juice industry cannot grow (Narnoliya and Jadaun, 2019; Pangallo et al., 2021). Debittering processed juices looks to be the most favorable method, and some other global citrus juice industries are already furnished with debittering procedures (Curci et al., 2021).

Naringin is a flavanone glycoside, a type of flavonoid. It is glycosylated by a disaccharide at position seven to give a flavanone glycoside. Since naringin and limonin

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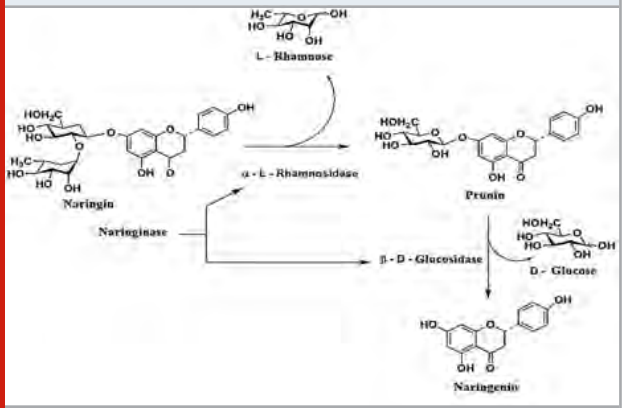
is the principal bitter component of citrus juice, thus, its hydrolysis with a concomitant decreases the bitterness becoming of great industrial importance (Luo et al., 2019). Presently maximum production goes to the fresh fruit market. It is notable that due to poor post-harvest infrastructure, wastage of citrus fruits is around 25-30% and that only a small percentage of the total production is processed due to its problem of bitterness (Sharma et al., 2021).

Figure 1: Biochemical puri of naringinase enzyme was two steps reaction where naringin α-L- rhamnosidase first split the naringin to prunin (aglycone and D-glucose) liberating on the molecule of L-rhamnose and β-D-glucosidase hydrolysis prunin into non-bitter naringenin liberating one molecule of D glucose (Puri et al. 2010: Rubio 2011; Yadav et al. 2021).



To control juice quality and improved commercial market value of the citrus juices, namely of grapefruit, maintaining their health properties and increasing consumer acceptance, the reduction of naringin concentration by naringinase hydrolysis is one promising technique having wide industrial application (Gudimella et al., 2021). Limitations of physicochemical technology can be overcomed by introducing the biotechnological methods in fruit juice processing by using naringinase or using whole microbial cells with the ability to produce naringinase as a debittering enzyme, was used in the industrial production of citrus juice (Yu et al.,2021). Biochemically, the naringin of certain fruits has sugar complexes (a-L-rhamnose and b-D-glucose) and an aglycone (Naringenin) part. The b-D-glucosidase (EC.3.2.1.23) and a-L- rhamnosidase (EC.3.2.1.40) are collectively known as Naringinase (Beekwilder et al. 2009). The enzyme a-L- rhamnosidase acts on the sugar complex, release prunin, and rhamnose whereas the b-D-glucosidase acts on prunin to release naringenin and glucose (Carqueijeiro et al., 2021).

Naringinase acts on many other natural glucosidases which include hesperidin, diosmin, quercitrin, rutin, naringin, and terphenyl glycosides (Zheng et al., 2021). These substrates have potential chemicals with important properties in the fields of healthcare, food, and agriculture as they are recognized as antioxidant, anti-inflammatory, anti-ulcer, neuroprotective, hypocholesterolemic effects (Torabizadeh et al. 2018; Wang et al., 2021). Microbes are mainly exploited in the industry for naringinase enzyme production. Furthermore, naringinase enzymes are supplied well standardized and market by several competing companies worldwide in recent time (Sheldon et al., 2021). This review focuses on the microbial origin of the naringinase enzyme and its applications. Microbial Origin of Naringinase Enzymes: A considerable amount of work has been reported from across the globe in the isolation, and purification of naringinase enzymes from different sources of nature. Different microbial sources reported for the naringinase production have been listed in Table 1.

This enzyme has existence in nature and has sources from animal tissues, bacteria, fungi, yeasts, and plants (Yadav et al.,. 2021). However, for the source of availability, the only process based on microbial naringinase is practicable. There are several reports on the microbial production of naringinase and several production methods from them have been patented, (Puri 2010). Although microorganisms have been reported as the main sources for naringinase enzyme production, these enzymes were first found from a plant source as the first naringinase enzyme was isolated from celery seeds (Hall 1938; Yadav et al., 2021).

Production of Naringinase from Fungal Species: Kishi (1955) first reported fungal naringinase, eventually studied through a 10-L bioreactor to enhance the production of naringinase enzyme by *Aspergillus niger* (Kishi 1955; Bram and Solomons 1965). Later on, Ito et al. (1970) reported and registered a patent for naringinase production from *Cochiobolus miyabeanus* Phanopsis citri, and *Rhizoctonia solani*. For the research by Okada et al., (1973) Penicillium sp. was used for the production of naringinase enzyme (Okada et al. 1973; Yadav et al., 2021).

Naringinase enzyme from *Penicillium decumbens* has been isolated by (Nourouzian et al., 2000). Whereas, Puri and Karla (2005) purified an extracellular naringinase of *Aspergillus niger* MTCC 1344 by ion-exchange chromatography. This purified naringinase molecular mass was investigated by SDS-PAGE and found to have 168 kDa. Busto et al., (2007) has reported naringinase production from *Aspergillus niger* CECT 2008, whereas Thammawat et al., (2008) reported *Aspergillus niger* BCC 25166 as the most potential naringinase-producing fungi out of 348 fungi isolated from 128 samples, which were collected from 11 different origins in China and Thailand (Thammawat et al., 2008; Yadav et al., 2021). Later on, Kumar et al., (2010) reported *Aspergillus niger* VB07 isolates from citrus fruit, producer of extracellular naringinase under various environmental conditions.

These authors have studied several inducers used in production for naringinase from *Aspergillus niger* MTCC-1344 and indicated that naringins are the best inducer for the production of naringinase in a medium. Chang et al. (2011) has isolated *Aspergillus sojae* from a traditional Korean fermented soybean product. Chen et al., (2013) also

reported naringinase enzyme from *Aspergillus aculeatus* JMUdb058. Later on, Radhakrishnan et al., (2013) isolated *Aspergillus flavus* and its enzyme naringinase was used for removal of the bitter taste from the juice. Sinthuja et al., (2016) studied the optimization of naringinase production by *Rhizophus stolonifer* in a solid-state fermentation medium followed by more researches that characterized the enzyme from *Aspergillus oryzae* (Zhu et al., 2017a; Borkar et al., 2020).

Table 1. In worldwide isolation of different microbial strains production of naringinase enzyme

Sources	Microorganisms	References
Plants	Celery seeds (*Apium graveolens*)	Hall, (1938)
	Grapefruit leaves	Ting, (1958)
	Buckwheat (*Fagopyrum esculentum*)	Bourbouze et al. (1976)
Gastropod	*Turbo cornutus*	Kurosawa et al. (1973)
Mammal	Pig liver	Qian et al. (2005)
Fungi	*Aspergillus niger*	Kishi (1955)
	Rhamnus dahurica	Suzuki (1962)
	Aspergillus niger	Bram and Solomons (1965)
	Cochiobolus miyabeanus and *Phanopsis citri*	Ito and Takiguchi (1970)
	Penicillium decumbens	Fukumoto and Okado (1973)
	Penicillium sp. and	Tsen and Tsai (1988)
	Aspergillus niger *Penicillium decumbens*	Young et al. .,(1989)
	Penicillium sp.	Hoescht (1994)
	Penicillium decumbens PTCC 5248	Norouzian et al. .,(1999)
	Aspergillus niger MTCC 1344	Puri and Karla (2005)
	Aspergillus niger CECT 2088	Busto et al. .,(2007)
	Aspergillus niger BCC 25166	Thammawat et al. (2008)
	Aspergillus niger VB07	Kumar et al. (2010)
	Aspergillus sojae	Chang et al. (2011)
	Aspergillus flavus	Radhakrishnan et al.(2013)
	Aspergillus aculeatus JMUdb058	(Chen et al. 2013)
	Aspergillus oryzae 1125	Zhu et al. (2017a)
	Trichoderma longibrachiatum ATCC18648	Housseiny and Aboelmagd (2019)
	Aspergillus niger van Tieghem MTCC 2425	Borkar et al. (2020)
	Aspergillus sp. isolate mk156394	Kumar et al. (2020)
	Aspergillus niger KMS	Bodakowska et al. (2020)
	Aspergillus niger van Tieghem MTCC 2425	Borkar et al. (2020)
	Penicillium purpurogenum	Patil and Dhake (2020)
	Aspergillus niger	Gao et al. 2021
	Aspergillus niger	Gupta et al. (2021)
Bacteria	*Bacteriodes distasonis*, JY-1 *Thermomicrobium roseum*	Jang & Kim, (1996)
	Staphylococcus xylosus MAK2	Puri et al. (2010)
	Serratia Sp.	Pavithra et al. 2012
	Micrococcus sp.	Kumar et al.(2015)
	Bacillus amyloliquefaciens 11568	Zhu et al. (2017)
	Bacillus cereus-K1	Pegu et al. (2019)
	Lactobacillus and *Bifi -dobacterium*	Tran et al. (2020)
Yeast	*Williopsiscali fornica* Jmudeb007	Ni et al. (2011)
	Cryptococcus albidusa	Borzova et al. (2017)

Recently, Borkar et al. (2020) characterized naringinase from *Aspergillus niger* van Tieghem MTCC 2425. According to Kumar et al., (2020) naringinase has been isolated and studied from Aspergillus sp. isolate mk156394. Similarly, Bodakowska et al., (2020) has reported naringinase enzyme which was immobilized by *Aspergillus niger* KMS. The production of naringinase by *Aspergillus tubingensis* MN589840 has also been recently reported that deermines extracellular naringinase production from *Aspergillus niger* 426 (Xia et al., 2021);

Fd et al., 2021). Similarly, Gupta et al., (2021) have characterized naringinase from *Aspergillus niger* (Gupta et al., 2021).

Bacterial Origins of Naringinase Enzyme: In the previous studies, almost all studies on naringinase targeted fungi as the source, while research on bacterial naringinase was rare. Recently, naringinase produced from bacteria has received more attention, still, only a few naringinase of bacterial origin have been reported (Gupta et al., 2021). Puri et al., (2009) investigated naringinase production from *Staphylococcus xylosus* MAK2 in a stirred-tank reactor and reported the maximum naringinase production of 8.25 IU/mL at 34th hr. Mukund et al., (2014) discovered new naringinase-producing bacteria *Bacillus methylotrophicus* commonly found in rhizospheric soil. This study was conducted under optimized culture conditions and obtained the highest naringinase activity of 12 U/L which was 50% more than the activity obtained without optimization. Pavithra et al., (2014) studied the importance of C and N sources for enhancement of naringinase productivity by newly isolate *Serratia*. Sp (Pavithra et al., 2014; Gupta et al., 2021).

Amena et al., (2015), reported the purification and characterization of naringinase from Micrococcus sp. (Amena et al., 2015). Zhu et al. (2017b) reported the naringinase from a *Bacillus amyloliquefaciens* 11568 (Zhu et al., 2017b). They have identified, purified, and characterized the enzyme and the purified enzyme molecular weight was found 32 kDa. Tran et al. (2020) carried out a detailed study on four probiotic bacteria strains in which Lactobacillus and *Bifidobacterium naringinase* gave the maximum production from grapefruit juice fermentation (Tran et al., 2020). Besides this, only a little literature was found on naringinase production from yeast strains. Ni et al., (2011), reported the naringinase enzyme production from *Williopsis californica* Jmudeb007 (Ni et al., 2011). Borzova et al.,(2017) studied the purification and characterization of naringinase enzyme from *Cryptococcus albidusa* by Ammonium sulfate fractionation and chromatography. Here, the enzyme had a purified molecular mass of 50kDa. and had an optimum pH at 5.0 as well as temperature of 600C respectively (Borzova et al., 2017; Tran et al., 2020).

CONCLUSION

Naringinase enzyme is a multi-complex enzyme, in general, is the one that catalyzes the hydrolysis of α-rhamnose and β-glucose. In addition to it, naringinase can also number other glycosides. One unique catalytic property of the naringinase, namely, steroids biotransformation, hydrolysis of glycosidase, renders it to catalyze the reaction in aqueous as well as in the non-aqueous environments. This property of the naringinase ascribes their ability to utilize a wide spectrum of substances, thus increasing the scope of exploiting their specificities in various reactions having importance in pharmaceutical, food, and many other industrial sectors. The industrial demands of naringinase having high catalytic specificities continue to stimulate the search for new enzyme sources. Most of the commercial naringinase are of fungal origins. However, the focus on bacterial and yeast has recently been laid due to their high stability, multifold properties, and abundant supply.

This review paper comprises a literature survey on the work done on naringinase during the last eight decades. Emphasis has been primarily given on the characteristics of naringinase, naringinase production, isolation, and purification. A survey on the recent advances in naringinase technology, such as the preparation of improved varieties of naringinase through molecular biology techniques has also been presented. Further, naringinase may be one of the target enzymes that could be undertaken for isolation from this region. This could be done1) by screening and use of new more potential naringinase producing microbial strains or 2) by optimizing the culture conditions of selected isolates for commercially viable naringinase production. Therefore, screening of microorganisms with higher naringinase productivity, further the discovery of novel naringinase producing microbial strains is appropriate to new industrial applications.

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Environmental
Communication

Modelling and Assessment of Oil Pollution Under Data Constraints

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ABSTRACT

Conducting effective targeted monitoring of land pollution by oil production and refining is a task that is relevant for all countries, especially for Russia. Much in the efficient solution of this problem depends on the types of pollutant and soil, protection technology. It is important to know the assessments of pollution risks, to localize them. The purpose of the work is system analysis and modeling of land pollution by oil under limited and uncertain data. The hypothesis of analysis and modeling under consideration: "an ecosystem is open, continuously developing and interacting with the environment". Used methods of analysis-synthesis, decision-making, optimization and simulation of systems, an evaluation technique and procedure, as well as expert and heuristic approaches. Multifactorial and uncertainty of the solved problem, which often complicates research and prediction, are proposed. The main result of our work is the methodology of modeling (forecasting) of the state of the land taking into account bifurcations, making managerial (for example, recreational) decisions. The study was conducted with a focus on predicting self-healing of the environment. The results can be used in the development of intelligent systems for assessing land pollution.

KEY WORDS: ASSESSMENT, DATA, MODELLING, OIL, POLLUTION.

INTRODUCTION

Soil is not a renewable resource; its degradation is almost irreversible or associated with duration comparable to human life. Pollution of the earth goes as a chain reaction; it reduces the diversity, stability and organics of the soil, its ability to self-repair. Pollutants from the soil enter on our table – through groundwater, plants, animals on pastures, birds and cause a "bouquet" of diseases, especially oncological ones. The yield of crops and their quality and the ability of the soil to decompose organic pollutants are reduced. Antibiotics, bacteria are getting into the soil, bacterial resistance of the fruiting part of

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the soil is falling. The polluting capacity of petroleum products and risk situations related to oil production or refining is greatly affected.

About 12.5% of all world oil production is produced in Russia (Pikovsky et al 2015). At the same time, the territories bordering oil production are at risk, sensitive to spills and other methods of pollution. Especially for effects that reduce the self-healing of the environment (ecological niche), which depends on the type and amount and the joint effects of pollutants that increase pollution ("summation effect"). The impact of pollution on land, human health is a multi-criterial and multifactorial problem, often with a lack of data, their uncertainty.

In Russia, estimates and models of risk situations with integral effects of pollutants on human health have only begun to be actively studied (Zaitsev et al., 2013).Non-compliance with environmental standards is associated with the risk of costs of oil producing and refining enterprises to compensate for losses in the quality and quantity of land used in the national economy. Now, remote soil sensing, satellite or UAV applications are also successfully used, followed by intelligent data analysis (Abramov et al, 2018, Van Opheusden, et al 2020). The environment is directly subjected to controlled and controlling influences in oil development (refining) systems and is considered by us as a self-organizing system for which various situational scenarios and evolution strategies are possible. Heuristic and expert methods are an effective method of systemic, complex analysis of environmental impacts. In the work, they are applied to the study of the problems of analyzing environmental pollution.

MATERIAL AND METHODS

Geo-environmental analysis relies, as usual, on monitoring, its results and their applicability to all objects of the environment, and not only, for example, to soil or air. In relation to soil, a complete set of factors is taken into account – erosion, salinization, swelling, waterlogging, etc., as well as self-organizational processes in the environment – migration of substances, restoration, accumulation, etc.The most dangerous factors for oil-contaminated soil are hydrochloric salts, hydrogen sulfide, sulfur, organic chlorides, small fractions, resinous and paraffin compounds, etc. Factors can be classified – according to the properties of the environment, relationships with the environment, the structure (composition) of pollutants and soil, water and air. Data sources, completeness and certainty also affect. For example, using the work data (Pikovsky, et al, 2015), the following table can be compiled.

The complexity of monitoring activities and data structures, the inability to adapt to their processing of procedures verified by formal means, leads to the need to attract non-traditional approaches. They help out heuristic and expert procedures, modeling methods that we actively use. As noted, their application is complicated by uncertainty and lack of data, without which the relevance of heuristic and expert procedures is impossible. But a tool for analytical processing of monitoring data has been developed to help researchers. Big Data in Computational Social Sciences and Humanities (2018) is processed by Data Mining (Zayar, Mykhaylov, Ye Thu, 2020), mathematical (Kaziev, Shevlokov, 2008) and situational modelling.

A certain pattern of soil pollution can be obtained by the method of bioindicators (bioremediation) or determinants of the degree of pollution of the environment with the help of living organisms –bacteria, algae, conifers, invertebrates, etc. They respond to many environmental factors and impacts, up to oil spills. It occurs according to the scheme "impact – response – information" (Buzmakov, Egorova, Gatina, 2017).But soil bioindication is a complex, thin instrumentation (Israel, Phillipova, Insarov et al, 1982). It uses the natural metabolic activation of microorganisms, plants and the activity of photosynthesis, catalytic reactions that lead to changes in the soil. The method is efficient and simple, without large attachments. But incorrectly selected or transferred to another area, it gives incorrect results. For example, "responds" in various ways depending on the type of soil, humidity, altitude, etc.

The impact of petroleum products on the soil is intensified by suboptimal (unsustainable) requirements, logistics, investments in risk forecasting and damage prevention, the development of infrastructure support for oil production and processing.The effect of the pollutant on the soil can also be non-specific, manifested in the reduction of its resistance, resistance to desertification and diseases due to petroleum products. The amplitude and the exposure intensity period, as well as the pollutant dose.

Table 1. Range of factors influencing oil pollution in Russia

N	Factor	Max (%)	Min (%)
1	Light pollutants	18.2	11.9
2	Paraffin	1.9	0.5
3	Pitches	27.0	19.5
4	Sulfur	2.05	1.55
5	Density (kg/m3)	907	896

RESULTS AND DISCUSSION

With fixed pollution, for example, releases of the pollutant into the soil with subsequent physicochemical compounds, pollution fields and concentrations of the pollutant in the medium are formed. The usual problem is the identification of parameters, pollution data or their lack, uncertainty. Here we propose the following approach.Let a pollutant with a concentration of u(x,y,h,t) enter the soil to the depth $h \in [0,H]$ and by the area determined by the coordinates x,y by the moment t.

To smooth out the lack of information and "compress" it, its possible inaccuracies, as well as reduce the number of variables, average as follows:

$$\bar{u}(x,y,h) = \frac{1}{T}\int_{t_0}^{t_0+T} \bar{u}(x,y,h,t)\,dt\,.$$

It's assumed that at the interval $[t_0;T]$, the function

$$u(x,y,h) \equiv u(x,y,h,t)$$

is little dependent on the initial moment. The pollution concentration field is a complex vector object. It can be associated with a scalar value – the average territorial (according to the ecological niche with area determined by the measure (Ω)).

$$\bar{U} = \frac{1}{mes(\Omega)}\int_{\Omega} \bar{u}(x,y,h)dxdydh.$$

The value of in the territory of Ω coincides with We call this value, by analogy with (Pikovsky, Ismailov, Dorokhova, 2015), the background value of the concentration of the pollutant in the territory of Ω. Evasion of U can occur under the influence of cleaning measures or natural, anthropogenic influences, forming "peaks". The very value of can greatly depend on the territory,. The most complete picture nevertheless does not give the average value, but the mathematical expectation and distribution function u over x, y, h and t.When modeling the state of soil contaminated with petroleum products, system models and principles of forecasting the response of land, soil to pollution are necessary.

Balance models of semi-empirical type, expert and heuristic procedures (Bestuzhev-Lada, 1982) can be used to obtain information on the degree of response of soil, land to changes in pollutant concentration with the above measure. Let us give an example of this approach. If we could receive optimum, for example, in size of residual dispersion, a form of dependences of pollution on each considered factor of $x_i, i = 1,2,\dots,n,\ \bar{u}_i = f_i(x_i)$, possible to define multiple-factor best dependence of a look.

$$u = \sum_{i=1}^{n} c_i f_i,(x_i),$$

where–weight coefficients of significance of the criterion of the type "pollutant concentration–pollution value (pollution changes)" for the i-th pollutant. This is an indicator of participation in the total pollution of the i-th pollutant. In the deterministic version, the traditional least squares method is used, and in the random nature of impacts, the maximum likelihood method is used, for example (Van Opheusden, Acerbi, Ma, 2020). This approach will take into account self-organization, adaptive management regimes based on evolutionary potential for self-cleaning of soil with the help of bioactive substances. Here, the methods of Social Mining (Kaziev, Kazieva, Khizbullin, Takhumova, 2019) should be applied, flexible models that take into account connections throughout the contaminated territory. Its state will be taken into account in the following evolutionary way. We define the state vector x, activity s(x), the functionality of the activity (or tension, fatigue) of processes in the system. They will provide additional or generalized information on the contaminated area. For example, if a pasture is restored or knocked out of circulation at a rate defined in following case.

$$s(t) = s_0 + s_1 x(t),$$

$$0 < t < T,\ \ 0 < x < X,$$

then the potential of this process and the entire system can be set by the functionality of the form:

$$E = \int_0^T s(\tau)exp\left(-\int_0^{\tau} a(z)dz\right)d\tau\,,$$

where α is the coefficient of natural land regeneration, x is spatial, t is temporal variables. Above the pace – above the potential, or vice versa. The soil cover specified at the initial moment will be depleted under E < 1.The threshold concentrations above which changes in soil occur, as well as the adaptive response to changes in oil concentrations, should first be determined. If there is contamination of land in the oil production zone, then we consider the vector of concentrations of pollutants (factor vector)–

$$x = (x_1, x_2, \dots, x_n)$$

and the vector of concentrations (MPC) –

$$y = (y_1, y_2, \dots, y_n), x_i \le y_i\,,\ i = 1,2,\dots,n$$

and the effectiveness of the effect of the pollutant (participation in pollution) soil:

$$R = \sum_{i=1}^{n} \frac{x_i}{y_i}\,.$$

Taking into account the "total" effect – the enhancement of the polluting capacity, its toxicity and the ability to act. To assess impacts, we propose a system of ordinary and fissile equations of the form:

$$\frac{dy_i}{dx_i} = m_{ij} \sum_{\substack{j=1 \\ j \neq i}}^{n} \frac{y_j}{y_{j0}}, \qquad i = 1,2,\dots,n,$$

where mij is the weight corresponding to the relative influence of pollutant i, enhanced (attenuated) by the presence of pollutant j in the soil, y10 – MPC of pollutant j in the absence of other pollutants.Weights can be set according to various methods. The simplest and most understandable:

1. m_{ij}=1, if the effects are summed up (amplify each other);
2. m_{ij}=0, if the effects do not affect each other;
3. m_{ij}=-1, if the effects weaken each other. If denoted

$$K_{10} = \sum_{\substack{j=1 \\ j \neq i}}^{n} \frac{1}{y_{j0}},$$

then the solution of the given system in logarithmic form can be represented in the following form:

$$\ln y_1 = \ln y_{10} - m_{12} K_{10} x_2,$$

$$\ln y_1 = \ln y_{10} - m_{13} K_{10} x_3,$$

$$\vdots$$

$$\ln y_1 = \ln y_{10} - m_{1n} K_{10} x_n.$$

At zero contamination (m_{ij}=0) MPC values are stable:

$$\ln y_i = \ln y_{i0}.$$

From the conditions of

$$K_{i0} > 0, \qquad x_i > 0$$

follows an increase in pollution at $m_{ij} = +1$ and $m_{ij} = -1$– and – its weakening. From the values according to calculations (forecast) we select the largest. Similarly, we find minimum values. They are used in situational modeling (simulation calculations) to make relevant design decisions, for example, agricultural ones. In the above approach to determining a multifactor dependency, it's important to know the forms of the basis dependencies To determine them in conditions of lack of data, we propose to use simulation simulations with a database of such functions, formed by experts.

For example, one can use the following types of functions:

$$f_1(x_1) = a_1 x_1 + b_1;$$

$$f_2(x_2) = \frac{1}{1 + exp(a_2 x_2 + b_2)};$$

$$f_3(x_3) = \frac{a_{31} x_3 + b_{31}}{a_{32} x_3 + b_{32}};$$

$$f_4(x_4) = \ln (a_4 x_4 + b_4)$$

and others.As a criterion for the adequacy of single-factor constituents, a residual dispersion is usually taken. But for the necessary testing of zero hypotheses, "sufficient process depth" and others, the probabilities should be identified, both of the pollution risks themselves, as well as the possibility of their localization and subsequent neutralization.Data are not always normally distributed, as, however, are their errors. Therefore, a preliminary statistical analysis should be performed. As a procedure for such an analysis, a working procedure can be used (Fetisov, Kolesnikov, 2018).We'll suggest another algorithm.

Let $x = (x_1(t), \dots, x_n(t))$ be a vector of the state of the medium (not necessarily pollution, as above), for example, x_1 is the sulfur content, x_2 is the acidity of the soil (PH), etc. The following procedure may then be used.

1. Entering experimental data .
2. We believe i: = 1.
3. We find the optimal form of communication for , for example, using a bank of dependencies (logistic, fractional-rational, lognormal, etc.).
4. If i < n, then i:=i+1 and go to item 3.
5. We divide the medium into cellular structures (discretization of space) according to the principles of cell neighborhood.
6. Leaving from the starting point (for example, the pollutant where the pollutant enters), we find pollution according to the pollutant transfer model.

Design of forecast models – after preliminary statistical analysis and application of Big Data and Data Mining, with the help of which we improve predictive analytics on structural, phase responses to the effects of the pollutant. Health risk monitoring (Deryabin, Unguryan, Buzinov, 2019) is associated with high costs and data redundancy. You can get rid of such costs using intelligent analytical systems, situational criteria for information and modeling (forecasting) with optimal termination of monitoring ("at the level of rationality and profitability"). The effect of petroleum products on the ecosystem is not additive by type of pollutants and their concentrations. If the impact is close to the state under normal, natural conditions, then intra-system changes, alterations are possible. If

the effect is significantly different from normal, then a transition to a new state of equilibrium is possible. Such is the process of evolution – the search for a new state of equilibrium and "balancing" (adjustment) near this point does not always go smoothly.

Quantitative indicators of soil, the entire ecosystem, necessary data for prediction, modeling, their certainty, sensitivity, etc. also change.When recultivating land, involving them again in economic circulation, without taking into account soil and bioclimatic restrictions (on land categories) on drilling wells, the use of acid-water and washing solutions cannot be dispensed with. As without forecasting, simulating their effects, without taking into account the effects of pollutants on the anthropogenic risk load of the ecosystem, system analytics and the technique of "squeezing maximum information from minimal data."

For example, the criterion for recoverability may be the ability to grow plants, their density and stability, pH in soil samples (up to 0.5).

Methods (procedures) for analyzing and evaluating data with restrictions on them, methods for classifying and ranking land should be developed. This will allow the transition to legal environmental assessment standards.In the course of the development of these studies, one can consider sources of random data of Markov observations, which may be the Markov observations, with a matrix of transition probabilities. This situation corresponds to the used statistical data processing procedure, for example, with a given quality function, if observations of the use of the environment are acting ahead of time. If a vector observations or experts polling is specified, then the observation corresponds to a Markov chain with an identifiable matrix of transition probabilities. The structure and complexity of data, monitoring determines the assessment of environmental pollution. Using evolutionary stochastic modeling, it's possible to reduce the complexity of obtaining and processing data, and to increase the efficiency of ecological decision-making.

CONCLUSION

Extraction and processing of oil resources can lead to irreversible consequences, risks. Monitoring, assessment and prevention of soil contamination should take into account the ranges of permissible concentrations, deviations from the possible residual content of harmful compounds in the soil. In our opinion, the main risks for land, soil cover are the practical alienation of land (desertification or salinization), accumulation of poorly neutralized and toxic impurities. The risk of bifurcations in the environment, during the operation of fields and oil refining sites, is associated with them (so far although theoretical).

The results of the proposed study and the expert heuristic procedure will allow to identify, predict risks and damage from pollution in conditions of insufficient statistical monitoring data (by volume, accuracy, structure).The results of the study will help in the development of predictive intelligent systems, for example, expert ones, as well as moving from traditional MPC to differentiated sanitary and epidemiological standards, which is very relevant during the pandemic COVID-19. This situation is relevant and should be taken into account.

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Technical Communication

Creation of 3D Cloud Models for Plants Using A Scanner and Walking Machine with Dynamic Stability

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ABSTRACT

Processes research of plant organisms involves many biometric parameters. Latest 3D scanning tech-nologies and computer vision systems makes it possible to evaluate such plant parameters as leaf width, length, plant height, green mass and volume. Thus, the chief purpose of the study is to examine the crea-tion of 3D cloud models for plants using a scanner and a walking machine with dynamic stability. To ful-fil that aim, for scanning plants in laboratory and in field conditions, a 3D scanner and stereo cameras are mounted on a walking machine with dynamic stability. To automate the collection of biometric parame-ters of plants, it is proposed to use a walking machine with dynamic stability equipped with high-resolution cameras and a 3D scanner. For scanning plants in the laboratory and in the field, a 3D scanner and stereo cameras are installed on a walking machine with dynamic stability. A 3D scanner creates a point cloud depth map by illuminating the surrounding space with an infrared laser with a structured light. Depth data is converted to 3D image by software.Laboratory experiments and field tests of a 3D scanner installed on a walking machine with dynamic stability, were created 3D point clouds models of plants. In the course of the research were revealed the features of scanning plants with aperture light.Small leaves and thin stems are difficult to scan if their size are less than 8 mm. Due wind condition imperfect scan algorithms create duplicated identical leaves. To reduce the distortion of the point cloud, it is necessary to apply stabilization methods, based on vibrations and orientation in space parameters of the walking machine body. And make scan action at exactly moment in time when the machine body has the lowest oscillatory speed.

KEY WORDS: WALKING MACHINE, WALKING MACHINE SENSORS, 3D POINT CLOUD, 3D SCANNING, COMPUTER VISION.

INTRODUCTION

Research of plant body's growth processes in-volves the measurement of multiple indicators. The need to reduce manual effort to measure plant biometrical indicators is long overdue and modern digital technology makes it possible to automate measurement processes. The use of the newest 3D scanning technologies and computer vision systems allows estimating such plant characteristics as leaf width and length, height of the plant, and the volume of green mass.At present, we have accumulated experience and developed methods for determining leaf area and plant height, and planimeters have been created for this purpose. Many of the methods for determining the indicators require manual work being performed by an agronomist. In order to automate the collection of plant biometric parameters, it is proposed to use a walking machine with dynamic stability equipped with high-resolution cameras and a 3D scanner. It is planned that, while moving in open and protected ground, the machinewill be an excellent assistant for agronomists in their plant monitoring (Asgharzadeh and Manda, 2021).

Nowadays,with the advent of computer technology, automation of the plant growth and devel-opment monitoring processes allows us to re-ceive plants parameters, to systematize them, and make prognoses

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and give recommendations to agronomists on the basis of mathematical models. Foreign specialists have been actively using the newest technologies of plants scanning (Sheikhshoaie et al, 2018). Databases of colour 3D plant models are being created. Decreasing the cost of 3D scanners and optical systems makes it possible to create inexpensive auto-mated monitoring systems. High resolution laser scanners are used for monitoring wheat growth (Adam et al., 2017). Digital models of plants are being created based on graphs and triangula-tion; they allow creating models based on the growth points of leaves, flowers and fruits on the plant stem (Kjaer & Ottosen, 2015; Lancelot et al., 2017; Asgari, 2021).

Nowadays researchers face difficulties in soft-ware development, precision of optical instru-ments and there is a search of balance between high cost of the equipment and practical impor-tance of data collected by them (Wang et al., 2019). Few studies have been conducted so far, and their results are difficult to replicate because each study uses many types of equipment and also non-standard and often self-written soft-ware not suitable for other research projects. Since 3D technologies have been actively used in plant growing relatively recently, it is safe to say that this area is understudied (Panjvani et al., 2019).

Optical methods for creating plant models are also actively studied, and specialized scanners and software are created (Paulus, 2019). Neural network algorithms are also used in modelling (Nguyen et al., 2016). Research and design of walking machines is being carried out not only abroad, but also in Russia (Petrov, 2019; Chaudhury et al., 2020). The purpose and importance of this survey is demonstrating the possibilities of 3D plant scan-ning by means of scanner and computer vision intended to define values of plants' biometric indicators, to accumulate an experience of scan-ner operation, to reveal the best methods of scanning and processing point clouds.

MATERIAL AND METHODS

For scanning plants in laboratory and in field conditions, a 3D scanner and stereo cameras are mounted on a walking machine with dynamic stability. 3D scanners generate a depth map in the form of a point cloud by illuminating the surrounding area with an infrared laser with structured illumination. This makes it possible to track and identify objects in three dimensions. For this task, inexpensive and reliable diffractive optical elements are well suited (Sheikhshoaie et al, 2018; Chaudhury et al., 2020).

A Kinect device made by Microsoft is used as a 3D scanner. This device allows us to capture a colour image at a speed of 30 fps with a field of view of 57°x43° and a resolution of 640x480 pixels. The colour image is encoded in RGB format with 8 bits per channel and contains depth information with an accuracy of 11 bits. The unit's laser projector illuminates the entire scene simultaneously using a diffraction grating to create a digital pattern. The distance from the lens is calculated by correlating and triangulating an IR image and the reference pattern diagram stored in the device's memory. This method allows moving parts to avoid, and also to reduce electric power consumption, device dimensions and sensor cost in comparison to LIDAR scanners.

Depth data can be converted into a three-dimensional representation by means of soft-ware. Sensor transmits coordinates of points and their colour (x,y,z, R, G, B). The practical sensor accuracy is ±10 mm at 2m distance and ±70 mm at 5m (Paulus, 2019; Chaudhury et al., 2020). The manufacturer's stated optimum distance is 1.2 - 3.5 m. This distance is too long for meas-urements between rows of plants, so optical lenses from spectacles are used to correct the camera's "far-sightedness". A special lens holder on the sensor allows easy selection of the necessary dioptre value. The measurement accu-racy is also affected by many factors: bright sun-shine, the reflectivity of leaf surfaces, and movement of plants in windy weather.

Point clouds were collected and processed using the open-source Point Cloud Library (PCL). Blender, an open-source editor, was used to edit and visualise the point cloud. Point clouds were acquired using Kinect. The data was processed in several steps: noise reduction, smoothing and removing artefacts, selecting individual plants, calculating the volume and dimensions of the plants. Scanning was carried out using a walking machine, and further processing and analysis was performed in the laboratory using a desktop computer, see Figure 1. In the field conditions, a laptop computer can be used to adjust the mo-tion parameters, to record telemetry, and also to make prompt changes to the walking machine software.

Figure 1: Experimental walking machine

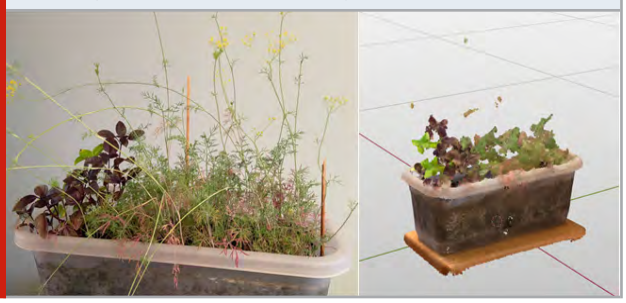
Communication between the laptop and the machine's on-board computer was car-ried out using a wireless Wi-Fi connection. Scanning was carried out by means of the equipment installed on the walking machine. The measurement accuracy made with the scanner was checked by comparing the digital data values and linear dimensions measured with a ruler. The experimental plot, where measure-ments were carried out, is located on the terri-tory of the Educational Scientific and Production Centre "V.I. Edelstein Experimental Vegetable Growing Station" at K.A. Timiryazev RSAU-MAA in Moscow (coordinates: 55°49′40″N, 37°33′3″E).

RESULTS AND DISCUSSION

In the course of the research, data was obtained in the form of a plant's colour point cloud. In the course of the research, the peculiarities of plant scanning by means of aperture illumination were revealed. As the scanner and on-board computer scan the surrounding space slowly, at a speed of 3-10 fps, some leaves of the plants were blurred. The plants were moved by the wind during the scanning process. Contrary to expectations, bright sunlight had no effect on the laser light, and the accuracy and quality of the measure-ments were the same in both shaded and lit areas. To improve scanning quality and speed, it is advisable to increase the number of scanners mounted on the walking machine. This will widen the viewing angle, as well as reduce the blind spots in the surrounding space, where there are no point clouds.Plant scanning was carried out under laboratory and field conditions.

The following plants were scanned under laboratory conditions with artificial light in statics: common basil (lat. *Ocimum basilicum* L.), hot pepper (lat. *Capsicum annuum* L.), zonal pelargonium (lat. *Pelargonium hortorum* L.), jade tree (*Crassula ovate* L., another name is *Crassula* or "money tree"), spurge (lat. *Euphorbia leuconeura*), dill (*Anethum graveolens* L.). The plants were placed on a high stool, around which the scanner was moved manually. The purpose of scanning was to identify peculiarities of plant scanning and to work out methods of collection, processing and visualization of the obtained data. The results showed that small stems and leaves were difficult to scan and were often not detected by the scanner. Dill was the most difficult plant to scan. Its thin stems and leaves, like a cloud of dots,were a jelly of dots. It was not possible to distinguish the stem, the individual leaves, or to separate one plant from another, see Figure 2. In the cloud of dots, there were groups of dill flowers that hang separately in space.

Figure 2: Plants of great basil (lat. *Ocimum basilicum* L.) and fragrant dill (lat. *Anethum graveolens* L.)



Scanning of the pepper showed that leaves with a circumference of 8 mm or more on their surface were confidently detected by the scanner at a distance of 50 cm. Thin stems with a diameter of 4-5 mm at the root were absent from the dot cloud, see Figure 3. An attempt was made to scan a plant with a thick, pronounced stem and leaves. The objects selected were a Crassula and aspurge plant. The thick stems and leaves of the Crassula are well suited for scanning, while the stems of the milk thistle are hidden behind the leaves. Plants with a small gap between the leaves are difficult to scan, see Figure 4. A scan of the geranium also revealed areas inaccessible to the scanner in the blind spots between the leaves of the plant. The stems and soil in the pot were not detected by the scanner, see Figure 5. An interesting result was obtained by scanning a flowering basil plant. The scanned image clearly shows flowers from 8 mm in size, Figure 6.

Figure 3: Plants of hot pepper (lat. *Capsicum annuum* L.)

Figure 4: Crassula (lat. *Crassula ovata* L.) and spurge (lat. *Euphorbia leuconeura*)

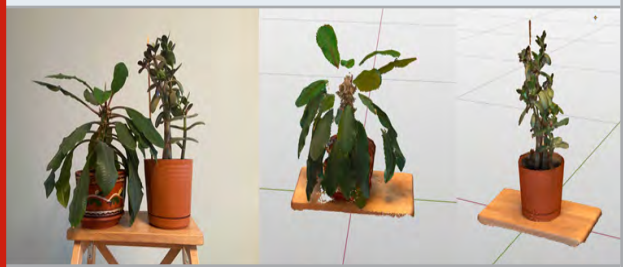
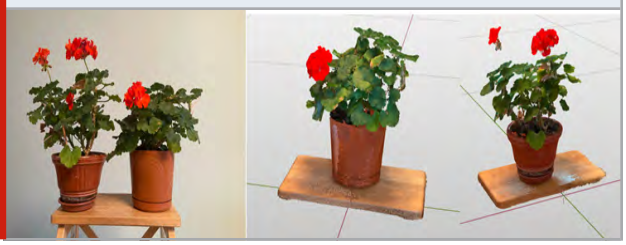


Figure 5: Plants of geraniums (lat. *Pelargonium hortorum* L.)

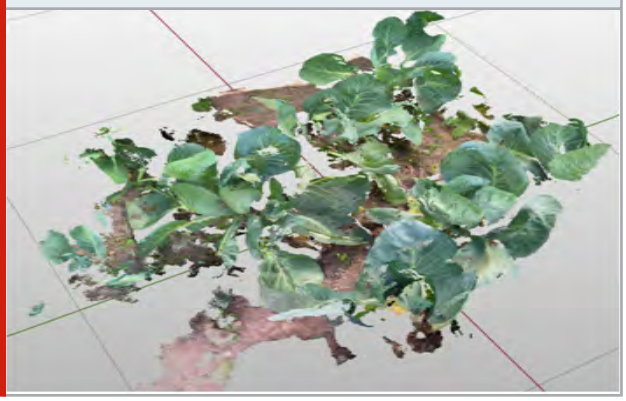


The experience gained with the scanner in the laboratory made it possible to carry out the experiment in the field conditions. A scanner was installed on a walking machine, see Figure 1. It was found in the process of scanning that the software, which provides orientation in space of a lot of pictures with data on depth, is very important parameters of feedback on vibrations and oscillations of the machine body, and also it is necessary to synchronize movements on shifting supports with the process of scanning. Abrupt movements cause blurring of the point cloud. White cabbage (lat. *Brassica oleracea* L.) plants were scanned under field conditions. Growing conditions, row spacing, and leaf size matched the specifi-cations formulated in the laboratory tests, see Figure 7.

The field experience with scanning revealed a number of observations. The scanning algorithms used proved ill-prepared for moving plants. Leaves moving in the wind in the resulting point cloud look like duplicated leaf surfaces at a distance of about 10 mm from each other.

Figure 6: Plants of great basil (lat. *Ocimum basilicum* L.)

Figure 7: The fragment of points cloud for cabbage (lat. *Brassica oleracea* L.)



During laboratory experiments and field testing of a 3D scanner installed on a walking machine with dynamic stability, 3D models of plants in the form of a point cloud were created. Plant scanning showed that small leaves and thin stems are difficult to scan if their size is smaller than 8 mm. Movable plant leaves in the wind look like duplicate identical leaves due to imper-fect algorithms in the point cloud (Petrov 2019; Chaudhury et al., 2020). In order to reduce distortion of the point cloud it is necessary to apply stabilization methods, to take into account vibrations and orientation in space of the walking machine body, as well as to scan at a given point in time, at which the ma-chine body has the lowest speed of oscillatory motion.

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Biotechnological Communication

In-vitro Biocontrol Activity of a Novel Soil Strain *Streptomyces albidoflavus* Against *Fusarium oxysporum* as Causal Agent of Fusarium wilt in Banana Plants

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ABSTRACT

Fusarium oxysporum f. sp. *Cubense* is causal agent of *Fusarium* wilt disease in Banana. The activity of antagonistic bacterial strain was studied for the low-cost and eco-friendly management of *F. oxysporum* in banana. *Streptomyces* S128 was identified by 16S rRNA sequence analysis and resulted as *Streptomyces albidoflavus*. Bioassay activity showed inhibition diameter of 22.5mm against *Fusarium oxysporum* f. sp. *Cubense*. Optimum combination of factors in the fermentation medium was obtained as: soluble starch 4%, peanut flour 2%, $(NH_4)_2SO_4$ 0.2%, $CaCO_3$ 0.8%, NaCl 0.8%. Identification of active compounds were performed via GC/MS chromatographic techniques. There were total of nineteen compounds identified in the extract. On the base of area percent (18.10) Phenol, 2, 4-bis (1, 1-dimethylethyl) and (18.71) morphine were the major constituents of extract. This study concluded that *Streptomyces albidoflavus* effective against *Fusarium oxysporum* f. sp. Cubense, which might be introduced as an effective biocontrol agent for sustainable agriculture.

KEY WORDS: BANANA FUSARIUM WILT, STREPTOMYCES ALBIDOFLAVUS, GC-MS, MORPHINE.

INTRODUCTION

Over 400 million people rely on Banana (*Musa* spp.) as major subsistence (Dale et al. 2017). Fusarium wilt or Panama is one of the destructive diseases of banana. In the past century in the early days, it has caused heavy economic loss (Ploetz 2015). The production of Banana (*Musa* spp.) is severely in danger because of the infection of the soil-borne fungus q f. sp. cubense (Foc) and is commonly referred to as Panama disease (Dita et al. 2018). Due to complex nature of soil zone, it's quite challenging to combat soil-borne diseases. Soil-borne fungus had severe effects on crops which leads to heavy economic loss (Jayaprakashvel et al. 2019). Chemical practices in disease management of Fusarium wilt can make the soil unfit (Siamak and Zheng 2018). It also caused negative effects on health, so biocontrol is an alternative measure (Rajaofera et al. 2019). In several mechanisms of disease management, this approach has gained significant value, (Bubici et al. 2019).

Currently, the demand for natural bioactive substances has increased because of the potential effects in clinical practices and as well as in crop protection (Singh et al. 2017). In recent years, bioactive compounds are in high demand in the pharmaceuticals and naturopathy, due to their health benefits to human and plants. *Actino* bacteria are a major source to obtain novel compounds, which could be utilized in clinical practices, pharmaceutical industry and agricultural applications, (Barka, et al. 2016; Chater 2016). Mostly *Actinomyces* obtained have been from the soil (Guo et al. 2015) as production of natural compounds from microbes requires optimal growth conditions besides the nutrient medium (Rajnisz et al. 2016).

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Nowadays different statics models are applied to optimize the fermentation mechanisms to extend the production of bioactive compounds, (Latha et al. 2017). There are several approaches in use, such as GC-MS, LC-MS, and NMR, for identification and structural characterization of the bioactive compounds (Tiwari et al. 2015). GC-MS is an innovative approach to identify the compound (Awla et al. 2016). In the current study, we have identified a novel potent *Streptomyces* strain for the biological control of banana *Fusarium* wilt. This study was aimed to produce secondary metabolites by optimizing the nutrient medium through Orthogonal Array Design, extraction and identification of bioactive compounds by GC-MS.

MATERIAL AND METHODS

Microorganisms: All the microbes, Banana *Fusarium* wilt pathogen and bio-agent were obtained from plant pathology lab, College of Plant Protection, Shenyang Agricultural University, China. *Streptomyces* strain 128 and other comparative antagonistic microbes (*Bacillus subtilis, Trichoderma harzianum, Paenibacillus polymyxas, Bacillus licheniformis, Streptomyces cacaoi, Bacillus laterosporus*, and *Bacillus mucilaginosus*), were maintained on nutrient and Gausses medium. *Fusarium oxysporum* was maintained on PDA.

Identification of Streptomyces strain: PCR amplification of the 16S rRNA gene of strain S128 was performed using the universal primer: 27(5'AGTTTGTCMTGGCTCAG-3) and 1492R (5′-GGTTACCTTACGACTT-3) (verity TM 96-well PCR, Applied Biosystems, Singapore). The PCR products were sent to Sangon Biotech (Shanghai, China) Co., Ltd for sequence determination. Phylogenetic analysis was conducted by using Mega version 6 (Ahsan et al. 2017).

Inoculum development: Fermentation was performed in two stages, seed growth and production of the active antifungal substance. Strain S128 was grown on plates of gausses medium (Gao et al. 2016) at 28 °C for 5 days after spore production in the liquid fermentation medium. Two spore cakes (5 mm) of Strain 128 were used to inoculate 40ml medium in a 250 mL flask volume and incubated at 28 °C with a shaking speed of 160 rpm for 48 h.

Fermentation process: From seed culture, 5% (v/v) were inoculated aseptically into 250 mL flask containing 40 mL of fermentation medium The medium comprised of [47 g soluble starch, 3 g yeast extract, 22 g peanut meal, 2.7 g $(NH_4)_2SO_4$, 2.7 g NaCl, 2.7 g $CaCO_3$ dissolved in 1 L distilled water and pH were adjusted to 6.8–7.2] and incubated at 28 °C in a rotatory shaker (HZQ-F16 Harbin Dong Lian Electronic Technology Production. Co., Ltd., China) at the speed of 160 rpm for 96 h. After that, the fermented culture was centrifuged the supernatant was stored at 4 °C for further study. The antifungal activity was determined by measuring the diameter of inhibition zones (Ahsan et al. 2017).

Experimental design for optimization of nutrients: The main nutrient factors affecting the fermentation of the strain and its concentration range were determined by a single factor test. On the basis of this, the nutritional formula of the strain was optimized by orthogonal design test. Based on the average value, the optimal fermentation medium formulation was determined by orthogonal analysis.

Effect of KH_2PO_4 on antibiotic production: KH_2PO_4 was added in the optimized medium in the amounts of 0.01%, 0.02%, 0.03%, and 0.04%, respectively, without KH_2PO_4 as control, and cultured at 28 °C, 140 r / min constant temperature shaker for 96 h. The Oxford cup plate method was used to determine the antifungal activity of the fermentation broth.

Effect of inorganic salts and trace elements on bioactive compound production: On the basis of optimizing the medium, a certain amount of inorganic salts and trace elements were added respectively, that is, K_2SO_4, ZnSO4, $MnSO_4$, $MgSO_4$, $CuSO_4$, and $FeSO_4$ were added with a concentration of 0.01%, 0.02%, 0.04%, and 0.08%, respectively, at 28 °C and 140r/min shaking speed for 96 hours. The antifungal activity of the fermentation broth was determined by Oxford cup plate method and without adding trace elements was considered as a control value.

Biochemical profiling for stability test of fermentation broth: Using the dinitrosalicylic acid, reducing sugar in the fermentation batch was measured and total sugars were measured by Phenol–sulphuric acid method. Amino nitrogen was determined using ninhydrin reagent. pH values of the fermentation batch were determined at different interval of time using pH meter. Dry cell weight analysis was done by the method of (Ahsan et al. 2017).

Separation, extraction, and identification of active compounds: The fermented broth was further treated with different organic solvents by the solvent extraction method. Four different solvents were used to separate fermented broth, i.e. Ethyl acetate, Diethyl ether, Ethanol, Methanol, and N-Butanol, with 1:1ratio and check the activity. Potent separated extract further purified by Silica gel column chromatography. The column was packed with silica gel (60–120 mesh). The sample to be separated was loaded on the packed column and eluted with the ethanol solvent at the flow rate of one drop per 30 seconds. Collected the fractions in test tubes and check the antifungal activity of the fractions. Most potent fraction selected to identify the active compound. GC-MS was used to identify the active compound (Ahsan set al. 2017).

Antifungal assay: Purified Silica gel column chromatography fractions were utilized for antifungal activity of *Streptomyces* against the *Fusarium oxysporum* f. sp. Cubense. Make a serial dilution concentration of most potent fraction, i.e., 0.5, 2, 4, 6, 8, 10 µg/ml. Oxford cup method was used.

Effects of inhibition rate among *S. albidoflavous* and other biocontrol agents against *Fusarium oxysprum*: A comparative antifungal activity was conducted against *Fusarium*. *S. albidoflavnous* and other test biocontrol agents (*Bacillus subtilis, Trichoderma harzianum, Paenibacillus polymyxas, Bacillus licheniformis, Bacillus laterosporus*, and *Streptomyces cacaoi* were evaluated against *Fusarium* wilt. Antifungal activity was performed by oxford cup plate method.

Statistical analysis: Statistical analysis was performed with Minitab software version 0.7.

Table 1 Results of $L_{25}(5^6)$ orthogonal test for the production of antifungal compounds

Run No.	Peanut flour (%)	Soluble starch (%)	Sodium chloride (%)	Yeast extract (%)	$(NH_4)_2SO_4$ (%)	$CaCO_3$ (%)	Diameter of inhibition zone (mm)
1	0	0	0.2	0.4	0.6	0.2	9.51
2	2	0	0. 6	0.8	0.8	0.8	19.21
3	4	0	0	0.6	0	0.6	22.20
4	6	0	0.4	0	0.4	0	10.51
5	8	0	0. 8	0.2	0.2	0.4	25.55
6	0	2	0.6	0.6	0.4	0.4	22.01
7	2	2	0	0	0.2	0.2	31.11
8	4	2	0.4	0.2	0.6	0.8	31.10
9	6	2	0.8	0.4	0.8	0.6	25.09
10	8	2	0.2	0.8	0	0	22.81
11	0	4	0	0.2	0.8	0	0
12	2	4	0.4	0.4	0	0.4	21.98
13	4	4	0.8	0.8	0.4	0.2	25.00
14	6	4	0.2	0.6	0.2	0.8	23.90
15	8	4	0.6	0	0.6	0.6	25.10
16	0	6	0.4	0.8	0.2	0.6	21.76
17	2	6	0.8	0.6	0.6	0	20.45
18	4	6	0.2	0	0.8	0.4	23.95
19	6	6	0.6	0.2	0	0.2	17.00
20	8	6	0	0.4	0.4	0.8	17.50
21	0	8	0.8	0	0	0.8	15.14
22	2	8	0.2	0.2	0.4	0.6	11.80
23	4	8	0.6	0.4	0.2	0	11.00
24	6	8	0	0.8	0.6	0.4	10.00
25	8	8	0.4	0.6	0.8	0.2	8.00
k1	17.510	13.780	18.630	16.800	18.852	17.880	
k2	26.467	20.910	18.413	19.801	14.976	22.398	
k3	18.824	22.400	16.400	19.087	19.818	20.395	
k4	20.150	17.160	18.775	21.274	17.042	12.989	
k5	10.756	19.912	21.896	16.898	23.356	20.875	
k_{max} - k_{min}	15.654	8.616	5.431	4.980	8.340	9.547	

RESULTS AND DISCUSSION

Identification of Streptomyces strain: A partial 16S rRNA gene sequence (1435 nucleotides) of strain S128 was determined and deposited in the Gene Bank database (Waiting for Accession number). Comparative 16S rRNA gene sequence analysis using BLAST showed that the strain could be classified as a member of the genus *Streptomyces* and shared sequence identity (99%) with *Streptomyces albidoflavus* Eu257268. A 16sRNA gene-based phylogenetic tree was constructed with the maximum likelihood method with the different *Streptomyces* reference species available in the Gen Bank database (Fig-1). Phylogenetic analysis indicated that strain S128 closely clustered with the strain *Streptomyces albidoflavus* (Eu257268).

Submerged fermentation: Based on the single factor optimization, the number of influencing factors and the different levels of each factor was determined. In order to eliminate the interaction between various factors and the differences between different test batches, and determine the optimal ratio of various factors in the fermentation medium, soluble starch, peanut cake powder, sodium

chloride, yeast extract, ammonium sulfate and carbonic acid Calcium was the most influencing factor. Orthogonal design test L25 (56), results were mentioned in (Table 1). Based on the results of 3 trials, the optimal fermentation medium formulation was determined by orthogonal analysis.

In the table, ki (i = 1, 2, 3, 4, 5) represents the average value of the diameter of the inhibition zone of the fermentation broth when the i-th level of a factor is combined with other factors, and the highest ki value is selected as the optimal level of the factor, that is, if a factor k2>k1, k3, k4 and k5, the second level of the factor is selected as the optimal fermentation level. So the best combination of factors in the fermentation medium was obtained as: soluble starch 4%, peanut flour 2%, $(NH_4)_2SO_4$ 0.2%, $CaCO_3$ 0.8%, NaCl 0.8%. Kmax-kmin represents the extreme difference between the average values of different factors, and its size represents the degree of influence of different factors on the antibacterial activity of the fermentation broth. An increase in the values; cause an increase the degree of influence.

Conversely, the smaller the values decrease the degree of influence. The smaller, so it can be seen that the degree of influence of the six factors on the activity of the fermentation broth is: peanut flour > calcium carbonate > soluble starch > ammonium sulfate > sodium chloride > yeast powder. The shake flask fermentation test was carried out with the best medium formula and the original medium formula under the same culture conditions. The results showed that the diameter of the inhibition zone of the optimized fermentation broth was higher than that of the original medium %.

Table 2. Effect of mineral salt and trace element on yield of *S. albidoflavus*

Concentration (%)	Diameter of inhibition zone (mm)					
	$K2SO_4$	$ZnSO_4$	$MnSO_4$	$MgSO_4$	$CuSO_4$	$FeSO_4$
0(CK)	30.54	29.10	28.54	28.98	25.58	29.14
0.01	28.38	27.3	28.62	27.92	0	28.70
0.02	27.60	26.62	28.82	27.78	0	28.24
0.04	27.36	0	30.24	27.12	0	28.00
0.08	27.36	0	30.24	28.88	0	28.00

Table 3. Metabolism of *S. albidoflavus* during fermentation in shaking flasks

Parameter	Culture time (h)										
	0	12	24	36	48	60	72	84	96	108	120
Total sugar (mg/mL)	84.7	78.5	76.1	64.2	50.7	40.3	34.3	27.6	19.8	14.2	11.3
Reducing sugar (mg/mL)	0	3.2	4.8	5.6	15.7	18.4	20.3	17.5	14.3	8.9	8.3
Amino nitrogen (mg/mL)	0.63	0.61	0.62	0.59	0.56	0.50	0.38	0.15	0.12	0.11	0.09
pH value	6.4	6.5	6.5	6.5	6.5	6.5	6.1	5.9	5.9	5.3	5.3
Dry mycelium weight (mg/mL)	0	4.1	6.3	14.5	19.3	20.9	20.9	22.4	21.7	19.5	18.1

Effect of inorganic salts and trace elements on the yield of active compound: Microorganisms require certain inorganic salts, and trace elements such as iron, magnesium, zinc, manganese and potassium during growth and reproduction and secondary metabolite synthesis. The effect of many metal ions on the physiological activity of microorganisms is related to their concentration, low concentrations tend to be stimulating, and high concentrations show inhibition. In order to further determine whether the trace elements contained in the fermentation medium of *Streptomyces* S128 can meet the needs of the synthesis of secondary metabolites, six different inorganic salts such as K_2SO_4, $ZnSO_4$, $MnSO_4$, $MgSO_4$, $CuSO_4$, and $FeSO_4$ were added to the optimized medium to study the pair of elements and impact of compound production.

The results showed that the yield of the bioactive compounds was increased after adding proper amount of $MnSO_4$ in the medium; while the yield of agricultural anti-SNO_3 was decreased after adding $ZnSO_4$, when the content exceeded 0.02%, the fermenting cells could not produce the bioactive compound. After adding $CuSO_4$, the bacteria could not produce compound; after adding $FeSO_4$ and K_2SO_4, the capacity of the cells was reduced to some extent; after adding $MgSO_4$, there was no effect on the yield of extract (Table 2).

Biochemical changes during fermentation: Biochemical analysis during fermentation process showed a stable production of secondary metabolites. Results showed in (Table 3), after inoculation to the fermentation medium, the reducing sugar content increases with the degradation of the starch. After that, the content of reducing sugar

decreased due to the growth of mycelium and energy activities such as metabolism. At the same time, carbon sources and nitrogen sources were continuously consumed, and the total sugar content and amino nitrogen content also showed a downward trend.

Table 4. GC-MS chromatograph of identified compounds from the extract of *S. albidoflavus*

Peak #	Retention time	Area %	Name of the Compound	Chemical Formula	Molecular Weight
1	4.563	3.9	Stannane, trimethyl propyl-	$C_5H_{14}Sn$	192.87
2	4.845	4.1	Nonadecane	$C_{19}H_{40}$	268.5
3	8.030	7.110	1,3,5,7,9-pentaethyl cyclopentasiloxane	$C_{10}H_{25}O_5Si_5$	365.73
4	15.327	5.21	Tetracosane, 1-bromo-octadecane	$C_{24}H_{49}Br$	417.5
5	17.372	18.10	Phenol, 2,4-bis(1,1-dimethylethyl)	$C_{16}H_{26}O_3$	266.381
6	17.895	0.810	Benzyl alcohol, .alpha.- (1-aminoethyl)-m-hydroxy	$C_9H_{14}ClNO_2$	203.66
7	18.394	1.06	Hexadecanoic acid , methyl ester	$C_{17}H_{34}O_2$	270.4507
8	18.841	0.63	Pinacolyl alcohol, TMS derivative	$C_6H_{14}O$	102.174
9	18.929	2.01	3H-pyrazol -3-one, 2,4 –dihydro-2, 5-diphenyl	$C_{15}H_{14}N_2$	222.28
10	19.258	0.98	Cyclononasiloxane, octadecamethyl-	$C_{18}H_{54}O9Si_9$	666
11	20.086	6.3	1,2-Benzenedicarboxylic acid, bis (2-methylpropyl) ester	$C_{16}H_{22}O_4$	278
12	20.504	18.71	Morphinan, 7, 8-dihydro	$C_{17}H_{19}NO_4$	301.342
13	20.351	5.31	Dibutanyl morphine	-	-
14	21.643	1.25	2-Ethylacridine	$C_{18}H_{19}N_3O$	293.4
15	22.689	1.98	Benzoic acid, 2, 5-bis (trimethylsiloxy) -, trimethylsilyl ester	$C_{16}H_{30}O_4Si_3$	370.66
16	22.830	6.96	Phenol, 1`,1 dimethyl-ester	$C_8H_{10}O_3$	154.16
17	23.917	2.45	Benzoic acid, 2,3 methyl-ester	$C_{11}H_{14}O_2$	178.23
18	25.104	6.00	Cyclotrisiloxane, hexamethyl	$C_6H_{18}O_3Si_3$	222.46
19	26.485	2.5	Tetracosane, 1-bromo-octadecane	$C_{18}H_{37}Br$	333.4

Table 5. Inhibition Effects of *S. albidoflavus* and Test Antagonistic agents against *Fusarium oxysporum* f. sp. Cubense

Antifungal Biocontrol Agents	Concentration (Cfu/ ml)	Colony diameter (mm)	Inhibitory rate (%)	Significance of Differences	
				5%	1%
Bacillus subtilis	1.0×10^{9}	9.92	92.65	a	A
Streptomyces albidoflavus	1.0×10^{9}	10.79	89.53	a	A
Trichoderma harzianum	1.0×10^{9}	29.88	71.42	b	B
Paenibacilluspolymyxas	1.0×10^{9}	22.97	83.09	bc	BC
Bacillus licheniformis	1.0×10^{9}	26.76	74.97	bcd	BCD
Streptomyces cacaoi	1.0×10^{9}	13.07	91.83	cd	CD
Bacillus laterosporus	1.0×10^{9}	19.56	80.11	cd	CD
Bacillus mucilaginosus	1.0×10^{9}	13.00	91.00	d	D

The concentration of the bacteria increased continuously, and the growth of the bacteria reached a peak at 84 hours. After that, due to the large consumption of nutrients, the products inhibiting the metabolic activity of the bacteria continued to accumulate, the growth rate of the cells decreased, and the death of the growth phase was entered. From the utilization of the nitrogen source and the carbon source, both can satisfy the growth and metabolism requirements of the bacteria throughout the fermentation process, and therefore, no intermediate

feed was required. During the fermentation process, the pH value decreases with the accumulation of secondary metabolites and other metabolites. So the results indicted a stable fermentation broth was manufactured.

Separation, Purification, and identification of compound: Fermented broth was separated by solvent extraction with the 1:1 ratio. Among four solvents (Ethyl acetate, Diethyl ether, Ethanol, Methanol, and N-Butanol) Diethyl ether had significant antifungal effects as in (Fig-2). The inhibition zone was 20 mm while all other low inhibition effects. Selected this potent extract for Silica gel column chromatography. Silica gel column chromatography purified fractions were analyzed by antifungal activity and selected the most potent for further analysis. At different concentrations of the potent fraction (0.5, 2, 4, 6, 8, 10 µg/ml) antifungal activity was varies.

Figure 1: Phylogenetic tree of *Streptomyces albidoflavus*

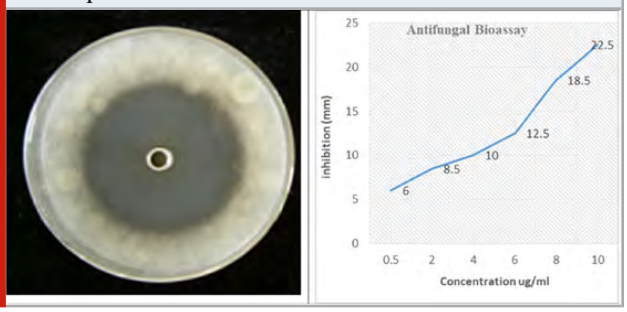
Figure 2: Bioassay effects of different solvent extracts against *Fusarium oxysporum*. All the inhibition zone values are mean of 3 replicates.

As the concentration of fraction increased from 0.5 to 10 there was increased in the activity as shown in (Fig-3). At 0.5 10 µg/ml the inhibition zone was 6mm and later on 10 10 µg/ml inhibition zone was 22.5mm. The data presented in the graph was mean of three replicates. Later on Silica gel column chromatography purified potent fraction was selected for biochemical profile identification by GC-MS technique. There were 19 compounds identified in GC-MS spectrometer profile as shown in (Fig-4). On the base of area percent, there were two compounds considered as a major composite in this extract. Morphinan, 7, 8-dihydro and Phenol, 2, 4-bis (1, 1-dimethylethyl) with the area percent of 18.71 and 18.10 respectively (Table 4).

Inhibition Effects of *S. albidoflavus* and Test Antagonistic agents against Fusarium: Among these seven agents (including *S. albidoflavus*) of biocontrol in the inhibition test, almost all of them exhibited potent inhibitory effects against *F. oxysporum*, while *Bacillus subtilis* had exhibited the most significant effect at the inhibiting rate of 94.26 %, whereas *S. albidoflavus* displayed the effects of inhibition rate of 90%. As it was observed the second most potent antagonistic agent (Table 5). From the results it indicated this novel strain have potent effects against *Fusarium oxysporum* f. sp. cubense by comparison to other test antagonists.

In this study, a soil bacterial isolate were screened against *Fusarium oxysporum* f. sp. Cubense, the causal agent of Fusarium wilt in Banana. Molecular analysis indicated the strainS128 belongs to *Streptomyces* bacteria identified as *S. albidoflavus*. It indicated from the results (Fig-1), concerned strain had potent antifungal effects against *F. oxysporum*. In previous studies indicated that, *Actinomycetes* are the major source of bioactive compounds (Hug et al. 2018). Several studies founded that *Streptomyces* can control the fungus phytopathogens likewise *Rhizoctonia solani* (tobacco target spot) (Ahsan et al. 2017), Ginseng damping-off (Van et al. 2017) and *Streptomyces plicatus* on the oomycete *Phytophthora capsici* (Chen et al. 2016). So this novel *Streptomyces* strain could be potent biocontrol antagonist against *Fusarium oxysporum* f. sp. Cubense. Recently reported that *Streptomyces* sp. AC-19 and *Bacillus* sp. BS-20 were successfully controls the Banana *Fusarium* wilt (Anusha, et al. 2019).

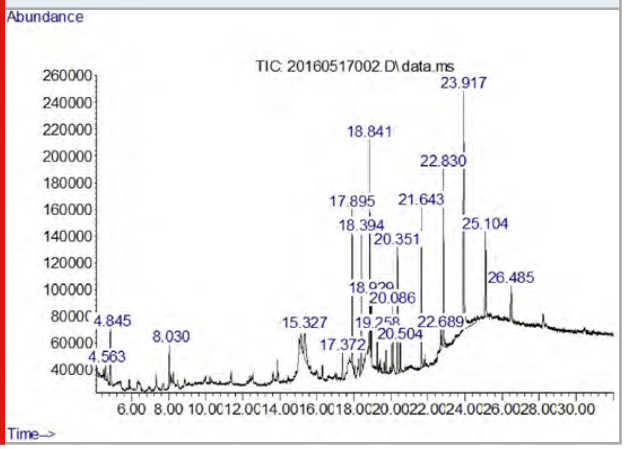
Figure 3: Antifungal bioassay of purified fraction by silica gel column chromatography against *Fusarium oxysporum* f. sp. Cubense. In the graph antifungal values are the mean of 3 replicates



For the production of active compounds in fermentation batch the best combination of factors in the fermentation medium was as followed, soluble starch 4%, peanut flour 2%, $(NH_4)_2SO_4$ 0.2%, $CaCO_3$ 0.8%, and NaCl 0.8%. The results indicated that it is necessary to make a well-balanced combination to develop the bioactive compounds. Well, balanced combination of nutrients helps out to produce maximum yield of active compounds. Optimization of nutrients not only caused in an increased of efficiency but also give the understating of nutrient

components (Gao et al. 2016). From the results, it was indicated that orthogonal design helps out to optimize the best nutrient parameter in less experiment for the production of active compounds. Experimental designs decrease the labor and cost to produce active substance from submerged fermentation. During the process of fermentation statistical designs, not only reduced the cost and work but also improved the quality and production (Elibol 2004). Solvent extracts from fermented broth showed efficacy against the pathogen. The strong activity exhibited by Diethyl ether. As diethyl ether are virtuous solvents for a comprehensive range of polar and nonpolar organic compounds (Ouellette and Rawn 2015).

Figure 4: GC-MS Profile from the extract of *Streptomyces albidoflavnous*



Potent extract of Diethyl ether from fermented broth of strain further purified by silica gel column chromatography. Several fractions assayed against the pathogen *F. oxysporum* then selected the most potent fraction. The purified fraction exhibited inhibition zone against the pathogen, which indicated from the results that the strain have the potential to control the pathogen. In earlier reports investigated that *Streptomyces* strain could control the soil borne fungus pathogens (Anusha, et al. 2019). The current study indicated that purified fractions from *S. albidoflavnous* produced 19 compounds. Production of antibiotic substances greatly depends on natural resources. In natural resources, *Streptomyces* is a major source of bioactive compounds for pharmaceutical products (Jakubiec et al. 2018).

Among 19 different compounds, there are 2 compounds of phenol were identified, one of them had a high area of percentage (18.10) Phenol, 2,4-bis(1,1-dimethylethyl) 18.0 while other had low (6.96) Phenol, 1`,1 dimethyl-ester. Phenol compounds have potent antimicrobial effects (Al-Youssef and Hassan 2015). GC-MS analysis revealed that Phenol, 2,4-bis(1,1-dimethylethyl) could be an active compound as it constitutes the major portion of this extract. This compound previously reported as antimicrobial to combat biofilm formation (Padmavathi et al. 2014). From the GC-MS analysis, there was another compound Morphinan, 7, 8-dihydro with the highest 18.71 area percentage was investigated. As previously reported that derivatives of morphine have no antimicrobial effects, but caused mammalian seizures (Jalodia et al. 2018). From the results, it indicated that S. albidoflavus had significant effects on the pathogen. In conclusion, *S. albidoflavus* is a strong antagonistic agent against Fusarium oxysporum fungus and could have broad-spectrum capability to control Fusarium wilt in banana.

CONCLUSION

This study concluded that *Streptomyces albidoflavus* identified a novel antagonistic agent against soil borne fungus pathogen *Fusarium oxysporum* f. sp. Cubense. This strain might be introduced as an effective biocontrol agent for sustainable agriculture. The results indicated that, extract from the Streptomyces strain would be a substitute to chemical substances for the disease management of Banana *Fusarium* wilt. *S. albidoflavus* strain have broad spectrum potential against fungus pathogens.

Competing Interest: There is no Competing interest.

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Authors' contributions: TA; did the experiments, write, analyze and drafting of the manuscript, WY; plan the work, supervise the work, provide funds, analyze, and proof read. All the authors have read and approved the manuscript", and ensure that this is the case.

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Pharmaceutical
Communication

Establishment of *In vitro* Adventitious Root Cultures, Analysis of Phenolics and Curculigoside Contents in *Curculigo orchioides*

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ABSTRACT

Traditionally, Curculigo orchioides was used for aphrodisiac and many other effects. Curculigoside is the principal bioactive component of the medicinal plant *Curculigo orchioides*, to which various diverse pharmacological properties are attributed. Adventitious root culture of leaf explants of *Curculigo orchioides* was studied using different strength MS medium supplemented by different concentrations of sucrose and pH different values for growth and secondary compound (phenolic and curculigoside) production with 3.0 mg/L NAA in liquid culture. Adventitious roots grown in modified ¾ strength of MS medium showed the highest root growth (2.499 g/g FW), as well as the highest amount of curculigoside (76.521 µg/Treatment) as compared with roots grown in different treatments. HPLC analysis displayed the presence of curculigoside from the *in vitro* adventitious roots of *Curculigo orchioides*. The growth characteristics (biomass) together with phenolic and curculigoside content from the established *in vitro* adventitious roots of *Curculigo orchioides* was successfully enhanced by the manipulation of culture conditions involving ¾ strength of MS medium and the addition of 4% (w/v) of sucrose. Hence, it is important to study other strategies in order to enhance the maximum production of secondary metabolites in *in vitro* systems.

KEY WORDS: *CURCULIGO ORCHIOIDES*, ADVENTITIOUS ROOTS, CURCULIGOSIDE, HPLC, MEDIUM STRENGTH.

INTRODUCTION

Curculigo orchioides Gaertn. (Amaryllidaceae), which grows in subtropical regions of Asia, has been used as a traditional herbal medicine in China, India and Vietnam It is believed to be a tonic for the treatment of declined physical strength. Curculigoside, one of the main bioactive phenolic compounds in the rhizome of *Curculigo orchioides* Gaertn., has been shown to have significant antioxidant properties by scavenging superoxide radicals in the normal systems, and anti-apoptotic activities in H_2O_2- treated vascular endothelial cells (Tang et al. 2004; Wu et al., 2005; Wang et al., 2010).

Curculigoside reduced the oxidative damage and induced proliferation and differentiation of osteoblasts under oxidative stress status, as well as inhibited bone resorption via its anti-oxidative character in ovariectomized rats (Wang et al., 2012; Jiao et al., 2009; Liu el at., 2012). Curculigoside increased the proliferation and alkaline phosphatase (ALP) activity of osteoblasts, inhibited osteoclast bone resorption, osteoclast formation and tartrate-resistant acid phosphatase activity (Jiao et al., 2009). Curculigoside protected osteoblasts against oxidative damage and promoted osteoblastic differentiation via inhibiting extracellular signal-regulated kinase (ERK) and ERK dependent nuclear factor-kB (NF-kB) signaling pathways and stimulating p38 signaling pathway under oxidative stress conditions (Wang et al., 2012). In addition, Curculigoside can also improve the learning and memorizing ability of aged rats by decreasing cerebral acetylcholinesterase activity and inhibiting the expression of β-site APP cleaving enzyme 1 in the hippocampus (Wu et al., 2012). Curculigoside

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effects on Aβ deposition induced memory deficit, bone loss and the potential proximate mechanisms (Zhao et al., 2015).

The quality and quantity of the secondary metabolite collected from wild and fieldgrown plants are often fluctuating and heterogeneous depending upon the environmental conditions. Infestation, diseases and the application of pesticides additionally decrease the quality of the plant materials (Gerth et al., 2007). One is hence listed in this category. Therefore, there is a need to develop an alternative strategy to full-fill the ever increasing demand. Few of the known strategies are *in-vivo* cultivation, micropropagation, direct rhizogenesis and root organ culture. By these techniques, secondary metabolites are isolated and produced in large quantities, as well as of good quality and are stable and produced rapidly in an adapted culture medium. Therefore, production of secondary metabolites under *in vitro* conditions has become an active field of research (Sato et al., 2001).

dventitious roots have been successfully induced in many plant species and cultured for the production of high value secondary metabolites of pharmaceutical, nutraceutical and industrial importance (Mehrotra et al., 2007; Murthy et al., 2008). In this study, we describe the effects of the different strength of media, concentrations of sucrose and pH different on growth, phenolics and curculigoside production in adventitious root culture *Curculigo orchioides* Gaertn.

MATERIAL AND METHODS

Plant material and adventitious root induction: Wild plants were collected in the An Giang province in Vietnam and were identified by Dr. Son of Botanical Museum (Institute of Tropical Biology, Vietnam Academy of Sciences and Technology). Young leaves were used as explants for adventitious root induction in winter. Leaf explants were washed with 70% ethanol for 30 s. And then they were transferred into the bottles with 100 mL of 50% (v/v) sodium hypochlorite solution and 5–6 drops of tween 80.

The bottles were shaken for 20 min. Leaf explants were cut into 0.5 cm×0.5 cm sections after being washed with sterilized distilled water for five times. They were inoculated on MS (Murashige and Skoog, 1962) medium containing 3.0 mg/L NAA (1-naphthaleneacetic acid), 3% (w/v) sucrose and 0.6% (w/v) agar. All the initial pHs of the medium were adjusted to 5.80 with 0.1 M of NaOH or 0.1 M of HCl before autoclaving.The cultures were maintained in a growth chamber at different temperatures 25 °C in the dark. The initiation ratios and lengths of the adventitious roots were recorded after incubation for eight weeks.

Effects of Culture Conditions on Root Biomas: The well established *in vitro* adventitious roots that were cultured onto MS medium supplemented with 3.0 mg/L NAA was used as the initial material. Liquid cultures were established by inoculating 1.0 g (fresh weight, FW) adventitious roots into 100 mL Erlenmeyer flasks containing 75 mL of different strength liquid MS medium with NAA (3.0 mg/L). The flask was kept under continuous agitation at 80 rpm on an orbital shaker at 25 ± 1°C under dark condition.

The adventitious roots were transferred onto MS medium with different MS strengths (¼, ½, ¾, and 1), sucrose concentrations (0%, 2%, 3%, 4%, 5%, 6% and 7%) and the initial pHs of the medium was adjusted (to 5.5; 6.0; 6.5; 7.0; 7.5) with 1.0 M sodium hydroxide (NaOH) and/or 1.0 M hydrochloric acid (HCl) prior to autoclaving. Growth of the adventitious roots was quantified on the basis of initial weight and final weight of fresh weight, dry mass, concentration of phenolic (mg/g DW), and concentration of curculigoside (µg/g DW) after four weeks.

Statistical analysis: All *in vitro* experiments were conducted in 3 replications. The data were analyzed by analysis of variance (ANOVA) to detect significant differences between means using MS Excel. Means differing significantly were compared using the LSD at the 1% probability level by SAS program (ver. 6.12). Quantification of Phenolic and Curculigoside Content from Adventitious Root Cultures

Plant Extraction: The adventitious roots were cleaned, sliced, oven dried at 38 °C, ground into powder and extracted with the method 50% of the ultrasonic vibration. The mass of the methanol extract was recorded and re-dissolved in methanol HPLC grade at a ratio of 1 mg of extract to 100 µl methanol HPLC grade. This methanolic solution of the extract was filtered through 0.45 µm PTFE filter (Sartorius 13 CR) prior to quantification of phenolic and curculigoside content.

Quantification of Phenolic Content: The total phenoliccontent (TPC) in *Curculigo orchioides* adventitious roots crude extracts was determined by using the Folin–Ciocalteu method (Chang et al., 2002). Standard solutions of gallic acid of concentration 1.56–100 µg/ml were prepared in water. 50 µl of extract (1 mg/ml) or standard solution were added to 50 µl of distilled water. 50 µl of 10% Follin–Cicocalteu's (F–C) phenol reagent and 50 µl of 1 M sodium carbonate solution were added to the mixture in a 96-well plate. Distilled water was used as blank. Reactions were incubated for 60 min at room temperature and protected from light. The absorbance was measured at 750 nm with a Microplate Reader (Biotek, USA.). Total phenolic contents were expressed as µg Gallic Acid Equivalents (GAE) per mg of dry plant material.

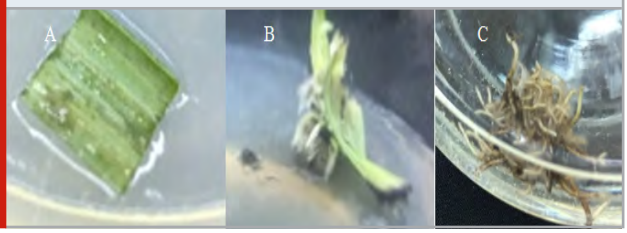
HPLC Analysis: The curculigoside was extracted from the crude medicine with the method 50% of the ultrasonic vibration. Using Sep-Pak C18 cartridges to purify the solution, the curculigoside was detected by HPLC. C18 (250 mm x 4,6 mm; 5 µm) chromatographic column was used, mobile phase of water-Acetonitril (78:22) and detect

wavelength was set at UV 284 nm (Lu et al., 2002). The curculigoside was detected and quantified by matching their retention times and spectral characteristics with known standards that had been identified previously.

RESULTS AND DISCUSSION

Adventitious root formation has a complex molecular process involving numerous endogenous and exogenous physiological factors (Sorin et al. 2005). According to Praveen et al. 2009, the process of induction and differentiation in the physiological stages of rooting can be triggered by changes in endogenous auxin concentrations and external addition of specific auxins (Praveen et al. 2009). *Curculigo orchioides* leaf samples were grown on MS medium supplemented with 3 mg/L NAA to stimulate adventitious roots. The generated adventitious roots were grown on liquid MS medium to multiply biomass and used as raw materials to investigate the effects of some factors on the growth and accumulation of secondary compounds in adventitious roots (Fig. 1).

Figure 1: Diverse *in vitro* events of adventitious root culture of *Curculigo orchioides*; A. Samples of *Curculigo orchioides* leaves for 10 days after sterilization; B. Performance Modified MS +3 mg/L NAA; C. growth of adventitious roots in MS + 3 mg/L NAA liquid medium.



Effect of Culture Conditions on Adventitious Root Growth of Curculigo orchioides: The effect of MS strength, pH and sucrose concentration were evaluated in term of fresh weight, dry weight of adventitious roots produced for each treatment as shown in Table 1. pH affected the root development through an ion exchange process through its membrane. According to "developing hypotheses about the acid", the size of cells were manipulated by its environment's pH, where it decreases with decreased pH (Cosgrove, 1999). Winch and Pritchard (1999); Evans (1976); Edwards and Scott (1974) concluded that environemtal pH induced root elongation. The effects of pH on the development of adventitious roots were proved by Link et al. (2009) while culturing *Orthosiphon stamineus* roots. pH 6.0 was the best among pH 4.0; 5.0; 5.8 và 7.0 and pH 6.0 was also appropriate for the adventitious roots development and saponin accumulation in *Panax ginseng* (Kim et al., 2005). The results showed that, the culture medium with pH = 6.5 is the best compared to the environment with pH under investigation for growth of *Curculigo orchioides* adventitious root (Fig 2.), with a mean value of 1.833 g FW and 0.147 g DW. Phenolics and Curculigoside content of *Curculigo orchioides* adventitious root were not statistical difference in the culture medium with variable pH (Table 2).

Figure 2: Adventitious roots produced in different pH medium; (a) 5.5; (b) 6.0; (c) 6.5; (d) 7; (e) 7.5

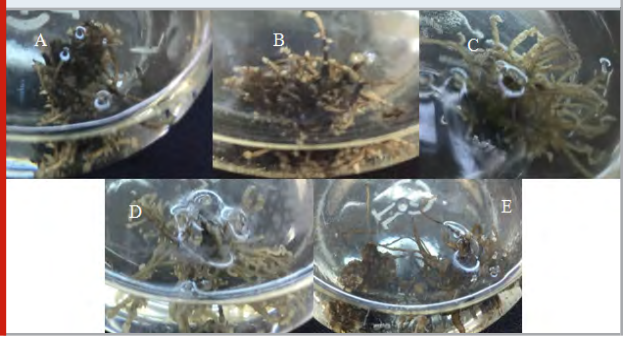
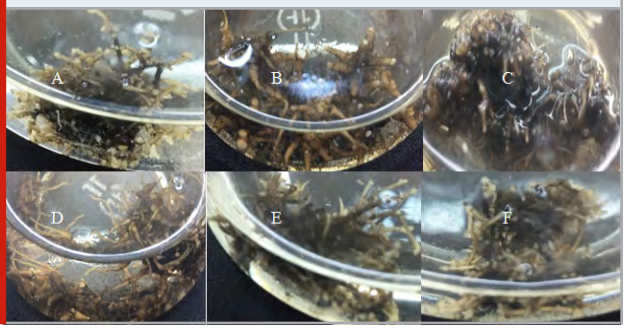


Figure 3: Adventitious roots produced in differentsugar content; (a) 2%; (b) 3%; (c) 4%; (d) 5%; (e) 6%; (f) 7%.



Carbohydrate was essential for plant metabolism *In vitro*, therefore sugar content in the media had a crucial impact on the root induction and development. Sugar plays an important role in the regulation and expression of the transcription of photosynthesis genes (Sheen, 1990) and also to signals of abscisic acid and ethylene (Leon and Sheen, 2003). Cheng et al. (1992) reported sacrose concentration at 2-3 % positively affected the root induction of *Eucalyptus sideroxylon*. Moreover, sugar concentration was reported to have effects on adventitious roots induction of *Orthosiphon stamineus* and *Scopolia parviflora* at concentration 30% and 50%, respectively (Ling et al., 2009; Min, 2007). *Echinacea angustifolia* adventitious roots were cultured on bioreactor on MS media supplemented with 2 mg/l IBA and 50 g/L sucrose. The results showed that, the culture medium supplemented with 40 mg/L sucrose is the best compared to the environment with pH under investigation for growth of *Curculigo orchioides* adventitious root (Fig 3.), with a mean value of 2.471 g FW and 0.198 g DW. Moreover, the optimum sucrose concentration for highest production of secondary metabolites may be varied from every plant species (Koch, 2004).

The best sucrose concentration for production of phenolics, flavonoids and chlorogenic acid of *E. angustifolia* root suspension cultures was found to be 5% (w/v) (Wu at el., 2006) while for *Hypericum perforatum* adventitious root cultures, 5% to 9% (w/v)

of sucrose concentration demonstrated the increment of secondary metabolites production (Ganefianti et al., 2017). Similar observation also displayed in the present study of adventitious root culture of *Curculigo orchioides* where the highest curculigoside content (75.330 µg/ treatment) was enhanced in MS medium supplemented with 4% (w/v) sucrose concentration (Table 2). These results were supported by the detection of curculigoside content through chromatogram peaks according to the sucrose concentrations treatments using HPLC (Fig. 5).

Figure 4: Adventitious roots produced in different strength of MS medium; (a) ¼; (b) ½; (c) ¾; (d) 1

These results may be due to the osmotic stress that occurred during the accumulation of high carbon ion in a nutrient medium that boost the production of secondary metabolite in plant cells (Cui et al., 2010; Praveen and Murthy, 2012; Wang and Weathers, 2007 Kusuma et al., 2016). In terms of nutrients requirement, different plants entail varying content of nutrients for growth. Based on Table 1, data recorded after four weeks of culture revealed that between all MS medium strengths, the fresh weight of adventitious roots cultured on the ¾ strength of MS medium was found to produce the highest root biomasses with a mean value of 2.499 g FW and 0.200 g DW. Nutrients content has an important regulatory role on repressing the transcription of photosynthetic genes (Sheen at el., 1999) and interacting with abscisic acid and ethylene signaling (Yanaglsawa at el., 2003). Medium salt strength also induced changes in growth, physiology and secondary metabolite content in adventitious roots of *Morinda citrifolia* (Baque at el., 2010).

In vitro adventitious root culture showed a high rate of proliferation and active secondary metabolism (Hahn at el., 2003; Yu at el., 2005). Therefore, a substantial increase in phenolic and curculigoside content was detected in the adventitious roots grown in modified MS medium that had the lower macro salt concentration. The results are in agreement with earlier studies on *Lobelia inflata* (Yonemitsu at el., 1990), *Fagopyrum esculentum* (Lee at el., 2007), and *Withania somnifera* (Murthy at el., 2008). Adventitious-root cultures of *Iris germanica* on liquid medium accumulated 4-5 fold higher contents of isoflavone, aglycones and glucosides in 3-week-old liquid cultures (Akashi at el., 2005). The

Table 1. The fresh weight and dry weight of adventitious root produced from each treatment with different MS strength, pHs and sucrose concentration after four weeks in culture.

Culture condition	Treatment	Fresh weight (FW) (g)	Dry weight (FW) (g)
pH	5.5	1.680^{bc}	0.134^{bc}
	6.0	1.766^{ab}	0.141^{ab}
	6.5	1.833^{a}	0.147^{a}
	7.0	1.718^{bc}	0.137^{bc}
	7.5	1.610^{c}	0.129^{c}
	CV	2.44	2.48
	$LSD_{0.01}$	0.11	0.01
Sucrose concentration	2%	1.731^{cd}	0.138^{cd}
	3%	1.837^{c}	0.147^{c}
	4%	2.471^{a}	0.198^{a}
	5%	2.107^{b}	0.169^{b}
	6%	1.732^{cd}	0.139^{cd}
	7%	1.607^{d}	0.129^{d}
	CV	3.49	3.46
	$LSD_{0.01}$	0.17	0.01
MS strength	¼	1.877^{d}	0.150^{c}
	½	2.101^{c}	0.168^{d}
	¾	2.499^{a}	0.200^{a}
	1	2.384^{b}	0.191^{b}
	CV	1.82	1.90
	$LSD_{0.01}$	0.11	0.01

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 amongtreatments by t Tests (LSD).

present study is the first detailed description for testing different MS medium strengths for *In vitro* curculigoside production from adventitious root cultures. The produced curculigoside content were 76.521 (µg/Treatment) on the ¾ strength of MS medium (Table 2).

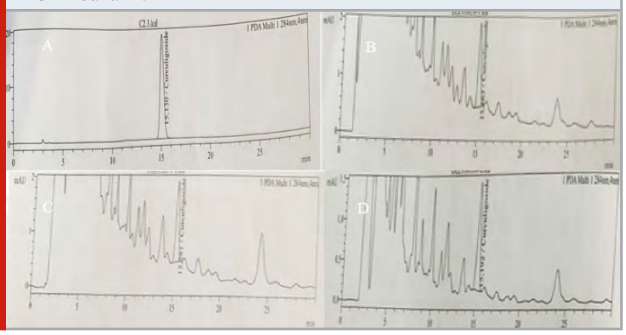
Meanwhile, the root growth (fresh weight and dry weight of adventitious roots) showed a significant reduction in MS medium supplemented with 4% (w/v) sucrose. This may be due to higher osmotic pressure in the medium with the rising of sucrose concentration that gave deleterious effects to the root induction (Cui at el., 2010; Zhang et al., 2012; Tewtrakul at el., 2003). These could be observed through obvious adventitious root growth in Fig. 4. Therefore, it could be specified that reasonably low concentration of sucrose was suitable for adventitious roots growth whereas too low or too high sucrose concentration was not appropriate as it might not supply sufficient energy for root initiation which soon reduces the adventitious roots growth characteristics respectively.

Table 2. The Phenolic and Curculigoside contents of adventitious root produced from each treatment with different MS strength, pHs and sucrose concentration after four weeks in culture.

Culture condition	Treatment	Concentration of Phenolic total (mg/g DW)	Concentration of Curculigoside (µg/g DW)	Concentration of Curculigoside (µg/Treatment)
pH	5.5	31.716[a]	388.112[a]	52.174[ab]
	6.0	31.994[a]	378.601[a]	53.508[ab]
	6.5	32.290[a]	382.625[a]	56.105[a]
	7.0	31.650[a]	390.017[a]	53.587[ab]
	7.5	31.575[a]	377.512[a]	48.640[b]
	CV	2.83	1.95	3.83
	$LSD_{0.01}$	2.34	19.35	5.24
Sucrose concentration	2%	32.018[a]	381.134[a]	52.769[cd]
	3%	32.865[a]	382.405[a]	56.187[c]
	4%	30.435[b]	381.013[a]	75.330[a]
	5%	31.823[a]	380.848[a]	64.200[b]
	6%	31.993[a]	382.111[a]	52.937[cd]
	7%	31.833[a]	382.677[a]	49.186[d]
	CV	1.61	0.47	3.91
	$LSD_{0.01}$	1.27	4.44	5.70
MS strength	1/4	29.036[c]	367.849[b]	55.229[d]
	1/2	29.667[bc]	377.046[a]	63.364[c]
	3/4	31.041[ab]	382.767[a]	76.521[a]
	1	31.849[a]	383.870[a]	73.201[b]
	CV	1.85	0.88	1.72
	$LSD_{0.01}$	1.54	9.11	3.15

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 (LSD).

Figure 5: HPLC chromatogram for curculigoside content in adventitious roots of *Curculigo orchioides* cultured on (A) Standart, (B) pH 6.5, (C) 4% sucrose, (D) 3/4 strength MS medium.



CONCLUSION

HPLC analysis displayed the presence of curculigoside from the *in vitro* adventitious roots of *Curculigo orchioides*. The growth characteristics (biomass) together with phenolic and curculigoside content from the established *in vitro* adventitious roots of *Curculigo orchioides* was successfully enhanced by the manipulation of culture conditions involving 3/4 strength of MS medium and the addition of 4% (w/v) of sucrose. Hence, it is important to study other strategies in order to enhance the maximum production of secondary metabolites in *in vitro* systems.

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Dental
Communication

Parental Comprehension About Use of Fissure Sealant and Fluoride for their Children

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ABSTRACT

Children's oral health is the foundation on which preventive education and dental care must be built to increase the opportunity for life-time freedom from preventable oral diseases. The purpose of this study was to assess the comprehension of Saudi parents towards the use of fluoride and fissure sealants, and to determine the factors that influence their comprehension. This was a cross-sectional study of Saudi parents. A self-administered questionnaire was collected from 206 parents of outpatients attending the pediatric dentistry clinic of King Saud University in Riyadh, Saudi Arabia. In addition to the demographic questions, we investigated the knowledge and attitude of Saudi parents toward their children's use of fluoride and fissure sealants. In the present study, most of the parents a high-school education. Most of the parents (68.4%) had a favorable attitude toward the use of fluoridated gels for their children, while only 39.8% had a positive opinion regarding fluoridated water. The satisfaction levels were very high regarding fluoridated mouth rinses and fluoridated gels (69.4% and 68.4%, respectively). Satisfaction with fissure sealant was split almost equally (55.3% were "pleased" and 44.7% "not pleased"). The most important source of parental oral health knowledge was dentists (82%). The present study found that parents have a low opinion of fluoride and fissure sealants for their children. Therefore, greater effort should be made by professional organizations and government agencies to inform parents of the benefits of sealants and fluoridated products to prevent dental caries in children.

KEY WORDS: CARIES, FISSURE SEALANTS, FLUORIDES, PEDIATRIC DENTISTRY, PREVENTIVE DENTISTRY.

INTRODUCTION

Children's oral health is the foundation on which preventive education and dental care must be built to increase the opportunity for life-time freedom from preventable oral diseases (Nagarajappaet al., 2013). Preventive procedures must be started in early years of life (Thakareet al., 2012). The use of preventive treatment modalities in European and other developed countries is more than 50%, whereas there are few published reports on the use of preventive dental modalities in Saudi Arabia (Al-Shalan and Wyne 2002; Hamasha et al., 2019).

In Saudi Arabia, a significantly high prevalence of dental caries has been reported in children, adults, and older individuals. These groups have shown a higher prevalence and severity of caries over the past few decades (Al-Ansari, 2014). The use of caries preventive approaches, such as community water fluoridation, topical fluoride therapy, plaque control, and dietary sugar control has been generally seen to be the cause of the overall decline of caries prevalence, which in turn has had a greater effect on smooth-surface caries reduction (Kitchens, 2005). Exposure to fluorides plays a major role in preventing and reducing caries experience, with strong evidence for the effectiveness of both fluoridated water and toothpastes (Gussy et al., 2008).

In recent years, the importance placed on the systemic protective effect of fluoride against caries has significantly waned. Re-analysis of data from water fluoridation trials supports the presence of the post-eruptive effect of fluoride. It appears that teeth erupting during a period of water supplementation receive a measure of caries protection that would most likely be topical in nature (Adair, 2006). The primary caries preventive effects of fluoride result from its contact with enamel and through

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its antibacterial properties. Therefore, therapeutic use of fluoride for children should focus on regimens that maximize topical contact, preferably in low-dose, high-frequency approaches (Adair, 2006). Topical application of fluoride by a dentist four times a year has been reported to result in an 86% reduction in dental caries (Donald et al., 2000). The plaque-retentive nature of pits and fissures make them difficult to clean, causing them to be more susceptible to caries than smooth surfaces and possibly not to be protected by fluoride administration (Kitchens, 2005). The and, since fluoride exposure is mostly on smooth surfaces and more than 50% of dental caries in under-20-y-olds occurin the dental grooves, the use of fissure sealants (FSs) is recommended as another preventative (Ahovuo-Salorantaet al., 2016).

It is well documented that sealants are more effective than topical fluoride in preventing occlusal caries (Jafari et al., 2010). Pit-and-fissure caries accounts for about 90% of caries in permanent posterior teeth and 44% of caries in primary teeth (Beltran-Aguilar et al., 2005) Moreover, the application of resin-based FSs on permanent teeth (first molars) has been reported to reduce caries from 86% in the first year, to 78.6% in the second year, and 58.6% in the fourth year (Beauchamp et al.,2008). Sealant application is a preventive conservative approach involving the introduction of sealants into the pits and fissures of caries-prone teeth. This sealant then bonds to the tooth micromechanically, providing a physical barrier that keeps bacteria away from their source of nutrients (Simonsen, 1978). Despite the overall increase in sealant use and the efficacy and caries-preventive effect of pit and fissure sealants being well documented, they are still considered to be underused worldwide(Riley et al., 2010;Tellez et al., 2011).

Since preventing dental caries is a huge challenge for the public, increasing parental knowledge and using preventive methods, as practiced in developed countries, may lead to decreased dental caries and improved health of children (Daly et al.,2002).Parents are responsible for their child's oral health care. Preschool children are not capable of brushing themselves and lack the manual dexterity and the psychological maturity to understand the importance of maintaining oral health. With changing lifestyles, a trend of having a single child, and increased cost of living, most parents are working with very little time left for performing day to day oral health care practices in their child's early years (Maniet al., 2011). Especially in preschool children, the parental role is the most important aspect of maintaining good oral health (Castilho et al., 2013; Hamasha et al., 2019).

In view of the high caries prevalence in Saudi children, it is important to carry out such studies evaluating the comprehension of Saudi parents about various caries preventive modalities available (Al-Ansari, 2014). However, such studies are rare in Saudi Arabia. The objective of the present study was to assess the comprehension of Saudi parents about the use of fluoride, fissure sealants and other preventive modalities for their children in Saudi Arabia.

MATERIAL AND METHODS

This cross-sectional study was conducted among the parents of children who attended the Pediatric Dentistry Clinics of King Saud University College of Dentistry in Riyadh, Saudi Arabia. The research proposal was submitted to the Institutional Review Board and Ethics Committee of the College of Dentistry Research Center (CDRC) and approvals (19/0315/IRB) were obtained. The sample size for the study was estimated through power 0.89 and $\alpha = 0.05$ (maximum difference, 0.9). The sample size was determined to be a minimum of 200. Participation in the research was on a voluntary basis. Informed consent was obtained from each participant before commencement of the study, and no risks to the participants were anticipated. Inclusion criteria were Saudi parents who were able to answer the questionnaire, and whose children were patients at pediatric dentistry clinics. The exclusion criterion was parents not agreeing to participate in the study.

The questionnaire was constructed in English before being translated into the local language (Arabic) and then back to English to ensure accuracy. The parents were asked to complete a 23-item questionnaire to elicit information in the following areas:a. Demographics (age, gender, number of years of education of both parents, number of children).b. Dental history (last dental visit of their children and reason for visit). c. Personal use of fluoridated mouth rinses.d. Source of information about oral health (media, Internet, dentist, friends).e. Attitude toward fissure sealants and fluoridated water, toothpastes, gels, mouth rinses, and other fluoridated products.f. Satisfaction with fluoridated water, fluoridated gels and mouth rinses, and fissure sealants.

A pilot study was conducted on 10 parents not participating in the main study to check the validity and reliability of the questionnaire; changes were made accordingly. The pilot study responses were not considered in the main study.The data were analyzed using SPSS pc+ version 22.0 statistical software (IBM Inc., Chicago, Ill, USA). Descriptive statistics (mean, standard deviation, frequencies, and percentages) were used to describe the quantitative and categorical variables. The chi-square test was used to determine the significant difference between the responses.Confidence was kept at 95% and a P-value ≤ 0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

A total of 206 parents consented and then completed the study questionnaire. Table 1 summarizes the demographic information and their children dental office visits. Mothers mostly (60.2%) completed the questionnaires. The average age of most (52%) of the parents was 30 to 39 years. About three-fourths (73.3%) of the parents had university-level education. Three in every four (75.2%) families had three or more children.

Table 1. Summary of main demographic and their children dental office visits		
Parents Gender	Females	124 (60.2%)
	Males	82 (39.8%)
Parents Age	20-29	39 (18.9%)
	30-39	107 (51.9%)
	40 and older	60 (29.1%)
Education level of parents	Below high school	22(10.7%)
	High school	33(16%)
	University degree	151(73.3%)
Number of children in family	1	15(7.3%)
	2	36(17.5%)
	More than 3	155(75.2%)
Time of last dental visit for child	Less than year	104 (50.5%)
	Between one and two years	44(21.4%)
	More than two years	58(28.2%)
Reason for last visit	Check-up	50(24.3 %)
	Emergency	33(16%)
	Routine Treatment	123(59.7%)
Source of parents' oral knowledge	Dentist	169(82%)
	Internet	19(9.2%)
	Media	8(3.9%)
	Friends	10(4.9%)

Table 2. Knowledge of parents for definition of fissure sealants

Which one is the definition of fissure sealants	Father n (%)	Mother n (%)	Total n (%)
Covering carious fissures of tooth crown by mercury	1(0.5%)	2(1%)	3(1.5%)
Covering deep carious fissures by tooth color material	10(4.9%)	14(6.8%)	24(11.7%)
Covering deep normal fissures of tooth crown by tooth color material as a foundation	7(3.4%)	24(11.7%)	31(15%)
Covering all of the tooth crown by metal sheets to prevent dental caries	7(3.4%)	13(6.3%)	20(9.7%)
I do not know	57(27.7%)	71(34.5%)	128(62.1%)
Total n (%)	82(39.8%)	124(60.2%)	206(100%)

Almost half the children (50.5%) had visited a dentist during the past year. The main reason for the dental visits was routine dental treatment (59.7%).Also shows various sources of parental oral heath knowledge.

Dentists were the source for most of the parents (82%), followed by the Internet (9.2%), friends (4.9%), and media (3.9%). Overall knowledge of parents about the definition of fissure sealants was poor. However, mothers had better knowledge than did the fathers (Table 2). Most (72.8%) of the parents supported the use of fluoridated toothpastes for their children (Table 3). Almost half the parents did not know about fluoridated water or fissure sealants. Nevertheless, a majority (59.7%) supported the use of topical fluoride products.

Table4 shows the parents' satisfaction levels about the four methods of caries prevention. With the exception of fluoridated water wherein most (60%) parents were "not pleased" by the other three methods, most showed "pleased" results, that is, 55.3% for fissure sealants, 68.4% for fluoridated gels, and 69.4% for fluoridated mouth rinses. The parents' own dental experience had no significant effect on their attitude towardthe use of fluoride and fissure sealants for their children (Table 5). Parents who visited their dentist recently tended to have a higher satisfaction for fissure sealants, fluoride gels, and mouth rinses. Parents who visited the dental clinic for routine examinations demonstrated a higher level of satisfaction for fluoride gels and mouth rinses than those whose reason for their last dental visit was a checkup or

emergency treatment. There was a tendency for higher satisfaction with fissure sealants among parents who visited their dental clinic for a routine examination (Pearson chi-square, P = 0.05, Table 5).

Table 3. Parents' personal attitude towards the use of fluoride for caries prevention in children

Variables	Responses			
	For n (%)	Against n (%)	Do not Know n (%)	Did not replay n (%)
The parent's Personal attitude to Topical Fluoride products	123(59.7%)	7(3.4%)	58(28.2%)	18(8.7%)
The parent's Personal attitude to Water Fluoridation	51(24.8%)	23(11.2%)	103(50%)	29(14.1%)
The parent's Personal attitude to Fluoridated toothpaste	150(72.8%)	14(6.8%)	30(14.6%)	12(5.8%)
The parent's Personal attitude to Fissure Sealant	84(40.8%)	6(2.9%)	96(46.6%)	20(9.7%)

Table 4. Parents' satisfaction level from (four) means of caries prevention.

Variables	Responses	
	Pleased n (%)	Not Pleased n (%)
The parent's satisfaction level from Water Fluoridation	82(39.8%)	124(60.2%)
The parent's satisfaction level from Fissure Sealant	114(55.3%)	92(44.7%)
The parent's satisfaction level from Fluoridated gels	141(68.4%)	65(31.6%)
The parent's satisfaction level from Fluoridated mouth rinse	143(69.4%)	63(30.6%)

The attitude toward preventive measures was related to parents' gender, age, and level of education. More mothers (61%) supported the use fissure sealants for their children than did the fathers (39%), and the difference was statistically significant (P = 0.019, Pearson chi-square). Only 41% of the parents who had university degrees and 45.5% who had less than a high school diploma supported the use of fissure sealants. Families with three or more children demonstrated higher support for topical fluoride products than small families (69.1% vs. 30.9%), but the difference was not statistically significant (P = 0.269, Pearson chi-square). Larger families were also correlated with their parents' satisfaction with fluoride gels. Most parents with three or more children were satisfied with fluoride gels than were families with a smaller number of children (72.3% vs. 27.6%), but the difference was not statistically significant (P = 0.324, Pearson chi-square). Further, most parents with large families were satisfied with mouth rinses (73.4% vs. 26.6%), but the difference was not statistically significant (P= 0.568, Pearson chi-square).

Studies about parents' comprehension of their children's oral health are scarce in Saudi Arabia. Increasing parental comprehension about their children's oral health in developed countries has led to a decrease in dental caries and improved health of their children (Khan et al., 2009). The aim of this study was to evaluate Saudi parents' comprehension of preventive dental measures. Health behaviors established in childhood have implications not only for their current oral health, but also as they grow up into adulthood (Saldunaite et al., 2014). Considering parents' central role in ensuring the well-being of young children, it is important to explore their attitude toward preventive oral health measures.

The results of the present study have provided important information in this area.Fissure sealants serve as important caries-preventive measures for children. It is recommended by the American Dental Association (ADA) and the American Academy of Pediatric Dentistry (AAPD) that dental fissure sealants be placed on a primary or permanent tooth when it is determined that the tooth is at risk for developing dental caries in these sites (Crall and Donly, 2015). The present study showed that most of the parents had limited knowledge aboutfissuresealants as a preventive measure. Results of a similar study were the same (Tahaniet al.,2017).

It has been reported that the most significant factors in sealant awareness are dentists—the main source of dental information for parents and frequency of visits for children (Mafeni and Messer ,1994). Most of the parents in this study were highly educated, and they had better knowledge about their children's oral health. Other studies have reported a significant correlation between parents' educational level and their knowledge about preventive dental measures(Jafari et al., 2010; Tahaniet al.,2017). This observation might be explained by the fact that people with a higher education have more of a chance to receive and understand information about preventive dental programs (Kazemi, 2012).

In the present study, the majority of parents (82%) received preventive dental information from dentists; those receiving the information from dentists had better preventive knowledge. This is in agreement with the results of other studies (Mafeniand Messer,1994; Jafari et al., 2010). This could be attributed to the effectiveness of face-to-face education by the dentists (Kay and Locker ,1998). Considering the proven effectiveness of media in oral health education as reported by several studies, use of thissource of information should also be encouraged was poor (Mårtensson et al., 2006; Gholami et al., 2014; Hamasha et al., 2019).

Table 5. Relationship between the parents' dental experience for their children and opinion about the four methods of caries prevention.

Variables	Parents opinion about the four methods of caries prevention.																				Satisfaction to four methods of caries prevention.											
	The parent's Personal attitude to Topical Fluoride products					The parent's Personal attitude to Water Fluoridation					The parent's Personal attitude to Fluoridated tooth paste					The parent's Personal attitude to Fissure Sealant					Parent's satisfaction level from Fluoridation Water			Parent's satisfaction level from Fissure Sealant			Parent's satisfaction level from Fluoridated gels			Parent's satisfaction level from Fluoridated mouth rinse		
	against		Don't know	Did no replay		Against		Don't know	Did no replay		Against		Don't know	Did no replay		Against		Don't know	Did no replay	p-value	Pleased	Not Pleased	p-value	Pleased	Not Pleased	p-value	Pleased	Not Pleased	p-value	Pleased	Not Pleased	p-value
Last dental visit																																
less than one year	63	2	30	9		31	11	50	12		71	8	17	8		50	3	41	10		45	59		63	41		74	30		67	37	
between 1-2 year	24	3	10	7	0.274	9	6	22	7	0.717	34	3	4	3	0.611	13	2	25	4	0.400	14	30	0.429	25	19	0.151	29	15	0.700	32	12	0.275
More than 2 years	36	2	18	2		11	6	31	10		45	3	9	1		21	1	30	6		23	35		26	32		38	20		44	14	
Reason for last visit																																
Check-up	32	1	12	5		13	3	26	8		37	2	8	3		24	1	23	2		18	32		31	19		35	15		33	17	
Emergency	21	0	11	1	0.584	12	4	14	3	0.510	28	3	1	2	0.430	15	2	13	3	0.460	13	20	0.800	12	21	0.050	21	12	0.805	19	14	0.661
Routine treatment	70	6	35	12		26	16	63	18		85	9	21	8		45	3	60	15		51	72		71	52		85	38		91	32	

*Statistical significant difference at P<0.05

About half the children visited the dentist during the past year, encouraging dentists to provide oral health education about preventive measures.A great majority of parents with three or more children were satisfied with fluoridated products, including topical fluorides and fluoridated mouth rinses. A higher number of siblings is known to be one of the risk factors for high caries experience in children (Sujlana and Pannu, 2015). The satisfaction rate among parents with large families is encouraging and symbolizes the change in their state of mind and a better understanding of the advantages of preventive measures.

Parents who are aware of their own oral health and visit their dentist on a regular basis exhibited greater satisfaction from the use of topical fluorides for their children. Parents who visit their dentists frequently are exposed to preventive information that may affect their children's oral health. Therefore, it is essential to increase dentists' awareness toward educating parents about the ways of preventing dental diseases in their children. More research is needed to determine parental comprehension of the issues related to their children's oral health.

Due to the low knowledge of parents about the four methods of caries prevention, it is necessary for professional organizations and government agencies to inform them of the benefits of sealants and fluoridated products to improve the oral health of their children.

Most parents support using resin fissure sealants as an overall acceptable procedure, with their acceptance improving with increased treatment experience. On the other hand, since the main source of preventive information comes from the dentists, it appears that increasing dentists' knowledge and asking them to offer prevention education to parents would be a sensible approach. Greater effort should be made by health care providers and government organizations to impart primary dental care knowledge to parents, as they have greater influence on their children.

CONCLUSION

The parental comprehension about fluoride and fissure sealants was low. Greater effort should be made by the professional organization and governmental agencies to inform parents of the benefits of fissure sealants and fluoridate products in prevention of dental caries in children was inadequate.

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Biotechnological Communication

Utilization of ITS-Based DNA Bar code for Classification of Different *Panax* species in Vietnam

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ABSTRACT

Several species in *Panax* genus have been identified and utilized as important traditional medicines in Vietnam for decades. Due to the varying degrees of scarcity and value of medicinal herbs, different *Panax* types are often blended to provide illegal benefits. Current morphology-based identification of *Panax* species is low accuracy. In this study, Internal Transcribed Spacer (ITS) regions of 23 *Panax* samples in Vietnam were amplified and sequenced. The obtained sequences were then searched for homology in NCBI Genbank to identify Latin names. The genetic relatedness of ITS sequences were then analyzed by phylogenetic analysis. The results show that 23 studied samples belong to four *Panax* species. The sequence alignment also reveals the distinct variation in ITS regions of *Panax* species in Vietnam in the comparison to ITS sequences of *Panax* species from other countries. The obtained results show high effectiveness of using ITS in distinguishing Panax species in Vietnam. Furthermore, the variation within this region could be developed into specific marker for authenticating ginseng-derived products.

KEY WORDS: DNA BARCODE, GINSENG, IDENTIFICATION, ITS, PANAX SPECIES.

INTRODUCTION

Ginseng is the common name of the species in the *Panax* genus Araliaceae. This plant has been used as medicine for a long time as its outstanding medicinal properties include stimulating metabolism in the body, regulating blood sugar in the body, and protecting the activity of the central nervous system, counteract the formation and inhibit the growth of tumors, increase the body's resistance to diseases (Ali et al., 2012; Peng et al., 2012; Lee et al., 2015). High medicinal activity of ginseng is due to the plant's high ginsenoside content, it is also rich in other valuable ingredients such as anti-oxidant compounds, peptides, polysaccharides, and vitamins (Lee et al., 1995; Hong et al., 2012). So far, at least 17 *Panax* species have been reported worldwide (Zhang et al., 2000) and medicinal compounds are variable from species to species (Harkey et al., 2001; Liu et al., 2016, Hyeonah et al., 2021).

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Currently, ginseng identification and classification is mainly based on morphological characteristic since it is straight forward to perform and feasible to carry out on the field at a low cost (Proctor et al., 2011; Hong et al., 2012). Nevertheless, significant limitations of this method have been addressed such as low number, complex inheritance pattern, and vulnerable to changes in the environment (Ahmedand and Mohamed, 2014). Consequently, ginseng authentication and cultivar identification based on this method are unreliable.

With the rapid development in molecular biology, DNA-based markers have been used intensively for characterizing the genetic relationships among plant species due to their fast, specific, reliable, and sensitive features (Shim et al., 2003; Serrone et al., 2006; Kim et al., 2016). Recently, the DNA barcode has been used intensively for classifying at the species level (CBOL Plant Working Group, 2009). This marker type has been applied to discriminate at species level for different organism such as plants, animals, fungi and bacteria (Hollingsworth et al., 2011; Barcaccia et al., 2016; Hu et al., 2019).

Among several DNA barcode loci, Internal Transcribed Spacer (ITS) region has been proposed as a standard region for plant identification because the ITS region is highly conserved among interspecies but variable between inter Species and is easy to amplify even from limited DNA quantity (Chase et al., 2007; Giudicelli et al., 2015). Numerous researchers reported that discrimination power of ITS is higher in the comparison to other plastid markers (Muellner et al., 2011; Yang et al., 2012; Zhang et al., 2014). So far, several studies have exploited the ITS-based DNA barcode in the investigating the genetic composition and developing protocols to differentiate species in *Panax* genus. In 2011, a study in India showed the effectiveness of ITS in clustering analysis of *Panax assamicus* populations according to geographic locations (Panday and Ali, 2011).

In Saudi Arabia, Ali and colleagues applied ITS2 sequence to control ginseng-derived products (Ali et al., 2012). Bang and colleagues used ITS to differentiating and authenticating *Panax* species in Korea (Bang et al., 2015). More recently, this barcode region was also applied to characterize genetic diversity of 24 ginseng samples collected at Lai Chau province of Vietnam (Pham et al., 2018). The present study describes the effectiveness of ITS-based DNA barcode in classifying 23 *Panax* samples belong to different species collected in different geographical locations in Vietnam. The obtained data could be applied for classification, identification, and authentication activities in *Panax* plants of Vietnam.

Figure1: Map showing locations for collecting Panax samples.

MATERIAL AND METHODS

Sample collection: Total of 23 *Panax* samples were collected from North to South of Vietnam, samples were collected from different sources such as *Panax* traders, research institutes, universities, and companies in different locations (Figure 1 and Table 1). Collected samples were then store in cool and dry condition and targeted for DNA extraction.

DNA extraction: DNA from *Panax* samples extracted by cetyltrimethyl ammonium bromide (CTAB) method followed Li et al. (2013). After extraction, DNA quality was examined by electrophoresis on 1% agarose then spectrophotometer (Optima SP 3000 nano UV-VIS, Japan) was used to determine DNA concentrations. The DNA samples were then kept at -20 °C freezer until use for PCR reactions.

Table 1. List of collected Panax samples used in this study.

No.	Sample ID	Local name	Collection location
1	TTB1	Tam that bac	Quang Nam province
2	TTB2	Tam that bac	Quang Nam province
3	TTH1	Tam that hoang	Lao Cai province
4	TTH2	Tam that hoang	Yen Bai province
5	TT1	Tam that	Quang Nam province
6	TT2	Tam that	Quang Nam province
7	TT3	Tam that	Quang Nam province
8	TT4	Tam that	Quang Nam province
9	TT5	Tam that	Quang Nam province
10	TT6	Tam that	Quang Nam province
11	TT7	Tam that	Quang Nam province
12	TT8	Tam that	Quang Nam province
13	TT9	Tam that	Quang Nam province
14	TT10	Tam that	Quang Nam province
15	SLC1	Sam Lai Chau	Lai Chau province
16	SLC2	Sam Lai Chau	Lai Chau province
17	SNL1	Sam Ngoc Linh	Ho Chi Minh city
18	SNL2	Sam Ngoc Linh	Quang Nam province
19	SNL3	Sam Ngoc Linh	Quang Nam province
20	SNL4	Sam Ngoc Linh	Quang Nam province
21	SVD1	Sam vu diep	Yen Bai province
22	SVD2	Sam vu diep	Lao Cai province
23	SVD3	Sam vu diep	Yen Bai province

PCR reactions and DNA sequencing: The composition of PCR reactions to amplify ITS region as follows: 7.5 µL 2X Mytaq Red Mix (Bioline, UK), 20 ng DNA, 0.2 µM of each primer (C26A 5' AGGAGAAGTCGTAACAAG3' and N-nc18S10 5' GTTTCTTTTCCTCCGCT 3') (Wen and Zimmer, 1996), and PCR water for a final volume of 15 µl. The PCR cocktails were run in thermal cyclers SureCycler 8800 Thermal Cycler (Agilent, USA) with following conditions: initial denaturation at 94 °C for 2 minutes; then repeated by 35 cycles of 30 seconds at 94 °C, 30 seconds at 55 °C, 50 seconds at 72 °C, and finally one minute at 72 °C to complete the reaction. The PCR products were visualized on gel electrophoresed and use 1 kb ladder (Bioline, UK) to determine amplification length.

Correct PCR products were sequenced by Sanger methods at Nam Khoa Company (Ho Chi Minh City, Vietnam). Each sample were sequenced for both sense and antisense

directions. Nucleotide sequences of both DNA strands were obtained and compared the forward and reverse sequence to ensure accuracy. The finally assembled sequences were submitted to NCBI GenBank to obtained accession number (Table 2) and then used for following analysis. Data analysis: The homology identification of each sequence were implemented by using Basic Local Alignment Tools (BLAST) function in NCBI (National Center for Biotechnology Information, USA) database. To evaluate the capacity of ITS based DNA barcode in distinguish *Panax* accessions from Vietnam and abroad, additional ten ITS sequences of related *Panax* species retrieved from Genbank. All sequences were aligned using the Clustal method in Molecular Evolutionary Genetics Analysis (MEGA) 6 software (https://www.megasoftware.net). Evolutionary trees were constructed based on two methods consisting of Maximum Likelihood (ML) and Neighbour Joining (NJ) since they represent for discrete character methods and distance methods, respectively. For satisfactory reliability in phylogenetic construction, bootstrap value was set at 1000 replicates and bootstrap support was classified as Kress et al. (2002).

Table 2. Genbank accession numbers of samples and corresponding Latin names.

No.	Sample ID	Length (bp)	Accession number	Latin name	Percent identity (%)
1	TTB2	704	MZ149934	*Panax notoginseng*	100
2	TTB1	707	MZ149935	*Panax notoginseng*	99.86
3	TTH1	703	MZ149936	*Panax stipuleanatus*	99.72
4	TTH2	631	MZ149937	*Panax stipuleanatus*	99.68
5	TT1	705	MZ149938	*Panax japonicus* var. *bipinnatifidus*	99.43
6	TT2	702	MZ149939	*Panax japonicus* var. *bipinnatifidus*	99.57
7	TT3	702	MZ149940	*Panax japonicus* var. *bipinnatifidus*	99.57
8	TT4	702	MZ149941	*Panax japonicus* var. *bipinnatifidus*	99.57
9	TT5	702	MZ149942	*Panax japonicus* var. *bipinnatifidus*	99.57
10	TT6	702	MZ149943	*Panax japonicus* var. *bipinnatifidus*	99.57
11	TT7	701	MZ149944	*Panax japonicus* var. *bipinnatifidus*	99.43
12	TT8	702	MZ149945	*Panax japonicus* var. *bipinnatifidus*	99.57
13	TT9	702	MZ149946	*Panax japonicus* var. *bipinnatifidus*	99.57
14	TT10	702	MZ149947	*Panax japonicus* var. *bipinnatifidus*	99.57
15	SLC1	700	MZ149948	*Panax vietnamensis* var. *fuscidiscus*	99.86
16	SLC2	630	MZ149949	*Panax vietnamensis* var. *fuscidiscus*	99.52
17	SNL1	704	MZ149950	*Panax vietnamensis*	100
18	SNL2	701	MZ149950	*Panax vietnamensis*	99.71
19	SNL3	702	MZ149951	*Panax vietnamensis*	99.72
20	SNL4	705	MZ149952	*Panax vietnamensis*	99.86
21	SVD1	698	MZ149953	*Panax stipuleanatus*	100
22	SVD2	703	MZ149954	*Panax stipuleanatus*	99.72
23	SVD3	642	MZ149955	*Panax stipuleanatus*	99.53

RESULTS AND DISCUSSION

PCR and DNA sequence: Although three are few study reported the difficulty in amplifying and sequencing ITS region (Gonzalez et al., 2009; Wang et al., 2016), all PCR and sequencing reactions in this study were performed successfully. The sequences length is 693 bp on average, varying from 631 bp to 707 bp. The similarity of obtained sequences to homologous sequences in NCBI Genbank ranges from 99.52 to 100%. The Latin names of collected samples were identified and presented in Table 2.

After identification, 23 samples were found belonging to four species consisting of *Panax notoginseng, Panax stipuleanatus, Panax japonicus* var bipinnatifidus, *Panax vietnamensis* var. *fuscidiscus*, and *Panax vietnamensis* Thus, two different plant groups in Vietnam local names consisting of "Tam that hoang" including TTH1 and TTH2 samples and "sam vu diep" including SVD1, SVD2 and SVD3 samples were identified as *Panax stipuleanatus*. This confirm the low accuracy in traditional method for *Panax* classification due to several uncontrollable factors such as changes of environmental conditions and developmental stages (Ali et al., 2012).

Sequence alignment and Phylogenetic analysis: Together with 23 obtained sequences from this study, additional 10 ITS sequences from different species in *Panax* genus were downloaded from NCBI Genbank and combined for sequence alignment and the alignment summary is presented in Table 3. Totally, up to three 33 variation sites were detected within analyzed sequences.

Table 3. Variable sites in 33 ITS sequences from different Panax species.

Consensus	A C A T G G A C G T G C C A C A A C A T C C G C C C A G C G C C C
TTB2	. . . . .
TTB1	. . . . .
TTH1	. . . C A A . T A . T T . . T G . . C G . T . . . G . T A . . .
TTH2	T . . C A A . T A . T T . . T G . . C G . T . . . G . T A . . .
TT1	. T C . . . . . A . . . T . . . . . T . . . T T . . . . .
TT2	. T C . . . . . A . . . T . . . . . T . . . T T . . . . .
TT3	. T C . . . . . A . . . T . . . . . T . . . T T . . . . .
TT4	. T C . . . . . A . . . T . . . . . T . . . T T . . . . .
TT5	. T C . . . . . A . . . T . . . . . T . . . T T . . . . .
TT6	. T C . . . . . A . . . T . . . . . T . . . T T . . . . .
TT7	. T C . . . . . A . . . T . . . . . T . . . T T . . . . .
TT8	. T C . . . . . A . . . T . . . . . T . . . T T . . . . .
TT9	. T C . . . . . A . . . T . . . . . T . . . T T . . . . .
TT10	. T C . . . . . A . . . T . . . . . T . . . T T . . . . .
SLC1	C T . . . . . T G . . G T . . . . . A . . . . .
SLC2	T T . . . . . T G . . G T . . . . . A . . . . .
SNL1	C T . . . . . T G . . . T . . . . . T . .
SNL2	. T . . . . . T G . . . T . . . . . T . .
SNL3	. T . . . . . T G . . . T . . . . . T . .
SNL4	. T . . . . . T G . . . T . . . . . T . .
SVD1	. . . C A A . T A . T T . . T G . . C G . T . . . G . T A . . .
SVD2	. . . C A A . T A . T T . . T G . . C G . T . . . G . T A . . .
SVD3	C . . C A A . T A . T T . . T G . . C G . T . . . G . T A . . .
AJ786235.1*	C T . . . . . A . . A T . . . . . T . . T . . . . . T .
MH711079.1*	T T . . . . . A . . . T . . . . . A . . . . . T T
U41683.1*	G T . . . . . A . . . T . . . . . T T
U41686.1*	G T C . . . T . A . . . T . . . . . T . . A . . . . .
HQ112438.1*	T . . C A A . . A C T T . . G . . C G . T . . . . . T . . T .
HQ112440.1*	T T . . . . . A . . . T . . . . . T . . . . . T .
AY271920.1*	G T . . . . . A . . . T . . . . . A T . . . . . T .
HQ112445.1*	T . . C A C G . . C . T . . . T . . C G . . C . . . . .
MK408809.1*	T T C . . . . . A . . . . . T . . . . .
MK408808.1*	T T . . . . . T G . . G T . . . . . A . . . . .

(*: sequences were retrieved from NCBI Genbank)

Previously, several research groups have employed the nucleotide variations to develop molecular makers for plant identification such as in *Taxus* (Liu et al., 2011) or onion (Ipek et al., 2014). Thus, these variations are valuable for developing specific markers or diagnosis KIT for discriminating different *Panax* species. The genetic distances among 23 *Panax* samples were calculated based on Kimura-2 parameter (K2P) method of MEGA 6 software. The lowest genetic distance is 0.000 indicating the sequence similarity among samples, while the highest is 0.052 this result showing the close genetic relationship among these accessions when using ITS regions. The phylogenetic trees constructed by either neighbor-joining (NJ) or Maximum Likelihood methods are shown in Figure 2 and Figure 3, respectively.

In general, the results show the high similarity between Neighbor-Joining and Maximum Likelihood methods. *Panax* samples collected from Vietnam in this study is clustered into several groups corresponding to its identified species using NCBI BLAST. Interestingly, Vietnam ginsengs are also separated from different ginseng species collected from different countries. The grouping of *Panax* species in obtained phylogenetic trees of this study is slightly different with previous study of Yang and colleagues in Korea in which they reported the closer clustering of *P. notogingsen and P. pseudoginseng* as well as *P. omeiensis* and *P. zingiberensis* (Yang et al., 2001).

Figure 2: Neighbor-Joining tree with 1000 bootstrap replicated based on ITS sequences (Star symbols indicating sequences retrieved from NCBI Genbank and the availability of common names are shown in parentheses).

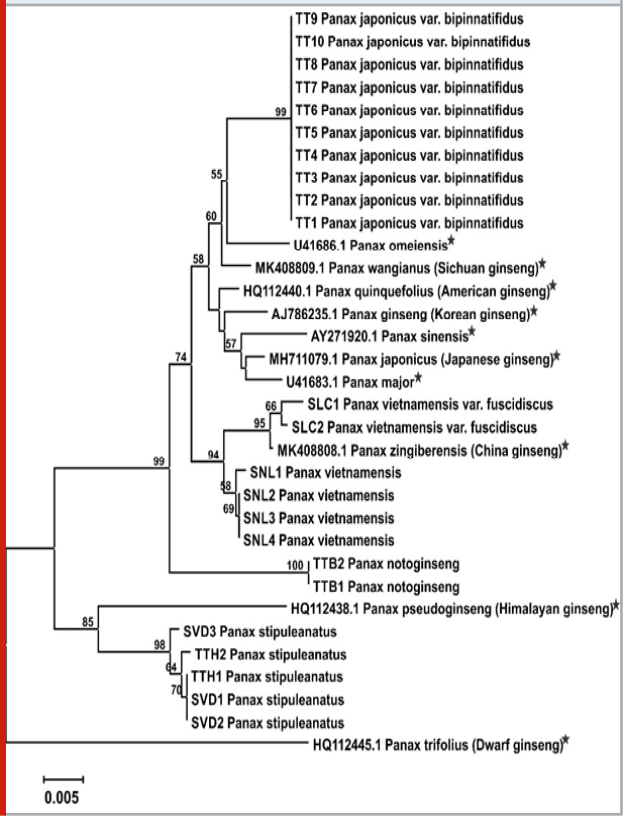
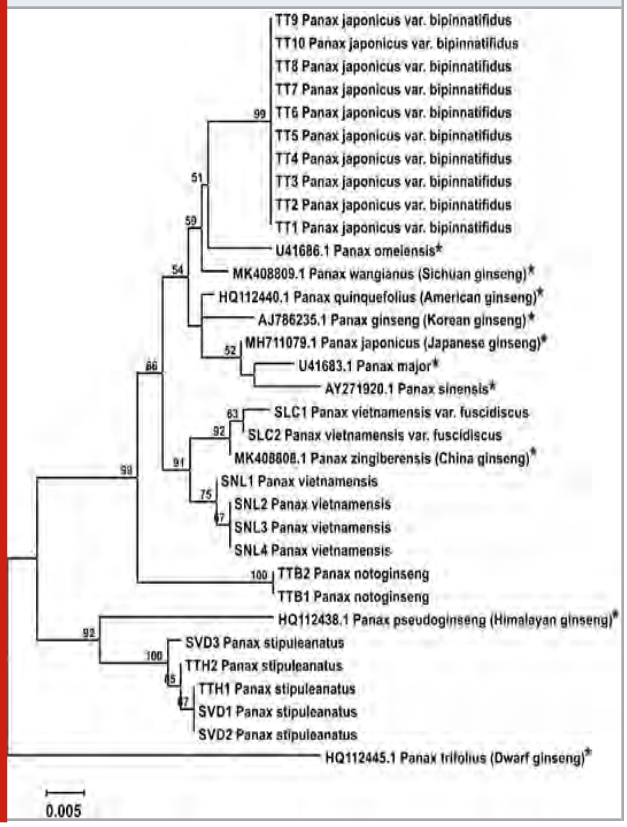


Figure 3: Maximum Likelihood tree with 1000 bootstrap replicated based on ITS sequences (Star symbols indicating sequences retrieved from NCBI Genbank and the availability of common names are shown in parentheses).



CONCLUSION

The obtained data from this study confirms the effectiveness of using ITS marker to classify *Panax* samples in Vietnam. Furthermore, this marker also shows high discrimination power to distinguish *Panax* species from Vietnam and from abroad. Future study should focus on develop specific primer pairs or diagnosis KIT to serve for identification and authentication activities of *Panax* plants.

Ethics approval and consent to participate: Not applicable.

Conflict of Interests: The author has no conflict of interest.

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Ecological
Communication

Peculiarities of the Population Dynamics of Brown-Tail Moth *Euproctis chrysorrhoea*, in the Plantations of Penza Region of Russia

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ABSTRACT

Overall, the study of the population dynamics of brown-tail moth (*Euproctis chrysorrhoea* L.) in the Penza region can help identify the main factors which affect the change in the number of the pest. This scientific approach is necessary for the development of effective methods for the protection of forest plantations.Hence, the chief purpose of this study is to investigate the peculiarities of the population dynamics of brown-tail moth in the plantations of the Penza region. In order to meet the purpose of the study, the examination of the population dynamics of brown-tail moth was carried out in 2018-2020 on permanent stationary test plots laid in various forest inventory and forest growing conditions in Penza region. The solution to this issue was on the basis of quantitative counts with a simultaneous assessment of the identified mortality factors at different age intervals. Arrangement and sampling of trial units was random and systematic according to the existing statistical methodology. Furthermore, the authors applied generally accepted and specially developed techniques. As a result of the study, examining the population dynamics of brown-tail moth in the plantations of the Penza region led to recognizing 19 different factors of the biotic environment. The regulating role of each of them was established at a specific stage of development of brown-tail moth during ontogenesis. In conclusion, it can be inferred that these results and findings of the study can grant a considerable chance to consider and perform forest protection measures against phytophagous organisms. Some feasible and practical measures are recommended at the end of the article that can be applied in future researches.

KEY WORDS: ENTOMOPHAGES; POPULATION DYNAMICS; POPULATION; SURVIVAL RATE; THE BROWN-TAIL MOTH.

INTRODUCTION

The brown tail moth (BTM) is a forest and ornamental pest in Russia and the United States. Its ultimate polyphagia and documented phonological shift related to host use recommend the appearance of distinct host races ((Iliiinskii, 1965; Wu et al., 2020). Population genetic variety indices reveal a higher genetic diversity in Russia, predominantly in the samples from Penza region in Russia (Sheikhshoaie et al., 2018; Ali et al., 2020). The region has all the conditions for the development of brown-tail moth, and in the event of its massive foci, it can cause significant damage to trees and shrubs. Based on the literary sources, brown-tail moth is a poorly studied species throughout the growing area of deciduous plantations (Dubrovin, 2011; Dubrovin, 2016; Sajjadi and Moosavi, 2019; Ramachandran and Rosen, 2020). In the Penza region, this species has practically not been studied (Tachi et al., 2021). Given the necessity of this matter, the objective of this work is to study the factors of the population dynamics of brown-tail moth population that affect change in the insect population per generation to use the data obtained in the development of effective methods for protecting forest plantations.

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MATERIAL AND METHODS

As mentioned earlier, to carry out this survey, the study of the population dynamics of brown-tail moth was conducted over the years 2018-2020 on permanent stationary test plots laid in various forest inventory and forest growing conditions in Penza region. Field survey

data were supplemented by laboratory raising of insects. At the same time, a fixed number of the studied phase of ontogenesis was taken, and the percentage of transition of pests to the next phase of development was determined during the observation periods.The transition to 10% of individuals was noted as the beginning of the next phase of ontogenesis, the transition to 50% of individuals - the mass appearance of the phase, the transition to 80% of individuals - the end of the development period of the phase under study.Thus, the egg mortality was determined by the ratio of hatched caterpillars to the total number of eggs taken from the natural population. Caterpillars were kept in 0.5-liter jars, 2 to 5 caterpillars of II - IV ages each before the emergence of adult insects. The number of caterpillars died from parasites, diseases, and unidentified causes was considered.

The number of caterpillars in the nest was determined using the first developed method, by measuring the circumference of the nest in its widest part and calculating its volume. Further, the actual number of the caterpillars was calculated. The result obtained was converted into a certain unit of the nest volume (Dubrovin & Mladentsev, 2019; Ali et al., 2020). The mortality of caterpillars in wintering nests from various factors, including damage from birds by characteristic pecks, was also determined in laboratory settings. Pupal mortality in natural conditions was registered once a week according to external signs. When a butterfly flew out of the pupa, it formed a large triangular opening on the shell along the borders of the antenna inmantle and chest segments (Dubrovin, 2016; Tachi et al., 2021).

After the emergence of ichneumons, large or small (depending on the type of parasite) rounded holes remained on the shell of the host pupae. The pupae, eaten by tahinas, had irregular holes. The pupae, eaten by the beauties larvae and other predators, had large irregular holes in their different parts. At the end of the survey, the total mortality from parasites, diseases and unidentified causes was determined. The mortality of insects from unspecified causes was determined by the difference in the total number of dead individuals and individuals that died from established causes.

Mortality from interspecies competition was determined in the natural population of pests by keeping caterpillars in isolated sleeves located on the branches of model trees. Feeding caterpillars of the investigated and accompanying insect species, as well as the investigated species alone, were studied in the sleeves.The dead caterpillars without signs of infection and disease, and the difference in mortality were counted to record the total mortality from interspecies competition (Dubrovin, 2016; Ramachandran and Rosen, 2020).

Table 1. Identified factors of mortality affecting the population dynamics of brown-tail moth

No.	Mortality factor	Phase when an insect dies	Mortality, %
	Fam. Chalcidoidae		
1	Telenomus laeviusculus Rtzb.	Egg	18.1
	Undefined causes		17.2
	Fam. Braconidae		
3	Meteorus versicolor Wesm.	Caterpillar	16.2
4	Meteorus ictericus Nees.		9.1
	Fam. Chalcidoidae		
5	Eupteromalus nidulans Toer	Caterpillar	5.3
	Fam. Tachinidae		
6	Zenillia libathrix Panz.	Caterpillar	25.4
7	Blondelia nigripes Fall		18.4
8	Pteromalus puparium L.		9.3
9	Pareudora praeceps Mg.		6.8
	Fam. Chalcidoidae		
10	Brachymeria secundaria Rast.	Caterpillar	7.3
	Fam. Carabidae		
11	Calosoma inguisitor L.	Caterpillar	3.4
12	Calosoma sycophanta L.		5.0
13	Birds	Caterpillar	21.0
	Fam. Entomophthoraceae		
14	EntomophthoraaulicaeReich. родРодBeauveria	Caterpillar	11.2
15	Beauveria bassiana (Bals) Vuill.		16
	Fam. Braconidae		
16	Microgaster calceatus Hal.	Pupa	5
	Fam. Carabidae		
17	Calosoma inguisitor L.	Pupa	8.3
18	Calosoma sycophanta L.		12.4
	Fam. Entomophthoraceae		
19	Entomophthora aulicae Reic h.	Pupa	13.6

Analysis of the dynamics of the number converted the results of the counts to a unified accounting unit equal to 100 growth points. Material processing and compilation of survival tables were carried out according to the methods (Ali et al., 2020; Ramachandran and Rosen, 2020). Analysis of survival tables determined statistical relationships between population density and survival at different age intervals. The factors of the highest mortality per generation were identified. Mortality from one group of factorsover an elementary.

periodoftime: $g=n_d/n_t$ [1]

where n_d – the number of dead individuals,
n_t – the total number of individuals counted.

The survival rate from one group of factors was calculated by formula:

$$W=1-q \quad [2]$$

The survival rate from one group of factors for the entire observation period was:

$$W_f=W_1+W_2+W_3+...+W_n \quad [3]$$

Mortality over the entire observation period:

$$Q=1-W_f \quad [4]$$

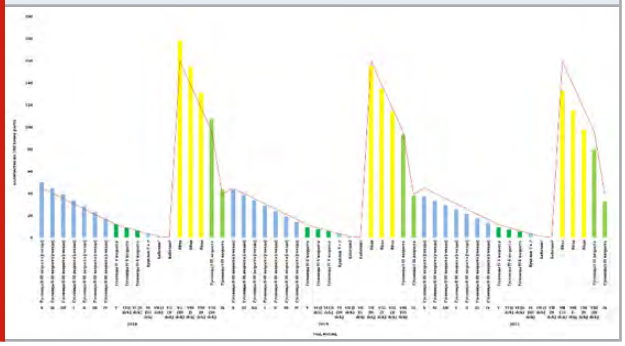
Survival tables assumed that all factors acting on the populations of the studied insects acted independently of each other.

To identify parasites, predators, and diseases of insects during the research the authors used "A review of braconid ichneumons (Hymenoptera, Braconidae) of the fauna of the USSR", Iliinskii A.I. Supervision, accounting, and forecast of mass reproduction of needle- and leaf-eating insects in the forests of the USSR / A.I. Iliiinskii, I.V. Tropin. - M.: Forest industry, 1965. - 525 p. (Iliiinskii, 1965), as well as with the help of the staff of the Department of Plant Protection and Horticulture, N.I. Vavilov Saratov State Agrarian University.

Table 2. The survival rate of brown-tail moth over the age intervals

	Unified unit of account (100 growth points)			
	2018	2019	2020	Mean
Eggs	178.00	154.97	132.97	155.31
I-III-age caterpillars	107.15	93.29	80.05	93.50
IV-V-age caterpillars	12.02	9.41	9.28	10.24
Pupae ♀ + ♂	3.44	3.68	3.59	3.57
Sex ratio (I)	0.58	0.46	0.43	0.49
Pupae ♀	1.98	1.71	1.55	1.75
Butterflies ♀	1.14	0.98	0.89	1.01

Figure 1: Changes in the abundance of brown-tail moth by development phases in a generation



RESULTS AND DISCUSSION

The research identified the factors of the natural environment influencing the size of the brown-tail moth population. Among them there are 19 species of entomophages and diseases that affect the decline in the number of brown-tail moth at all stages of development (Table 1). For a comparative analysis of the factors of population dynamics affecting the brown-tail moth population, the role of each of them was studied. Data obtained were converted to unified accounting units, i.e., per 100 growth points, which facilitated the analysis of population dynamics (Table 2).

In 2018-2020, there was a high number of pests in the studied plantations, the percentage of damage in some cases reached more than 80%. Currently, there has been a decline in the insect population (Dubrovin & Mladentsev, 2019; Ramachandran and Rosen, 2020; Wuet al., 2020). One of the main factors was the biotic factors of the environment, described in the article. Analysis of Table 2 shows the changes the brown-tail moth population undergoes in a generation. From the egg to adult phase, the population size decreases by about 76 times, but due to the high fertility of females the population is restored.

The greatest loss of abundance occurs during the over wintering period of caterpillars and the transition to active feeding, approximately 9 times. In this period of time, there is high mortality from inter- and intraspecific competition.The sex ratio decreased over the years of the study and averaged 0.49, which indicates a gradual transition of the gradation cycle to the phase of population decline.The presented analysis of the survival rate of brown-tail moth over the age interval made it possible to construct a curve of the number of the pest by years of study (Fig. 1). Based on the factors

of population dynamics identified in the course of the study, a table of the survival rate of the regulation of the brown-tail moth population over the time interval was compiled (Table 3).

Table 3. The survival rate of brown-tail moth in the plantations of the Penza region

Age interval x	Initial number of live individuals, lx	Factors causing population decline, dx F	Number of individuals died during the interval, dx	100 qx (dx to lx ratio, %)
1	2	3	4	5
Egg	155.31	Parasites	54.82	35.3
		Undefined causes	6.99	4.5
		Total	61.81	39.8
I-III-age caterpillars	93.50	Parasites	21.89	23.4
		Predators	23.66	25.3
		Diseases	10.47	11.2
		Birds	19.66	21.0
		Intra- and interspecies competition	7.58	8.1
		Total	83.26	89.0
IV-V-age caterpillars	10.24	Parasites	4.49	43.8
		Predators	0.54	5.3
		Intra- and interspecies competition	0.50	4.9
		Diseases	1.64	16.0
		Total	7.17	70.0
Pupa	3.57	Predators	0.92	25.7
		Diseases	0.49	13.6
		Undefined causes	0.11	3.2
		Total	1.52	42.5
Butterflies ♀	1.01	Undefined causes	0.65	64.5
		Total	0.65	64.5

Figure 2: Relationship in the number of I-III-age caterpillars per 100 growth points and their survival

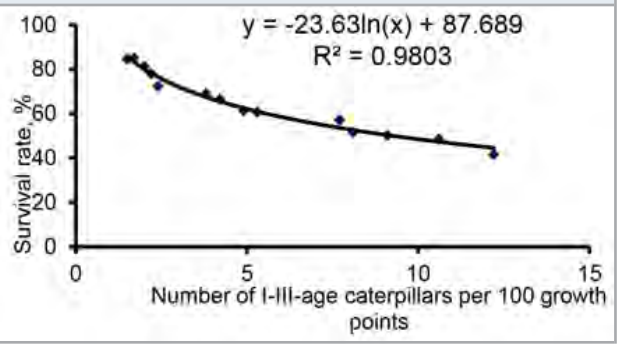
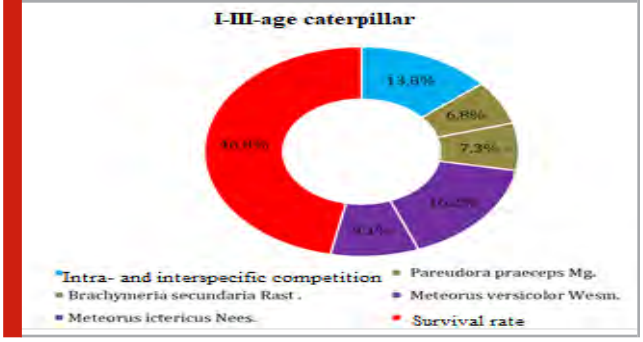


Figure 3: Mortality factors for caterpillars of I-III age



Speaking about the total mortality of brown-tail moth in the caterpillar phase, it should be noted that the younger caterpillars died much more often than the older ones. Statistical analysis of the influence of factors of the biotic environment on young caterpillars revealed an inverse relationship between their abundance and survival in this age interval (Fig. 2). In particular, (Iliiinskii, 1965; Ali et al., 2020; Tachi et al., 2021), considering the influence of the parasites of brown-tail moth, the authors established an inverse relationship of the effectiveness of their action on the survival of the pest. This pattern was found during the parasitization of *Eupteromalus nidulans* and *Apanteles lacteicolor* Toer, tachin *Zenillia libathrix* Rapz, *Blondelia nigripes* Fall. The data of the listed authors are consistent with our studies. However, as further studies show, the percentage of infection of the caterpillars of brown-tail moth from the listed parasites was maximum during the crisis phase and in the first year

of the depression. The research results made it possible to make a similar analysis of the influence of the factors of the dynamics of the brown-tail moth population using diagrams (Fig. 3-6).

Figure 4: Mortality factors for caterpillars of IV-V age

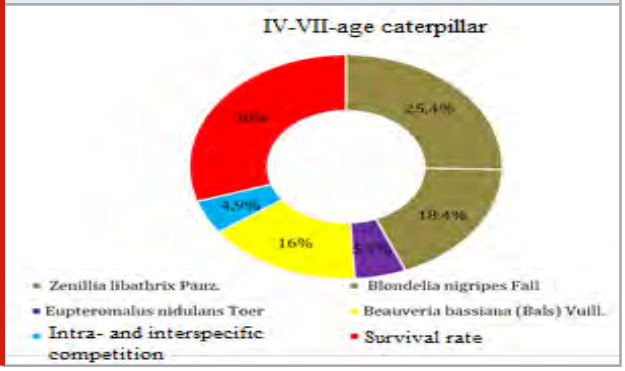


Figure 5. Mortality factors for caterpillars of IV-V age

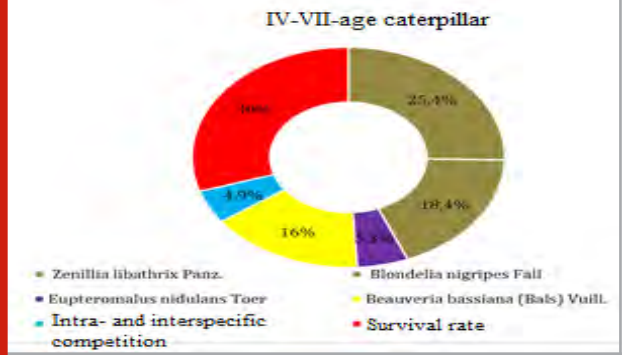
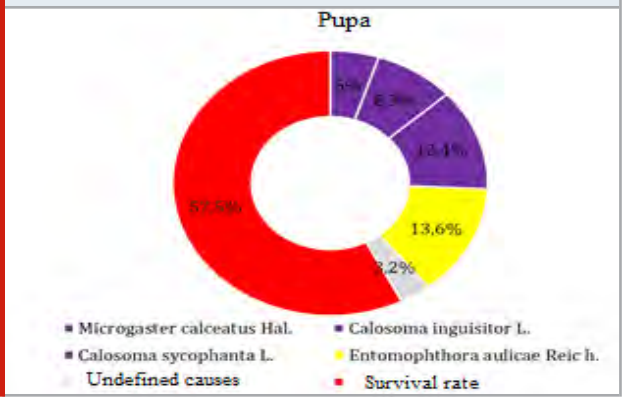


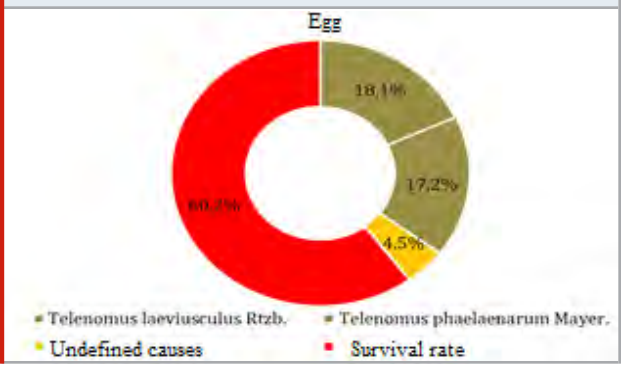
Figure 6: Pupa mortality factors



We found that during active feeding, mortality from intraspecific competition of young caterpillars that left the winter nest was 13.8%. The influence on the survival rate of caterpillars from parasites of the tachin family *Pareudora praeceps* Mg. - 6.8%, *Brachymeria secundaria* Rast - 7.3%, as well as the braconid family *Meteorus versicolor* Wesm. - 16.2%, *Meteorus ictericus* Nees. - 9.1 %, The role of birds in the decrease in the number of caterpillars in the nest was 21%. Mortality was also recorded from intraspecific and interspecific competition - 13.8% and diseases of *Entomophthora aulicae* Reich. Thus, the total mortality from the listed number of factors at this stage of the pest development was 88.0%.

The total mortality of the pest for the given interval of development was 51.6%. A decrease in the number of caterpillars of older ages was caused by parasites of the tachin family *Zenillia libathrix* Papz. - 25.4%, *Blondelia nigripes* Fall - 18.4%, and the braconid family *Eupteromalus nidulans* Toer - 5.3%. Diseases of *Beauveria bassiana* (Bals) Vuill played an important role in the number of caterpillars - 16.0%. Their death from intra- and interspecific competition was 4.9%.

Figure 7: Egg mortality factor



Microgaster calceatus Hal was bred from brown-tail moth pupae with a mortality rate of 5.0%. Predators of pupae *Calosoma inguisitor* L. and *Calosoma sycophanta* L. caused death in this phase - 25.7% of pupae. The death of pupae from diseases of *Entomophthoraceae*, *Entomophthora aulicae* Reic h. was about 13.6%, of unknown causes - 3.2%. The survival rate of brown-tail moth pupae was 57.5%.

Chalcids *Telenomus laeviusculus* Rtzb and *Telenomus phaelaenarum* Mayer greatly influenced the survival rate of brown-tail moth in the egg phase. Total mortality from factors was 35.3%. Initial mortality from abiotic factors was 4.5%. The survival rate of caterpillars in egg-laying after overwintering was 60.2%.

CONCLUSION

Study of the population dynamics of brown-tail moth in the plantations of the Penza region identified 19 different factors of the biotic environment. The regulating role of each of them was established at a specific stage of development of brown-tail moth during ontogenesis. The data obtained will serve as the basis for further study of this pest in the plantations of the Penza region. They will provide an opportunity to plan and effectively carry out forest protection measures against phytophagous organisms.

Conflict of Interests: the authors state that there is no conflict of interest in this study.

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Sports Science Communication

On the Role of Physical Education Programs in the Extracurricular Hours to Improve Physical Fitness for 14–15 Year Old Boys

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ABSTRACT

This study aimed to find and evaluate an experimental approach to developing the strength of 14–15-year-olds in extracurricular fitness and health classes at the gymnasiums. Classes based on a circle training form, were held three times a week, taking into account the students' age-specific strength development and performing complex strength exercises and how they were used. The randomized method was used to select a group of students aged 14-15 years (60 participants) to participate in experimental classes in the school's physical education system (supplemental). The positive influence of the experimental program on the training and development of strength competencies (dynamic, static) of students (male) aged 14-15 in the context of supplementary physical education at school has been being established. By the end of the trial period, the number of students with "average" and "above average" physical health increased and the number of young men with "low" strength development levels decreased. The proposed test method increased the student's fitness level, as evidenced by the increase in the motor test score values. The value of the elastic index of young men increased by 20%, the speed was 12.48%, the endurance being 10.26%. The speed-strength was found to increase by 7.75%. The number of young men with "average" and "high" levels of the value of the motor test indicators also increased. It is concluded that the proposal of experimental methods to develop the physical capabilities of 14-15-year-old students in school gymnastics is highly effective and can be encouraged to improve the fitness and physical capacity of the students elsewhere.

KEY WORDS: STRENGTH CAPACITY, STRENGTH TRAINING, GYMNASTICS, PHYSICAL FITNESS.

INTRODUCTION

In recent years, an increasing number of students in Vietnam, and some other countries are interested in forms of extracurricular physical activity, (Thuc, 2019; Zori et al., 2018). This indicates a decrease in motivation for traditional forms of classroom organization and the need to modernize physical education in educational institutions (Andrieieva et al., 2020). One of the forms of organizing advocacy activities according to the student's choice is supplementary physical education, (Dugnist, et al., 2020).

In this regard, in our opinion, the mission is to develop methods and technologies to develop the strong competencies of high school students in physical education, sports in sports, and sports groups. Physical enhancement is essential. This issue has not been fully addressed in the scientific literature, which reduces the effectiveness of 12th-grade capacity development training sessions. A certain level of strength development is required in all major sports. In young men, a high degree of strength development is a prerequisite for successful service in the military, (Thuc,2018; Roman 2018). Meanwhile, according to various studies, about 80% of young men and women, after graduating from high school, have low physical characteristics.

Improving health and developing healthy lifestyle habits are priorities in the physical education of

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young people in many different countries (Karol and Reineke, 2020). Reduction Youth advocacy activities in recent decades in several countries (Olafsdottir et al., 2016) and physical educational performance (Hortigüela et al., 2015). Therefore, the issue of youth actively participating in physical training and sports is one of the State's policy priorities in many countries. Research on physical culture shows that physical development, one of the main motor qualities of a child's body, has a significant impact on overall physical development. This fact shows the need to improve the effective educational and referral process technology and curriculum for regular and extracurricular activities that develop the strong competencies of high school students. Scientific literature analysis has shown that a lot of material has been accumulated on the choice of media and methods to develop the strengths of high school students. Physical training of students is done in many forms of physical education (regular, extracurricular). The research purpose of the topic is to find and evaluate an experimental method to develop physically effective 14-15-year-old students during class time in the practice room.

Table 1. Contents of the program to develop male strength abilities with the use of exercise equipment

	Basic exercises	Sets per exercise	Amount of repetition	Loading (kg)
1 st day (Monday; from 15-00 till 16-30.)	Alternate standing dumbells curl with hand supination	2	20	8
	Standing row	3	10-12	35
	Alternate standing dumbells curl with the neutral position of hand «hammer»	2	20	8
	Abdominal raise from the support position on elbows on bars	3	10	Sole weight
	Walk (6 min.) on a treadmill 1	1	-	Sole weight
	Lat pulldown	4	8	50
2nd day (Wednesday; from 15-00 till 16-30)	Dig-up on the bars	3-4	4-5	Sole weight
	Lying tricep extension on a horizontal bench	4	12	25
	Lifting the torso from the supine position	3	15-20	Sole weight
	Pec deck in «Butterfly» training simulator	2-3	12-15	30
	Standing lateral raise with dumbells	4	10-12	8
	Walk (6 min) on a treadmill	1	-	Sole weight
3rd day (Friday; from 15-00 till 16-30)	Push-ups	3	20	Sole weight
	Leg extension in a training device	3	10-12	50
	Superextention (hyperextention)	3	12-15	Sole weight
	Walk on a treadmill	1	-	Sole weight
	Squats (front squat, overhead squat, back squat)	4	10-12	20
	Leg curl in a training device	3	12-15	30

MATERIAL AND METHODS

A randomized method was used to form a 14-15-year-old group of 60 people to practice in the school gym using an experimental approach to improving strength. Before and after the pedagogical experiment, the male students' endurance abilities were assessed by dynamic endurance tests: pull-up on a high crossbar, (times); pull-over, (times); dig-up on the bars, (times); lifting the torso from the supine position, (times/min); standing long jump, (cm); throwing a 3 kg stuffed ball with two hands sitting from behind the head, (cm).

Static strength tests were performed: exercises needle, (s); bun (Ball), (s).; half-squat, (s); plank, (s); handgrip and deadlift dynamometry tests (kg). Motor tests were used to assess speed, endurance, speed and strength abilities, and flexibility: running 100 m, (s); running 1500 m, (min, s); jumping rope 30 sec, (times); leaning forward from a standing position with straight legs on a gymnastic bench, (cm). The training sessions are held in 4 phases, three times a week, for 90 minutes, from August 2019 to March 2020. Fitness is selected to suit individual teenagers' possibilities. Classes follow the circular method of training. In the main part of the unit, students are asked to do groups of local and regional empirical exercises with recommendations Table 1).

In the early stage (first 2 months), circular exercise is used to strengthen the musculoskeletal system and increase the functioning of the male body, as well as to provide a basis for increased load. The impact intensity is 40-45% of the maximum, the number of repetitions in the approach – 15-25 for the basic development of endurance, number

of stations – 6-12, number of rounds – 1- 3. The work phases are arranged as follows: 15 seconds. – work, 45 seconds. – rested; 15 seconds – work, 30 seconds. -rested; 30 seconds. – work, 30 seconds. – rested. For the next 2 months (phase II), we used intensive alternate training methods to develop strength with local exercises. At this stage, the load intensity is 50-65% is the maximum, the working time in each exercise is 15-30 seconds, the number of repetitions in the approach is 8-12 reps. The interval between approaches is 50-90 seconds, the station number is 4-10, and the lap number is 1-2.

In the third stage, to increase the load intensity and the differential effect on the lagging muscle groups, the successive series method is used. When doing exercises with local weights, we use weights 50-70% maximum, increasing the number of approaches and repeating with a 40-60 second resting interval. When doing area exercises, you should do 2-4 repetitions 12-15 repetitions with a pause between sets of 60-120 seconds. In the final stage IV, a combination of circular and repetitive exercise methods was used to produce distinct effects on muscle groups. The young men perform 2-3 series of exercises at stations 4-6. (World Medical Association Declaration on Helsinki, 2013). Consent from the boy's parents to conduct the survey. To statistically analyze the obtained results, the applications of software Microsoft Excel and SPSS 20.0. were used.

RESULTS AND DISCUSSION

After the experiment, the boys showed statistically significant increases in all indicators of dynamic and static strength, handgrip, and deadlift dynamometry (Table 2).

Table 2. Young men's strength indicators values before and after the experiment (M±SD)

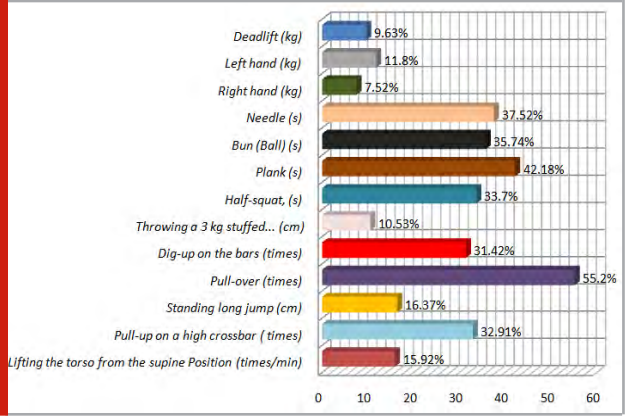
Tests		Indicators				t	P	(W%)
		Before the experiment (M±SD)		After the experiment (M±SD)				
Dynamic strength Position(times/min)	Lifting the torso from the supine	42.2	4.96	49.5	5.21	2.28	<0.05	15.92
	Pull-up on a high crossbar (times)	8.2	3.52	11.43	4.22	2.49	<0.05	32.91
	Standing long jump (cm)	184.5	7.22	217.4	6.52	3.06	<0.05	16.37
	Pull-over (times)	1.6	1.39	2.82	1.73	2.26	<0.05	55.20
	Dig-up on the bars (times)	7.94	3.42	10.9	4.25	2.31	<0.05	31.42
	Throwing a 3 kg stuffed ball with two hands sitting from behind the head (cm)	258.2	3.75	286.9	4.45	2.82	<0.05	10.53
Static strength	Half-squat, (s)	52.3	4.12	73.5	4.38	2.45	<0.05	33.70
	Plank (s)	29.78	2.46	45.7	2.98	2.31	<0.05	42.18
	Bun (Ball) (s)	38.6	2.87	55.4	2.95	2.01	<0.05	35.74
	Needle (s)	40.4	3.56	59.06	4.23	2.45	<0.05	37.52
Dynamometry	Right hand (kg)	38.89	2.86	41.93	3.59	2.44	<0.05	7.52
	Left hand (kg)	34.38	4.68	38.69	4.97	2.28	<0.05	11.80
	Deadlift (kg)	156.45	4.31	172.28	4.98	2.56	<0.05	9.63

The highest value (55.20%) of the increase in the young men's dynamic strength was set in the test Pull-over (time). The lowest value (10.53%) of the increase was in the test Throwing a 3 kg stuffed ball with two hands sitting from behind the head (cm). The values of the static force indicators increase exceeded 35% of the initial level. The values of the increase in handgrip and deadlift dynamometry indicators were about 9%.At the end of the trial, the number of young adults with a growth rate is shown in (Fig 1). The experimental method of training strength abilities had a positive impact on the students' physical fitness (Table.3), as evidenced by significant changes in the indicators values in all motor tests.

The indicators of youth mobility increased by 20%, speed by 12.48%, endurance increased by 10.26%, and speed and strength increased by 7.75%.The number of young men with "medium" and "high" mobility test scores has increased. The number of young men with a 'low' degree of the 'speed' index decreased from 17 (56.67%) to 5 (16.67%), the number of young men with a 'moderate' degree increased from 13 (43.33%) to 19 (63.33%) and

The "high" rate was from 0 (0%) to 6 (20%).The number of students with "low" levels of endurance development decreased twice (from 9 to 4), and three young people with "high" levels of endurance appeared. Number of young men with an 'average'. The level of development of endurance remains unchanged.

Figure 1. The number of young men with different levels of dynamic and static strength development before and after the experiment (%)



Results of the speed and endurance test showed that after the trial, the number of young men with a "high" level of fitness quality increased 3 times (from 4 to 12). The number of young men who have a «medium» speed endurance does not change. Young men with "low" qualifications were not registered. After the trial, the number of young men with a "high" flexibility index tripled, and the number of young men with an "average" increased by 12.5%. No young male with a "low" level of flexibility is registered.

The search for new and improved traditional approaches to increase the efficiency of fitness classes for students continues to be relevant (Kolumbet, & Dudorova, 2016; Natal'ya et al., 2020) It confirms the importance of research. choose ours. Since students' motivation for generally accepted methods of physical activity is still low (Drachuk et al., 2018), some researchers propose organizational methods. is different from the student's physical education. Researchers have proposed to use time-based intensive training (Yessica Segovia & David, 2020) and fitness technology (Valery Zhamardiy, et al., 2020) more widely in school physical education. Increasing physical activity, in addition to regular fitness classes, has a positive impact on students' physical health (Talovic et al., 2015), according to data from our study.

Table 3. Students' physical condition tests indicators' values before and after the experiment (M±SD)

Physical condition tests	Before the experiment (M±SD)		After the experiment (M±SD)	t	P	W%	
Running 100 m (s)	14.98	3.22	13.22	3.19	2.28	<0.05	-12.48
Running 1500 m (min, s)	7.58	4.36	6.84	3.89	2.39	<0.05	-10.26
Jumping rope 30 sec. (times)	62	3.75	67	3.65	3.15	<0.05	7.75
Leaning forward from a standing position with straight legs on a gymnastic bench (cm)	9	6.23	11	7.09	3.26	<0.05	20.00

The results of using the proposed program to develop strength for boys aged 14-15, using exercise equipment showed that by the end of the trial, the number of students with average physical health had ups and downs among young people, as there was a 'low' level of power development. Using the circle exercise method in the proposed program we tried to enhance male strength, which was found to increase along with the motor and emotional density of the participants of the classes, making them varied and enjoyable. We believe that one of the reasons that increase the mobility, static, and strength of the arms and body is the value of the indices in the motor tests at the end of using our test method. The proposed increase is an increase in young men's physical motivation associated with an additional form of extracurricular physical education. This is consistent with survey results of Hispanic students, studying 1-2 years in secondary education institutions. They point out in their survey of important temporal activities outside of the classroom, regarding a growing interest in this form of a physical education organization (Zorio et al., 2018).

Other researchers especially recommend spending significant time on extracurricular physical activity (Codina et al., 2016), which increases not only the physical but also the mental as a result. other authors observed. an increase in the indicators of students' cognitive and physical functions (Berrios et al., 2017). This statement is consistent with data obtained by other researchers like that of Natal'ya et al, (2020). We believe that researching capacity development in the physically complementary learning environment of students is a promising direction of the program to enhance the fitness of modern students.

CONCLUSION

The experimental method we have developed and tested to assess the physical capacity of 14-15-year-old boys using exercise equipment in the supplementary physical education system at school has become a useful method, which has significance in enhancing students' mobility and stillness of hand development and measuring dynamics. At the end of the test, an increase in the values of the indexes of the motor tests in terms of speed, general endurance, speed endurance, and active flexibility of the spine has been found. The proposed program to develop the competencies and endurance of 14-15-year-old males expands theoretical knowledge in the field of physical and sports education and it can be introduced for use in institutions other than physical education.

Conflict of Interest: The author state that there is no conflict of interest.

Ethical Clearance Statement: The Current Research Work Was Ethically Approved by the Institutional Review Board (IRB) of University, Ho Chi Minh City, Vietnam.

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Agricultural Communication

Production of Wheat Seed Through Various Plantation Practices in Maternal Environment

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ABSTRACT

Several studies have investigated the effects of maternal environment on various characteristics of seed quality; but no previous study has been done ondifferent plantation practices in maternal environment and its effect on the produced seed quality. Therefore, in the present study, a field and laboratory study was performed aimed at investigating the effect of three various plantation practices (conservation till age (CT), minimum tillage (MT), and no- tillage (NT)) in maternal environment on germination of the produced wheat (*Triticumaestivum* L. cv. Shiraz) seed in the College of Agriculture, Shiraz University (Shiraz, Fars Province, Iran) during 2011-2012 growing season. The results demonstrated that plantation practice significantly influenced grain yield in field; as well as seed germination and seedling growth. CT treatments caused the highest grain weight and yield. The results of laboratory experiment indicated different seed germination and early growth in the seeds developed under various plantation practices. CT as a type of plantation practice in maternal environment significantly reduced characteristics of seed germination, radicle and plumule length, as well as, vigor index of next generation. Although,according to the results, the negative effect of MT and NT on the produced seeds could be an important factor regarding adoption of CT; however this needs to be further studied. Thus, more research is required in this area.

KEY WORDS: CONSERVATION, GERMINATION, MATERNAL FACTOR, VIGOR INDEX.

INTRODUCTION

A decrease has been reported in growth and yield of crops in residues, especially in heavy wheat residues. Reduction of grain yield can be due to several factors including climatic conditions, pathogens in soil, unavailability of nitrogen, toxic effects caused by decomposing of surface residues, and/or poor seedling establishment (Khayatnezhad and Gholamin, 2021a, Gholamin and Khayatnezhad, 2020d, Karasakal et al., 2020b). For conservation tillage (CT), tillage modifications can be used to improve soil conditions. CT practices include the decreased types of plantation practices, such as minimum tillage (MT) and no-tillage (NT) practices, to elevate soil cover with crop residues from the previously cultivated crop (conservation technology information center (CTIC), (Alayi et al., 2020, Arjaghi et al., 2021, Esmaeilzadeh et al., 2020, Aletor, 2021). Improvement of soil surface cover usually leads to enhancement of water uptake and retention. NT as a promising practice for croplands located on the Mediterranean basin can increase water use efficiency (Si et al., 2020).

For achieving yield potential, quality, and also profit in wheat production, attention should be paid to rapid seed germination and uniform emergence of seedlings as essential prerequisites. For achieving an optimal seedling establishment and better productivity, there is a critical need for greater and better synchronized germination (Huang et al., 2021, Farhadi et al., 2020, Fataei, 2017). The two most important environmental problems faced by the crops are unsuitable quality and inadequate germination and establishment. Many factors influence seed quality including cultivar, genetic purity, physical purity, germinability, and vigor index. Although, there are other factors, such as genetic structure, environmental conditions, and maternal environment that highly influence seed quality (Hewitt, 2021).

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Several studies have reported about the effects of maternal environment on different characteristics of seed quality including germinability, dormancy, size, and composition. Temperature, water availability, light (quality and photoperiod), altitude, and mineral nutrition are some environmental factors that have been frequently studied (Li et al., 2021, Huma et al., 2021). However, no study has been conducted about different plantation practices in maternal environment and its effect on the produced seed quality. Thus, in the present study, the effect of different plantation practices (conservation, minimum, and no tillage) was evaluated in maternal environment on seed germination of wheat cv. Shiraz.

MATERIAL AND METHODS

This study was performed in field and laboratory of the College of Agriculture, Shiraz University (Shiraz, Fars Province, Iran) during 2011-2012 growing season. The experimental field was located in a semi-arid region (52° 46'E, 29° 50'N, altitude 1810 m ASL). Table 1 shows physico-chemical characteristics of the soil in the experimental field. The irrigated wheat was cultivated in the experimental site. The treatments were composed of conservation tillage (CT), minimum tillage (MT), and no-tillage (NT). The field and laboratory experiments were arranged in randomized complete block (RCB) and completely randomize design (CRD) types of design, respectively; with three replications in both kinds of experiments. Moldboard plow, twice-disc plow, and leveler were applied in CT treatment. In MT treatment, a combination of tillage tools including sweep plow, disc plow, and roller were used. Row planter was used to sow wheat seeds in CT and MT treatments; while for NT treatment, direct planter was used to sow the seeds.

Row and plant spacing were equal to 20 and 2 cm, respectively; and it was expected to cultivate 2.5 million plants per ha^{-1} (87.5 kg seeds ha^{-1}). Viable wheat seeds (Shiraz cultivar) were sown in plots with dimensions of 3 × 6 m in November 6, 2011. The fertilizer broadcasting was done using 150 kg ha-1 of triple superphosphate at sowing time and also 250 kg ha^{-1} of urea (half of which was used at sowing and the other half was used at stem elongation). Manual weed control was done. Harvesting was performed in June 16, 2012, and grain in each plot was separated for laboratory experiment.

Petri and solution dishes were put in oven for 24 h at 110°C before performing the experiments. Surface of the seeds was sterilized using 5% NaOCl (sodium hypochlorite) for 5 min to prevent fungal invasion, and then, they were washed with distilled water (Karasakal et al., 2020a, Sun et al., 2021). In each petri dish, 25 seeds were placed. Seeds were placed in 9 cm-diameter petri dishes on two layers of filter paper (Whatman No.1). The petri dishes were irrigated with distilled tap water during the experiment. Dishes were put in a germinator at 23 ± 2°C. The filter papers of each petri dish were replaced every 2 days for preventing salt accumulation Muhibbu-din, 2020, Kabir et al., 2021).

Seed germination was recorded daily up to 8th and 15th days after sowing for 25/20 and other regimes, respectively; at the time of lack of germination. Seed germination was considered when radical emerged by about 2 mm in length (Khayatnezhad and Gholamin, 2012b, Karasakal et al., 2020a, Gholamin and Khayatnezhad, 2020a, Sun et al., 2021). In each recording, 10 seedlings were randomly chosen from each petri dish, and sample data were obtained from their averages. The characteristics including germination percentage (Equation 1; (Khayatnezhad and Gholamin, 2020a, Omrani and Fataei, 2018), germination rate (Equation 2; (Omrani and Fataei, 2018)) radicle and shoot length, and vigor index (Equation 3; (Omrani and Fataei, 2018, Ren and Khayatnezhad, 2021)) were measured.

$$\text{Equation 1: } GP = \frac{n}{N} \quad \text{Equation 2: } EI = \frac{\sum n}{Dn} \quad \text{Equation 3: } VI = (RL+SL) \times GP$$

In Eq. (1); GP is germination percentage, n is number of the germinated seeds, and N is total number of the planted seeds. In Eq. (2); GR is the germination rate, n is the number of the germinated seeds on a specific day, and D is the number of days passed from beginning of experiment. In Eq. (3); VI, RL, SL, and GP are vigor index, radicle length, shoot length, and germination percentage, respectively. Data were analyzed by analysis of variance and significant differences were detected between treatment means by the least significant difference test at $P < 0.01$ level using the SAS v. 9.1 computer software.

Table 1. Some physico-chemical characteristics of the soil in the experimental field

EC (dS m^{-1})	pH	OM (%)	N (%)	P (mg kg^{-1})	K (mg kg^{-1})	Texture
0.62	7.04	1.03	0.25	13.45	693	Silty loam

RESULTS AND DISCUSSION

According to the results, plantation practice significantly influenced grain weight such that, the grains developed in CT treatment were heavier than those in NT treatment; however, there was no significant difference between the developed grains in MT treatment and those of two other treatments (Fig. 1(a)). Although, this difference caused a variation in grain yield between treatments so that, the highest and lowest grain yield were obtained in CT and NT treatments, respectively (Fig. 1(b)). Heavy residues of the irrigated crops left on the soil surface (e.g., in NT) have been indicated to reduce kernel weight of wheat and/or grain yield due to poor crop establishment, disease transmission, and unavailability of nitrogen (Radmanesh, 2021).

Plantation practices in maternal environment influenced germination percentage of wheat so that; CT treatments

caused the highest germination of seeds, while seed germination was significantly lower in MT and NT treatments. Germination percentage of wheat seed had no significant difference between MT and NT treatments (Fig. 1(a)). Germination rate in CT treatment was also significantly higher than MT treatment; whereas the lowest germination rate was observed in NT treatment (Fig. 2(b)). Germination and seedling establishment can be mentioned as critical phases in life cycle of many plant species (Gholamin and Khayatnezhad, 2020b, Rodríguez, 2021).

Figure 1: Seed weight (A) and grain yield (B) influenced by three plantation practices in the field

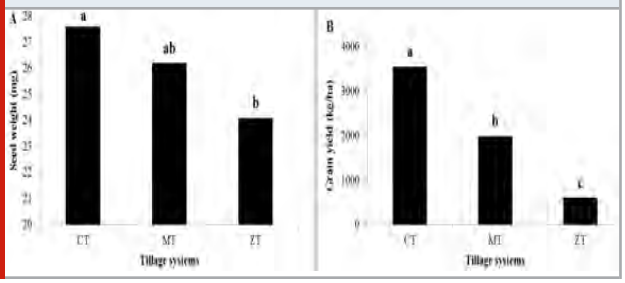
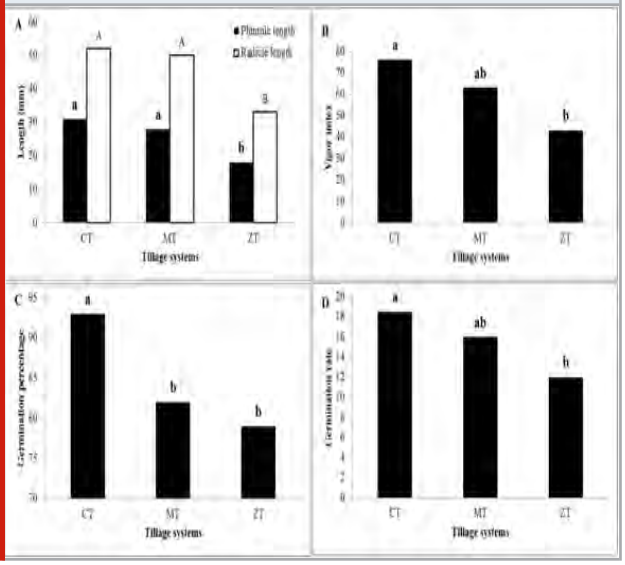


Figure 2: Germination percentage (A) and rate (B), plumule and root length (C),and vigor index (D) influenced by three plantation practices in maternal environment



Maternal environment has been shown to have strong effects during seed development percentage and germination rate in terms of various environmental factors; however no study has been conducted on plantation practices. In the study by Gholamin et al., (2021), it was found that maternal effects were predominant in determination of progeny seed size and germination characteristics. Enhancement of germination rate and percentage in CT treatment could be related to greater storage of seed. Khayatnezhad et al., (2021b) in a study reported early and better germination in heavy seeds, which can be attributed to bigger storage reserves of these seeds.

Higher seedling growth was found in CT treatment, which was not significantly different with MT treatment so that, the highest radicle and plumule length was observed in CT and MT treatments, while the lowest radicle and plumule length was obtained in NT treatment (Fig. 2). Furthermore, similar trend was observed for vigor index, where CT and NT treatments caused the highest and lowest vigor index, respectively (Fig. 3). Greater radicle length might be due to greater seed size. Seed size has been also shown to influence other characteristics of seedling growth, such as plumule length, root and shoot dry weight (Gholamin and Khayatnezhad, 2020c, Khayatnezhad and Gholamin, 2020b).

Based on the results, MT and NT treatments negatively influenced the produced seed so that, the seed developed in these treatments had lower germination percentage and rate. Thus, this effect should be considered along with other disadvantages of CT practices including low temperature in soil surface and incidence of weeds and diseases. Reduction of seed germination and seedling growth under the influence of CT practices could be due to lower seed size (as observed in our study), or higher level of inhibitory components. Thus, more studies are needed to understand these probabilities.

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Communication

Fish Reproduction Conditions of Volgograd Reservoir Near the Villages of Akhmat and Zolotoe Russia in 2020 in Comparison With Previous Years

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ABSTRACT

The article considers the conditions for the reproduction of fish in the waters of the Volgograd reservoir of the villages Akhmat and Zolotoe, such as level and temperature regimes, as well as production processes (the rate of gross photosynthesis, the rate of respiration of the plankton community, net primary production, which can serve as indicators of the state of the natural food base). According to the results of the fry survey in 2020, the conditions for the fish reproduction in the waters of the Volgograd reservoir of villages Akhmat and Zolotoe should be recognized as very unfavorable, which could be predicted by the dynamics of the level and temperature regimes. Black Sea sprat (Clupeonella cultriventris (Nordmann, 1840), as in previous years, is reproduced in the water area of the village of Zolotoe and is not reproduced in the water area of the village of Akhmat. The high food capacity of the studied water areas has a noticeable effect on the efficiency of reproduction of certain fish species in years with a more favorable level regime but is not able to compensate for the damage caused to the reproduction of fish by a particularly unfavorable regime of the water level in general.

KEY WORDS: PLANKTON, REPRODUCTION, TEMPERATURE, VOLGOGRAD RESERVOIR, WATER LEVEL.

INTRODUCTION

According to the literature, the success of fish breeding depends on the level and the duration of water standing at high elevations and the synchronization of water heating with the rise in the level, which ensures the maturation of reproductive products following the conditions for spawning, are very important. Successful reproduction and feeding of juvenile fish in the Volgograd reservoir require a slow rise in the water level to optimal levels by the end of April – early May (16.5-17 m of the Baltic System (BS)), a long standing (for 30-35 days) at maximum marks from the subsequent slow (from the beginning of June) lowering of the level to low-water levels in July (Biological substantiation of maintaining the optimal water level in the Volgograd reservoir to increase the productivity of the population of the main commercial valuable fish species, 2005).

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Globally, wildlife is disappearing faster than ever before, largely due to habitat degradation and fragmentation (Atlas of freshwater fish of Russia, 2002). The factors of such degradation and fragmentation include the operation of hydroelectric power plants. In a market economy, when the regulation of the operation of hydroelectric power plants is unprofitable for the shareholders of the energy sector, the problem of ensuring the success of fish reproduction can be solved by the efforts of the agricultural sector by influencing subordinate factors that vary in individual water areas (Tiulin et al., 2018; Tiulin et al., 2019; Tiulin et al., 2019; Ermolin et al., 2019 Tiulin et al., 2020; Kostin et al., 2021).

Correct assessment of the breeding conditions of fish is possible using an integrating indicator – fry survey data. The yield, which falls within the interval between 37.2 and 11.5 thousand ind./ha, corresponds to the average yield (the conditions for the reproduction of fish are average); the interval between 11.5 and 5.2 thousand ind./ha indicates low yield (breeding conditions are unfavorable); 37.2-79.4 thousand ind./ha – high yield

(breeding conditions are favorable). All values less than 5.2 thousand ind./ha correspond to a very low yield (very unfavorable breeding conditions), more than 79.4 thousand ind./ha – to a very high fry yield (highly favorable breeding conditions) (Ermolin et al., 2019; Tiulin et al., 2020; Boldyrev et al., 2021).

In 2020, the fish reproduction conditions in the waters of the villages Akhmat and Zolotoe were studied again. The purpose of this work is to assess the state of natural reproduction of commercial fish in the waters of the Volgograd reservoir of the villages Akhmat and Zolotoe based on observations of fry yield in 2020. To achieve it, the following tasks were completed: the study of such conditions for the reproduction of commercial fish as the temperature and level regimes of the Volgograd reservoir during the spawning period, production processes, the rate of gross photosynthesis, the rate of respiration of the plankton community, net primary production, and analysis of the data of fry survey in the studied water areas.

MATERIAL AND METHODS

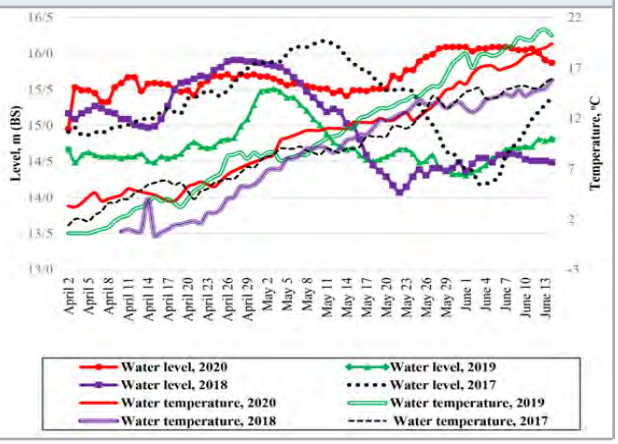
The curves of temperature and level regimes of the Volgograd reservoir were compiled based on data from the meteorological station near the city of Saratov. To study production processes in water areas, the daily flask exposure method was used (Phytoplankton and its products, 1984). In this case, three flasks are used. Oxygen in the control flask was fixed immediately after taking a water sample. Two other flasks (darkened with opaque material and light) were fixed on a pole and kept in a reservoir for 24 hours. Net primary production is the difference between the oxygen concentration in the light and control flasks, the respiration rate of the plankton community is the difference between the oxygen concentration in the control and dark flasks, and the gross photosynthesis rate is the difference between the oxygen concentration in the dark and light flasks. For greater accuracy, 9 flasks were used in each experiment.

The concentration of dissolved oxygen in the water was determined by the Winkler method (Muravyov, 2011) and using a Samara-2 oximeter. The data on the production processes were compared with the previously published data on the fry yield in the studied water areas (Tiulin, 2017; Tiulin et al., 2018; Tiulin et al., 2019; Tiulin et al., 2020). The material in terms of the fry yield was sampled from July to September 2020. The species of fry was established according to the A.F. Koblitskaya identification key (Koblitskaia, 2014). Samples were taken with a 10-m-long fry trawl, a 2-m-high wing, with an 8-mm wing mesh, and a 4 mm belly mesh. In total, in 2020, 33 fry hauls were carried out with a trawl, about five hundred specimens of juvenile fish were analyzed and measured. The relative abundance was calculated by bringing the data on the catches of fry trawl per unit area (Minin et al.,2007). The Brodskaia and Zenkevich index of dominance (ID) and the Shannon index of species diversity were calculated (Chief, 2005).

RESULTS AND DISCUSSION

In spring 2020, the Volgograd reservoir lacked any favorable conditions. Despite the relatively high, in comparison with 2019, water level, its rise towards optimal levels began only by the end of May; at the beginning of May, there was a prolonged decline, which threatens with drying out of eggs. These are significantly more unfavorable conditions in comparison with 2017-2019 (Tiulin, 2017; Tiulin et al., 2018; Tiulin et al., 2019 Tiulin et al., 2020). (Figure 1).

Figure 1: Changes in the water level and temperature in the Volgograd reservoir in spring 2017-2020 (according to the weather station near the city of Saratov)



A more correct assessment of the conditions of fish reproduction in the waters of the Volgograd reservoir near the villages Akhmat and Zolotoe can be obtained with an integrating indicator – fry yield (Ermolin et al., 2009). The fry survey (Table 1) in the water area near the villages of Zolotoe and Akhmat in 2020 found 12 and 8 species of fish, respectively (in 2019 – 11 and 10, respectively). The Shannon species diversity index has significantly decreased in comparison with 2019 for the water area near the village of Zolotoe and amounted to 1.82 (in 2019 – 2.26 (Tiulin, 2017)) and increased for the water area near the city of Akhmat – 2.47 (in 2019 – 1.36). In both water areas, the catches were dominated by commercial fingerlings: near the villages of Akhmat and Zolotoe their share was 72.8% and 50.1%, respectively (Tiulin et al., 2019; Tiulin et al., 2020; Boldyrev et al., 2021).

As in 2017-2019, Black Sea sprat (*Clupeonella cultriventris* (Nordmann, 1840)) is found in the water area of the village of Zolotoe in 2020. This species, as before, is absent in the water area near the village of Akhmat (Tiulin, 2017; Tiulin et al., 2018; Tiulin et al., 2019; Tiulin et al., 2020; Rudskaya et al., 2021). On a five-point fry yield scale (Ermolin et al., 2009), the fish reproduction conditions in the waters of the villages of Akhmat and Zolotoe in 2020 should be recognized as highly unfavorable. The intensity of production processes can serve as an indicator of the state of the natural food base, as evidenced by laboratory studies of samples of

phytoplankton, zooplankton, and zoobenthos (Tiulin et al., 2020; Popova and Pavlova 2021).

Figures 2 and 3 show the highest values of production indicators in 2017 and 2019, and the lowest – in 2018 and 2020. In this case, in terms of the level and temperature regimes, the most favorable years are 2017 and 2018, while the least favorable is 2020. Table 2 presents the values of the fish yield in different years in the considered water areas, on a five-point scale (Ermolin et al., 2009). High indicators of zooplankton biomass determined an outbreak of reproduction of the Black Sea sprat (Clupeonella cultriventris (Nordmann, 1840)) in the water area of the village of Zolotoe in 2017, which formed the basis of the catch with fry trawl (145,333 ind./ha) (Tiulin, 2017). At the same time, the main factor determining the efficiency of fish reproduction remains the hydrological regime of the reservoir.

The decline in the yield of juvenile Black Sea sprat and the yield of juvenile fish in general in 2018 may be due to the undermining of its natural food base – zooplankton which, in turn, is probably due to an unfavorable oxygen regime. In July 2018, in the shallow waters of the village of Zolotoe, an extremely low concentration of dissolved oxygen in the water was observed. In the same month, the biomass of phytoplankton (including diatoms and green algae) reached a minimum, which serves as food for many zooplanktons, including crustaceans of the genus Bosmina, which are the basis for feeding of fingerlings of the Black Sea sprat (Guliev and Meliakina, 2014; Tiulin et al., 2017; Tiulin et al., 2020; Popova and Pavlova 2021). A peak in the concentration of nitrate ions was also observed, which indicates that the water was polluted with organic matter.

Table 1. Composition of caught fry in the waters of the Volgograd reservoir near the villages Akhmat and Zolotoe in 2020.

Fish species	Catch composition									
	Akhmat					Zolotoe				
	ind./ha	ind., %	g/ha	g, %	ID	ind./ha	ind., %	g/ha	g, %	ID
Common roach	1	1.23	2.85	1.06	1.88	13	2.18	1.1	0.23	1.56
Common chub	15	19.75	33.11	12.34	25.57	252	43.83	335.9	68.41	121.33
Perch	22	28.40	159.66	59.48	67.33	14	2.42	25.5	5.18	7.85
Crucian carp	16	20.99	43.10	16,06	30.08	7	1.21	11.1	2.27	3.67
Asp	2	2.47	19.12	7.13	6.87	-	-	-	-	-
Common bream	-	-	-	-	-	1	0.24	8.9	1.81	1.47
Silver bream	-	-	-	-	-	1	0.24	0.8	0.17	0.45
Common bleak	4	4.94	1.33	0.50	2.56	242	42.13	88.6	18.05	61.09
Black Sea sprat	-	-	-	-	-	10	1.69	3.2	0.65	2.33
Broadnosed pipefish	15	19.75	3.04	1.13	7.76	28	4.84	6.7	1.36	5.69
Monkey goby	2	2.47	6.18	2.30	3.91	4	0.73	4.7	0.96	1.85
Bighead goby	-	-	-	-	-	1	0.24	2.4	0.48	0.76
Tubenose goby	-	-	-	-	-	1	0.24	2.1	0.42	0.71
Commercial	56	72.84	257.85	96.07	137.05	288	50.12	383.3	78.07	138.61
Non-commercial	21	27.16	10.56	3.93	16.94	287	49.88	107,6	21.93	73.28
Total	77	100	268.41	100	-	574	100	491.0	100	-
Shannon index	2.47	1.82								

The combination of these factors could lead to a collapse of the biomass of zooplankton in July 2018 in the water area of the village of Zolotoe, which, probably, together with an unfavorable oxygen regime, negatively affected the populations of fingerlings of the Black Sea sprat. The sprat eggs are pelagic (Atlas of freshwater fish of Russia, 2002) therefore, these fish are less in need of additional areas of spawning grounds, although the areas flooded together with the flood are important for the development of zooplankton, which is the basis for the feeding of juvenile sprat. Probably, the decisive importance in reducing the population of juveniles of the Black Sea sprat in the water area of the village of Zolotoe in 2018 compared to 2017 was played by limiting factors: an unfavorable oxygen regime and a critically low concentration of zooplankton, which is the basis of nutrition for this fish species.

The feeding of fish, which form the basis of catches in the water area of the village of Akhmat is mixed or consists of benthivorous fish (Tiulin et al., 2017; Rudskaya et al., 2021). Fingerlings of such fish have more opportunities to feed themselves, and the productivity of juvenile fish in this area in 2018 turned out to be moderate, due to a favorable level regime during the spawning period. The unfavorable situation with the natural food base in 2018 was also reflected in the production curves.

Table 2. Fish reproduction efficiency in the waters near the villages Akhmat and Zolotoe

Years	Fish yield (ind./ha)	
	Akhmat	Zolotoe
2017	770 (Very low)	146 833 (Very high)
2018	12 640 (Moderate)	11 748 (Moderate)
2019	636 (Very low)	498 (Very low)
2020	77 (Very low)	574 (Very low)

Figure 2: Production processes in the water area near the village of Akhmat in 2017-2020

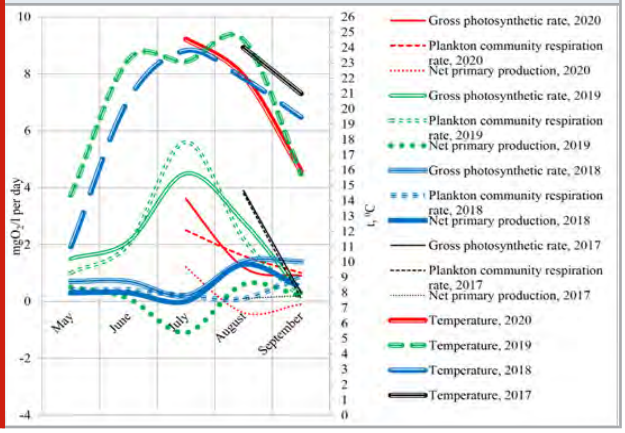
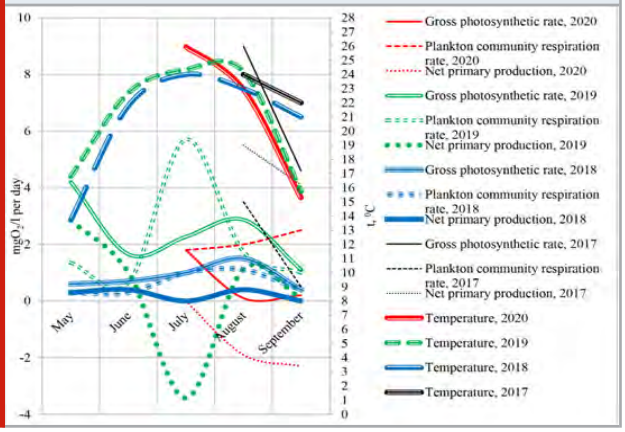


Figure 3. Production processes in the water area near the village of Zolotoe in 2017-2020



CONCLUSION

In 2020, the waters near the villages Akhmat and Zolotoe had very unfavorable fish reproduction conditions developed, which could be predicted by the dynamics of the level and temperature regimes. Black Sea sprat (*Clupeonella cultriventris* (Nordmann, 1840)), as in previous years, is reproduced in the water area of the village of Zolotoe and is not reproduced in the water area of the village of Akhmat. The high food capacity of the studied water areas has a noticeable effect on the efficiency of reproduction of certain fish species in years with a more favorable level regime but is not able to compensate for the damage caused to the reproduction of fish by a particularly unfavorable regime of the water level in general.

Conflict of Interest: Authors declares no conflicts of interests to disclose.

Ethical Clearance Statement: The Current Research Work Was Ethically Approved by the Institutional Review Board (IRB) of Agrarian University, Saratov, Russian Federation Russia.

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Microbiological
Communication

Microbiota of Cattle Buildings in the Northern Trans-Urals

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ABSTRACT

Microbiocenoses of livestock buildings affect not only animals, reducing their resistance and reactivity, causing diseases of various etiologies, but the staff and nearby residents. The objective of the research was to study the state of livestock breeding, the reasons for the withdrawal of animals, the likelihood of infectious diseases and the composition of microbiocenoses in livestock buildings for various purposes. The studies were conducted at an industrial cattle breeding enterprise (breeding reproducer) in the Northern Trans-Urals (Tyumen region) in 2018-2019. The subject of research was the microbial content of the air in the premises of the pedigree breeding unit with cattle of various technological groups of the Holstein breed. The main reasons for the withdrawal of young cattle are digestive and respiration disorders – 43.21% and 41.60%, respectively. Withdrawal of adult cattle is due to digestive diseases, metabolic disorders, and orthopedic problems and injuries – 25.6%, 25.4%, and 17.2%, respectively. Cattle leukemia and rabies have long been the problems for the Tyumen region. There is a likelihood of particularly dangerous diseases such as anthrax, infectious dermatitis nodosa, tuberculosis, Pasteurellosis, brucellosis, and foot and mouth disease. The composition of the microflora of the surveyed livestock buildings has been found to consist of three types of bacteria – *Staphylococcus aureus, Streptococcus faecalis,* and *Escherichia coli* and three genera of fungi – *Mucor, Candida* and *Aspergillus.*

KEY WORDS: CATTLE, MICROBIOCENOSES, EPIZOOTIC SITUATION, LIVESTOCK DISPOSAL, OPPORTUNISTIC MICROFLORA, LIVESTOCK BUILDINGS.

INTRODUCTION

The most important factor in the high quality of work, biological safety, and resulting products is the well-organized veterinary service of farms. An inadequate sanitary condition poses a risk of various diseases that can compromise the rhythm of production, and cause economic losses. An essential factor influencing the development of agricultural enterprises is the quality of air, an integral part of the habitat of most living organisms (Seedorf et al., 1998; Feingold et al., 2012; Masclaux et al., 2013; Mkrtumyan et al., 2018; Alvarado et al., 2019; Kochetova et al., 2020; Dhiman et al., 2021). On going through the literature it becomes it imperative to monitor the condition and control the degree of air pollution in the context of intensification of animal husbandry. Achieving a high level of sanitary condition of the industrial complex is one of the main tasks in animal husbandry. To predict more accurately the development and spread of various diseases, both the qualitative and quantitative composition of the populations of microorganisms, as well as the elements of the external environment that affect the production and processing of livestock products should be considered.

To ensure the biological safety of animal husbandry, it is necessary to control the number of pathogenic microorganisms in the air and reduce their number by means of veterinary and sanitary measures (Dungan et al., 2011; McEachran et al., 2015; Sancheza et al., 2016; Morozov et al., 2017; Navajas-Benito et al., 2017 Saleeva et al., 2018; Sintiurev et al., 2020). Unfortunately, various livestock enterprises underrate the composition of the community of air microorganisms (spores of microscopic fungi, bacteria, saprophytes and various exotoxins), which adversely affects the body of animals and humans (Casey et al., 2016; Borlee et al., 2017; Schaeffer et al., 2017; Borlee et al., 2018; Glazunova et al., 2018; Stolbova et al., 2018; Myrna et al., 2019; Stolbova, 2019; Domatsky

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et al., 2020; Glazunova et al., 2020; Kochetova et al., 2020; Stolbova, 2020; Bogado et al., 2021).

The factors affecting the quality of livestock products are both the sanitary condition of the premises and the environment, the resistance and reactivity of the animal organism, and the epizootic situation of the agricultural enterprise. Therefore, part of the preventive measures is medical examination of animals and monitoring of infectious diseases of animals at a modern livestock enterprise. Another fact to consider is the people who are constantly exposed to the microflora in the contaminated premises, which in turn can cause sensitization, asthma, atopy, allergic rhinitis, pneumonia, exacerbation of chronic infections and many other pathologies (Hiranuma et al., 2011; Smit et al., 2014; Casey et al., 2015; Borlee et al., 2017; Zomer et al., 2017; Borlee et al., 2018; Freidl et al., 2019; Domatsky et al., 2020; Gagarin et al., 2021). This is another reason for a detailed study of the microbiota of livestock buildings and the development of methods for its correction.

MATERIAL AND METHODS

The studies were conducted at an industrial cattle breeding enterprise (breeding reproducer) in the Northern Trans-Urals (Tyumen region) in 2018-2019. The subject of research was the microbial content of the air in the premises of the pedigree breeding unit with cattle of various technological groups of the Holstein breed. Microbiological and bacteriological studies were conducted in an accredited laboratory. The object of the study was various livestock premises: a dairy building, a building for replacement heifers, a maternity pen and a calf barn.

Air in each room was sampled in the morning, when the animals were at relative rest (before feeding, changing bedding, feeding calves and milking cows), and in the daytime, when the listed activities were carried out. Bacterial species were differentiated based on their morphological, tinctorial, cultural, and biochemical properties. Microbiological studies were carried out in compliance with the methodological manual and (Masclaux et., et al 2013; Kochetova et al., 2020). The identification of the isolated cultures was carried out in compliance with the requirements set out in the Bergey's Manual of Systematic Bacteriology (1997). For inoculation, diagnostic media were used: enterococcus agar (for *Streptococci*), salt agar (for *Staphylococci*), Saburo (for fungi), Endo's medium (for *E. coli*). Biochemical studies of the isolated cultures were carried out on Api test systems (bioMérieux, France).

QMAFAnM – Quantity of Mesophilic Aerobic and Facultative Anaerobic Microorganisms was determined by pour plate method. The numerical data were processed using BIOSTAT and Microsoft Excel. All manipulations with animals were in compliance with Directive 2010/63/EU of the European Parliament and the Council of the European Union "On the protection of animals used for scientific purposes". During the studies, we used the generally accepted methods of scientific knowledge, such as interrelation and interdependence; synthesis and analysis; generalization and comparison; observation, measurement and interpretation; and special methods: bacteriological, clinical, biochemical, and hematological. The results were analyzed using the statistical and mathematical methods to ensure the reliability and objectivity of the data.

RESULTS AND DISCUSSION

The second most developing industry in the Tyumen region is the agro-industrial sector; despite the harsh climate, both crop production and animal husbandry are actively developing. Over the past fifteen years, the number of cattle has been stable and varied within 250-265 thousand heads. For many years, the average duration of the use of cows at dairy enterprises has been very low and averaged 2.4-2.6 lactations (Seedorf et al., 1998; Dorozhkin et al., 2018; Sheveleva et al., 2020; Patra and Kar, 2021). This testifies to the colossal economic losses of livestock enterprises and the industry in general. Such a difficult situation with the disposal of animals is characteristic of intensive livestock farming technology, where the concentrate type of feeding is practiced, animals are subject to physical inactivity, technogenic stress and receive less solar insolation. These factors and many others lead to profound metabolic disorders and immunodeficiency states (Sidorova et al., 2020; Sintiurev et al., 2020; Dhiman et al., 2021).

Losses of cattle occur due to the high culling of young animals from the herd. 75.4% of the culled head of cattle are young. The livability of young animals in the Tyumen region was 82.1%. The main reasons for the withdrawal of young cattle are digestive and respiratory diseases – 43.21% and 41.60%, respectively. The number of adult cattle is the most stable; however, there are much more factors leading to culling in this group of animals than in young animals. The main reasons for the withdrawal of adult cattle in the Tyumen region are digestive and metabolic disorders – 25.6% and 25.4%, respectively. Every sixth cow in the region (17.2%) leaves the herd due to orthopedic problems and injuries. Due to respiratory and reproductive diseases, 11.8% and 10.9% of animals, respectively, are withdrawn. The problem of animal poisoning in production remains urgent, which has caused the withdrawal of 9.1% of animals. In addition, the resulting immune deficiency states determine the infectious and invasive susceptibility of animals.

Epizootic situation in the Tyumen region is tense, characterized by constant problem of rabies (including cases in farm animals) and leukemia. Diseases such as anthrax (2016 in the Yamal-Nenets Autonomous Okrug), Pasteurellosis (2018), infectious nodular dermatitis (2019), brucellosis (2020) are sporadically recorded; tuberculin-positive animals are regularly detected. In addition, there is a high likelihood of FMD, which can be introduced from border areas. Considering the multifactorial nature of infectious diseases, we have studied the microbiota of cattle breeding premises to identify the likelihood

of diseases caused by opportunistic microflora. The microbial composition of the studied livestock premises consists of three types of bacteria – *Staphylococcus aureus, Streptococcus faecalis*, and *Escherichia coli* and three genera of fungi – *Mucor, Candida*, and *Aspergillus* (Table 1).

Table 1. Qualitative and quantitative composition of the microbiota of the cattle-breeding premises of the industrial enterprise

Microorganisms	The total number of microbial colonies in five Petri dishes, sampled from...		
	maternity pen and calf barns	dairy building	replacement calf building
Total viable count	535.5±11.23	409.3±8.44	263.1±6.02
Staphylococcus aureus	215.1±9.28	454.0±18.09	126.0±6.11
Streptococcus faecalis	95.5±2.06	76.1±2.62	82.4±3.01
Escherichia coli	17.1±0.41	5.3±0.33	9.3±1.33
Aspergillus spp.	32.5±2.00	5.0±0.33	49.9±1.12
Mucor spp.	8.1±0.20	3.0±0.12	5.4±0.14
Candida spp.	1.1±0.04	2.2±0.08	1.1±0.07
Total colonies	904.9±7.42	954.9±11.07	537.2±6.89

The total number of colonies of microorganisms in five Petri dishes sampled from the maternity pen and calf barns where calves were kept from 0 to 6 months was 904.9 ± 7.42 colonies, in the dairy building with cows aged two years and older the total number of colonies was 954.9 ± 11.07, and the rearing building for replacement calves aged from 6 to 12 months showed the lowest quantitative indicator 537.2 ± 6.89. Staphylococcus aureus dominated in the microbial community, it was found in the air of the housing where dairy cows were kept – 454.0 ± 18.09 colonies, in the maternity pen and in the rearing building – 215.1 ± 9.28 and 126.0 ± 6.11, respectively. *Streptococcus faecalis* sub dominated in the air of livestock buildings; the quantitative indicators of the total number of colonies differed slightly in different rooms and amounted to 95.5 ± 2.06, 82.4 ± 3.01, and 76.1 ± 2.62 colonies in the maternity pen, rearing building, and dairy building, respectively.

Colonies of *Escherichia coli* were least represented, while the total number of colonies also had small fluctuations – 17.1 ± 0.41; 9.3 ± 1.33 and 5.3 ± 0.33 colonies, respectively. Among the representatives of fungi, the growth of *Aspergillus* was most abundant, with the total number of colonies in the air of the rearing building was 49.9 ± 1.12, the maternity pen and the calf barn – 32.5 ± 2.00, and the dairy building – 5.0 ± 0.33 colonies. The number of colonies of fungi *Mucor* and *Candida* did not exceed 10 colonies in five Petri dishes. Considering that livestock buildings must be disinfected only when completely free from animals, which is practically impossible under intensive agriculture, enterprises often neglect the preventive disinfection. This approach to preventive measures does not provide biological safety for both animals and the staff, as well as those living nearby. Therefore, it is necessary to develop acceptable methods of disinfection in the presence of animals.

CONCLUSION

The main reasons for the withdrawal of young cattle turned out to be digestive and respiratory diseases – 43.21% and 41.60%, respectively. Withdrawal of adult cattle is due to digestive diseases, metabolic disorders, and orthopedic problems and injuries – 25.6%, 25.4%, and 17.2%, respectively. Cattle leukemia and rabies have long been the problems for the Tyumen region. There is a likelihood of particularly dangerous diseases such as anthrax, infectious dermatitis nodosa, tuberculosis, pasteurellosis, brucellosis, and foot and mouth disease. The composition of the microflora of the surveyed livestock buildings has been found to consist of three types of bacteria – *Staphylococcus aureus, Streptococcus faecalis*, and *Escherichia coli* and three genera of fungi – *Mucor, Candida*, and *Aspergillus*.

The study has shown differences in the quantitative indicators of microorganisms based on the purpose of the premises. The constant presence of a significant number of opportunistic microorganisms in livestock buildings increases the likelihood of respiratory and digestive diseases, and undermines the natural resistance of animals. Given that most manipulations with animals are carried out at the same place of their keeping, this casts doubt on the compliance with the rules of asepsis and antisepsis during any surgical procedures, and especially during surgical interventions. In addition, the animal-care staff, being in constant contact with opportunistic flora, is exposed to significant risk of occupational diseases. The data obtained dictate the need to develop a universal method for disinfection of livestock buildings in the presence of animals.

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Biomedical
Communication

Serum Lipid Profile in Patients Visiting King Khalid General Hospital in Majmaah, Saudi Arabia

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ABSTRACT

Cardiovascular diseases, coronary heart diseases, and other noncommunicable diseases are leading causes of high mortality worldwide. Different studies have shown that these diseases are caused by abnormal lipid levels in the blood, dyslipidemia, obesity, and less physical activity. The lipid profiles of 59 patients were analyzed. Information about these patients was collected from the King Khalid General Hospital Laboratory in Majmaah City, Saudi Arabia. The disctiptive statistaical analysis was used to present the data for the parameters including low-density lipoprotein (LDL), high-density lipoprotein (HDL), triglyceride (TG), and total cholesterol levels.Women had higher levels of total cholesterol and LDL than men due to their sedentary lifestyle. However, women had higher levels of HDL than men, which can reduce the risk of dyslipidemia. Abnormal total cholesterol, LDL, HDL, and TG levels were observed in the following age groups: 0-25, 26-35, and 36-45 years. More abnormalities were detected in women than in men. Women are at greater risk of developing certain comorbidities, including cardiovascular diseases, due to their unhealthy lifestyle and less physical activity. Certain chronic diseases, particularly heart diseases, are associated with abnormal lipid levels, obesity, and lifestyle habits with less physical activity. Huge variations in HDL, LDL, TG, and total cholesterol levels have been observed in men and women with respect to age. Variations in lipid levels have also been observed in men and women living in different regions of the world. Moreover, this present study has shown that high levels of LDL-cholesterol are mostly present in the female population as their lifestyle habits involve less physical activity and an unhealthy diet, which leads to obesity. This study provides insight into future regulation and awareness campaigns by health authorities in Saudi Arabia regarding the effect of dyslipidemia on human health.

KEY WORDS: AGE, DYSLIPIDEMIA, GENDER, LIPID PROFILE, SEDENTARY LIFESTYLE.

INTRODUCTION

Noncommunicable diseases, mostly of the circulatory and endocrine systems, caused by sedentary lifestyle habits are major health problems worldwide. Other related diseases that cause mortality are dyslipidemia, cardiovascular disease (CVD), and coronary heart disease (CHD). Therapeutic interventions play a significant role in managing these diseases (Cotte et al., 2019). People of all ages, genders, and races are susceptible to various CVDs, including stroke, cardiac arrest, and hypertension. Cardiac arrest risk is higher in men than in women and in individuals over 45 years of age. These individuals

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are at greater risk of developing comorbidities such as cancer, diabetes mellitus, and chronic obstructive pulmonary disease. Fluctuations in lipid levels cause dyslipidemia, which affects the normal blood levels of low-density lipoprotein (LDL) and triglycerides (TGs). Dyslipidemia ultimately results in CHD due to atheroma. Accumulation of fat in the arterial walls also leads to atherosclerosis (Khalil et al., 2018, Al Amri et al., 2019, Alasnag et al., 2020).

Lipids perform important functions such as energy storage, cell signaling, cell membrane formation, and hormone production. However, excess quantities of lipids cause adiposity, which leads to some metabolic diseases. Hyperlipidemia has a severe effect on the body, particularly on the liver, leading to nonalcoholic fatty liver disease (Maciejewska et al., 2019). Two types of lipids, cholesterol and TGs, are present in the human body. Cholesterol is a fat-like substance classified into two types: LDL and HDL. LDL is considered a bad cholesterol that leads to atherosclerosis and affects cardiac muscle (Bijker et al., 2017). HDL is considered a good cholesterol that removes LDL deposits from arteries (Tauseef et al., 2020).

Low levels of LDL and high levels of HDL are ideal for good health. Another type of fat is TG, which is stored in the skin. Excess TGs cause adiposity, particularly affecting the liver, which metabolizes fat. Adiposity also leads to other chronic diseases, including hypothyroidism, fatty liver, polycystic ovarian syndrome, and metabolic disorders such as diabetes, insulin resistance, renal malfunction, and osteoarthritis (Liou and Kaptoge, 2020).LDL, HDL, total cholesterol, and TG levels are examined using a combination of tests resulting in a lipid profile.

A lipid profile is usually conducted to check the risk of CHD in individuals who have unhealthy and sedentary lifestyle habits. Usually, healthy LDL levels should be below 135 mg/dL, and HDL levels should be above 40 mg/dL. Fluctuations in these levels increase the risk of CVDs. Lipid profiles indicating dyslipidemia are observed in obese individuals with hypertension and type II diabetes (Belay et al., 2014). Hereditary factors, a family history of obesity, and CVDs are also associated with dyslipidemia (Alzaheb and Altemani, 2020). There are four types of dyslipidemia: hypercholesterolemia, hypertriglyceridemia, hypo-HDL-cholesterolemia, and hyper-LDL-cholesterolemia. Hypercholesterolemia is a major problem. In recent years, the lifestyle of people has included less physical activity due to socioeconomic development. A sedentary lifestyle leads to obesity, which increases the risk of comorbidities (Al Amri et al., 2019). Obesity has led to several CVDs in the population of Saudi Arabia (Osman and Al-Nozha, 2000).

The risk of CVD has increased by 4%, and the prevalence of hypertension and associated diseases has increased by 15% since 2010 (Al-Hazzaa, 2018). Almost 35% of Saudi people are obese and have a high risk of developing CHD. Dyslipidemia risk has increased by 8%, and >50% of cases of hypercholesterolemia are still undiagnosed (Al-Hazzaa, 2018). In Saudi Arabia, dyslipidemia is more prevalent among women than among men owing to women's lower physical activity (Al-Hazzaa, 2018). Total cholesterol and LDL levels can be lowered by long-term statin therapy (Habte et al., 2020). The severity of heart disease symptoms can be reduced by increasing HDL levels (Al Qahtani et al., 2015). Adopting a healthier lifestyle by eating low-fat food and increasing physical activity can help to prevent the risk of heart diseases (Osman and Al-Nozha, 2000). Here, we report and analyze the serum lipid profile status among patients visiting King Khalid General Hospital (KKGH) in Majmaah, Saudi Arabia.

MATERIAL AND METHODS

Subjects, data collection, and ethical approval: Fifty-nine patients were enrolled as study subjects between January 2019 and January 2020. Their mean ± standard deviation age was 34.73 ± 20.09 years for male subjects and 41.15 ± 14.79 years for female subjects (range <1-80 years). Patient information was collected from the logbook of the KKGH laboratory. The following parameters were included in the laboratory data: total cholesterol, TG, HDL, and LDL levels. The following ratios were determined: total cholesterol: HDL, LDL: HDL, and TG:HDL. All the standard ranges for the examined parameters were obtained by the hospital laboratory's standard working techniques. Ethical approval was obtained from the Saudi Ministry of Health (number 2019-0015E).

Data analysis: The data was recorded in a Microsoft Excel spreadsheet and investigated utilizing SPSS (version 25.0) for Windows. The data were exported to a Microsoft Excel sheet, checked for possible missing values, and prepared for analysis. Descriptive statistics were used to calculate the mean, standard deviation, and frequency of the variables. Tables are used where appropriate.

RESULTS AND DISCUSSION

The 59 patients included in the study were divided into different groups according to age; LDL, HDL, total cholesterol, and TG levels; and total cholesterol:HDL and LDL:HDL ratios. Table 1 shows the distribution of patients according to sex and age. Of the 59 patients who were selected for this study, 19 (32.2%) were men and 40 (67.8%) were women. The study subjects were divided into different age groups. There were 6 men (10.2%) and 5 women (8.5%) aged 0-25 years, 5 men (8.5%) and 10 women (16.9%) aged 26-35 years, 2 men (3.4%) and 9 women (15.3%) aged 36-45 years, 5 men (8.5%) and 11 women (18.6%) aged 46-59 years, and 1 man (1.7%) and 5 women (8.5%) aged 60-80 years. The overall distribution of patients in the different age groups was as follows: 0-25 (18.6%), 26-35 (25.4%), 36-45 (18.6%), 46-59 (27.1%), and 60-80 (10.2%).

Table 2 presents the distribution of the different parameters of the lipid profiles according to sex and age. A prevalence of high total cholesterol was detected in the female group. The lowest total cholesterol level (<5.2 mmol/L) was observed in 15.3% of the men and in a higher proportion (42.4%) of the women. The highest total cholesterol level (>6.2 mmol/L) was found in 3.4% of the men and in a higher proportion (18.6%) of the women. The lowest LDL level (<2.6 mmol/L) was found in the same proportion (1.7%) of men and women. High LDL levels (3.3. mmol/L) were observed in 25.4% of men and 33.9% of women. Due to the high LDL levels, there is a greater risk of dyslipidemia in women. Similarly, high levels of TG were observed in women. The lowest TG level (<1.7 mmol/L) was found in 23.7% of men and 37.3% of women. The highest TG level (>2.2 mmol/L) was observed in 5.1% of men and 6.8% of women. We found that approximately 2/3 of the population had low HDL levels (77.9%). The lowest HDL level (<1.3 mmol/L) was found in 28.8% of men and 49.2% of women. Similarly, the highest HDL level (>1.5 mmol/L) was observed in 15.25% of women and in no men.

Table 1. Distribution of patients by age, group and sex

Age, years	Male, n = 19 (32.2%)	Female, n = 40 (67.8%)	Total, n = 59 (100%)
0-25	6 (10.2%)	5 (8.5%)	11 (18.6%)
26-35	5 (8.5%)	10 (16.9%)	15 (25.4%)
36-45	2 (3.4%)	9 (15.3%)	11 (18.6%)
46-59	5 (8.5%)	11 (18.6%)	16 (27.1%)
60-80	1 (1.7%)	5 (8.5%)	6 (10.2%)

Table 2. Lipid profile of the study population adjusted for sex and age

Variable	Men, n (%)	Women, n (%)	Total, n (%)
Total cholesterol, mmol/L			
<5.2	9 (15.3%)	25 (42.4%)	34 (57.6%)
5.2-6.2	8 (13.6%)	4 (6.8%)	12 (20.4%)
>6.2	2 (3.4%)	11 (18.6%)	13 (22%)
LDL cholesterol, mmol/L			
<2.6	1 (1.7%)	1 (1.7%)	2 (3.4%)
2.6-3.3	3 (5.1%)	19 (32.2%)	21 (35.6%)
>3.3	15 (25.4%)	20 (33.9%)	36 (61%)
TG, mmol/L			
<1.7	14 (23.7%)	22 (37.3%)	36 (61%)
1.7-2.2	2 (3.4%)	14 (23.7%)	16 (27.1%)
>2.2	3 (5.1%)	4 (6.8%)	7 (11.9%)
HDL cholesterol, mmol/L			
<1.3	17 (28.8%)	29 (49.2%)	46 (77.9%)
1.3-1.5	2 (3.4%)	2 (3.4%)	4 (6.8%)
>1.5	0 (0%)	9 (15.25%)	9 (15.3%)

HDL, high-density lipoprotein; LDL, low-density lipoprotein; TG, triglyceride.

Table 3 shows the prevalence of lipid profiles in men according to age group. We observed that a higher total cholesterol percentage is present in the age group 0-25 years (21%), whereas a reduced prevalence was detected in age groups 26-35 (10.5%), 36-45 (5.2%), 46-59 (5.2%), and 60-80 (5.2%). The highest level of total cholesterol was found in age groups 0-25 and 26-35 at a prevalence of 5.2% but not at all in the other age groups. Likewise, high LDL levels were found in age groups 0-25 (26.2%), 26-35 (26.3%), 46-59 (21%), and 36-45 (10.5%). A high LDL level was not found in any patient in the age group 60-80 years. Low LDL levels were not found in any group. Similarly, higher TG percentage found in age groups 0-25 (26.2%) and 46-59 (21%), but a lower prevalence was found in the age group 60-80 (5.2%). Furthermore, low HDL levels in men were found in age groups 0-25 (31.4%), 26-35 (21%), 36-45 (10.5%), 46-59 (21%), and 60-80 (5.2%). Normal HDL levels were found in age groups 26-35 and 46-59 (5.2%). High HDL levels were not detected in any sample, which led to a greater risk of dyslipidemia. Similarly, a high total cholesterol:HDL ratio was observed in age groups 0-25 (21%), 26-35 (10.5%),

46-59 (10.5%), and 60-80 years (5.2%). Moreover, a high LDL:HDL ratio was found in age groups 0-25(26.2), 26-35 (15.7%), and 46-59 (15.7%). A low TG:HDL ratio was observed in age groups 0-25 (26.2%), 26-35 (15.7%), 36-45 years (10.5%), 46-59 years (26.3%).

Table 4 shows the prevalence of lipid profiles in women according to age group. We found high total cholesterol levels in age groups 0-25 (2.5%), 26-35 to 46-59 (7.5%), and above 60 (2.5%). Likewise, high levels of LDL were found in age groups 0-25 (2.5%), 26-35 (12.5%), 46-59 (12.5%),–36-45 (12.5%), 46-59 (17.5%), and >60 years (5%). However, in women, the increased level of TG is less than that in men of the same age. The low TG levels were found in age groups 0-25 (12.5%), 26-35 (15%), 36-45 (12.5%), 46-59 (10%), and >60 (5%). Furthermore, we found low HDL levels in women in the age groups 0-25(10%), 26-35 (17.5%), 36-45 (15%), 46-59 (25%), and 60-80 (5%).

Table 3. Lipid profile of the male population by age group

Variable	Age group (%)				
	0-25	26-35	36-45	46-59	60-80
Total cholesterol, mmol/L					
<5.2	4 (21.0%)	2 (10.5%)	1 (5.2%)	1 (5.2%)	1 (5.2%)
5.2-6.2	1 (5.2%)	2 (10.5%)	1 (5.2%)	4 (21%)	0
>6.2	1 (5.2%)	1 (5.2%)	0	0	0
LDL cholesterol, mmol/L					
<2.6	0	0	0	0	0
2.6-3.3	1 (5.2%)	0	0	0	1 (5.2%)
>3.3	5 (26.2%)	5 (26.3%)	2 (10.5%)	4 (21%)	0
TG, mmol/L					
<1.7	5 (26.2%)	2 (10.5%)	2 (10.5%)	4 (21%)	1 (5.2%)
1.7-2.2	0	1 (5.2%)	0	1 (5.2%)	0
>2.2	1 (5.2%)	2 (10.5%)	0	0	0
HDL cholesterol, mmol/L					
<1.3	6 (31.4%)	4 (21%)	2 (10.5%)	4 (21%)	1 (5.2%)
1.3-1.5	0	1 (5.2%)	0	1 (5.2%)	0
>1.5	0	0	0	0	0
Total cholesterol:HDL ratio					
<3.5	0	0	0	0	0
3.5-5	2 (10.4%)	3 (17.5%)	1 (5.2%)	4 (21%)	0
>5	4 (21.0%)	2 (10.5%)	0	2 (10.5%)	1 (5.2%)
LDL:HDL ratio					
<2.5	0	0	0	0	0
2.5-3.3	1 (5.2%)	2 (10.5%)	1 (5.2%)	2 (10.5%)	0
>3.3	5 (26.2%)	3 (17.5%)	1 (5.2%)	3 (17.5%)	1 (5.2%)

HDL, high-density lipoprotein; LDL, low-density lipoprotein; TG, triglyceride.

We observed a relatively high HDL in females than in males, which reduces the risk of dyslipidemia. A high total cholesterol: HDL ratio (>5) was found in age groups 0-25 (5%), 26-35 (12.5%), 36-45 (12.5%), 46-59 (12.5%), and 60-80 (5%). Moreover, high LDL:HDL ratios (>3.3) were observed in age groups 0-25(5%), 26-35(12.5%), 36-45 (12.5%), 46-59(12.5%), and 60-80 (5%). These levels were lower than those in men. Similarly, a low TG:HDL ratio (<2) was found in women aged 0-25 years (12.5%), 26-35 years (17.5%), 36-45 years (10%), 46-59 years (25%), and 60-80 years (5%). Although a high level was observed in the age group 46-59 years (5%). Obesity, diabetes, and hypertension can be caused by abnormal blood lipid levels, and these diseases cause coronary artery disease, leading to mortality and morbidity in developed countries (Murray et al., 2003). A high prevalence of diabetes, hypertension, and obesity has been observed in Saudi women in a review (Alshaikh et al., 2016). A high and low level of total cholesterol was observed in women than in men in the present study. A higher prevalence of hypercholesteremia was detected in women (19.9%) than in men (18.7%) in a national report from the kingdom (Al-Kaabba et al., 2012). In the Taif region of Saudi Arabia, a prevalence of 44% of high HDL levels has been reported (Al Amri et al., 2019).

It has been observed that there is a higher prevalence (21%) (Table 3) of low total cholesterol level men aged 0-25 years and only 10% (Table 4) in women in same age group. Although in patients aged 0-25 and 26-35,

a higher prevalence of high total cholesterol level was found in women (5.2%) than in men. Furthermore, 40.5% of men and 37.5% of women have been observed in Guadeloupe in a similar study (Foucan et al., 2000). In an Indian study, 8.3% of men and 7.5% of women have been reported, which differs from our results (Dhok and Dubey, 2018). In our study, low LDL cholesterol levels (<2.6 mmol/L) were observed in 1.7% of both men and women. The prevalence of the high LDL level (3.3. mmol/L) was 33.9% in women and 25.4% in the men. Therefore, there is a higher risk of dyslipidemia in women than in men. However, the same prevalence (1.7%) of low LDL levels was observed in both sexes (Table 2). In Portugal, 16.8% of men and 21.8% of women have been observed to have high LDL levels in a similar study (Cortez-Dias et al., 2013). In the Saudi population, there is a higher prevalence of LDL level in men (30.7%) than in women (29.8%) (Al-Kaabba et al., 2012).

Table 4. Lipid profile of the female population by age-group

Variable	Age group (%)				
	0-25	26-35	36-45	46-59	60-80
Total cholesterol, mmol/L					
<5.2	4 (10%)	5 (12.5%)	5 (12.5%)	9 (22.5%)	2 (5%)
5.2-6.2	0	2 (5%)	0	1 (2.5%)	1 (2.5%)
>6.2	1 (2.5%)	3 (7.5%)	3 (7.5%)	3 (7.5%)	1 (2.5%)
LDL cholesterol, mmol/L					
<2.6	1 (2.5%)	0	0	0	0
2.6-3.3	3 (7.5%)	4 (10%)	4 (10%)	6 (15%)	2 (5%)
>3.3	1 (2.5%)	5 (12.5%)	5 (12.5%)	7 (17.5%)	2 (5%)
TG, mmol/L					
<1.7	5 (12.5%)	6 (15%)	5 (12.5%)	4 (10%)	2 (5%)
1.7-2.2	0	3 (7.5%)	2 (5%)	7 (17.5%)	2 (5%)
>2.2	0	1 (2.5%)	1 (2.5%)	2 (5%)	0
HDL cholesterol, mmol/L					
<1.3	4 (10.0%)	7 (17.5%)	6 (15%)	10 (25%)	2 (5%)
1.3-1.5	0	0	0	1 (2.5%	1 (2.5%)
>1.5	1 (2.5%)	3 (7.5%)	2 (5%)	2 (5%)	1 (2.5%)
Total cholesterol:HDL ratio					
<3.5	0	1 (2.5%)	0	0	0
3.5-5	3 (7.5%)	3 (7.5%)	2 (5%)	8 (20%)	1 (2.5%)
>5	2 (5.0%)	6 (15%)	7 (17.5%)	4 (10%)	3 (7.5%)
LDL:HDL ratio					
<2.5	2 (5.0%)	2 (5%)	1 (2.5%)	1 (2.5%	1 (2.5%)
2.5-3.3	1 (2.5%)	3 (7.5%)	2 (5%)	7 (17.5%)	1 (2.5%)
>3.3	2 (5.0%)	5 (12.5%)	5 (12.5%)	5 (12.5%)	2 (5%)

HDL, high-density lipoprotein; LDL, low-density lipoprotein; TG, triglyceride.

Likewise, high levels of LDL have been found in patients aged 0-25 (26.2%), 26-35 years (26.3%), 46-59 years (21%), and 36-45 years (10.5%). No sample with a high LDL level was found in the age group 60-80 years. In southern India, a higher prevalence of LDL level in men than in women in aged 40-60 years was observed in a similar study (Gupta et al., 2017). In Saudi Arabia, a 44% prevalence of high LDL levels has been observed in the nonobese population in a parallel study (Al Amri et al., 2019). In the Hofuf region of Saudi Arabia, a 12.85% prevalence of LDL levels has been reported in both genders (Al-Hassan et al., 2018).

In the present work, we have found that low HDL levels were more prevalent in women than in men (49.2% and 28.8%, respectively). High HDL levels were found in 15.3% of women, whereas no sample with low HDL was observed in the men (Table 1). In another study conducted in Portugal, the prevalence of high HDL was found to be high (40.6% in women and 20.2% in men) (Cortez-Dias et al., 2013). In the Indian population, a higher prevalence of high HDL levels was observed in women (7%) than in men (5.6%) in a similar study (Karki et al., 2004). Low HDL levels in men were 0-25 (31.4%), 26-35 (21%), 36-45 (10.5%), 46-59 (21%), and 60-80 (5.2%). Normal HDL levels were detected only in age groups 26-35 and 46-59 (5.2%) years (Table 3). High HDL levels were not observed in any age group among men.

According to research conducted in the Hofuf eastern region of Saudi Arabia, high levels of HDL were detected in various age groups: <20 (32.5%), 20-29 years (32.4%), 30-39 years (41.15%), 40-49 years (45.3%), 50-59 years (37%), and 60-60 years (39.8%) and above 60 years (40%) (Al-Hassan et al., 2018).Similarly, the low HDL levels were observed in women of different age groups: 0-25 (10%), 26-35 (17.5%), 36-45 (15%), 46-59 (25%), and 60-80 (5%). Likewise, high HDL levels were as follows: 0-25 (2.5%), 26-35 (7.5%), 36-45 (5%), 46-59 (5%) and 60-80 (2.5%). Similarly, higher levels of TG were found in women (6.8%) than in men (5.1%). According to a similar study conducted in the Nepalese population, the high TG levels in men and women were 35.7% and 35.2% (Karki et al., 2004). The high levels of total cholesterol:HDL that we found in women according to age were as follows: 0-25 (5%), 26-35 (15%), 36-45 (17.5%), 46-59 (10%), and 60-80 (7.5%). According to a similar study conducted in Saudi Arabia, high total cholesterol:HDL levels in women were 17.5% and 30.6%, respectvily (Al-Kaabba et al., 2012).

The high LDL: HDL ratio that we observed in men according to age was 0-25 (26.2%), 26-35 (15.7%), 46-59 (15.7%), and 5.2% in the 36-45 and 60-80 age groups. Likewise, the high LDL:HDL ratios observed in the female population were 0-25 (5%), 26-35 (12.5%), 36-45 (12.5%), 46-59 (12.5%), and 60-80 (5%). Similar percentages were recorded in another study conducted in the Saudi population (Al-Kaabba et al., 2012). Low TG:HDL ratios were observed as follows: 0-25 (26.2%), 26-35 (15.7%), 36-45 (10.5%), 46-59 (26.3%), and no sample was detected in the 60-80 age group. Normal levels of the TG:HDL ratio were only 5.2% in the group 0-25, 26-35, and 60-80 years. High levels were found in 26-35 (5.2%) age groups.

CONCLUSION

Certain chronic diseases, particularly heart diseases, are associated with abnormal lipid levels, obesity, and lifestyle habits with less physical activity. Huge variations in HDL, LDL, TG, and total cholesterol levels have been observed in men and women with respect to age. Variations in lipid levels have also been observed in men and women living in different regions of the world. Moreover, this study has shown that high levels of LDL-cholesterol are mostly present in the female population as their lifestyle habits involve less physical activity and an unhealthy diet, which leads to obesity. This study provides insight into future regulation and awareness campaigns by health authorities in Saudi Arabia regarding the effect of dyslipidemia on human health.

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Biomedical Communication

Effect of Tele-Rehabilitation Exercise Program on Pain and Functional Ability in Patients with Neck Pain

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ABSTRACT

Tele-rehabilitation plays a major role in reducing pain and functional ability of a patient with neck pain. It is the best way to communicate with patients to know their improvement status and moreover it works through video conferences and online chat services, using a cloud-based peer-to-peer software platform. The rapid development of telecommunication technologies requires continuous studies in order to establish the efficacy of these innovations. The present study was aimed to assess the impact of tele-rehabilitation exercise program on pain and functional ability of the patients with neck pain of the Western region Orthopedic Clinics in Saudi Arabia. A purposive sampling technique was used to collect the data from patients, which were all males where parameters like the neck disability index and numeric pain scale (NPS) were studied. The data was analyzed using IBMSPSS9 Statistical package for social science version.21 and also a person correlation coefficient was used to explain the association between the variables. The pre-numerical pain scale score (N=4) was associated with M=4.5(SD=2.03) by comparison of post-test (N=4) was associated with M=3(SD=2.03).to the t-test hypothesis that the pain score. The pre- neck disability index score (N=4) was associated with M=6.75(SD=4) by comparison of post-test (N=4) was associated with M=5(SD=4). All data were secured and locked for three years and then will be destroyed finally there was a highly significant correlation between the pain score and neck index from pre and post-test and great improvement with Tele-rehabilitation of neck pain. These results indicate the tele-rehabilitation can be a promising potential alternative standard of care, although some factors like unavailability of internet and materials always can negatively affect the therapy. The exercises program is important to treat patients with neck pain and furthermore the treatment sessions should focus on specific cervical muscles and their rehabilitation.

KEY WORDS: NECK DISABILITY INDEX, NECK PAIN, NUMERICAL PAIN SCALE, PATIENT PREFERENCE, TELE –REHABILITATION.

INTRODUCTION

Musculoskeletal disorders are considered as life threatening and more over having the potential to restrict daily activities, cause absence from work, and result in a change or discontinuation in employment. Neck pain is one of major problems that are affecting people in several parts of their life and a lot of studies have recommended that the neck pain is most popular in the middle age with marked variations in its prevalence. The pain begins from cervical region and can rise to be of more complex nature; and may impact seriously the psycho-social lifestyles of patients, causeing economic problems on health organizations. Mechanical neck pain is most common kind of neck pain disorder . The revalence of neck pain is one the major challenging issues, because it directly affects the quality of life. There are more factors related to neck pain, including muscle strains, nerve compression, stiffness leading to

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traumatic injuries (Bulk et al 2018, Suvarnnato. 2019 Dias et al 2021).

The neck pain usually does not subside within days but can persist for months and be a signal of an underlying medical cause that needs to be urgently treated. Patient-centered design that addresses patients' preferences and needs is considered important aim for improving health care systems. In the current study, we have found that the field of pain rehabilitation including patients' preferences regarding tele rehabilitation remains unexplored and little is known about the optimal combination between human and electronic contact from the patients' perspective. The assessment of patients' preferences important regarding telemedicine because it is the step toward the design of effective patient-centered care, (Bulk et al.2018 Fiani et al 2020). The importance of tele-rehabilitation is focused to circumvent physical barriers ,transportation concerns and financial limitations, concurrently, improving the quality of the health care environment, and giving more attention to reduce the number of such cases. Studies have shown that tele rehabilitation should be developed and implemented with well planned strategies particularly pointed towards ensuring an adequate level of rehabilitation, (Dias.2021).

MATERIAL AND METHODS

A quasi-experimental study approach was used including 4 patients with neck pain at western region orthopedic clinics in Saudi Arabia during the period of 2021 January to May. The subjects were assigned in to one group pre-test-post-test design and the study intends to assess the effect of Tele rehabilitation exercise program on pain and functional ability of patients with neck pain. The sample size was calculated with using an online sample size calculator (American Association for Public Opinion Research.2015). A purposive sampling technique used to collect the data including patient's demographic data such as age, gender, educational level and moreover the data were collected by the researchers personally for a period of three months to recruit as many eligible patients as possible.

The patients who met the inclusion criteria were approached with explaining the study purpose and assurance to protect their information and received signature on a consent form. The numerical pain scale score and neck index used in the assessment sheet of patient to assess the neck pain and the quality of patient life via Tele rehabilitation or zoom videos conferences to conduct the tele-sessions with using personal computers. The exclusion criteria for sample selection included history of cervical and thoracic spine fracture and dislocation, surgery of the cervical and thoracic spine, spinal infections and the intake of analgesic medication to reduced pain. (Morphine, Paracatamol). Pre-test and post-test analysis were conducted personally by the researchers and placed into large envelopes for confidentiality. Each session took around 30 minutes for completion.

The data were collected through the use of ZOOM video conference (Zoom Video Communications. 2019) to evaluate by assessment sheet treat the patients, neck disability index to measure the functional ability for Neck. Numerical Pain Scale to measure pain intensity (Schofield.2018) and more over Personal computer used to keep all data. The Tele-rehabilitation exercises program session was 2 per week. The participants were instructed to repeat the same exercises program daily at home. The exercises program started with active range of motion exercises, flexed and extended their neck slowly without holding at the end ranges. Participants rotated their neck slowly to right and left without holding at the end ranges, bending their neck in bilateral side without holding at the end ranges. The repetition was 10 times in 3 sets, in each direction, with adequate rest between of about 30 seconds in each set, followed by stretching exercises, which they performed by stretching toward lateral flexion and general through stretching exercises for extensor neck muscles. The exercises were performed for 30 seconds /10 times with a 30 second rest between each set, thus finishing 10 isometric strengthening exercises: The strengthening exercises were performed as isometric neck strength exercises with holding for 10 seconds /10times in each direction sets, (Ylinen.2007).

Figure 1. Distribution of patients based on age group

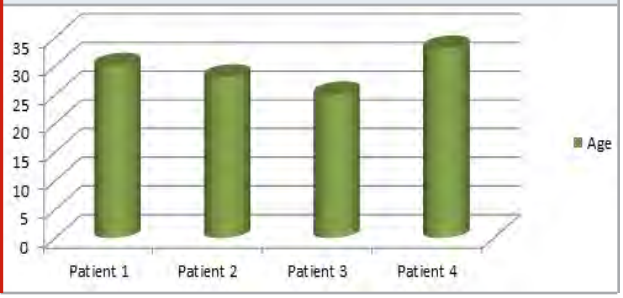
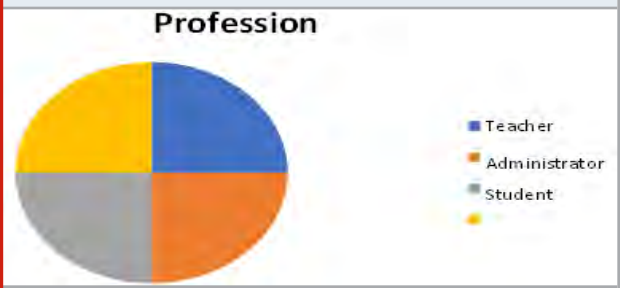


Figure 2: Distribution of patients based on profession



RESULTS AND DISCUSSION

The statistical values provided an explanation of the relationship between demographic characteristics of the patient and factors that influence neck pain as well as the quality of tele rehabilitation. All statistical analysis were conducted using IBM SPSS Statistics version 21.Data, which was presented as descriptive analysis, frequencies, percentages and mean ±standard deviation. The results of the study are presented in this section .showing the

descriptive analysis of socio demographic details where a paired t-test was used to analyze the difference between the mean of groups. It is expected that the results of this study will add to the existing database of body knowledge regarding tele-rehabilitation in KSA.

Figure 3: Distribution of patients numerical pain score based on pre and post-test

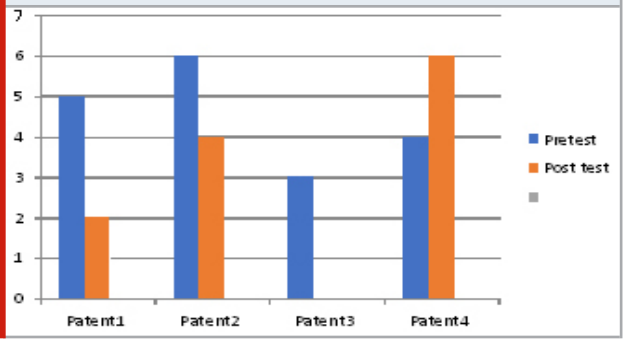
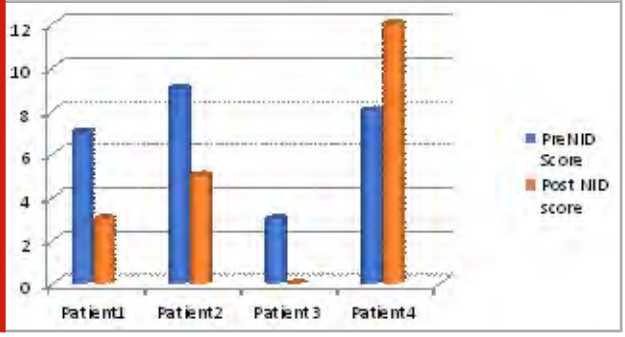


Figure 4: Distribution of patients neck disability index score based on pre and post test



The pre-numerical pain scale score (N=4) was associated with M=4.5(SD=2.03) by comparison of post-test (N=4) was associated with M=3(SD=2.03).to the t-test hypothesis that the pain score. The pre- neck disability index score (N=4) was associated with M=6.75(SD=4) by comparison of post-test (N=4) was associated with M=5(SD=4).

Tele rehabilitation has become more expanded and moreover the people are trying to understand the benefits of the same rapidly. The fast development of telecommunication technologies require continuous study in order to establish the efficacy of technologies. Data of the present work show that the neck exercises are one of important treatment modalities in treat neck pain and play prime role in reducing pain and improving the functional ability in neck patients. It strengthens the weak muscles and improves neck posture as well. Suvarnnato, et al (2019) have also shown that excurses are highly beneficial and have effect on specific deep cervical muscles decreasing the functional disability and pain intensity in a randomized controlled trial.

The overall aim of the present study was to measure the effectiveness of tele rehabilitation exercise program on pain and functional ability in patient neck pain. The socio demographic data revealed that all patients responded were males and the age between 25 to35 years. Also the current study showed there was an improvement in the statistical difference at $p<0.1$ between pre and post- test in both numerical pain score and neck disability index. From the Findings of the study includes, 4 patients were participated and three among the patients showed improvement were as one among them pain score and neck index became 0 after post- test and one patient increased pain and neck index score after the session. The result of the present study showed more significance among the score of both pre and post -test in numerical pain score as well as neck disability index .The use of tele-rehabilitation was associated with minimizing the time, expense and inconvenience of receiving rehabilitative care These results indicate the tele rehabilitation can be a promising potential alternative standard of care although some factors like unavailability of internet and materials always negatively affect the therapy.

Table 1

Variable	Mean pre test	Mean post test	P-value	T-value	Inference
Numerical Pain scale	4.5	3	0.48345	2.044	Not Significant
Neck disability index	6.75	5	0.2158	1.225	Not Significant

The exercises program is important to treat patients with neck pain and furthermore the treatment sessions should focus on specific cervical muscles. The study was limited to the patients who came to participate in this study additionally the small sample size was another challenge because of COVID 19 crisis and more over the study could only choose the patients with neck pain, although we did not observe for other diagnosis. Furthermore the study was conducted among male patients only and during the sample collection the patients were willing to participate only for the part of study due to covid-19 pandemic, the number of patients of this study was quite low.

CONCLUSION

In this part the conclusion of the present study in addition their recommendation for further study of practice, education, and research implication was discussed. The result of the present study showed more significance among the score of both pre and post -test in numerical pain score as well as neck disability index .The use of tele-rehabilitation was associated with minimizing the time, expense and inconvenience of receiving rehabilitative care These results indicate the tele rehabilitation can be a promising potential alternative standard of care

although some factors like unavailability of internet and materials always negatively affect the therapy. The exercises program is important to treat patients with neck pain and furthermore the treatment sessions should focus on specific cervical muscles.

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Technical Communication

Role of Collaborative Network Learning in Developing the Effectiveness of Research Self-Identity Among College Students of Education of King Khalid University Abha Saudi Arabia

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ABSTRACT

The present study aimed at investigating the effectiveness of collaborative network learning in developing the effectiveness of the research self-identity in the "Research Seminars" course for the seventh-level students of the College of Education at King Khalid University Abha Saudi Arabia. The study followed the semi-experimental approach, which is the design of pre-measurement and post-measurement for two groups, the experimental group and the control group. The experimental group uses the method of networked collaborative learning through course codes to activate the research self-identity with the pre and post application of the research tool on the two research groups. The researcher applied the tool of Research Self Effectiveness Scale on a sample consisting of (25) students from the College of Education. The study confirmed the learning effectiveness of the networked collaborative learning in developing the research effectiveness of the experimental group.

KEY WORDS: EFFECTIVENESS – COLLABORATIVE NETWORKED LEARNING – RESEARCH SELF-IDENTITY.

INTRODUCTION

The current era is witnessing rapid and changing technological progress in all areas of life in general and particularly in the field of education, and to keep pace with this, e learning has become an entry and a starting point for the strategic development of the educational process and educational institutions. Thus, new concepts emerged based on scientific and theoretical foundations related to e-learning such as virtualization, network, personality, and the use of various web technologies and tools to provide a collaborative electronic learning environment of a personal and social nature that increases the effectiveness of communication between teachers and students. These electronic environments have been indispensable in our lives, as the intensive use by some students has helped them to meet their social, academic, and psychological needs. (Ozad & Uygarer 2014)

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Zaitoun (2005) referred to the modern trend in e-learning. He explained that it applies e-learning to a group of learners in a collective, collaborative manner called the "collaborative e-learning style", and with the emergence of the new generation of the Web (Web2), which includes many social programs such as "blogs", forums, wikis, and others, the concept of e-learning and how to interact with it has changed to be more interactive and more specialized. These collaborative e-learning environments contribute to improving individuals' awareness of their own effectiveness and their research skills. Self-efficacy is one of the important matters for the individual; it is through the individual's personal beliefs about his own efficacy that he can achieve the goals he seeks. Therefore, the individual with high effectiveness has more persistence, endurance, and perseverance to accomplish tasks. It makes him more balanced, less stressed, more self-confident, and obtaining his goals without attacking others or infringing moral and legal rules (Al-Ma'aytah, 2000).

More recently, the concept of self-efficacy appeared in the field of scientific research, so the definition of "Forester et, al., 2004" explained that it is "the effectiveness of the research self-identity as the confidence in the performance of tasks associated with conducting research successfully. However, " Unrau & Beck, 2005" determined that the effectiveness of Research self-identity is defined as confidence in the implementation of research activities from organizing a research plan to carrying out a research process from office research to reading, writing and publishing research. In light of the above, the researcher considered the need to encourage female students in the collaborative learning groups, and to provide appropriate support and conditions that help them raise the level of effective research self-identity. This is the starting point for the researcher in studying the effectiveness of collaborative-networked learning in developing the research self-identity, including scientific research skills among students of the College of Education for Girls at King Khalid University.

The study of Hamada and Ismail (2014) aimed at measuring the impact of designing a collaborative e-learning environment based on some Web 2 tools in accordance with the principles of communicative theory on developing personal knowledge management skills among computer students by conducting a research experiment on students of the fourth year in the Department of Educational Technology. They were randomly distributed into two groups: the first experimental group is taught through a collaborative e-learning environment based on Web 2.0 tools that was designed through the Moodle learning management system. It is linked to Web 2.0 tools like wiki, Facebook, and blogs. The second experimental group is taught through a traditional e-learning environment designed using the Moodle system Learning Management Chat tool for synchronous communication and Forum tool for asynchronous communication.

The results of the current research confirmed the effectiveness of both the collaborative e-learning environment based on some Web 2.0 tools and the traditional e-learning environment in developing personal knowledge management skills for computer students in favor of post-performance. The results also showed that the collaborative e-learning environment based on some proposed Web 2.0 tools outperformed the traditional e-learning approach in developing personal knowledge management skills for educational technology students. The study of Al-Yateem and Al-Sayyad (2015) aimed at finding out the effect between two types of educational designs (blogs) on gaining knowledge in mathematics among secondary school students in Bisha. The chosen sample was divided into two experimental groups with one design assigned to each group – one of the two designs is based on Hypertext, the other design is based on super graphics in the engineering unit in mathematics. The results showed that there is a change in the two experimental groups in knowledge acquisition after applying the two designs.

The study of Abu Hashim, Abu Al-Layl, Allam and Kamel (2016) aimed at revealing the effect of using a collaborative e-learning environment on developing some self-organization skills and mathematics achievement among high school students. The research relied on the semi-experimental method. The research group consisted of (30) students from the second grade of secondary school in one of the Al-Azhar institutes in Ismailia Governorate. The research tools were represented in an achievement test in the dynamics unit of the second grade secondary school students. The theoretical framework of the research was based on several elements such as the collaborative e-learning environment, the characteristics of the collaborative e-learning environment, the importance of employing the participatory e-learning environment in teaching and learning mathematics. In addition, it also focused on the collaborative e-learning environment, the characteristics of blogs, the justifications for using blogs in the collaborative e-learning environment, the importance of educational practices to employ blogs within the collaborative e-learning environment, and the considerations that must be taken into account when designing the collaborative e-learning environment. The results of the research indicated that there were statistically significant differences at the level of significance of (0.01) between the arithmetic average scores of the control students group and the experimental group in the post measurement of the achievement test in favor of the experimental group.

The research recommended the necessity of using the collaborative e-learning environment in different school subjects. It also raises the need to prepare training programs for teachers to develop the skill of using the collaborative e-learning environment, and the need to spread awareness of the importance of activating the collaborative e-learning environment because of its effective role in building the educational process and developing achievement. The research suggested studying the effect of using the collaborative e-learning environment on developing achievement in other school subjects. The study of Arnout (2017) also aimed at identifying the level of research effectiveness of the self-identity among graduate students and revealing the differences in the effectiveness of the research self-identity according to the influence of some demographic variables. The researcher used the descriptive and comparative causal approach. A random sample of (671) students was selected. The results of the application showed a decrease in the effectiveness of the research self-identity.

Ismail's study (2019) sought to measure the effect of the interaction between the design of the evaluation method and the pattern of corrective feedback through digital platforms and its effect on developing the effectiveness of the research self-identity and making professional decisions among graduate students. The semi-experimental approach was used based on the global design (2 x 3). The research sample consisted of (90) master's degree graduate students in the field of educational technology. They were randomly divided into

(6) experimental groups of (15) members each. The two tools of the study were a measure of the effectiveness of the research self-identity consisting of (5) dimensions namely research initiative and perseverance, research planning, research effort, seeking research assistance, and research writing effectiveness.

It included (40) items, and the professional decision-making scale included (40) items. The results related to both the effectiveness of the research subject and the professional decision-making showed the effectiveness of the discussion method compared to the video interview method via the digital platform, and the effectiveness of corrective feedback after discussion with peers compared to the corrective feedback after discussion with the teacher and direct corrective feedback. The existence of statistical significant differences due to the basic effect of the interaction between the design of the evaluation method and the pattern of corrective feedback via digital platforms. The study of Abu Bakr and Ahmed (2020), which dealt with identifying the relationship between organizational conflict management methods like cooperation, negotiation, avoidance, coercion, settlement and job bliss and self-efficacy of research among faculty members at Minia University. The researchers prepared three measures namely organizational conflict management methods, job bliss, and research self-efficacy. The basic study sample consisted of (252) faculty members in some colleges of Minia University. The results of the study found that there is a positive correlation between the methods of cooperation, negotiation, and settlement as well as job blissfulness and the effectiveness of the research self-identity among faculty members at the university.

Research Problem: Through e-collaborative learning, students learn through collaborative groups via the Internet, which is a type of e-learning based on the participation of each group in learning lessons and implementing educational projects, and includes synchronous and asynchronous collaborative learning (Loo, 2004).

Here, the vital role of interaction in the collaborative e-learning environment emerges, which encourages students to be active and pushes them to continuous education and engage in learning and actively participate in multiple learning activities through various e-learning tools in collaborative interactive environment. The latter is characterized by the abundance of open learning resources, open access and open search seeking to build knowledge autonomy and the acquisition of learning experiences that enable students to achieve learning goals on their own. In addition, interaction in e-learning via the web is a communicative dialogue between the learner and the e-learning program and gives him/her a degree of freedom to actively participate in learning and building information (Sheikh, 2013). Self-identity is one of the most powerful processes of self-regulation. When self-efficacy is of a high level, one gains confidence in his ability to perform behaviors that control a difficult circumstance and self-identity in this case can be considered a form of confidence. (Bim, 2010) According to Pandora, self-identity in the field of scientific research is confidence in the ability to carry out research activities including organizing research plan, library research, reading, writing, and publishing (Lei, 2008).

The collaborative e-learning environment via the web is one of the environments in which the various tools and capabilities of the Internet can be used in developing problem-solving skills if they are appropriately constructed. Employing Internet tools are the best employment to serve the collaborative learning environment (Al-Sayed, 2013). (Alsurehi & Youbi 2014) explained that many universities in the Western world are using a range of student social networking applications in order to facilitate and enhance communication, collaboration and research. Some foreign studies also indicated that social media has greatly benefited in meeting human needs for belongingness, self-confidence and self-realization. It allowed university students to satisfy many needs according to the "Maslow" hierarchy of human needs and help them improve academic achievement, complete academic tasks, and enhance social interaction with classmates and members of the teaching staff as in the study of (Ahmad 2012).

In light of the above, the researcher found during the teaching of a "Research Seminars " course for seventh-level students at the College of Education for Girls at King Khalid University, that many students prefer electronic participation through various blogs and forums such as discussion forums on the blackboard system to perform the required tasks and information with their peers in that course. In fact, the course of the research circle is advanced and has a special nature that requires research with high skills and the generation and application of knowledge so that students can understand and apply their knowledge in preparing research plans. The tasks and educational activities in this course needs to build knowledge and effective communication through collaborative learning via the Internet in the blackboard e-learning system to complete those tasks and activities in the course. The researcher stressed the need to examine the effectiveness of network collaborative among these students and measure its role in developing the effectiveness of their research self-identity in the course of Research Circle by trying to answer the following question: What is the effectiveness of collaborative networked e-learning in developing the research self-identity among the students of the College of Education at King Khalid University?

In order to answer this main question, the validity two hypotheses will be tested. The first of which states, "there is a statistically significant difference between the pre and post measurement of the experimental group on the measure of the effectiveness of the research self-identity in favor of the post measurement". The second hypothesis states, "there is a statistically significant difference between the two groups of experimental and control research on the measure of the effectiveness of the research self-identity in the course forums after using

the collaborative learning method in the network in the post-measurement for the benefit of the experimental group.

METHODOLOGY

The semi-experimental approach was used, which is the design of the pre-measurement and the post-measurement for two groups, the experimental group and the control group. The experimental group uses the collaborative learning method in the network through the course codes to activate the research self-identity with the pre and post application of the research tool on the two research groups. The researcher applied the tool of the effectiveness of the research self-identity scale on a sample consisting of (25) female students from the College of Education at King Khalid University by conducting the apparent validity of each of them to verify the validity of the content.

It was presented in its initial form to a group of referees including professors specialized in educational psychology, psychometrics, pedagogy, curricula and teaching methods, and educational technology. In light of their opinions and directions, some modifications were made, including deletion and addition, in line with the nature of the sample in the current research until the research tool reached its final form to be applied to the current research sample. The researcher determined a measure of the effectiveness of the research self-identity in the following dimensions: The ability to succeed In the course of the research circle – the ability to choose a research problem – the ability to collect literature – the ability to choose a systematic design –

Table 1. Shows the significance of the differences between the pre and post measurement for the experimental group on the research self-efficacy scale

Dimensions of the Research Self Effectiveness Scale	Comparison group	Arithmetic Average	Standard Deviation	T value	level of significance
The ability to succeed in the course of the research circle	Pre	7.72	1.43	8.68	Significant at 0.01
	Post	12.84	3.11		
The ability to choose a research problem	Pre	6.92	1.85	30.64	Significant at 0.01
	Post	11.56	1.85		
The ability to collect literature	Pre	13.24	1.66	17.20	Significant at 0.01
	Post	20.68	1.82		
The ability to choose a systematic design	Pre	7.36	1.50	37.29	Significant at 0.01
	Post	12.40	1.38		
Efficient data analysis, interpretation and recommendations	Pre	7.04	1.43	20.31	Significant at 0.01
	Post	12.08	1.73		
Total marks	Pre	42.28	3.39	34.51	Significant at 0.01
	Post	69.56	4.75		

Data analysis competence, interpretation, and recommendations. It was formulated in (28) phrases. After that, she calculated the validity and reliability of the scale, and the results showed that the scale has its ability to distinguish between individuals. As for reliability, the Alpha Cronbach equation was used and the results showed that the reliability coefficient is high and statistically significant at a level of significance (0.01). The study community consisted of female students from the College of Education at King Khalid University. The research sample consisted of (50) female students of the seventh level who are studying the " Research Seminars " course at the College of Education for Girls in Abha at King Khalid University. The sample was divided into two groups, the experimental group of (25) female students and the control group of (25) female students. The researcher believes that the selection of female students at King Khalid University in this research was done on the basis that they are a distinguished age group in the Saudi society characterized by maturity, vitality and the ability to assume responsibility. Moreover, they are taught in the final stage of university education and use the tools of collaborative networking represented in Blogging to develop and activate their research self-identity.

Statistical Results: The first hypothesis validity test: To verify the validity of this hypothesis, the research self-efficacy measure was applied in the " Research Seminars" course on the members of the experimental group before and after the use of the networked collaborative learning method. The arithmetic average scores of the experimental group in the pre and post measurements were compared on this scale as a total grade. The following table shows the results related to this hypothesis.

It is evident from the data of table (1) that there is a statistically significant difference between the pre and post measurement of the experimental group on

the measure of the effectiveness of the research self-identity before and after the use of the collaborative learning method in the network in the overall score and dimensions by (0.01) in favor of the post-measurement of the experimental group. This result can be explained by the superiority of the experimental group members in the post-measurement on the measure of the effectiveness of the research self-identity due to the use of the collaborative learning method on the network through the course notes on the blackboard system.

Here, students independently implement the steps of scientific research, organize the research plan and display information while they have a high degree of confidence in their capabilities and capacities to perform the tasks required in the " Research Seminars " course codes with high skill. They can identify the research problem and write it distinctly and search for books and references by themselves in the electronic library on the King Khalid university website and other electronic resources such as "Google". This scientific activity related to scientific research skills that students perform on their own in the " Research Seminars " course blogs increases significantly the effectiveness of the research self-identity. This result is consistent with the findings of the studies of (Vaccaro, 2009), (Amin and Muhammad, 2009), (Al-Dukhani, 2015), (El Desouki, 2015), and (HeidariGorji et. al, 2015) whose results agreed that there is a strong relationship between the motivation to learn and the effectiveness of the research self-identity.

The second hypothesis validity test: To verify the validity of this hypothesis, the research self-efficacy measure was applied to all the sample members (the experimental group – the control group) after completing the use of the networked collaborative learning method, and the arithmetic average scores of the two groups were compared on this scale as a total degree. The following table shows the results related to this hypothesis. It is evident from the data of table (2) that there is a statistically significant difference between the two groups of experimental and control research on the measure of the effectiveness of the research self-identity after the completion of the use of the collaborative learning method in the network in the overall degree and dimensions, where the values of "T" ranged between (6.588 – 19.815), which are significant values at A level (0.01) in favor of the experimental group, and this indicates that the third hypothesis has been fulfilled.

Table 2. Illustrates the significance of the difference between the experimental and control groups On the research self-efficacy scale after completing the networked participatory learning method

Dimensions of the Research Self Effectiveness Scale	Comparison group	Number	Arithmetic Average	Standard Deviation	T value	level of significance
The ability to succeed in the course of the research circle	Control	25	8.08	1.82	6.588	Significant at 0.01
	Experimental	25	12.84	3.11		
The ability to choose a research problem	Control	25	6.96	1.86	9.419	Significant at 0.01
	Experimental	25	11.56	1.58		
The ability to collect literature	Control	25	13.08	1.52	16.006	Significant at 0.01
	Experimental	25	20.68	1.82		
The ability to choose a systematic design	Control	25	7.08	1.75	11.904	Significant at 0.01
	Experimental	25	12.40	1.38		
Efficient data analysis, interpretation and recommendations	Control	25	7.08	1.38	11.290	Significant at 0.01
	Experimental	25	12.08	1.73		
Total marks	Control	25	42.28	4.97	19.815	Significant at 0.01
	Experimental	25	69.56	4.75		

Whereas, the students of the experimental group, as indicated by the statistical evidence shown in Table (3), have benefited from the use of the collaborative learning method in the network to acquire scientific research skills in the course of the "Research Seminars" and were able to satisfy their psychological needs (such as a sense of self-worth and freedom of expression), academic needs (Such as participating in the topics of the course decision and increasing motivation and improving the level of academic achievement), intellectual needs (such as exchanging opinions and ideas easily, developing critical thinking and enriching intellectual outcomes) in the course forums on the blackboard system. This contributed to the development of their research self-identity in a positive and effective tangible manner as compared to the control group that did not use the networked collaborative learning method and their research self-identity was very low. This confirms the effectiveness of networked collaborative learning in developing the research self-identity in the " Research

Seminars " course among the seventh-level students of the kindergarten department at the College of Education at King Khalid University. Moreover, this is in agreement with the findings of the studies of (Hamada, 2014), (Al-Rehaily, 2014), and (Abu Hashem, 2016), which agreed that the use of the e-learning collaborative environment has a great impact on learners.

Findings and Recommendations: Through the application of the study methodology and its tools, the study confirmed in its results the effectiveness of using the collaborative network learning method in developing the effectiveness of research among the students of the experimental group, which led to the enhancement and acquisition of scientific research skills, the satisfaction of their psychological and academic needs, the increase of motivation and the improvement of academic achievement. This result can be explained by the fact that the superiority of the experimental group members in the post-measurement over the research self-efficacy scale is attributed to the use of the collaborative learning method on the network through the course codes on the blackboard system. At this level, each student independently implements the scientific research steps from organizing the research plan and presenting information while she is of a high degree of confidence in her capabilities and capacities to perform the tasks required in the "Research Circle" curriculum blogs with high skill. She can identify the research problem, write it distinctly, and search for books and references by herself in the electronic library at the King Khalid University website and other electronic resources such as "Google scholar".

The scientific activity related to the scientific research skills practiced by each student on her own in the " Research Seminars " course blogs significantly increases the effectiveness of their research self-identity. They have benefited from the use of the collaborative network learning method in acquiring scientific research skills in the course of the " Research Seminars " and they were able to satisfy their psychological needs (such as a sense of self-worth and freedom of expression), academic needs (such as collaborative in the subjects of The research circle course, increasing motivation, improving academic (and intellectual) achievement (such as exchanging opinions and ideas easily, developing critical thinking and enriching intellectual outcomes) on the blackboard system course blogs.

This contributed to the development of their research self-identity in a positive, tangible and effective way compared to the control group with whom the network collaborative learning method was not used, so their research self-identity was very low. This confirms the effectiveness of networked collaborative learning in developing the research self-identity in the "Research Circle" course among the seventh-level students of the Kindergarten Department of the College of Education at King Khalid University.

The study recommends, based on its results, to make use of the participatory network learning method in developing critical thinking skills, self-development, and developing personal, academic and skillful abilities among university students, as well as the necessity of holding training courses for university faculty members to develop the skills of using online collaborative learning tools such as blogs and discussion forums in the educational process. Moreover, there is a need for educating faculty members about the importance of networked collaborative learning in developing the research self-identity among university students, also encouraging university students to have confidence in their abilities and capabilities to implement scientific research steps with skill and accuracy, as well as supporting the method of online collaborative learning in teaching electronic courses to achieve high quality learning outcomes.

Conflict of Interest: Author declares no conflicts of interests to disclose.

Ethical Clearance Statement: The Current Research Work Was Ethically Approved by the Institutional Review Board (IRB) of King Khalid University, Abha Saudi Arabia.

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Biotechnological Communication

Production of Bioethanol from Sugarcane Juice, Molasses and Paddy Straw using *Saccharomyces cerevisiae*

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ABSTRACT

The non-renewable sources of energy are depleting all over the globe at an alarming rate. Bioethanol is considered as the fuel of modern era as it contributes around 10-14% of the total global energy supply. Researches are in continuous search of renewable resources for energy production. The use of biodegradable waste as a source of energy is gaining interest. In the present study, different renewable resources viz. sugarcane juice, molasses, and paddy straw were tested for their efficacy to produce bioethanol. Three different yeast strains designated as Y1, Y2, and Y3 were isolated from over-ripened fruits and were used to ferment the different substrates used in the present study. Sugarcane and molasses were directly used for ethanol production while paddy straw was pre-treated biologically and chemically before using it as substrate for ethanol production. For biological treatment, cellulolytic microorganisms were used to degrade paddy straw while acid and alkali treatments were given to paddy straw during chemical treatment. Thereafter, ethanol was produced using selected yeast strains. The study showed that the strain Y3 produced maximum ethanol with all three substrates viz. sugarcane juice (9.4%), molasses (9%) and paddy straw (4.21%). Paddy straw yielded very little ethanol as compared to sugarcane juice and molasses. The present study showed that sugarcane and molasses are very good substrates for the production of alcohol but lignocellulosic agricultural waste like rice straw can also be used for the production of alcohol but they need to be pre-treated. This research is expected to act as a milestone for future studies providing with credible baseline work.

KEY WORDS: BIOETHANOL, MOLASSES, PADDY STRAW, SACCHAROMYCES CEREVISIAE, SUGARCANE JUICE.

INTRODUCTION

Ethanol is the important fuel used in automotive industries and in other potable purposes. It is considered as a renewable alternative to fossil-based fuel. So, its production needs to be enhanced in the coming time by using modern techniques. Ethanol is basically produced by the fermentation of sugar or starch from agricultural crops by yeast or bacteria. A variety of substrates are used for the production of ethanol. These substrates include molasses, sugar beet pulp, and waste newspapers (Xin et al., 2010; López et al., 2012; Kasavi et al., 2012; Amid et al., 2021) etc. Substrate, strain, nutrient, and physiological factors like temperature and pH affect the fermentation process and ethanol production.

The availability and cost of the substrate need to be assessed before beginning fermentation. The use of cheap and readily available substrate is recommended to make the fermentation process cheaper. The production cost can be further reduced by using renewable sources like molasses, potato starch, sugarcane, and paddy straw (Oscar and Carlos 2008; Amid et al., 2021). Molasses are the most commonly used and easily available substrates and cheapest too but the increased demand has reduced the availability, thereby increasing the cost (Schweinitzer and Josenhans 2010; Amid et al., 2021).

So it is the need of hour to focus on alternative substrate for ethanol production. Recently, focus has shifted from

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molasses to paddy straw. Sugarcane is regarded as a good source of ethanol production because of its high sugar content and even infected plant juice can also be used for ethanol production. Moreover, the energy accumulated in the sugarcane biomass is directly from the products of photosynthesis, due to carbon dioxide fixation, which decreases the crop's overall contribution to global warming. Alternatively, paddy straw is also a very good substrate for the completion of ethanol requirements because it contains 32-47% cellulose and 19-27% hemicellulose. However, some problems are also associated with paddy straw if used for fuel production. One of the major problem is the presence of lignin, cell wall polysaccharides, and cellulose crystallinity. Lignin makes enzymatic degradation difficult as it covers cellulose and hemicellulose and hence protects polysaccharides from degradation. So removal of lignin is necessary so that cellulose becomes accessible to the enzymes and yeast action. Lignin can be hydrolyzed by using some chemical and biological methods (Krishna and Chowdary 2000; Malik et al., 2021).

The cellulose and hemicellulose become fermentable sugars by pretreatments either it is chemical or biological means, the later employing enzymes like cellulases and hemicellulases. Although, some advantages and limitations are there for both methods. Chemical hydrolysis though advantageous by being rapid but is limited by low sugar recovery efficiency, formation of furfural and other degradation products are poisonous to the fermentation microorganisms and raised environmental concerns due to disposal of acid. The biological (enzymatic) methods, on the other hand, have the advantage of being highly specific, ecofriendly, and no degradation products of glucose are formed (Sukumaran et al., 2010; Hou et al., 2021). The present study, aims to utilize alternative renewable resources for ethanol production using both chemical and enyzymatic means of degradation. The use of paddy straw for ethanol production is a potential and a low-cost method that can be employed at commercial scale. Further, it will also help to solve the paddy straw burning problem in northern India.

MATERIAL AND METHODS

The present study was carried out in the Department of Biotechnology, Govt. College, Hisar (Haryana). All the material for yeast isolation and substrates for ethanol production was taken from the local market and field of Hisar. The chemicals and media ingredients were of AR or GR grade and procured from either M/s E. Merck or BDH or HI- MEDIA, India. The glassware used was of high-quality Borosil. For the yeast was isolated on YEPD medium from honey, cheese, tomato, curd, sugarcane, jaggery, and molasses procured from the local market of Hisar (Haryana) and then characterized. The isolated colonies of yeast were tested for their ethanol-producing abilities. These were tested on different substrates like sugarcane, paddy straw, and molasses (Kreger-van Rij 1984).

For the isolation of cellulolytic microorganisms, one gram of rice straw powder was taken and suspended in 9 ml of sterile distilled water. After serial dilution of this suspension (10-1 to 10-6 times), 100 µl from each dilution was spread on carboxymethyl cellulose (CMC) agar plates (1% CMC, 0.1% KH_2PO_4, 0.1% K_2HPO_4, 0.04% $MgSO_4$, 0.005% NaCl, 0.000125% $FeSO_4$, and 1.8% agar, pH 7.0) and incubated at 37°C for 24-48 h which is suitable temperature for the growth of yeast. The isolated bacterial colonies forming clear-zones after application of 1% congo red dye solution were selected as cellulase producers. Bacterial isolates producing significant clear zone on CMC agar were identified based on cultural, morphological, and biochemical characteristics (Cowan and Steel 1974; Kasana et al. 2008). For observations, plates were stained with 1% Congo red dye (15 min), followed by de-staining with 1M NaCl solution for 20 min. Cellulolytic strains were selected on the basis of the hydrolysis zone surrounding the colonies. The cultures were identified based on the cultural, morphological, and biochemical characteristics (Rifai 1969; Cowan and Steel 1974; Teather and Wood 1982). For the ethanol production from sugarcane, the samples of sugarcane juice were collected from local hand-operated cane crushers from different locations of Hisar. Different pretreatments like filtration, sterilization, and concentration were given to sugarcane juice. Then it was inoculated with a previously isolated yeast strain and incubated at 30 °C for 24 h for fermentation.

For the ethanol production from paddy straw, the paddy straw saccharification was conducted by microorganisms. The isolated colonies of bacteria were then tested for their ability to grow and degrade rice straw. Rice straw was crushed and added into 100 ml of mineral salt medium in a 500 ml Erlenmeyer flask and autoclaved. The media was allowed to cool down and 3 ml of yeast suspension was added to it for fermentation. The cultures were incubated at 25°C for 14 days. After 14 days, culture aliquots were centrifuged (REMI) at 6000 rpm to remove solids. The supernatant was used as crude enzyme solution (Belal and El-Mahrouk 2010). Now the supernatants were assayed for their enzymatic activity. Cellulase activity was determined by incubating 0.5 mL of the supernatant with 1% CMC and it was incubated at 60°C for 30 min. After incubation, the reaction was terminated by adding3 mL of 1% 3,5-dinitrosalicylic acid (DNS) reagent to1mL of the reaction mixture and heated for 10 min at 100 °C. In these tests, reducing sugars were estimated calorimetrically using glucose as standard (Miller 1959; Belal and El-Mahrouk 2010).

For the alkali and acid treatment to the rice, paddy straw was procured from Ramray village of Jind district (Haryana). It was dried at 50 °C, comminuted to small pieces using grinder fitted with sieves of different mesh sizes. For alkali treatment, about 50 g chopped (>2 cm length) dried rice straw was suspended in 1, 2, 3, 4, and 5% NaOH in a ratio of 1:10 (w/v). Thereafter, the samples were incubated in water bath at 85°C for 1 h. Finally, hydrolysate was passed through cheesecloth. For acid hydrolysis, about 50 g chopped dried rice straw was

suspended in an 1:10 (w/v) sulfuric acid solution of 1, 3, 5, 7, and 9%. The mixtures were autoclaved at 121°C for 15 minutes and enzymatic treatment was given to slurry (1:10 of alkali-treated paddy straw in distilled water).

Commercial cellulose was added at a concentration of 7.5 FPU/g substrate and incubated at 35°C in an incubator. Reducing sugar was estimated by DNS method (Miller et al., 1959). For the ethanol production after treatment, the pretreated samples were inoculated with the isolated cultures of yeast for fermentation. After fermentation ethanol was estimated using dichromic method (Caputi et al., 1968). Absorbance was read at 600 nm against a suitable blank using spectrophotometer. The amount of ethanol was determined by referring to a standard curve plotted from different concentrations (1-7%) of absolute alcohol. For the statistical analysis, analysis of variance (ANOVA) was used to test the significant difference among the three strains viz. Y1, Y2, and Y3. Mean differences among the categories were separated by Tukey's test at a confidence interval of 95%.

RESULTS AND DISCUSSION

In the present study, isolated cultures were screened for primary identification from different samples and fermented products as described above. A total of ten cultures were isolated from these samples out of which three were identified as yeast. Apple, orange, banana, and other fruits were locally available and thus served as readily available raw materials for the separation of ethanol-producing yeasts. Various strains of indigenous yeasts capable of producing ethanol were isolated from local fermented pineapple juice by Eghafona (1999). These isolated strains were used for the production of ethanol from different substrates (Belal et al., 2013).

A comparative study on ethanol production from molasses using *Saccharomyces cerevisiae* and *Zymomonas mobilis* was performed by Bansal and Singh (2003) and Hossain et al. (2014). Different concentration of glucose (50, 10, 30, 50, and 70g/l) was used as a sole source of sugar in the MGYP medium; the consequences showed that the maximum yeast biomass and maximum ethanol yield was obtained at high glucose concentration. The cultures were identified as yeasts based on colony characters, microscopic examination, and budding formation. Colonies formed by yeast isolates were circular, smooth, and cream (Aguilar 2011).

Colony size varied from small to large (Table 1). Individual cells were oval, elongate, ovoid to spherical when young and hexagonal when aged. Cells showed oval, globose, spherical and ellipsoidal budding. Based on differences in colony morphology, color, appearance, size, and margin these strains were designated as Y1 to Y3 (Table 1) (Moaris 1996; Aguilar 2011). The isolated yeast strains were analyzed microscopically under 40X resolution of compound microscope (Olympus) using wet mount. Viability of *Saccharomyces* sp. also studied by Moaris (1996) and Aguilar (2011). In 50% glucose, reported viability of 10-98.8% in different strains of yeast (Moaris 1996; Aguilar 2011).

Table 1. Colony characteristics of yeast isolates

Yeast Isolate	Colony Color	Colony Nature	Appearance and Size	Elevation	Margin
Y1	Cream	Smooth	Circular & small	Raised	Entire
Y2	Cream	Smooth	Circular & Small	Raised	Entire
Y3	Cream	Smooth	Yeast like & medium	Convex	Entire

All the isolated strains were characterized on the basis of physiological parameters. All three selected yeast isolates were able to grow at 25°C-42°C. Therefore, the isolated yeast was considered to be thermo-tolerant. Ethanol tolerance of yeast was also checked and it was observed that yeast isolates can grow in liquid YEPD media containing up to 15% ethanol. Maximum growth was observed in 5% ethanol containing media. A very high or low concentration of ethanol is inhibitory for the growth of yeast. So, an optimum concentration is required for the growth of yeast. As far as we are concerned with sugar concentration, yeast was found to be resistant to sugar up to 10% (w/v) glucose concentration. The use of different concentrations of glucose (50, 10, 30, 50, and 70g/l) showed that the maximum yeast biomass and maximum ethanol yield was obtained at high glucose concentration (Nasreen et al., 2014).

Ethanol production from sugarcane bagasse was found to be 0.41g/L (Irfan et al., 2014). Rice straw (49 g/L) and wheat straw (34 g/L) also produced ethanol but their production was not good as compared to sugarcane bagasse. This difference in ethanol production was due to the availability of fermentable sugars from cellulose present in biomasses. Use of commercial enzyme for saccharification showed that treated rice straw gave better ethanol production (85 g/L) as compared to untreated (70 g/L) rice straw (Jalil et al. 2010). Pretreatment of sugarcane bagasse with 1 N NaOH resulted in 48% ethanol production by *C. cladosporoides* after 48 h of fermentation under static condition (Uma et al., 2010). A maximum ethanol production of 3.36 g/L was obtained from pretreated sugarcane bagasse under optimized process conditions in aerobic batch fermentation (Sasikumar and Viruthagiri 2010).

"Isolation of Cellulolytic microorganisms" Rotted rice straw residues were used as source for cellulolytic microorganisms in the present study. Only five microorganisms were isolated by using clear zone formation on MSA (mineral salt agar) containing carboxymethyl cellulose as a sole source of carbon. A preliminary classification based on cultural and morphological characteristics of the isolates revealed that the rice straw residues – degrading microorganisms belong to the group of fungi as well as to the group of bacteria, (Stella et al., 2015).

Figure 1: Percent ethanol produced from molasses after 24 h of fermentation

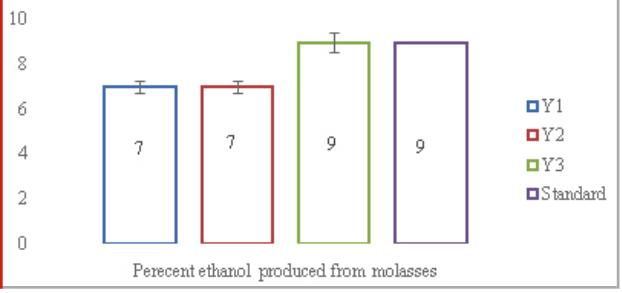
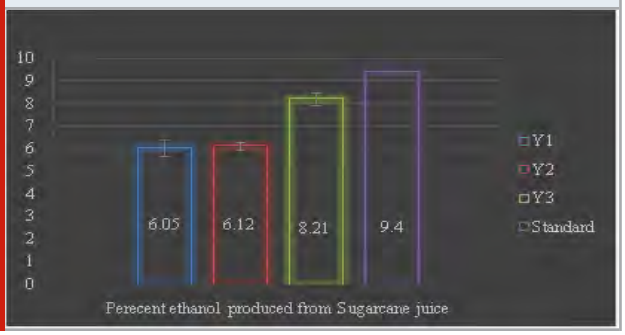


Figure 2: Percent ethanol produced from sugarcane juice after 24 h of fermentation



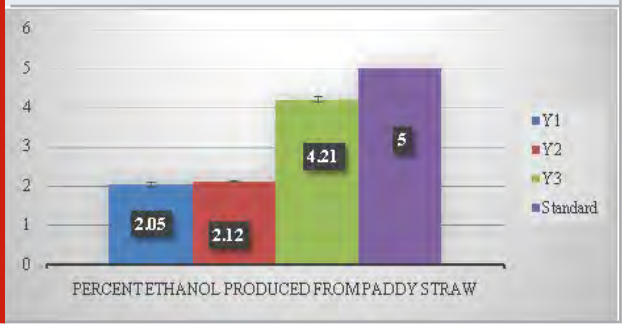
A total of three microorganisms were found to be gram-positive and rod-shaped. Results of identification showed that the rice straw degrading bacterial strain was identified as Bacillus. A microbial consortium consisting of 30 bacteria to study biodegradation of rice straw was developed by Stella et al., (2015). The microorganisms in the consortium were generally identified as *Proteus mirabilis, Raoutella planticola, Serratia sp., Pseudomonas viridilivida, Klebsiella oxytoca, B. fusiformis, B. cereus, Klebsiella sp., B. licheniformis, Corynebacterium urealyticum, Cellulomicrobium cellulans*, and *B. subtilis* using 16s rDNA molecular identification technique. "Ethanol production using molasses" Yeast isolate was examined for ethanol production using 25% (w/v) molasses, as substrate under the constant set of conditions. Ethanol production was estimated at 28°C and calculated after completion of fermentation. Yeast strains Y3 produced maximum ethanol (9%) followed by Y1 and Y2 (Figure 1) (Stella et al., 2015).

The data was statistically significant with a p-value of ≤0.02. Brooks (2008), isolated yeast strains from ripe banana peels for ethanol production and found, that isolates fermented 40% glucose at 30oC to yield 3.6 and 5.8% ethanol respectively. Molasses was used as a reference to check the production of ethanol in sugarcane and paddy straw. "Ethanol production using sugarcane juice" Sugarcane juice was explored for ethanol production. The process of ethanol production depends on the yeast strain employed (Stella et al., 2015).

The yeast strains differ considerably in the production of ethanol; therefore, it is essential to select suitable yeast strains for ethanol production from sugarcane. Y1, Y2, and Y3 yeast isolates of different morphology retrieved from different samples and sugarcane juice samples by dilution plating and enrichment culture technique were tested for ethanol production and their efficiency was compared against a standard culture of *Saccharomyces cerevisiae* (Giri 2008). Yeast strain Y3 give maximum ethanol production (8.21) but it was not higher than the standard culture of *Saccharomyces cerevisiae*. Y1 and Y2 produced almost similar i.e., 6.05% and 6.12% ethanol when sugarcane juice was used as substrate (Figure 2) (Giri 2008; Stella et al. 2015). The data was statistically significant with a p-value of ≤0.003. Sugarcane (*Saccharum officinarum*) is a C4 plant having high capability to convert solar radiation into biomass (Black et al., 1969). It is the most important feedstock grown in tropical and subtropical countries that can be used as juice or molasses (by-product of sugar mills) for fuel ethanol production. Total fermentable sugar content in sugarcane juice is about 12–17% in which 90% of this sugar is sucrose and the remaining 10% is glucose and fructose (Wheals et al., 1999). Sugar content in juice varies based on variety, maturity, and harvest time (Dhaliwal et al., 2011).

Sugarcane juice contains adequate amount of organic nutrients and minerals in addition to free sugars making it an ideal raw material for bioethanol production. "Ethanol production using paddy straw" An attempt was made to produce ethanol from paddy straw. In this experiment, paddy straw was de-lignified by using alkali and acid treatment. Thereafter, cellulosic enzymes were used for saccharification (Dhaliwal et al., 2011). After the treatment with cellulase, this solution was inoculated with three strains of yeast for fermentation. It was found that Y3 produced (4.21%) maximum ethanol followed by Y2 (2.12%) and Y1 (2.05%) (Figure 3). No statistical significance was observed in the data (p ≥2.99) (Dhaliwal et al., 2011). Lignocellulosic residues including rice straw, sugarcane bagasse, wheat straw, corn stover, spruce and municipal solid waste have been researched by several workers for microbial and enzymatic bioconversion with commercial or in-house produced cellulase into glucose employing various pretreatment protocols including acid, alkali and steam (Li et al., 2007; Patel et al., 2007; Kovacs et al., 2009; Rabelo et al., 2009; Yoswathana and Phuriphipat 2010).

Figure 3: Percent ethanol produced from paddy straw after 24 h of fermentation



Following pretreatment, plant cell wall polysaccharides are more susceptible to enzymatic hydrolysis that breaks them into monomeric (single) sugars that can be fermented into ethanol. The multiple mean comparisons (Tukey's test) of the average ethanol produced from molasses, sugarcane juice, and paddy straw using the three strains Y1, Y2, and Y3 showed that the average ethanol production from molasses was significantly different with paddy straw. A significant difference in ethanol production was also observed among sugar cane juice and paddy straw (Lynd et al., 1999; Yoswathana and Phuriphipat 2010).

CONCLUSION

Although the percentage of bioethanol produced during this study was not so high that can meet the increasing demand for fuel in modern time if parameters were optimized properly, this percentage can be improved. Clearly, the results exploring methodologies that allow the isolation of efficient ethanol-producing yeast strains from different source is limited and further research is needed. The opportunity exists to use such yeast strains to improve the efficiency of ethanol production from complex substrates like paddy straw. It should help reduce the current reliance on petroleum-based fuel. To take advantage of this opportunity, new approaches in the isolation of stable yeast strains capable of being used during high-temperature ethanol production will need to be developed.

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Biotechnological Communication

Evaluating the Effectiveness of Three DNA Bar code Loci to Classify Jewel Orchids Using *In silico* Approach

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ABSTRACT

Three DNA barcode loci consisting of ITS, *matK* and *rbcL* have been used intensively to discriminate jewel orchid species. Nevertheless, the discrimination ability of each locus is inconsistent among researches. Therefore, it is necessary to simultaneously check a large number of published DNA sequences to get an overview of the ability to recognize orchid varieties. In this study, total of 124 DNA sequences of these three loci from Genbank were evaluated for the discrimination ability on 114 species belonging to five genus of jewel orchids. The obtained data show the significant variability of barcode sequences at both taxonomy levels. Distinguishing ability descend from *matK*, ITS to *rbcL*. The obtained results suggest that the discrimination capacity of ITS, *matK* and *rbcL* DNA barcode loci are variable among different species of jewel orchid plants, in which *matK* and ITS loci reveal more potential for genetic classification at genus and species level of this herbal plants. In future, higher sequence number should be included in the analysis to give more reliable result. The information from this study could be useful in conservation and development programs of jewel orchid plants.

KEY WORDS: DNA BARCODE, ITS, JEWEL ORCHID, MATK, RBCL.

INTRODUCTION

Jewel orchid is a general term of different species belonging to Orchidaceae family. This plant can be found in large area from Southern China, Northeast India, Thailand, Vietnam, the Philippines, Malaysia, Indonesia and Myanmar. Beside ornamental use, jewel orchid is got more attention due to its high medical value. Different parts of plant are applied for treating numerous diseases such as abdominal pain, diabetes, nephritis, fever, hypertension, liver, and pleurisy (Du et al., 2008) since several medicinal compounds with strong biological activity have been identified in these plants (Liang et al, 1990; Lin et al., 1993; Panda et al., 2014). The term "jewel orchid" is used to refer to various species in Orchidaceae family having velvety brocade-like leaves with beautiful veins. Nevertheless, the medicinal and economical value are species dependent, thus, an accurate classification would be crucial importance as a basis for conservation and development. Traditionally, jewel orchids are discerned based on plant morphological characteristics such as leaves, stem structure, or flowers (Trinh et al., 2020).

While this method has been confirmed economically due to its ease and low cost, it is error-prone method due to identical external morphology features, large variable polymorphisms between adult and juvenile stages, and environmental factors as well as the plant growth development phases, all are leading to inaccuracy (Ahmedand and Mohamed, 2014). Furthermore, the inability to precisely identify specimens under damaged or processed conditions raises serious concerns about the medicinal value of the jewel orchid-derived products. Consequently, incorrect utilization will reduce effectiveness or harm the health of the patient due to the variation in medicinal compounds and application among different jewel orchid members (Du et al., 2008; Poobathy et al., 2018; Trinh et al., 2020).

Based on the rapid development of DNA sequencing technology, the application of a short standard DNA sequence, so-called DNA barcode, to identify target plant

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species has been used intensively. This method possesses several advantages in the comparison to traditional methods such as high repeatability and stability, applicability to any developmental stages of organism and ability to identify target organism being destroyed or processed (Barcaccia et al., 2016). Several barcode loci have been tried to investigate DNA barcode candidates for plant identification (Hollingsworth et al. 2011; Kress, 2017). However, two chloroplast gene namely maturase K (*matK*) and ribulose-1, 5-biphosphate carboxy lase (*rbcL*) have been proposed as preferred plant barcoding loci by consortium for the barcodes of life (CBOL, 2009). Beside *matK* and *rbcL* as standard DNA barcode, internal transcribed spacer (ITS) has also been used intensively for classification several plants recently such as *Talinum paniculatum* (Nguyen et al., 2017); *Astragalus* spp. (Zhang and Jiang, 2020); *Oryza* (Zhang et al., 2021).

In a short time, studies using these three DNA barcode loci for jewel orchid identification have been published (Chen and Shiau, 2015; Lv et al., 2015; Hu et al., 2019; Huynh et al., 2019; Sherif et al., 2020) suggesting the usefulness and potential of these three loci in plant classification. Despite the confirmed effectiveness of DNA barcode in plant identification, discrimination power and trending utilization of each barcode loci are variable. After careful survey 16 popular DNA barcode loci from 2005 to 2010, Hollingsworth and colleagues found the large difference in enthusiasm of using different locus (Hollingsworth et al., 2011). The species identification capacity of *rbcL* locus was higher than that of *matK* and ITS on mangrove plants (Wu et al., 2019). Nevertheless, another study in China at same time showed the inferior of *rbcL* to ITS and matK on *Astragalus* spp. (Zhang and Jiang, 2019). Therefore, the present study focused on evaluate the identification ability of ITS, *matK* and *rbcL* loci in jewel orchid plants by using DNA sequences available on the National Center for Biotechnology Information (NCBI). The obtained results could provide information to escalate the effectiveness in classifying and identifying different jewel orchid population at species and genus level.

MATERIAL AND METHODS

The sequences of ITS, *matK* and *rbcL* loci belonging to different jewel orchid species were downloaded from nucleotide database of NCBI (URL: http: //www.ncbi.nlm.nih.gov) by using corresponding Latin name and barcode locus as keywords. The sequences were selected for analysis based on criteria proposed by Suesatpanit and colleagues (1) sequences are not 'unverified' without a species name (2) contain <3% ambiguous base 'N' (Suesatpanit et al. 2017) and presented in Table 1.

Table 1. List of jewel orchids and number of retrieved sequences used in this study

	Sample code	Latin name	Genus	Sequence number		
				ITS	matK	rbcL
1	ARot	*Aenhenrya rotundifolia*	*Aenhenrya*	0	1	1
2	AA	*Anoectochilus albolineatus*	*Anoectochilus*	1	1	1
3	AE	*Anoectochilus elatus*		0	6	6
4	AF	*Anoectochilus formosanus*		5	1	0
5	AL	*Anoectochilus lylei*		2	2	1
6	ARox	*Anoectochilus roxburghii*		16	5	1
7	AS	*Anoectochilus setaceus*		6	0	0
8	DM	*Dossinia marmorata*	*Dossinia*	6	4	2
9	GH	*Goodyera hispida*	*Goodyera*	4	0	0
10	GP	*Goodyera pubescens*		2	6	6
11	GVir	*Goodyera viridiflora*		10	6	1
12	GVit	*Goodyera vittata*		2	1	0
13	LD	*Ludisia discolor*	*Ludisia*	8	2	6
14	MP	*Macodes petola*	*Macodes*	1	0	1

Sequences were converted into FASTA format and subjected to Multiple Sequence Alignment using Clustal W intergrated in MEGA 6 software (Tamura et al. 2013). This software was also applied to calculate the evolutionary divergence for each data set and pattern of nucleotide substitution. For phylogenetic analysis we used Neighbor-Joining tree method with 1000 bootstrap replicates and presented as circular cladograms (Ho and Nguyen, 2020). In order to estimate species resolution for a given barcode locus, the species/genus were resolved if con- specific individuals are grouped into one monophyletic branch in the phylogenetic tree with bootstrap support greater than 50% (Zhang et al., 2019). Contrarily, if separated in paraphyletic branches such species and genus are considered as identification failure (Sikdar et al. 2018).

RESULTS AND DISCUSSION

Sequence Retrieve: By using keyword "species names

+ITS/*matK*/*rbcL*" to find the sequences deposited in NCBI Gen Bank, after removal of unrealizable sequences as described by Suesatpanit and colleagues (2017), total of 124 sequences were obtained, there are 63, 35 and 26 DNA sequences of ITS, *matK* and *matK* regions, respectively (Table 1).

Table 2. Estimates of Average Evolutionary Divergence over Sequence Pairs within Group

Species	p distance of ITS	SE	p distance of matK	SE	p distance of rbcL	SE
Aenhenrya rotundifolia	NA	NA	NA	NA	NA	NA
Anoectochilus albolineatus	NA	NA	NA	NA	NA	NA
Anoectochilus elatus	NA	NA	0.000	0.000	0.000	0.000
Anoectochilus formosanus	0.002	0.002	NA	NA	NA	NA
Anoectochilus lylei	0.008	0.006	0.014	0.004	NA	NA
Anoectochilus roxburghii	0.003	0.002	0.023	0.004	NA	NA
Anoectochilus setaceus	0.044	0.008	NA	NA	NA	NA
Dossinia marmorata	0.000	0.000	0.002	0.001	0.000	0.000
Goodyera hispida	0.009	0.004	NA	NA	NA	NA
Goodyera pubescens	0.004	0.004	0.002	0.001	0.000	0.000
Goodyera viridiflora	0.009	0.003	0.012	0.003	NA	NA
Goodyera vittata	0.079	0.018	NA	NA	NA	NA
Ludisia discolor	0.003	0.003	0.000	0.000	0.004	0.002
Macodes petola	NA	NA	NA	NA	NA	NA

(The presence of n/c in the results denotes cases in which it was not possible to estimate evolutionary distances. Note: SE: standard error; NA: not available).

Table 3. Estimates of Evolutionary Divergence over Sequence Pairs between Groups for ITS

	AA*	AF	AL	AR	AS	DM	GH	GP	GVir	GVit	LD	MP
AA		0.006	0.003	0.005	0.004	0.016	0.019	0.022	0.018	0.016	0.016	0.016
AF	0.010		0.003	0.002	0.006	0.014	0.018	0.021	0.016	0.015	0.015	0.014
AL	0.004	0.006		0.003	0.005	0.015	0.018	0.021	0.017	0.015	0.015	0.015
AR	0.008	0.003	0.005		0.005	0.014	0.018	0.021	0.016	0.015	0.015	0.014
AS	0.023	0.028	0.025	0.026		0.016	0.019	0.022	0.018	0.016	0.016	0.016
DM	0.062	0.055	0.058	0.055	0.079		0.018	0.021	0.017	0.016	0.015	0.013
GH	0.086	0.079	0.082	0.079	0.104	0.090		0.009	0.009	0.010	0.016	0.017
GP	0.106	0.099	0.101	0.099	0.125	0.110	0.027		0.013	0.013	0.018	0.020
GVir	0.078	0.071	0.074	0.071	0.096	0.081	0.028	0.046		0.011	0.015	0.016
GVit	0.080	0.072	0.075	0.073	0.098	0.086	0.047	0.063	0.052		0.013	0.015
LD	0.066	0.059	0.062	0.059	0.085	0.059	0.069	0.084	0.069	0.066		0.013
MP	0.058	0.051	0.054	0.051	0.076	0.049	0.077	0.096	0.067	0.073	0.042	

(*: Species names are abbreviated corresponding to Table 1, standard error of comparison is presented in italics upper diagonal).

In general, ITS locus was more intensively studied with up to 63 sequences aquired, whereas m*atK* and *rbcL* are likely unattended with lower sequence number. Considering each species, the sequence availability also is varying, in which, *Anoectochilus roxburghii, Goodyera viridiflora, Ludisia discolor* and *Goodyera pubescen* show highest sequence abundance suggesting the economic importance of these species. Since previous studies highlighted the abundance of medical compounds detected in these species. *Anoectochilus roxburghii* is rich in polysaccharides, flavonoids, glycosides, organic acids, volatile compounds, steroids, triterpenes, alkaloids, and nucleosides (Ye et al., 2017); butanolide, goodyeroside A, butanolide in *Goodyera* genus (Du amino acids and anthocyanin content in *Ludisia discolor* (Poobathy et al., 2016).

Table 4. Estimates of Evolutionary Divergence over Sequence Pairs between Groups for matK

	AA*	AF	AL	AR	DM	GP	GVIR	GVIT	LD	AE	ARot
AA		0.004	0.002	0.004	0.007	0.009	0.006	0.007	0.006	0.000	0.009
AF	0.014		0.004	0.003	0.007	0.009	0.007	0.008	0.006	0.004	0.009
AL	0.007	0.017		0.004	0.007	0.008	0.006	0.007	0.006	0.002	0.009
AR	0.012	0.007	0.013		0.007	0.009	0.007	0.007	0.005	0.004	0.009
DM	0.031	0.037	0.035	0.035		0.008	0.007	0.007	0.005	0.007	0.008
GP	0.049	0.056	0.050	0.053	0.044		0.006	0.007	0.008	0.009	0.009
GVIR	0.035	0.041	0.037	0.039	0.034	0.031		0.002	0.006	0.006	0.007
GVIT	0.033	0.040	0.035	0.038	0.032	0.030	0.007		0.006	0.007	0.008
LD	0.022	0.024	0.025	0.023	0.021	0.041	0.027	0.025		0.006	0.008
AE	0.000	0.014	0.007	0.012	0.031	0.049	0.035	0.033	0.022		0.009
ARot	0.055	0.060	0.058	0.058	0.047	0.054	0.042	0.042	0.045	0.055	

(*: Species names are abbreviated corresponding to Table 1, standard error of comparison is presented in italics upper diagonal).

Table 5. Estimates of Evolutionary Divergence over Sequence Pairs between Groups for *rbcL*

	AA*	AL	ARox	DM	GP	GVir	LD	MP	AE	ARot
AA	0.000	0.002	0.002	0.003	0.000	0.002	0.008	0.000	0.003	
AL	0.000		0.002	0.002	0.003	0.000	0.002	0.008	0.000	0.003
ARox	0.002	0.002		0.003	0.003	0.002	0.003	0.008	0.002	0.004
DM	0.002	0.002	0.004		0.003	0.002	0.003	0.007	0.002	0.004
GP	0.004	0.004	0.006	0.006		0.003	0.003	0.008	0.003	0.004
GVir	0.000	0.000	0.002	0.002	0.004		0.002	0.008	0.000	0.003
LD	0.004	0.004	0.006	0.006	0.008	0.004		0.008	0.002	0.004
MP	0.028	0.028	0.030	0.026	0.032	0.028	0.032		0.008	0.008
AE	0.000	0.000	0.002	0.002	0.004	0.000	0.004	0.028		0.003
ARot	0.006	0.006	0.008	0.008	0.010	0.006	0.009	0.035	0.006	

(*: Species names are abbreviated corresponding to Table 1, standard error of comparison is presented in italics upper diagonal)

Table 6. Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution (in percentage)

	ITS				matK				rbcL			
	A	T	C	G	A	T	C	G	A	T	C	G
A	-	3.97	3.69	18.69	-	8.13	3.37	10.24	-	6.10	4.59	11.39
T	3.04	-	18.31	4.21	6.53	-	7.60	3.11	5.61	-	14.16	4.56
C	3.04	19.67	-	4.21	6.53	18.35	-	3.11	5.61	18.81	-	4.56
G	13.49	3.97	3.69	-	21.53	8.13	3.37	-	13.75	6.10	4.59	-

(Note: Each entry shows the probability of substitution from one base (row) to another base (column). For simplicity, the sum of r values is made equal to 100. Rates of different transitional substitutions are shown in bold and those of transversionsal substitutions are shown in italics)

Estimation of sequence divergence: The variation within and between species based on ITS, *matK*, and *rbcL* regions were calculated and the data representing by evolutionary divergence (p distance value) are shown in Table 2. Based on p distance, it suggests that there is the large variability of three regions within a specific species

of jewel orchid. *Anoectochilus setaceus* show highest variation divergence in ITS locus (0.044), *Anoectochilus lylei* show highest at *matK* (0.014) and *Ludisia discolor* at *rbcL* (0.004).

Figure 1: Neighbor-joining tree with 1000 bootstrap replicated based on *matK* sequences (Shaded areas and dot cycles present the completely resolved species and genus, respectively).

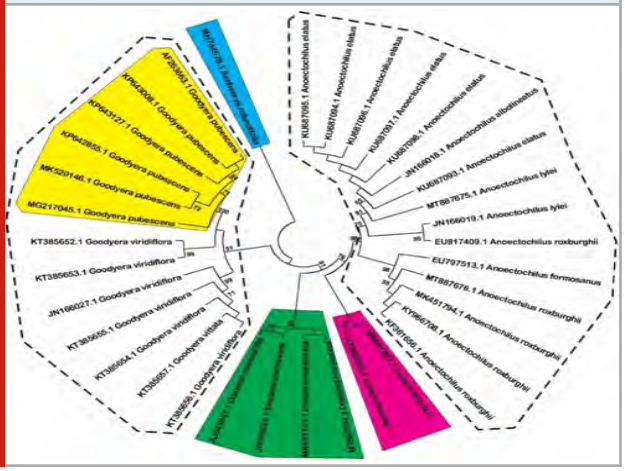
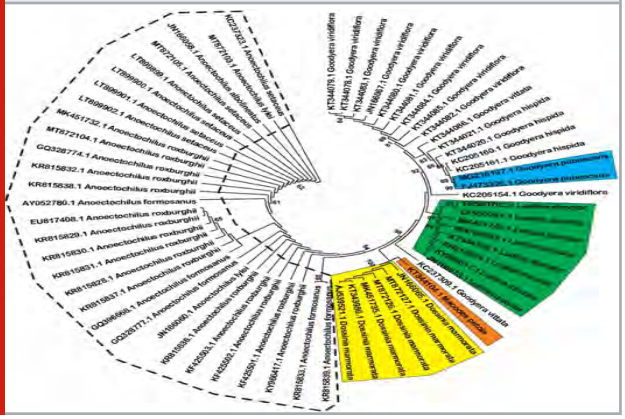


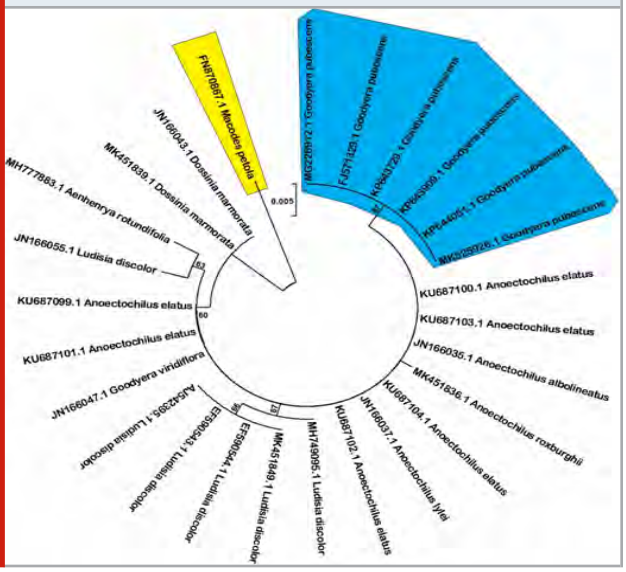
Figure 2: Neighbor-joining tree with 1000 bootstrap replicated based on ITS sequences (Shaded areas and dot cycles present the completely resolved species and genus, respectively).



The number of base substitutions per site from averaging over all sequence pairs within each group are shown in Table 3, 4 and 5. The variability among ITS, *matK* and *rbcL* regions is from 0.003-0.106, 0 - 0.06, and 0 – 0.035, respectively. Substitution bias consisting of transition and transversion at codon position for each luster could reveal the trend of evolution. In this study, the substitution of different bases in analyzed regions is evaluated on entire codon positions (1st+ 2nd + 3rd nucleotide) and shown in Table 6. In general, transitional substitution is higher than transversional substitution in all loci. Two chloroplast namely *matK* and *rbcL* show a higher transversionsal substitution than ITS.

Estimation of species resolution: Based on phylogenetic analysis, the species resolution is variable among three DNA barcode loci (Figure 1, 2 and 3). *MatK* show highest effectiveness in plant discrimination in which all of five genus are resolved (Figure 1), especially this locus is able to distinguish five per ten species consisting of *Aenhenrya rotundifolia, Goodyera pubescens, dossinia marmorata*, and *Ludisia discolor*. ITS locus could also discriminate four genus consisting of *Anoectochilus, Dossinia, Macodes* and *Lusidia*. DNA sequences from this locus could separate four species namely *Goodyera pubescens, Ludisia discolor, Macodes petola* and *Dossinia mamorata* (Figure 2). Although *rbcL* locus was reported as a good marker to differentiate species in *Prunus genus* (Singh et al. 2016), our data show that this is the poorest among three studied loci in term of discrimination power in which only *Macodes petola* and *Goodyera pubescens* are completely resolved (Figure 3). One of main reason of low discrimination power of this locus could be due tolimited number of examined sequences, Sikdar and colleagues proposed to increase sequence number to enhance the discrimination power (Sikdar et al. 2018).

Figure 3: Neighbor-joining tree with 1000 bootstrap replicated based on *rbcL* sequences (Shaded areas present the completely resolved species).



Even both *Anoectochilus* and *Goodyera* genus are rich in several medicinal compounds, the former is considered better than the later since some corresponding compounds extracted from *Goodyera* is less effective than those from *Anoectochilus* species (Du et al., 2008). Furthermore, *Anoectochilus* species are traditionally used for treating chest and abdominal pains, diabetes, nephritis, fever, hypertension, impotence, liver and spleen disorder, and pleurodynia whereas, *L. discoloris* used to reduce coughs and strengthening weak lungs (Poobathy et al., 2018). Thus, the high discrimination power of *matK* locus is highly valuable to identify, utilize and develop these two species for specific medicinal purposes.

CONCLUSION

The usefulness of three main DNA barcode loci in classify different jewel orchid plants is confirmed by *in silico* analysis. The obtained results suggest that the discrimination capacity of ITS, *matK* and *rbcL* DNA barcode loci are variable among different species of jewel orchid plants, in which *matK* and ITS loci reveal more potential for genetic classification at genus and species level of this herbal plants. In the future, higher sequence number should be included in the analysis to give more reliable result. The information from this study could be useful in conservation and development programs of jewel orchid plants.

Conflict of Interests: The author has no conflict of interest.

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Dental
Communication

Crown or Not to Crown Root Canal Treated Teeth Minireview

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ABSTRACT

Root canal treatment through cleaning, shaping and apical sealing are crucial for periapical healing. Moreover, Coronal restoration of the root canal treated tooth is required to prevent coronal leakage and to provide function and aesthetic. The quality and timing of the final restoration has its effect on the survival and success rate of endodontically treated tooth. It has been found that full coverage restorations show a higher long-term survival rate than direct restorations. Direct restoration has an excellent short-term survival rate comparing to crowns or onlays. Long term survival is the main criteria of successful endodontic treatment. Full coverage restorations show a higher survival rate than direct restorations. The definitive restoration should be placed as soon as RCT completed. It has been shown that time of crown placement after endodontic treatment affect the survival rate of endodontically treated teeth. Finally, no need for a post if the remaining tooth structure can withstand the core material.

KEY WORDS: ROOT CANAL TREATMENT, CROWNING, SURVIVAL RATE.

INTRODUCTION

Endodontic therapy has a success rate up to 86-98% (Song et al., 2011; Mustafa et al., 2021). However, a successful endodontic treatment does not depend only on a good root canal therapy, but good restorative treatment is crucial (Gillen et al., 2011). Completing the root canal treatment is not the end of the story, the tooth needs to be restored back to normal function, form, and aesthetic. The quality of the final restoration has its effect on the survival and success rate of endodontically treated tooth (Mannocci and Cowie., 2014; Bhuva et al, 2020). Well-sealed coronal restoration will prevent the ingress of microorganisms (Bayram et al., 2013). Swanson and Madison highlighted that coronal leakage is a major factor leading to endodontic treatment failure (Swanson and Madison., 1987; Mustafa et al., 2021). A meta-analysis published in 2008 stated that endodontically treated teeth with adequate restorations have a higher success rate comparing to poor quality restored teeth (Ng et al., 2008).

Effect of Endodontic Treatment on Teeth: A study done in 1992 investigated the physical and mechanical properties of dentine from vital and endodontic treated teeth at different hydration level. They found that neither root canal treatment nor dehydration has an effect on the properties of dentine (Huang et al., 1992). Another study found that endodontically treated teeth are not brittle compared to vital teeth (Sedgley et al., 1992).

On the other hand, it has been shown that the medicaments and irrigants used in endodontic treatment can change the physical properties of dentine. For example, long term use of calcium hydroxide can have an effect on the dentin, making it more brittle and prone to fracture (Grigoratos et al., 2001; Andreasen et al., 2002). Furthermore, it has been studied that using calcium hydroxide ($Ca(OH)_2$) dressing for 5 weeks or more will decrease the fracture resistance of the root (Andreasen et al, 2002; Batur et al, 2013; Zarei et al., 2013). They found also that irrigation and disinfection used in root canal therapy

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can interact with organic contents on the tooth lead to decrease in the modulus of elasticity of the dentine (Andreasen et al., 2002).

No doubt that sodium hypochlorite (NaOCl) and Ca $(OH)_2$ are important for adequate and successful root canal treatment, but they affect the strength of the remaining dentine (Ng et al., 2011). It has been found that using of NaOCl with concentration over 2% has a negative effect on collagen within the dentine (Kishen 2015). Recently, Bel Haj Salah et al., (2021) have showed the contribution of Bio Root Root Canal Sealing in the healing of periapical lesions, accordingly, bioceramic-based sealers seem to optimize the prognosis of root canal treatments. There are other factors that can lead to a weakening of the remaining tooth tissue such as a change in proprioception and tooth architecture (Eliyas et al., 2015). It has been found that access cavity preparation increased deflection of cusps during function and eventually increase the micro leakage and cusp fracture (Gutmann., 1992; Pantvisai and Messer., 1995).

Literature reports a huge reduction in tooth stiffness from 14-44% following occlusal cavity preparation, and 20-63% following mesio-occlusodistal (MOD) cavity (Geistfeld 1981). It is a fact that most teeth requiring endodontic treatment have had caries, restorations, cracks, and trauma which might be the reason behind the tooth weakness (Eliyas et al., 2015). Proprioception is affected by endodontic therapy. Non-vital teeth show a higher pain threshold and thus increased occlusal loading. It has been found that proprioception is decreased by 30% after endodontic treatment (Yu and Abbott., 1994).

Timing of restoration: It has been found that well sealed coronal restoration leads to successful endodontic treatment than good quality obturation (Ray and Trope., 1995). Before thinking of the final restoration, we should consider the following: pre-endodontic status of the tooth and surrounding structure, quality of the root canal filling, location of the tooth, and types of restoration to be used. If the root canal treatment satisfactorily completed and with no symptoms, it is sensible to place the final restoration immediately especially in a case of previously uninfected and vital tooth. However, if the tooth was symptomatic with periapical pathology, postpone the final restoration until symptoms subside is wiser until there is a clinical and radiographic prove of success. If the tooth had a small periapical lesion which is about 2mm or less, final restoration can be placed. Whereas, pre-operative large periapical radiolucency needs a review term before placing the definitive restoration (Mannocci and Cowie, 2014 ; Bhuva et al., 2020; Alserhan et al., 2021).

In questionable prognosis teeth, the dentist should protect the tooth direct after the root canal treatment with a good quality plastic restoration or stabilize the tooth with orthodontic band until there is a clear clinical and radio graphical evidence of success (Mannocci and Cowie, 2014). Placing an expensive coronal restoration such as gold crown or ceramic restoration might be the wrong decision if the prognosis of the root canal treatment is guarded. Periapical pathology may take several months or years, however, good quality permanent coronal restoration should be placed in order to guard the root canal system from any leakage and bacterial contamination (Bjørndal and Kirkevang., 2018, Alaki et al, 2021).

The dentist should be aware of the factors lead to endodontic failure which needs a review appointments and regular check-up before considering extra coronal restoration. Some of these factors are large periapical lesion, persistence of bacteria, overextended root filling, and complications during instrumentation such as ledges, and perforations (Tabassum and Khan et al., 2016; Bjørndal and Kirkevang., 2018; Bhuva et al., 2020). The definitive restoration should be placed as soon as the endodontic treatment finished. Endodontically treated teeth are more susceptible to fracture because of several reasons mentioned above: loss of proprioception, altered architecture of the tooth, and altered physical properties of the dentine. It has been found that the survival rate of RCT tooth received coronal coverage within 4 months of treatment was 85%, on the other hand, the survivability of tooth received crown after 4 months was 68% (Pratt et al., 2016).

Methods of restoring root canal treated (RCT) molars: The best plan for successful restoration is by a thorough examination of the tooth for caries or fracture before starting endodontic treatment. The dentist should assess the restorability, periodontal status, occlusal function, crown-root ratio, and biological width. If these factors are satisfactory, then treatment can be initiated. Practitioners should remove all existing fillings, build-ups, or crowns, when possible, before endodontic treatment. This will lead to more accurate assessment of the remaining tooth structure (Schwartz and Jordan, 2004; Alaki et al., 2021; Alserhan et al., 2021).

Direct Restoration

Amalgam: has been used for decades with long-term success. The use of amalgam decreased because of the patient concerns about toxicity and increasing demand for more esthetic restoration. However, amalgam is a safe material, has a high compressive strength makes it a good choice as a cusp coverage restoration (AFFAIRS ACOS, 1998).

Amalgam is not an adhesive restorative material; however, it has been found that bonding the amalgam restoration to tooth structure increases the fracture resistance of the tooth. Furthermore, they found that not statistically differences between bonded amalgam group and sound teeth in fracture resistance (Sagsen and Aslan., 2006; Alaki et al., 2021; Alserhan et al., 2021).

Composite: resin restoration can be used as a definitive restoration for posterior teeth especially when the access cavity is conservative (Mannocci and Cowie, 2014; Bjørndal and Kirkevang., 2018). Composite most

commonly used as a core material before crowning procedure. Composite has a lower compressive strength than amalgam restoration.

Glass ionomer (GI): has found to be as good coronal seal as an intact crown for eight weeks testing period. It has a good antibacterial effect and it bonds chemically to tooth structure (Bobotis et al., 1989). This makes it a good interim restoration until the definitive restoration placed.

Indirect Restoration

Gold Restorations: (crowns/onlays & inlays) are biocompatible material, long lasting restoration for posterior teeth. Gold restorations consider a conservative approach comparing to ceramic or zirconium which need more tooth reduction (Felden et al, 2000; Alaki et al, 2021; Alserhan et al, 2021). It has been found that cast gold restoration has a higher survival rate in comparison to amalgam and composite (Stoll et al, 1999). If esthetic is not a major concern, gold is still the first choice in posterior teeth. Porcelain Fused to Metal: crowns are the most commonly used indirect restorations in posterior teeth. Preparing the tooth for a PFM crown needs more reduction than gold crown especially in the buccal aspect of the tooth. The non-aesthetic aspect can be finished in metal by using metal collar or even a metal occlusal coverage (Mannocci and Cowie, 2014; Al Moaleem et al, 2017a).

Ceramic crowns, inlays, and onlays: are considered the best in mimicking the natural tooth (Griggs 2007). Ceramic materials come in different composition and strength and this should be taken into consideration when restoring posterior teeth (Raptis et al, 2006; Bhuva et al 2020; Al Moaleem et al., 2017b;). Posts: posts are indicated if the amount of the remaining tooth structure is not adequate to retain a core material. Using posts should be avoided when enough tooth structure is available to retain the core material. Post's materials available are: cast post and core, fiber post, ceramic, glass, and zirconium posts (Schwartz and Jordan, 2004; Bhuva et al 2020).

To crown or not to crown: It has been extensively discussed how to restore endodontically treated teeth, however, the best type of definitive restoration is still controversial (Willershausen et al, 2005; Dias et al, 2018; Bhuva et al., 2020). Full coverage crown with or without post was found to be the best choice as it protects the tooth from fracture, but crown restoration needs a preparation which leads to decrease the strength of the remaining tooth structure (Gupta et al., 2014; Alshiddi and Aljinbaz, 2016; Wang et al, 2016; Alaki et al., 2021; Alserhan et al., 2021). The main goal of conservative dentistry is to preserve the healthy tooth structure. Some dentists prefer to use direct composite restoration especially in case of conservative access cavity. Mannocci in 2002 found that no significant difference in success rate in his 3 years clinical trial between direct and indirect post-endodontic restoration. This study considers a short term and was done on premolars only which do not have as heavy occlusal load as molars.

It has been found that coronal coverage restoration decreased the possibility of tooth fracture under occlusal load (Sjögren et al, 1990; Vire., 1991; Eckerbom et al., 1992; Caplan and Weintraub., 1997; Bhuva et al 2020). Eckerbom stated that RCT teeth have the same survival rate as vital teeth (Eckerbom et al., 1992). Furthermore, Vire found that crowned RCT teeth with crowns show an increase in longevity than uncrowned RCT teeth (Vire DE., 1991; Al Moaleem et al, 2017 b). In a retrospective study by Aquilino and Caplan (2002) it was found that crowning the endodontically treated teeth promote higher longevity for posterior teeth. They placed a crown on 129 teeth and restored 74 teeth with either amalgam or composite restoration. The survival rate after 10 years was 89% for the crowned teeth, and 62% for the direct restorations.

In another study, 24 teeth with partial coverage gold crown survived without fracture with a mean period of 8.9 years. Teeth with crowns, bridges, individual post with crown and crowns and bridges with access cavity show a high survivability (>95%) in 10 years. In the same study a total of 235 teeth restored with direct restorations (GIC, Amalgam, Composite). 29.4% (n=69) of these teeth had to be extracted due to fracture. The 70.6 % survived during the observation period. The mean survival rate of composite was higher than amalgam and GIC. Also, it has been shown that cavitated teeth with one or two surfaces missing survived more than teeth missing three or more surfaces. This concludes that teeth with missing one or two surfaces can be restored with direct composite. However, this author used a small sample of 37 composite restorations which decrease the power of this study regarding the composite (Dammaschke et al., 2013).

Pratt et al in retrospective study found that the eight years survival rate of RCT teeth restored with crowns was 84%. Whereas in teeth restored with core build-up without crown the survival rate was 71%. Moreover, RCT teeth restored with composite or amalgam build-up were 2.29 more likely to be extracted (Pratt et al, 2016). According to a recently published systematic review, indirect restorations have a higher medium (>5 – <10 years) and long-term survival (>10 years) than direct restorations. However, in short-term (<5 years) no important difference was found between direct and indirect post-endodontic treatment (Shu et al., 2018).

Placement of post after endodontic treatment is a controversial matter. It has been shown that cementing a fiber post can improve the resistance to fracture of RCT anterior teeth (Abduljawad et al, 2016). However, the preparation for post space will increase the chance of root fracture (Peters et al., 2003). Posts should be avoided when we have adequate tooth structure to retain the core. Usually, molars do not need a post because the pulp chambers and canals can retain the core restoration.

Recently, Dallak et al (2020) and Al Ariqi et al, (2021) have concluded the importance of using the cone beam computed tomography (CBCT) during diagnosis, treatments, and before crowning of most of root canal treated teeth. Similarly, Bhuva et al, (2021) found that the survival of teeth and restorations following RCTs are affected by a large number of variables which include the residual volume of tooth structure, the presence of proximal contacts, tooth location, whether a cuspal coverage restoration has been provided (for molar teeth) and the use of a post. The following points should be in mind during evaluating of the RCT of all teeth particularly the molars;

1. Root filled teeth, particularly those undergoing RCT, when the remaining tooth size is less than 30%, there is a higher risk of endodontic failure. In-addition, teeth with one or less residual walls have less survival to those with two or more walls. The possibility of long-term tooth survival is maximum for teeth with two or more proximal contacts and lowest for those with no proximal contacts. Last molar teeth should be considered as having increased risk of failure.
2. The irresistible majority of retrospective studies report superior long-term survival for root filled posterior teeth restored with indirect cuspal coverage restorations when compared with those restored with direct restorations. The performance of root filled teeth restored with direct plastic restorations, such as composite resin, has rarely been compared with indirect cuspal coverage restorations in randomized clinical trials. Although the evidence is limited, a delay of more than four months to placement of the indirect restoration appears to be associated with a lower survival rate.
3. Cracked teeth which have undergone RCT showed to have good survival rates after four years. Whilst an increased periodontal probing depth associated with a crack is a negative predictive factor, the depth of the crack itself does not appear to be as relevant. Such factors should be measured when discussing treatment options with patients.
4. All of the available evidence indicates that for the restoration of posterior teeth, contemporary techniques such as all ceramic crowns, onlays and endocrowns are as durable as MC crowns. Prospective studies with longer follow-up periods are required to validate the performance of these restorations. Cementation protocols of these restorations seems to have a high relevant to their survival rates.
5. Teeth restored with fibre-posts have comparable survival rates to those restored with direct or indirect metal posts. The use of adhesive techniques for post placement that permit the preservation of the maximum amount of dentine is recommended. Adherence to appropriate bonding protocols, including the use of appropriate luting cement applications tips, is essential for predictable post placement. Though, based on the existing data, no definitive cementation technique can be suggested.

CONCLUSION

Long term survival is the main criteria of successful endodontic treatment. Full coverage restorations show a higher survival rate than direct restorations. The definitive restoration should be placed as soon as RCT completed. It has been shown that time of crown placement after endodontic treatment affect the survival rate of endodontically treated teeth. Finally, no need for a post if the remaining tooth structure can withstand the core material.

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Medical
Communication

Cytotoxic Study of *Albizia procera* and *Ailanthus altissima* Extracts on Human Tumor Cell Lines

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ABSTRACT

In nature, several drugs could be discovered as it is generous in phytochemicals that could be used as remedies. In fact, various cytotoxic drugs used against cancer arose from plants. Cancer is caused by genetic and epigenetic alterations that make the cells divide uncontrollably, ignoring the cell's normal machinery. In this study, seven methanol plants extracts from leaves, flowers, and branches of *Albizia procera have been analysed.*. White siris is the common name of such a tall, rapid-growing tree with an open canopy where all parts of the plant are reported to show anti-cancer activity. The bark and leaves of *Ailanthus altissima* which is commonly known as the tree of heaven in the family Simaroubaceae, were tested on three types of human cancer cell line: colorectal cancer (HCT-116) which is the third most frequent cancer, breast cancer (MCF-7) which is the most prevalent cancer among women, and hepatocellular carcinoma (HEPG-2) which is the third leading cause of death related to cancer worldwide. The investigation done evaluated the cytotoxicity of the extracts in a 2D cell culture, on the three cell lines, and a 3D cell culture on HCT-116, using MTT and Acid phosphatase assays respectively. The results reported one active extract on HCT-116 with an IC_{50} of 0.23 µg/ml in the 2D system and percentage inhibition of 72.0% using 100 µg/mL of the extract, accompanied by a regression in the proliferative peripherals in the 3D system. Regarding the MCF-7, four extracts were considered active having an IC50 of 2.53, 4.16, 11.8, and 13.4 µg/ml. Ultimately, the HEPG-2 cell line responded to two extracts, one having a percentage inhibition reaching 92% at 25 µg/ml, and the other having a percentage inhibition of 98.1% at 50 µg/ml.

KEY WORDS: HCT-116, MCF-7, HEPG-2, ALBIZIA PROCERA, AILANTHUS ALTISSIMA, MTT.

INTRODUCTION

Cancer is one of the most dreaded diseases of the 20th century and spreading further with the continuance and increasing incidence in the 21st century. The situation is so alarming that every fourth person is having a lifetime risk of cancer (Roy and Saikia, 2016). The evolution of the normal cell to a malignant one involves processes by which genes involved in normal homeostatic mechanisms that control proliferation and cell death suffer mutational damage which results in the activation of genes stimulating proliferation or protection against cell death, the oncogenes, and the inactivation of genes which would normally inhibit proliferation, the tumor suppressor genes. Finally, having overcome normal controls on cell birth and cell death, an aspiring cancer cell faces two new challenges: it must overcome replicative senescence and become immortal and it must obtain adequate supplies of nutrients and oxygen to maintain this high rate of proliferation (Bertram, 2000). Three types of cancer were investigated in this research: breast cancer, colorectal cancer, and hepatocellular carcinoma.

Breast cancer is one of the most common cancers and >10.5 new breast cancer cases per 100,000 individuals

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occur worldwide each year (Khoobchandani et al., 2009) while colorectal cancer, the third most commonly diagnosed cancer in the world, has a higher predominance in developed countries (Des Guetz et al., 2010, Adelstein et al., 2011, Ibrahim et al., 2014). The third type this study is subjecting to investigation is hepatocellular carcinoma (HCC) which is one of the most devastating malignancies worldwide (Maluccio and Covey, 2012, Wong et al., 2017). Despite recent advances in the diagnosis and treatment including hepatectomy and liver transplantation, the prognosis of patients with remains poor due to the high rates of recurrence and early metastasis (Omata et al., 2017). Despite progress in anticancer therapeutics, there are few efficient drugs with low toxicity available to treat cancer. Numerous plants have been previously used in cancer therapy (da Rocha et al., 2001). Throughout the centuries, certain plant extracts have been tested for antitumor potential (Ho et al., 2002.). In addition, plant products demonstrate fewer side effects compared with chemical drugs. There has been an increasing interest in identifying and isolating natural compounds from medicinal plants with an aim to develop novel anticancer drugs (Bishayee, 2012).

Albizia procera belongs to the angiosperms, from the family of Febaceae, a subfamily of Mimosoideae, and genus *Albizia*. *Albizia procera* is a native tree that is mostly found in America, Asia, Pakistan, India, and Australia. The common name of *Albizia procera* is white siris and is a medicinal plant, used for the stomachache, during pregnancy and healing of wounds (Rukunga and Waterman, 1996) but it exhibited cytotoxicity against HEPG2 cell line (Melek et al., 2007). It also possesses antioxidant properties and can be involved in medicinal plant category (Sivakrishnan and KottaiMuthu, 2014). *Ailanthus altissima* (Synonyms: *Ailanthus cacodendron* (Ehrh.) and *Ailanthus glandulosa* Desf.) belongs to Simaroubaceae family, native to China and Japan and has a peanut or cashew-like fragrance (Albouchi et al., 2013, Albright et al., 2010). Extract of *A. altissima* possess antimicrobial and antifungal activities (Khan, 2017) and considered as an anticancer agent (Wang et al., 2018). *Ailanthus altissima* is an example of a plant that has been used in tumor therapy (Efferth et al., 2007). The antitumor effect that has enabled the use of this plant in the treatment of colonic, cervical, and rectal cancer has been previously described by Wang et al., (2013).

The potential cytotoxicity of ethanol extract and its derived fractions chloroform, ethyl acetate, butanol, and aqueous) of *Adenosma bracteosum* Bonati. (*A. bracteosum*) on human large cell lung carcinoma (NCI-H460) and hepatocellular carcinoma (HepG2). Among these fractions, the chloroform showed significant activity in the inhibition of proliferation of both cancerous cells because of the presence of bioactive compoundsIn 3D models, the cultured cells form 3 dimensional spheres where the cells become arranged in numerous layers with the peripheral cells proliferating the most, and the core containing hypoxic and quiescent cells as they receive fewer growth factors, oxygen, and nutrients from the medium (Edmondson et al., 2014). This layout highly mimics the structure of the tumor masses, enabling a realistic a cell-to-cell and cell-ECM interactions where the cells are able to receive signals from their environment (Cawkill and Eaglestone, 2007, Lee et al., 2008).

The purpose of this research is to determine the cytotoxic effect of 7 methanol plant extracts from *Albizia procera* and *Ailanthus altissima* on 3 different human cancer cell lines including HCT-116, MCF-7, and HepG-2; as well as to examine the safety of the potential cytotoxic extracts on normal human cell line (BJ-1) and test the cytotoxicity on the 3D model of (HCT-116).

MATERIAL AND METHODS

Plant Material: Amount of the attainable wild and cultivated plants, *Albizia procera* and *Ailanthus altissima* were collected randomly from diverse habitat in Egypt. Voucher specimens were prepared and deposited in the Herbarium of the Pharmacognosy Department, National Research Centre, Egypt, and taxonomical nomenclature was performed (Boulos, 2002, 2005).

Preparation of extracts: The collected plants were separated into different parts. *Albizia procera*, leaves, flowers, and bark and *Ailanthus altissima*, leaves and bark samples were dried in solar ovens at 45°C and then ground. Sufficient weight of each plant powder was exhaustively extracted with 95% methanol. The methanol extracts were filtered, evaporated under vacuum at 35°C, freeze-dried and stored at -20 °C. The freeze-dried plant extracts were deposited at the Extract Bank of the In-Vitro Bioassay Laboratory, National Research Center, Egypt.

Cell Culture: HCT116 human colon carcinoma cell line was obtained from ATCC and was maintained in McCoy's 5A modified medium/10% FBS while hTERT-RPE1 cell line was obtained from CLONTECH. The human retinal epithelial cell line, hTERT-RPE1, is an immortalized cell line that stably expresses human telomerase reverse transcriptase (hTERT). It was maintained in DMEM: F12 Medium/10% FCS. Both cell lines were incubated at 37°C in 5% CO_2 and 95% humidity. Cells were sub-cultured using trypsin-versene 0.15%.

Cytotoxicity Bioassay on Monolayers: After 24 h of seeding 10000 cells per well (in 96-well plates), a 100-ppm final concentration of the test extracts were added in triplicates. The cells were treated for 48hrs. 1 µM staurosporine was used as positive control and 0.5% DMSO was used as a negative control. Cytotoxicity was ascertained using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay as described by Mosmann in 1983. Cytotoxicity was calculated according to the following equation:

As/ Ac x100 (El-Mostafa et al., 2014).

Where: As: absorbance of sample at 595 nm, Ac: absorbance of negative control.

Cytotoxicity bioassay on HCT116 multicellular spheroids: Spheroids were generated as previously described (Fayad et al., 2011, Awad et al., 2016). FBS was filtered through a 0.45 µm filter, to remove any particulates that cause deformation in the spheroids. 10 000 cells of HCT116 was added to each well of poly-HEMA-coated round bottom 96-well plates. The wells were overfilled by adding 170 µl media to obtain convex curvature. Plasticine spacers were placed in the four corners of plates to prevent the lids from touching the surface of the media. The plates were inverted and incubated for 24 h on a rotary shaker. Plates were flipped back to the normal position to allow the formed aggregates to settle down in the bottom of the wells. The excess medium was removed using a syringe needle connected to a pump and plates were incubated for more 4 days. By this time, mature spheroids were generated (~500 µm in diameter).

Before treatment, the media was changed and were adjusted to 200 µl. Test extracts were added in triplicates to a final concentration of 100 ppm and were incubated for 120 h. 1 µM final concentration staurosporine was used as positive control and 1 µl DMSO was used as a negative control. At the end of incubation, cytotoxicity was determined using the acid phosphatase method (Friedrich et al., 2009). After washing twice with 250 µl PBS buffer, 100 µl of 0.1M sodium acetate, 0,1% Triton X-100, p-nitrophenyl phosphate (Pierce Biotechnology Inc, Rockford, IL) were added to each well and incubated for 1.5 hours at 37°C. After incubation, 10 µl 1N NaOH stop solution was added to each well and absorbance was read at 405 nm. Cytotoxicity was calculated according to the following equation:

(1-(av(x))/ (av (NC))] *100.

Where: Av: average, X: absorbance of sample, NC: absorbance of negative control.

RESULTS AND DISCUSSION

Albizia procera (extract 1), leaves (extract 2), flowers (extract 3), and bark (extract 4) and *Ailanthus altissima* (extract 5), leaves (extract 6) and bark (extract 7) were obtained and their activities as antitumor agents were determined. The data were collected from the MTT assays that were done on the 3 cell lines HCT-116, MCF-7, and HEPG-2, using 7 methanol plant extracts from *A. procera* and *A. altissima*. The calculated percentage inhibition of the different concentrations was used to determine the IC_{50} and IC_{90} values of the 7 extracts by performing probity analysis using the SPSS program. One extract from *Albizia procera* which showed high cytotoxic effects was evaluated for its safety on normal cells using the cell line BJ-1. Extracts 1, 2, and 3 showed low cytotoxic effects in the 4 concentrations (100, 50, 25, and 12.5 µg/ml). The other 4 extracts exhibited a higher cytotoxic effect, especially the extract 4 which exerted an exceptional cytotoxicity on the cell line HCT-116 and showing a high percentage inhibition of 99.6% with a low concentration of 6.25 µg/ml. However, the 3 remaining extracts showed low cytotoxicity in low concentrations (25 and 12.5 µg/ml) (Table 1, Figure 1). The IC_{50} and IC_{90} values of the 7 extracts on HCT-116 were determined (Table 2) to evaluate the efficacy of the potential drugs on colorectal carcinoma. Only extract 4 is considered as very active and have an IC50 value less than 20.

Table 1. Mean percentage inhibition of the 7 extracts on HCT-116 cell line.

Methanol extract	Mean percentage inhibition							
	100 µg/ml	50 µg/ml	25 µg/ml	12.5 µg/ml	6.25 µg/ml	3.125 µg/ml	1.56 µg/ml	0.78 µg/ml
1	49.2	33.4	15.0	0.64	N/A	N/A	N/A	N/A
2	34.6	33.4	23.4	11.5	N/A	N/A	N/A	N/A
3	41.2	28.3	16.8	3.8	N/A	N/A	N/A	N/A
4	99.1	99.7	100	99.2	99.6	67.4	29.9	7.2
5	89.4	75	48.6	40.0	N/A	N/A	N/A	N/A
6	83.7	41.9	27.4	23.9	N/A	N/A	N/A	N/A
7	99.3	98.7	42.2	14.0	N/A	N/A	N/A	N/A

Albizia procera (extract 1) ,leaves (extract 2), flowers (extract 3), and bark (extract 4) and *Ailanthus altissima* (extract 5), leaves (extract 6) and bark (extract 7)

On the MCF-7 cell line, extracts 1, 2, 3, 5, 6, and 7 exhibited high to moderate percentage inhibition in the two concentrations 100 and 50 µg/ml, and moderate to low percentage inhibition in the two concentrations 25 and 12.5 µg/ml (Table 3, Figure 2). On the other hand, the methanol extract 4 showed high to moderate percentage inhibition down to the concentration 3.125 µg/ml with a percentage inhibition of 72.2%. The percentage inhibition was low in the two concentrations 1.56 and 0.78 µg/ml only, regarding this extract (Table 3, Figure 2). Regarding the HEPG-2 cell line, extracts 1, 2, 3, 5, and 6 had a minimal or negligible percentage inhibition in the 4 concentrations; while the extract 4 had a high percentage inhibition down to the concentration 25 µg/ml, and extract 7 showed a high percentage inhibition in the two highest concentrations 100 and 50 µg/ml (Figure 3).

Figure 1: Percentage inhibition of the 7 plant extracts with different concentrations on HCT-116 cell line.
Albizia procera (extract 1), leaves (extract 2), flowers (extract 3), and bark (extract 4) and *Ailanthus altissima* (extract 5), leaves (extract 6) and bark (extract 7)

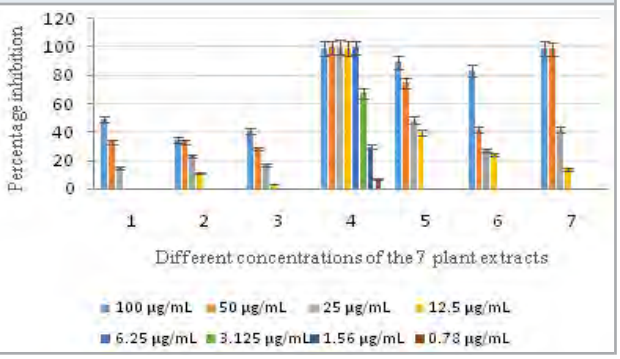


Table 2. IC_{50} and IC_{90} values of the 7 extracts on HCT-116 cell line.

Methanol extract	IC_{50} (µg/ml)	IC_{90} (µg/ml)
1	93.3	167.8
2	146.9	338.7
3	109.8	207.4
4	0.23	21.6
5	23.7	94.9
6	54.1	119.2
7	27.5	48.2

Albizia procera (extract 1) ,leaves (extract 2), flowers (extract 3), and bark (extract 4) and Ailanthus altissima (extract 5), leaves (extract 6) and bark (extract 7).

Table 3. Mean percentage inhibition of the 7 extracts on MCF-7 cell line

Methanol extract	Mean percentage inhibition							
	100 µg/ml	50 µg/ml	25 µg/ml	12.5 µg/ml	6.25 µg/ml	3.125 µg/ml	1.56 µg/ml	0.78 µg/ml
1	95.2	72.5	31.3	12.5	N/A	N/A	N/A	N/A
2	84.4	53.8	21.7	10.8	N/A	N/A	N/A	N/A
3	74.8	60.8	27.1	11.6	N/A	N/A	N/A	N/A
4	99.1	99.3	99.8	96.5	80.0	72.2	32.5	1.93
5	96.0	89.0	71.3	50.2	N/A	N/A	N/A	N/A
6	92.6	80.0	64.1	43.8	N/A	N/A	N/A	N/A
7	97.5	98.9	75.2	35.8	N/A	N/A	N/A	N/A

Albizia procera (extract 1) ,leaves (extract 2), flowers (extract 3), and bark (extract 4) and *Ailanthus altissima* (extract 5), leaves (extract 6) and bark (extract 7)

The IC_{50} and IC_{90} values of the 7 extracts on MCF-7 were determined (Table 4) to evaluate the efficacy of the potential drugs on breast cancer. Extracts 4, 5, 6, and 7 had an IC50 less than 20 showing high efficacy against the breast cancer cell line. The IC_{50} and IC_{90} values of the extracts against the cell line HEPG-2 could not be calculated due to their low cytotoxicity and percentage inhibition against this cell line. The extract 4 might have needed a secondary screening to be able to calculate these values.The highly cytotoxic extract 4 was evaluated for its safety on the cell line BJ-1, and showed a mean percentage inhibition of 4.3% with the concentration 100 µg/ml.

Spheroids of HCT-116 cells were studied under the microscope before and after treatment with a concentration of 100 µg/ml of the 7 examined extracts, as well as their percentage inhibition were calculated after measuring the absorbance of the plate at 405 nm. Before treatment, the spheroids had a length that was around 600 µm (Figure 4b). The positive control, using the chemotherapeutic drug cisplatin, showed a reduction in the spheroid length of approximately 100 µm (Figure 4b). On the other hand, the negative control exhibited a growth in the spheroid length of about 200 µm, with the core and the proliferative peripherals observed (Figures 4c). The wells treated cells with extracts 1 (Figure 4d) and extract 2 (Figure 5e) showed no inhibitory or cytotoxic effect and an increase in the spheroid's length of around 160 and 70 µm respectively. Figure 5 e showed HCT-116 spheroid treated with extract 2 (668.85 µm) while Figure 5f showed HCT-116 spheroid treated with extract 3 (660.59 µm). Similarly, Figure 5g showed HCT-116 spheroid treated with extract 4 (590.37 µm) and Figure 5 h- HCT-116 spheroid treated with extract 5 (577.03 µm). Figure 6i showed HCT-116 spheroid treated with extract 6 (609.80 µm), and Figure 6j showed HCT-116 spheroid treated with extract 7 (672.20 µm).

It is clear that, extracts 3, 5, 6 and 7 showed insignificant cytotoxic effects on the HCT-116 cells cultured in 3D model. On the other hand, extract 4 showed a decrease in the diameter of the spheroid, as well as a discontinuation in the proliferation of the peripherals of the spheroid was remarkable. Regarding the mean percentage inhibition

of the 7 extracts on the HCT-116 spheroids, extracts 1 and 2 had no cytotoxic effects at all, while extracts 3, 5, 6, and 7 had differential cytotoxic effects; however, none of them showed high cytotoxicity. The only extract that displayed an increased cytotoxicity is extract 4 (Table 6). According to the World Health Organization (2010), cancer is a disease that causes about 13% of total deaths worldwide. Chemotherapeutic or cytotoxic drugs can largely be discovered and developed from natural sources. In fact, drugs against infectious diseases could be discovered as well from natural products (Fayad et al, 2017). Plants and nature comprise an extensive variety of phytochemicals and chemical structures that offer several biological activities and hence could be used as remedies. Indeed, almost 60% of the currently prescribed drugs against malignant tumors originated and were developed from natural products (Newman et al., 2007). It has been vividly documented that different research projects have investigated the cytotoxic effects of *Albizia procera*, *Ailanthus altissima,* and other plants as well, and they have found that they have active cytotoxic effects on a variety of cell lines.

Figure 2: Percentage inhibition of the 7 plant extracts with different concentrations on MCF-7 cell line
Albizia procera **(extract 1), leaves (extract 2), flowers (extract 3), and bark (extract 4) and** ***Ailanthus altissima*** **(extract 5), leaves (extract 6) and bark (extract 7)**

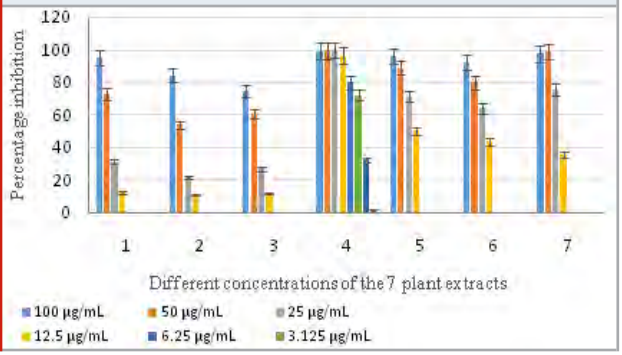


Table 4. IC_{50} and IC_{90} values of the 7 extracts on MCF-7 cell line.

Methanol extract	IC_{50} (µg/ml)	IC_{90} (µg/ml)
1	39.9	77.9
2	54.7	105.4
3	55.8	121.02
4	2.53	8.26
5	4.16	63.8
6	11.8	82.1
7	13.4	47.9

Albizia procera (extract 1) , leaves (extract 2), flowers (extract 3), and bark (extract 4) and *Ailanthus altissima* (extract 5), leaves (extract 6) and bark (extract 7)

Figure 3: Percentage inhibition of the 7 plant extracts with different concentrations on HEPG-2 cell line
Albizia procera **(extract 1), leaves (extract 2), flowers (extract 3), and bark (extract 4) and** ***Ailanthus altissima*** **(extract 5), leaves (extract 6) and bark (extract 7)**

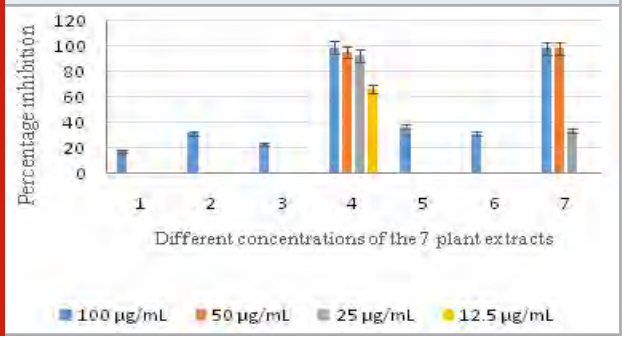


Table 5. Mean percentage inhibition of the 7 extracts on HEPG-2 cell line

Methanol extracts	Mean percentage inhibition (%)			
	100 µg/ml	50 µg/ml	25 µg/ml	12.5 µg/ml
1	17.5	0	0	0
2	31.7	0	0	0
3	22.6	0	0	0
4	98.9	95.3	92.6	66.2
5	36.8	0.8	0	0
6	31.1	0	0	0
7	98.5	98.1	33.1	0

Albizia procera (extract 1) ,leaves (extract 2), flowers (extract 3), and bark (extract 4) and *Ailanthus altissima* (extract 5), leaves (extract 6) and bark (extract 7)

The saponins which are isolated from *Albizia procera*'s bark exhibited cytotoxic effects on the HEPG-2 cell line with an IC_{50} of 9.13 µg/ml (Melek et al., 2007). However, another study that was done on the bark of *Albizia procera* included the isolation of triterpene glycosides with N-acetyl glucosamine units which displayed no significant cytotoxic effects on any of the cell lines MCF-7, HEPG-2, HT-29, or A-549. Additionally, experiments performed on oleanane-type saponins that were purified from the leaves of *Albizia anthelmintica* showed potent cytotoxic effects on HCT-116 and HEPG-2 cell lines with respective IC50 values of 4.75 and 3.60 µg/ml (Al-Sayed and Esmat, 2016).

An additional study was conducted on a β-carboline alkaloid, called 9-hydroxycanthin-6-one, was isolated from the bark of *Ailanthus altissima* and showed cytotoxic activity against ovarian cancer by increasing the intracellular levels of ROS followed by activating the caspases 3, 8, and 9 to induce apoptosis. This compound was found as well to repress the expression of RANTES and MCP-1 which are important determinants in the recruitment of macrophages at ovarian tumor sites; and

to decrease the levels of factors that promote cancer like VEGF.

Figure (4): a- HCT-116 spheroid before treatment (604.73 µm), b- HCT-116 spheroid treated with cisplatin (510.20 µm), c- HCT-116 negative control (783.23 µm), and d- HCT-116 spheroid treated with extract 1 (760.69 µm).

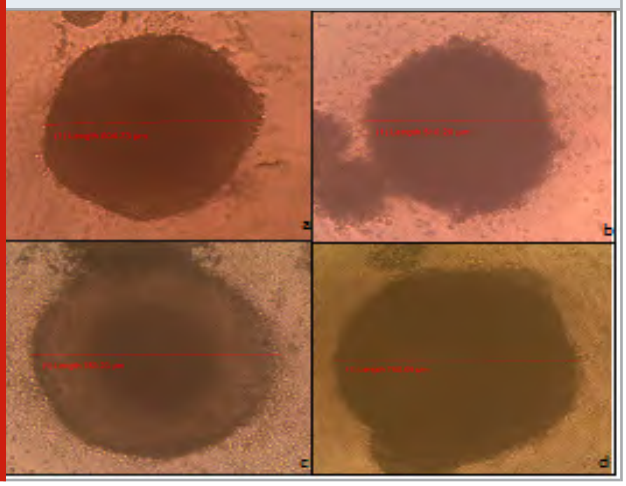
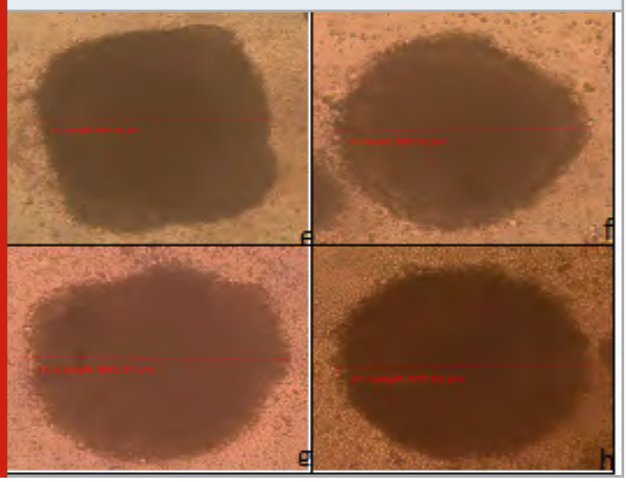


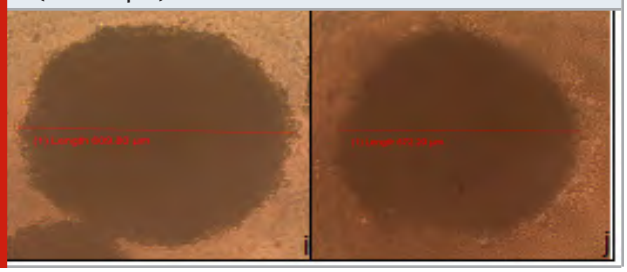
Figure (5): e- HCT-116 spheroid treated with extract 2 (668.85 µm), f- HCT-116 spheroid treated with extract 3 (660.59 µm), g- HCT-116 spheroid treated with extract 4 (590.37 µm), and h-HCT-116 spheroid treated with extract 5 (577.03 µm).



Similar findings were reported on studies done on the glioblastoma cell line U87; *Ailanthus altissima* was found to induce oxidative stress and endoplasmic reticulum stress sequentially which lead to the activation of the caspases, eventually leading to apoptosis of the malignant cells. Moreover, a chloroform fraction of *Ailanthus altissima* induced apoptosis on the human lung cancer cell line A549, as well as it was responsible for the upregulation of Bax, the pro-apoptotic factor, and the downregulation of the gene BCL-2 which encodes a protein that regulates the programmed cell death (Wang et al., 2011).

These anti-proliferative and cytotoxic effects are not restricted to the two plants *Albizia procera* and *Ailanthus altissima*, but they are seen among other plants as well. *Urtica pilulifera* and Lagenariasiceraria was found to inhibit the proliferation and induce apoptosis on the three cell lines HEP-3b (hepatocellular carcinoma), HeLa (cervical cancer) and PC-3 (prostate cancer). Meanwhile, the plant *Ficus carica* was ascertained to have cytostatic and anti-proliferative effects on the same 3 cell lines; it reduces the amount of metabolically active cells, leading to a decrease in the number of dividing cells. Another study done by (Réthy et al., 2007)) concluded that the plants E*lodea canadensis, Erigeron annuus, Ambrosia artemisiifolia, Helianthus annuus* and *Xanthium italicum* exhibited potent cytotoxic effects against the cell lines MCF-7, HeLa, and A431 (epidermoid carcinoma) with IC50 values that range between 1.84 and 19.91 µg/ml. Four other Malaysian plants were investigated in research in research done by Chu et al., (2013). The first one, *Acalypha wilkesiana*, had a GI50 of 15.9 µg/ml on breast cancer cells, as well as extracts of this plant, had the ability to impair the colony forming and cell survival mechanisms of the cancerous cells. The GI50 value demonstrates the concentration of the drug needed to reduce the proliferation of malignant cells by 50%. The second investigated plant was *Archidendron ellipticum*, whose leaf and bark extracts showed respective GI50 values of 9.3 and 1.7 µg/ml on the breast cancer cell line.

Figure (6): i- HCT-116 spheroid treated with extract 6 (609.80 µm), and j- HCT-116 spheroid treated with extract 7 (672.20 µm).



These extracts lead to the senescence of the cells primarily then induced apoptosis. The third plant was *Duabanga grandiflora*. Leaf and bark extracts of this plant showed a growth inhibition activity against the colorectal cancerous cell line HCT-116 by activating the protein caspase 3 to induce apoptosis. The last tested plant was *Pseuduvaria macrophylla* which inhibited the growth of the HCT-116 cells as well, with a GI50 1.6 µg/ml. The mechanism of action of this plant was found to activate the protein caspase 3 and induce apoptosis, similarly to *Duabanga grandiflora* (Chu et al., 2013).

In this study, 4 extracts from *Albizia procera* and 3 extracts from *Ailanthus altissima* were tested for their cytotoxicity effect on 3 human tumor cell lines colorectal cancer (HCT-116), breast cancer (MCF-7), and hepatocellular carcinoma (HEPG-2) in a monolayer cell culture; as well as they were tested on the colorectal

carcinoma cell line in a 3D model where the cells were cultured in spheroids. The investigation was done using 2 colorimetric-based assays: MTT assay for the monolayer cell culture, and acid phosphatase assay for the 3D model system.

Table 6. Mean percentage inhibition of the 7 extracts (100 µg/ml) on HCT-116 spheroids

Methanol extract	Mean percentage inhibition (%)
1	0
2	0
3	14.1
4	72.0
5	33.2
6	17.35
7	54.9

Albizia procera (extract 1), leaves (extract 2), flowers (extract 3), and bark (extract 4) and *Ailanthus altissima* (extract 5), leaves (extract 6) and bark (extract 7)

The results showed differential cytotoxicity effects on the 3 cell lines as well as there were significant differences between the monolayer and the spheroids cell cultures of the same cell line HCT-116. However, extract 4, leaves and flowers of *Albizia procera*) seems to have high cytotoxicity effects on all the tumor cell lines; while extracts 4, 5, 6, and 7 appear to have significant cytotoxic effects on the breast cancer cell line MCF-7. According to the National Cancer Institute Guidelines, plant extracts having an IC_{50} less than 20 µg/ml are considered as active extracts having a significant cytotoxic or anti-proliferative effect on the cancerous cell lines. In fact, IC_{50} is a pharmacokinetic measure that reveals the potency of the drug and lower IC50 value is the higher activity of the drug.

In the monolayer cell culture, extract 4 exhibited the highest efficacy (100% mean percentage inhibition) and potency (IC_{50} 0.23 µg/ml) on the HCT-116 cell lines, which means that only 0.23 µg/ml of the extract is needed to inhibit 50% of the colorectal cancer cells. It was the only active cytotoxic extract on this cell line with IC_{50} less than 20 µg/ml. The same extract had the highest efficacy and potency on the breast cancer cell line (MCF-7) as well, with a mean percentage inhibition reaching 99.8% and IC_{50} 2.53 µg/ml. Moreover, extracts 5, 6, and 7 demonstrated active cytotoxic effects on this cell line having IC50 less than 20 µg/ml. Meanwhile, their potency against this cell line is differential, with extract 4 having the highest potency, followed by extract 5, and extract 7 having the lowest potency. Nevertheless, extract 7 has the second highest efficacy after extract 4, with a mean percentage inhibition reaching 98.9%.

As HCC forms a therapeutic challenge and doesn't show a typical cytotoxic pattern, almost all of the extracts didn't show a significant cytotoxic effect against the HEPG-2 cell line, having no efficacy in the first place. Nevertheless, although extract 4 and 7 showed high efficacy using high concentrations, extract 7 is considered inactive as its IC_{50} value is more than 20; and the potency of the fourth extract couldn't be determined with the current results. However, extract 4 could conceal promising cytotoxic effects as it shows high mean percentage inhibition in 3 out of the 4 concentrations used, suggesting that its IC_{50} value could present an active, highly potent compound. The experiment done on the HCT-116 spheroids showed a significant decrease in the mean percentage inhibition using a concentration of 100 µg/ml with regard to the monolayer cell culture. However, extract 4, which was actively cytotoxic in the 2D culture model, inhibited almost three-quarters of the malignant cells in the 3D model, confirming its cytotoxicity against this cell line. The decrease in the cytotoxic effect of the extracts in the 3D system and the altered drug response in these two culture models is due to several facts; firstly, the unnatural and altered microenvironment of the cells in the monolayer cell culture leads to an altered cellular response to the drug, secondary, the varied gene expression in both models alters the cell's sensitivity to the drugs (Edmondson et al., 2014; Gurski et al., 2010).

Moreover, cells cultured in 3D models seem to be more resistant to drugs as cellular interactions and signals from the ECM and neighboring cells enter in the process, as well as the drug suffers from a restricted diffusion into the spheroid, accompanied by hypoxia which activates genes involved in the drug sensitivity and cell survival (Walker et al., 2003, Trédan et al., 2007). These resistance mechanisms which are present *in vivo* and observed in the 3D spheroids are not developed in the monolayer cell cultures, making the 3D spheroids a more reliable source to better understand the cellular behavior in response to a particular drug (Edmondson et al., 2014). This study showed 4 potential drugs against the 3 tested cell lines, one was potent against the all three tested cell line while the other 3 extracts showed efficiency against the breast cancer cell line only.

They could be used as direct drugs against these cancer types, or they could be used for the production of different compounds by semi-synthesis. However, the research regarding these 4 potential extracts should be extended to confirm their potency *in vivo*. Some of the limitations of the present investigation include the lack of secondary screening of extract 4 on the HEPG-2 cell line to be able to calculate its respective IC_{50} and evaluate its potency. Additionally, the safety of the 3 extracts 5, 6, and 7, showing potency against the breast cancer cell line, was not tested; their safety on normal cells was supposed to be evaluated on normal, non-cancerous, cells to confirm their possible use as a safe drug.

Hence, this research could be extended by undertaking the secondary screening of extract 4 on the HEPG-2 cell line and by testing the potential extracts on 3D cell

cultures of the 2 cell lines MCF-7 and HEPG-2 to have a closer look at their cytotoxic effect and their actual efficacy and potency on the respective cell lines *in vivo*, as the 3D cell cultures highly mimic the *in vivo* cells. Furthermore, the phytochemical compounds present in the potential extracts could be identified, isolated, and lastly produced to be used as direct drugs. Moreover, the molecular mechanism of the 4 cytotoxic extracts could be evaluated to determine the activated apoptotic pathways or the disrupted oncogenic pathways. Hence, in case a phytochemical compound caused the disruption of a particular oncogenic pathway, this compound could be used against other types of cancer where this pathway is activated. Moreover, since extract 7 showed the second highest efficacy against the MCF-7 cell line, the toxicity of this extract could be tested to determine the highest dose that remains safe and kills the malignant cells altogether. Lastly, the normal extension of this research is to test these 4 potential extracts in vivo, on mice or rats to confirm their cytotoxicity along with the physiological functions and responses.

CONCLUSION

After the screening of 7 plant extracts from *Albizia procera* and *Ailanthus altissima*, on the 3 cell lines HCT-116, MCF-7, and HEPG-2 in monolayer cell cultures and on HCT-116 on spheroids as well, 1 plant extract from A. procera exhibited privilege for its therapeutic effects against the 3 cancer types: colorectal cancer, breast cancer, and hepatocellular carcinoma; and 3 other plant extracts from *A. altissima* manifested potent cytotoxic effects against breast cancer only. These extracts could be investigated further to harness their therapeutic potential in medical achievements and benefits by being promising to be utilized as potential drugs against their respective cancer types.

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Ecological
Communication

Biosorption of Chromium Ions by *Streptomyces mutabilis* Isolated from Industrial Wastewater Treatment Plant

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ABSTRACT

The removal of heavy metals by actinomycetes has been the subject of many investigations as they are used as potential heavy metal sorbents. This study aimed to evaluate chromium biosorption activity of some actinomycetes isolated from wastewater sample collected from Industrial Wastewater Treatment Plant (IWWTP), Jeddah, Saudi Arabia. About 35 different isolates of actinomycetes were obtained on starch nitrate medium with 100 ppm of chromium ions. The removal of chromium from growth medium was maximum by isolate FM2 which was selected and identified as a species belonging to the genus *Streptomyces*. The 16S rRNA sequence of this isolate showed the highest similarity (98%) with *Streptomyces mutabilis* and identified as *S. mutabilis* FM2. The growth of the previous isolates was determined after 5 days at 30 ^{0}C in the presence of different chromium oxide concentrations, 50- 300 mg/l. The minimum inhibitory concentration (MIC) of this isolate for Cr (VI) was 135 mg/l. By dead biomass of the previous isolate FM2 (1g/l), the biosorption capacity measured by Plasma Atomic Emission Spectrometer ICPE-9000 of the bacterial strain for Cr (VI) ion was 800 mg/l which was 38% of the initial metal ion concentration. The maximum biosorption process for the tested isolate was recorded at pH 7, optimum temperature at 45 ^{0}C. Biosorbent mass of 0 – 1.5 g was tested for removal of chromium. The result showed that the adsorption capacities against the heavy metal Cr (VI) were increased with increasing the weight of the used dry biomass. The genus *Streptomyces* was the most potent in removing of heavy metals and *S. mutabilis* biomass has a good potential to be used in removal of chromium from wastewater. Their use in real life situation can alleviate pollution and increase the quality of water for human consumption and sanitary purposes. Initial concentration of metal ion, pH and cell biomass affect chromium biosorption process by the dried cells of *Streptomyces mutabilis*.

KEY WORDS: BIOSORPTION. MTC, BIOMASS, STREPTOMYCES MUTABILIS, BIOMASS, WASTEWATER, BIOREMEDIATION.

INTRODUCTION

One of the growing problem over the world is water pollution by chemical specially with heavy metals which showed serious side effects on human health and its environment. Several metals were highly toxic at low concentrations and through food chains, they accumulated in liver, kidney and other humans and animal tissues (Singh et al., 2011, Sahmoune, 2016). Physical or chemical methods like activated carbon adsorption, ion exchange, membrane filtration, and chemical precipitation can be applied to remove heavy metals from wastewater (Wang and Chen, 2009, Lakherwal, 2014). Nanocomposites adsorbents were applied to the removal of heavy metals from aqueous solution (Lu et al., 2017). Ion imprinted polymers has been used for elimination of heavy metal ions at low concentrations in complicated matrices (Cai et al., 2013, Fu et al., 2016). Heavy metals bioremediation methods received increasing attention because they are more safe and easily to be applied (Saiano et al., 2005, Yin et al., 2016).

Through the past two decades, biosorption method is efficient technique, cost effective and alternative for water and wastewater treatments (Ahmad et al., 2018). Biosorption methods were briefly studied by many authors

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(Al-Homaidan et al., 2014, Vendruscolo et al., 2017, Abed et al., 2020). Biosorption is a physicochemical process which involves the removal of various pollutants, such as heavy metals from solution by biological materials (Fadel et al., 2017, Yang and Wang, 2009). Different types of microorganisms, such as bacteria, algae, fungi and yeast cells can remove heavy metals from aqueous solutions (Vijayaraghavan and Balasubramanian, 2015, Podder and Majumder, 2016). The removal rate of Cr^{6+} has been explored by the optimized removal conditions by *Bacillus subtilis* strain SZMC 6179J and the initial pH, initial Cr^{6+} concentration (mg/l), time (hrs) and inoculation percentage (%) affect removal process. The optimal conditions for removal by the previous isolate were at pH 5.0, incubation time 24.0 hrs, inoculation percentage 4.6% (v/v) and initial concentration of Cr^{6+} 55.0 mg/l (Liu et al., 2020).

Actinomycetes, particularly *Streptomyces* species have been tested for uptake metals with very encouraging results (Gupta et al., 2015). *Streptomyces* species are stable and are not subject to the drastic treatments, and possess advantages such as low investment cost, and high treatment efficiencies (Mosbah and Sahmoune, 2013). Among the actinomycetes, the genus *Streptomyces* have produced useful secondary metabolites and the major source of a new bioactive molecule and good antibiotic producing organisms (Takahashi and Omura, 2003). *Streptomyces* are gram-positive filamentous bacteria with high guanine/cytosine content. *Streptomyces* strains characterized by a complex life cycle and the production of an amount of secondary metabolites which often find a use in medicinal applications (Hopwood, 2006). Streptomyces cells have excellent role in heavy metal removals and degradation of different pollutions. The aim of this study is to investigate the use of *Streptomyces mutabilis* biomass as an adsorbent for removing heavy metals from industrial wastewater solutions such as chromium which could reach to the underground water, causing harmful effects on human, animals, and plants.

MATERIAL AND METHODS

Collection of Soil Samples: For the present investigation, soil samples were collected from different contaminated soil area and were used for Actinomycete isolation. All samples were collected from Industrial Wastewater Treatment Plant located in Jeddah. Each sample was collected in sterile test tubes and stored at 4°C until used.

Isolation and Purification of Chromium Resistance Bacterial Isolate: About one gram of each soil samples was suspended in 9 ml of sterilized distilled water and serial dilution was done. One ml of each dilution was separately added to the surface of a plate containing starch nitrate agar medium and incubated at 30°C for 5 days. The obtained bacteria colonies were transferred to new plates until obtained of pure colonies which purified by streaking on soiled agar medium. The selected bacterial colonies were purified and transferred to slants on Starch nitrate agar for preservation at 4 ^{0}C. Long preservation of strains was in starch broth plus 50% sterile glycerol and stored at -20^{0}C until used (Ramesh and Mathivanan, 2009, Kannabiran, 2010).

Morphological, physiological and biochemical characterization of the bacterial isolates: Contaminated soil samples from Industrial Wastewater Treatment Plant, located in Jeddah, were used for actinomycetes isolation on Starch nitrate agar medium as described by Bakan et al., (2019). All actinomycete isolates were screened for Chromium resistant activity on starch nitrate agar medium containing 100 mg/l . After 5 days at 30 ^{0}C, the most resistance isolates were screened on medium contained different concentrations of Cr(VI) and minimum inhibitory concentration (MIC) was determined (Bakan et al., 2019). Strain FM2, the most resistant isolate, was preliminarily identified according to traditional morphological criteria, including morphology and growth pattern on starch nitrate agar.

Characteristics of the bacterial colonies on the agar plate, morphology of substrate and aerial hyphae, the morphology of spores, color of the produced pigment were carried out (Shirling and Gottileb, 1966). Also, the selected bacterial isolate FM2 was cultivated on different media plates for example; Yeast extract -malt extract agar (ISP-2), In-organic salts-starch iron agar (ISP-4), and Tyrosine agar (ISP-7) plates and identified according to morphology, physiology and biochemical characters. The cellular morphology of the bacterial isolate FM2 was examined under light microscope. Moreover, biochemical characterization of the isolate was carried out as described in International actinomycetes isolates Project including biochemical identification tests such as catalase, citrate oxidase, indole production and sensitivity to antibiotics (Pridham and Lyons, 1961).

Molecular Identification: Genomic DNA from each isolate was obtained using the QIAamp DNA Mini Kit (Weisburg et al., 1991). Then, the concentration and purity of DNA were determined. In princess Al-Jawhara Center of Excellence in Research of Hereditary Disorders, the 16S rDNA gene was amplified by PCR using the forward primer 5'-AGTTTGATCATGGTCAG-3' and the reverse primer 5'-GGTTACCTTGTTACGACT-3'. The DNA sequence was compared to the GenBank database at the National Center for Biotechnology Information (NCBI) using the BLAST program (Saitou and Nei, 1987).

Study of the Minimal Inhibitory Concentrations: The actinobacteria isolate FM2 was screened for heavy metal-resistant activity in starch nitrate agar medium, after preparing of the medium it was sterilized by autoclaving at 121°C for 15 min, then different concentration of each sterile metal salt solution was added a to molten cooled starch nitrate agar medium immediately before pouring to the plates and solidification. Microbial growth was used as the qualitative parameter of mean resistance after seven days of incubation at 30°C. Testing for tolerance of the microorganisms ended when complete inhibition of the growth was observed on the nutrient agar with metal

supplementation (Koushalshahi et al., 2012, Daboor et al., 2014). This method was used to determine the minimum inhibitory concentration (MIC) for each concentration of the heavy heavy metal at which there is no colony growth in the three copies. This method was used to give a rapid screening, but it is a qualitative estimation (Abbas and Edwards, 1989).

Biomass preparation of actinobacteria for biosorbent process: The biomass of actinobacteria (biosorbent) was prepared by the method of Saurav and Kannabiran (2011b), (Latha et al., 2015). The tested isolate FM2 was cultivated in 500 ml flasks containing 100 ml of starch nitrite broth medium and was kept shaking in an orbital rotary shaker at 130 rpm for 10 days at 30 ^{0}C. Then, the cultures were harvested by centrifugation at 4500 rpm for 15 min and were washed three times with distilled water. The pellet was kept in glass petri dishes and dried at 70 ^{0}C for 24 h. After the biomass was dried, it was crushed in a blender to powder. Then, it was used for further studies.

Preparation of Metal Solutions: The heavy metal used for the screening of metal resistance in actinobacterial isolate was chromium (Cr). The salt solution was prepared from analytical-grade Chromium (VI) oxide, sterilized separately for 15 min at 110°C (Saurav and Kannabiran, 2009) and preserved at 4^0 C until used. The concentrations of metal ion were prepared from stock solutions of 100,000 mg/l. Fresh dilutions were used for each study. The pH of each test solution was adjusted to the required value by using 1 M NaOH and 1N HCl (Latha et al., 2015).

Biosorption Experiments: The chromium removal ability of the actinomycete isolate FM2 was determined by measuring the level of chromium uptake following the method of Saurav and Kannabiran (2011b) with slight modifications. The isolated strain was tested to biosorption of Cr (VI) metal salt solution at different concentrations (50–300 mg/l) then in different concentrations (0.5, 1.0, 2.0, 2.5, 3.0 g/l of biomass obtained from tested strain. The dried biomass of the actinomycete isolate was suspended and the pH was adjusted to 7.0. Then the flasks were kept shaking in an orbital rotary shaker at 130 rpm for one week after that the filtrates were analyzed for chromium concentration by Plasma Atomic Emission Spectrometer ICPE-9000. The metal removal efficiency (MRE) was calculated by using the following equation.

$$\% \text{ of MRE or } \% \text{ Biosorption} = C_i - C_f / C_i \times 100$$

Where MRE: metal removal efficiency, C_i represents the initial chromium metal ion and C_f represents the final chromium metal ion concentration.

Effect of pH and temperature on Heavy Metal Removal: The dry biomass (0.25 g) of the Actinomycete isolate FM2 was incubated into a series of 100 ml conical flasks containing either 25 ml of distilled water with 320 mg/l of chromium (VI) oxide. The pH was varied from 4 to 10 (4, 6, 7, 8 and 10). On the other hand, the effect of different temperatures (24, 28, 30, 35 and 45 ^{0}C) on biosorption capacity was detected. The pH of the medium was adjusted using dilute HCl or NaOH. Then, all flaks were incubated at 30 ^{0}C and 130 rpm for 5 days. The percentage biosorption of metal ions was calculated at the all tested pH values.

Concentration of Heavy Metals Residuals in the Bacterial Biomass: To detect the residual of chromium found in the biomass of the isolate which they involved in bioremediation technique, the biomass fragments were collected after centrifugation at 4500 rpm in 15 minutes. then the samples pallets were collected and oven-dried at 70 ^{0}C about 24 h. After that, each sample (0.5 g) was then digested through addition of 5 ml of hydrochloric (HCl) acid and 5 ml sulphuric acid (H2SO4). After heating gently until the samples were digested (formation of a clear solution above the residue), the volume was adjusted to 10 ml with distilled water, pH was adjusted to 7 with 5 M NaOH and the solutions were analyzed for metals concentrations Cr (VI) using Plasma Atomic Emission Spectrometer (ICPE-9000).

Applied Biosorption Experiments: This experiment was detect the ability of dead bacterial biomass FM2 to removal heavy metal Cr(VI) from industrial wastewater. At first, the content of different heavy metals of waste water was analysis of by Plasma Atomic Emission Spectrometer ICPE-9000. Then, two conical flask (250 ml) containing 100 ml of waste water from Bani Malek Station, with 0.5 g of dry dead bacterial biomass, obtained from tested, was kept shaking at 130 rpm for one week at 3^0 C. After that the content of each flask was filtered through filter paper and the filtrate was analyzed for metal concentration by Plasma Atomic Emission Spectrometer ICPE-9000. The percentage biosorption of metal ions was calculated as follows:

$$\% \text{ of MRE} = C_i - C_f / C_i \times 100$$

RESULTS AND DISCUSSION

The actinobacteria with potential heavy metal biosorption ability were selected for identification. The morphological, culture and biochemical characteristics of the strains were investigated and recorded in Table 1. The isolate FM2 was Gram positive, not acid fast, with non-motile cells and substrate and aerial mycelia were well developed. The color of the isolate, on starch nitrate was gray. The growth on solid medium and examination under light microscope were recorded (Table 2, Figure 1). The growth and shapes on different agar media were appeared in Table 3 and Figure 2. The isolate FM2 grow well on starch nitrate agar, In-organic salt starch iron agar ISP4, and tyrosine agar was recorded on ISP-7, while moderate growth on yeast extract malt extract agar (ISP-2), E-Medium (ISP-9), Glycerol asparagine agar (ISP-5), and dextrose agar.

Table 1. The selected actinomycetes, color, growth, mycelia and production of melanin pigment

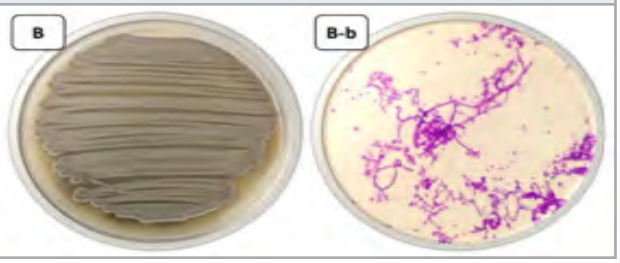
Isolates	Color	Growth (100 ppm Cr)		Substrate and aerial mycelia	Melanin pigment
		On agar medium	Dry weight g/l		
FM1	Pink	+	0.17±0.01	Well developed	+ve
FM2	Gray	+++	0.37±0.02	Well developed	+ve
FM4	Black	++	0.30±0.02	Well developed	+ve
FM9	Pink	++	0.23±0.02	Well developed	-ve
FM11	Gray	++	0.30±0.09	Well developed	-ve
FM15	White	++	0.21±0.01	Well developed	-ve

+: Poor growth, ++: Moderate growth, +++: Good growth, -ve: No pigment, +ve: Detected pigment

Table 2. Morphological characteristics of the selected isolate FM2

Characteristics	Results	Characteristics	Results
Gram stain	Gram-positive	Aerial mycelium	Present
Colony size	Discrete	Sporangia	Absent
Acid fast stain	Negative	Optimum temperature	30 °C
Motility	Absent	Optimum pH range	6.5 - 8.0
Respiration	Aerobic	Melanin pigment	Positive
Substrate Mycelium	Branched	Catalase	Positive
Spore chain	Positive	Penicillin	Sensitive
Motile spores	Absent	Cephalosporin	Resistance

Figure 1: The selected actinomycete isolate FM2, B: grown on starch nitrate agar medium, B-b: under light microscope x1000.



On the other hand, the isolate FM2 use different carbon and nitrogen sources, it was found that it used strongly the carbon sources, glucose, sucrose, lactose, and maltose while moderate utilization was recorded by isolate FM2 for starch, dextrose and fructose. Moreover, different nitrogen sources, yeast extract and peptone were well utilized by isolate FM2 table 4.

Molecular Identification: The selected strain shown the partial sequencing of 16S rRNA gene of the strain on both directions yielded 16S rDNA nucleotide sequence length with 1300 base pairs. The BLAST search of 16S rDNA sequence of the strain showed the highest (98%) similarity with *Streptomyces mutabilis* strain 64-LR13-2, *Streptomyces mutabilis* strain (HVA-18), and *Streptomyces* sp. Strain ELO60 (Figure 3).

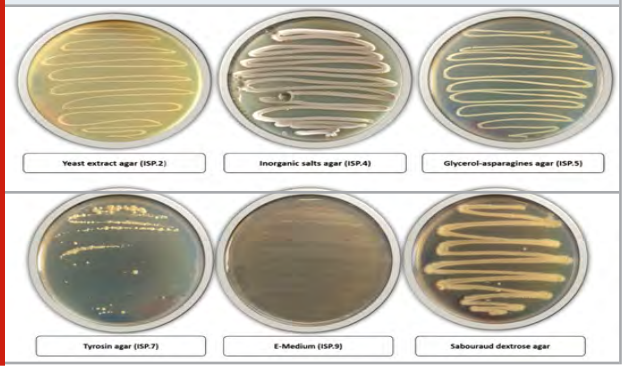
Minimum Inhibitory Concentration (MIC) of Chromium: In this study, the actinomycetes isolate from soil were explored for their bioremediation capabilities to prove that they have potential in bringing down the intensity of heavy metals in media. The inhibitory effects of Cr (VI) on bacterial growth were investigated on starch nitrate agar medium. The MIC was 135 mg/l for Cr (VI) as shown in Figure 4 and Table 5. Percentages of chromium adsorption by FM2 cells (0.1 g/l) added to different concentrations of chromium were determined. At 50 mg/l Cr, the removal percentage was 100% and decreased by increasing Cr concentrations (Figure 5). The percentage of adsorption was a function of the initial metal concentration. The amounts of metal uptake q (mg/g) by the dead biomass of FM2 at a different metal concentration Cr (VI) are calculated. Table 6 showed the amounts of chromium uptake q (mg/g) by the different concentrations of the dead biomass of isolate FM2. The amounts of metal uptake q (mg/g) by the dead biomass of isolate FM2 was ranged from 23.24% to 5.00% with a maximum adsorption of 34.86 % using 0.12 mg/l of the dry mass.

As the biosorbent weight of the tested isolate was increased from 0.12 to 1.50 g, the removal percentage increased from 34.86% to 93.91% for Cr (VI) but the chromium uptake q (mg/g) was decreased.

Table 3. Growth of FM2 isolate on different media growth for 5 days at 30°C.

Media	Growth	Color of aerial mycelium	Color of substrate mycelium	Soluble pigment
Starch Nitrate	Heavy	Grey	Grey	Light grey
Yeast extract-malt extract (ISP-2)	Moderate	Creamy	Creamy	Light brown
In-organic salt-starchiron (ISP4)	Heavy	Dark grey	Grey	Creamy
Glycerol asparagine (ISP-5)	Moderate	White	White	No- pigment
Tyrosine medium (ISP-7)	Heavy	Light grey	Light grey	No- pigment
E-Medium (ISP-9)	Moderate	Page	Page	No- pigment
Dextrose Sabouraud	Moderate	Creamy	Creamy	Yellow pigment

Figure 2: Growth of bacterial isolate FM2 on different agar media



The results showed the effect of pH on the adsorption of chromium oxide by dead biomass of the tested isolate. The highest biosorption capacity for chromium was at pH7 (Figure 6). Figure 7. showed the effect of different temperatures on the adsorption of chromium by the dead biomass of the tested isolate FM2. Biosorption capacity was analyzed over a temperature range from 24° C to 45° C. The highest biosorption capacity of chromium by the tested isolate was 100% at 37-45° C. Wastewater was collected from wastewater treatment plant and the selected bacterial isolate was used for chromium removal from wastewater. At the beginning the Chromium concentrations was 11 mg/l. After 7 days of incubation with the tested bacteria, the concentration of chromium decreased to 0.0 (as shown in Table 7).

Table 4. Growth of bacterial isolate FM2 on different carbon and nitrogen sources

Carbon sources	Glucose	Sucrose	Starch	Lactose	Dextrose	Maltose	Fructose
Results	+ + +	+ + +	+ +	+ + +	+ +	+ + +	+ +
Nitrogensource	Ammonium sulfate	Ammonium chloride	Sodium Nitrate	Potassium nitrate	Glycine Peptone	Vanillin	
Results	±	+	+	+	+ +	+	

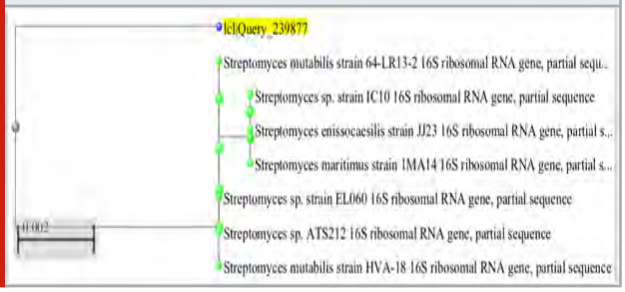
+++: high utilization, ++: moderate utilization, +: weak utilization, ±: very weak utilization

In Saudi Arabia, Jeddah is one of the biggest cities with about three million inhabitants. Large quantities of wastewater returned to the environment and accumulated in underground pools. Only 30 percent went wastewater treatment plants for purification before being dumped in the Red Sea. Most of the wastewater that is accumulated through pipes must be treated for heavy metal removal before dumping into the sea. Without purification, wastewater caused massive damage to the aquatic animals, beaches, air, human health and soil. Sewage treatment plants facilitate and improved wastewater management. The heavy metals such as Fe^{++}, Cu^{++}, Zn^{++}, Ni^{++}, Mn^{++}, Cd^{++}, Pb^{++} and Cr^{+++} are toxic pollutants of the environment, wastewater and aquatic live (Amundsen et al., 1997; Wong et al., 2001). Accumulation of these metals in soil, animal tissue is documented (Li et al., 2006, Okoye et al. 2011, Abduljaleel et al., 2012).

Thus, in recent decades removal of heavy metals using conventional physical and chemical methods are urgent but these methods are high expensive, low effectiveness and non eco-friendly. Biosorption methods by biological materials like live or dead microbes of non-pathogenic bacteria, fungi, gut microflora and actinomycetes can be used to remove detoxify and eliminate some heavy metals from soil and solutions. These methods are effective, little price, eco-friendly. easy to applied and had beneficial health effects (Wang and Chen, 2009). In different ways, actinomycetes played an important role in removal of harmful metals. Thus, in this study we tried to isolate and

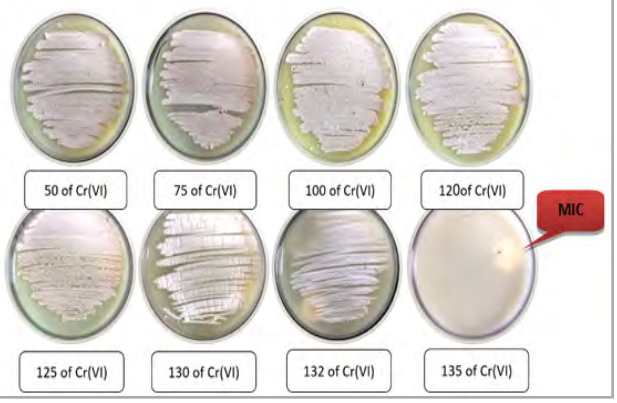
identify some actinomycete genera from contaminated area that can remove Chromium ions which showed high toxicity to living cells and pollute human environments (water, food, air and soils). Two forms of Chromium are recorded, trivalent (Cr III) which is less toxic to plants and animals and hexavalent (CrVI) which cause human cancer, genotoxicity, acute and chronic toxicity to skin and nervous and immune systems in addition to general environmental toxicity (Bagchi et al., 2002).

Figure 3: Phylogenetic tree based on 16S rRNA for *Streptomyces mutabilis* FM2.



As was reported before, actinomycetes showed exceptional resistance properties to some heavy metals and they adaptive themselves to adsorb these metals during continuous exposure (Abbas and Edwards 1989; Amoroso et al. 2000). Koushalshahi et al. (2012) reported that actinomycetes from contaminated samples are resistant to heavy metals compared to those from non-contaminated area. On minimal agar medium, out of

Figure 4: Effect of different concentration of chromium (50-135mg/l) on growth to the isolate FM2.



15 isolates, isolate FM2 was the most tolerant isolate (MIC value 135 mg/l). Latha et al., (2015) reported that the MIC was in the ranged from of <50–250 mg/l. It was identified as species belong to genus *Streptomyces* according to morphological, physiological, and biochemical characteristics. Identification was confirmed using molecular method. 16S rRNA sequence analysis is a tool used for confirmation of the identification of bacteria (Wallhausser et al., 1964, Muharram et al., 2013). Using 16Sr RNA, The isolate FM2 was identified as *Streptomyces mutabilis* FM2. The previous method was used by many authors (Dhanasekaran et al., 2012; Saha et al., 2013, Bahamdain et al., 2020, Aly et al., 2020).

Table 5. Actinomycetes isolates tolerance to different chromium concentrations.

Cr (VI) (mg/l)	50	70	100	115	125	120	125	130	135	150
FM 2	++ +	++ +	+ ++	+ ++	+ +	+ +	++	+	+	-

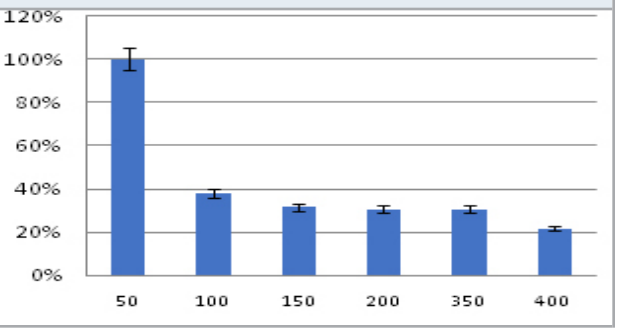
+++: high growth, ++: moderate growth, +: Low growth,-: no growth

Table 5. Effect of different concentrations of biomass on Chromium (VI)removal from a solution at 320 mg/l.

Biomass weight (g/l)	Final chromium concentration (mg/l)	% Biosorption	Specific metal uptake (mg/g)
0.00	320	0.00	0.00
0.12	208.41	34.86	23.24
0.25	172.91	45.96	14.70
0.50	77.90	75.65	12.10
0.75	64.65	79.79	8.51
1.00	51.25	83.98	6.71
1.50	19.48	93.91	5.00

* Specific metal uptake (Q)

Figure 6: Capacity of chromium adsorption by FM2 isolate(0.1 g/l) on different concentrations of chromium.



The biosorption capacity of *Streptomyces mutabilis* for Cr(VI) was found to be maximum (100%) at 50 mg/l metal ion concentration using 0.1 g/l of the cell biomass. At pH 7.0 at 37- 45^0 C, the higher biosorption ability (100%) was obtained using 0.12 mg/l of the dry material.

Increasing the concentration of Cr(VI) decreased biosorption capacity which may due to metal saturation on biosorption material while increasing the weight of the biosorption material, increased the biosorption capacity due to the highest contact between bacterial cell wall and metal ions (Saurav and Kannabiran, 2011a,b). They added that maximum Cr(VI) biosorption by *Streptomyces* sp. dry cells (3 g/l) was obtained at 100 mg/l at pH 7.

Figure 6: Effect of different pH rage on biosorption of Cr(VI) at 50 mg/l by dried cell of bacterial isolate FM2 (0.12 mg/l).

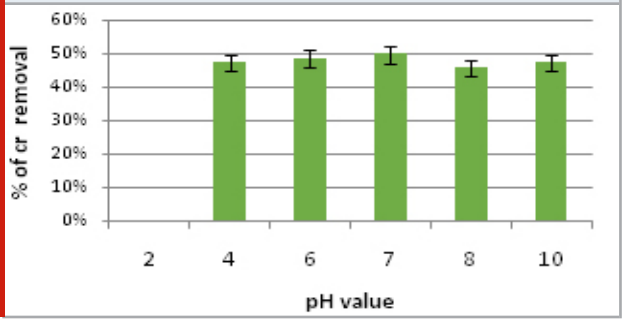


Figure 7: Effect of different temperatures on the biosorption of chromium at 50 mg/l by dried cells of bacterial isolates FM2(0.12 mg/l).

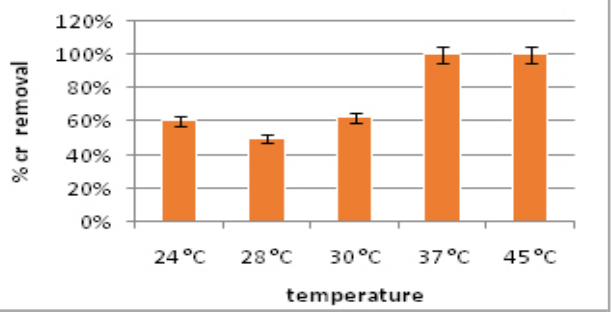


Table 6. Biosorption of some heavy metals from waste water using dry biomass of the isolate FM2.

Isolate	Chromium (VI) Concentration		
	At the start (mg/l)	After 7 days (mg/l)	% of Biosorption
FM2	11±0.7	0.00	100 %

Biosorption or removal capacity of heavy metal was affected by biosorbent weight, pH and initial metal concentration (Donmez et al. 1999, Yao et al. 2009, Saurav and Kannabiran 2011b) Cr(VI) ions react with several functional groups like the hydroxyl (OH), amine (NH2) and carboxyl (CO) groups, found on the bacterial biomass which play an important role in the metal ions biosorption process. Microbial cell walls contained, peptidoglycan polysaccharide, glycoprotein, glucan, chitin, mannan, phosphomannan and teichoic and teichuronic acids which may adsorb metal ions (Volesky 1990). The potent Cr(VI) biosorbent *Streptomyces mutabilis* was used to purify wastewater from heavy metals and to control the problem of bioaccumulation in living cells. Bakran et al. (2019) used two *Streptomyces* to remove lead from wastewater. In conclusion, *Streptomyces mutabilis* belong to actinomycetes and showed excellent activity to remove and resist hazardous heavy metals like Cr(VI) with maximum biosorption (100 %) at 0.12 g/l at pH7 and 37-45 ^{0}C after 7 days.

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Dental Communication

Influence of Upper Lip Curvature on Smile Attractiveness in Patients with Different Degrees of Gingival Smiles: A Study in Dravidian Population

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ABSTRACT

The importance of an attractive smile and its effect on positive self-image cannot be emphasized enough, which is why an attractive and balanced smile is a valued treatment goal in addition to creating a functional occlusion. Therefore, the aim of the current study was to Correspondence evaluate the influence of upper lip curvature on smile attractiveness in patients with different degrees of gingival smiles by a lay person, post graduate students and general dentists. A frontal photograph was digitally altered to generate 3 types of upper lip curvature shapes (upward, straight, and downward) with 3 different levels of gingival smile exposure (0 mm, 3 mm and 5 mm). Nine images were generated. Three groups of evaluators (10 dentists, 10 orthodontists, and 10 laypersons) assessed the images using a visual analog scale. One-way ANOVA to evaluate the difference in median aesthetic scores between postgraduate students, general dentist and lay persons was performed. A statistically significant difference was observed in median aesthetic scores between the general dentist and lay person when there was 3mm gingival exposure in upward curvature ($p<0.05$, p value 0.032). Upward or straight upper lip curvature shapes have a positive effect on the perceived smile aesthetic, while the downward curvature shapes of the upper lip have a negative effect on perception when different degrees of gingival smiles are rated. The study was conducted to produce results that can act as a milestone for future researchers helping them with establishing their findings using this as a credible reference.

KEY WORDS: AESTHETIC HARMONY, GINGIVAL DISPLAY, SMILE AESTHETICS, UPPER LIP CURVATURE.

INTRODUCTION

A pleasant smile not only plays a fundamental role in social interaction and personal development, it also improves the attractiveness of the face (Adams 1977; Feingold 1992). During interpersonal interaction, individuals focus on another person's eyes and mouth (Miller 1970). The importance of an attractive smile and its effect on positive self-image cannot be emphasized enough. Therefore, an attractive and balanced smile is a valued treatment goal, along with the creation of a functional occlusion (Petrungaro 2002). There are many factors that determine an attractive smile: a smile shows the entire length of the upper front teeth, shows an incisal curve of the upper teeth, that is, parallel to the inner part of the lower lip, and shows the upper front teeth and the premolar (Tjan, Miller and Josephine 1984). This depends not only on components such as the size, shape, color and position of the teeth, but also on the number of visible gums and the shape of the lips (Geld et al. 2007; Rozhkova et al. 2019).

Excessive gingival display or the "gummy smile" can interfere with an otherwise pleasing smile (Bedavanija, 2019). It is the result of a combination of factors such as the vertical excess of the upper jaw, increased overjet, increased overbite, a short upper lip, and a short incisor crown length (Allen, 1988). Tjan and Miller divided the smile line into three types: a high smile line that shows the complete upper incisors and a continuous band of the gingiva; an average smile line that shows 75 to 100 per cent of the upper incisors; and a low smile line that reveals less than 75 percent of the upper incisors (Machado et al. 2013; Rozhkova et al. 2019). Although it has been suggested in Western society that no more

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than 2mm of the maxillary gingiva should be visible when a person smile (Mew 1998; Rozhkova et al. 2019). There is no scientific evidence to support this point of view in the Dravidian population. Therefore, the aim of the present study was to evaluate the influence of upper lip curvature on smile attractiveness in patients with different degrees of gingival smiles by a lay person, post graduate students and general dentists.

MATERIAL AND METHODS

Construction of a series of images were carried out. A female frontal intraoral photo of ideally aligned teeth and a female extraoral photo showing aesthetically smiling lips were obtained from a subject. These ideally aligned teeth and lips together formed a standard composite smile, with all teeth up to the first molar and the upper lip touching the upper gingiva of the upper central incisors. The lower lip coincided with the curvature of the incisal edges of the upper incisors and canines. These images were modified with Adobe Photoshop CS2 (San Jose, California, USA) to create bilaterally symmetrical teeth and lips. 3 types of upper lip curvature were generated, that is lip curvature upwards, lip curvature straight and lip curvature downwards, in addition 3 different exposure levels of the gingival smile of 0 mm, 3 mm and 5 mm. Fig 1 represents the different curvature of upper lip and degrees of gingival smiles and fig 2 represents VAS scale with 1 score being the lowest and 5 score being the highest.

Figure 1: Represents the different curvature of upper lip and degrees of gingival smiles.

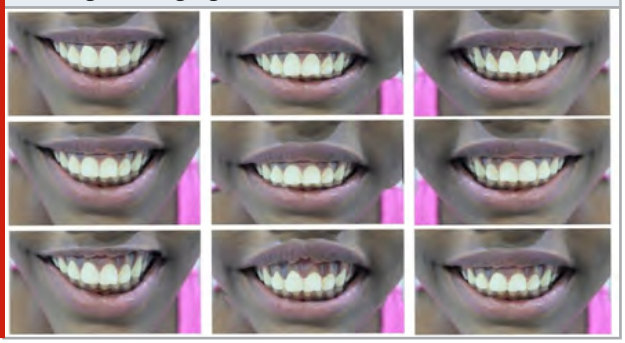
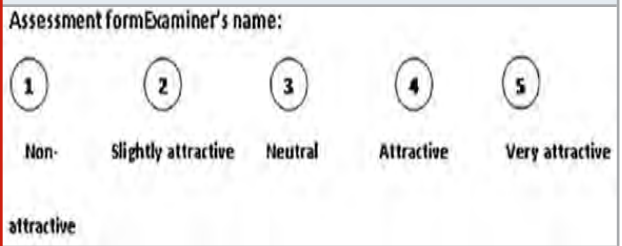


Figure 2: VAS scale with 1 score being the lowest and 5 score being the highest.



The smile raters were 10 Dravidian laypersons (4 males and 6 females; age 28 ± 7.6 years), 10 post graduate students (5 males and 5 females; age 24.8 ± 2.5 years) and 10 general dentists (7 males and 3 females; 29.6±2.66 years). Determination of the subjective aesthetic value of each smile was accomplished using a visual analogue scale (VAS). This rating scale was designed for minimal constraints and the most freedom to express a personal response style. The VAS was used to rank each smile from 'non-attractive attractive' to 'very attractive'. An aesthetic score was obtained by 1 being the least attractive (zero) and 5 being the most attractive. One-way ANOVA to evaluate the difference in median aesthetic scores between postgraduate students, general dentist and lay persons was performed using the Statistical Package for Social Sciences (SPSS, Version 23; Chicago, USA).

RESULTS AND DISCUSSION

A statistically significant difference was observed in median aesthetic scores between the general dentist and lay person when there was 3mm gingival exposure in upward curvature ($p<0.05$, p value 0.032) which is observed in the table below (Table 1). VAS has been used to assess pain intensity and has proven to be a valid, reliable, and reproducible method for measuring subjective pain (Ohnhaus and Adler 1975). Since many researchers used the VAS method to assess attractiveness, the VAS method became the Assessment used. The aesthetics should also provide simple, quick and reproducible results (Roden-Johnson et al. 2005; Martin et al. 2007; Krishnan et al. 2008). Excessive gingival display or gummy smiles are a common trait that has been reported to affect 7 percent of young adult men and 14 percent of young adult women (Tjan, Miller and Josephine 1984). In the present study it was observed that the lay person and general dentists preferred a smile with 3 mm of gingival exposure and high lip curvature which was found to be statistically significant ($p<0.05$) (Rozhkova et al. 2019; Valverde-Montalva et al. 2021).

The 0 mm gingival smile with straight upper lip curvature was considered the most attractive by postgraduate students (not statistically significant), while the 3mm gingiva smile with high curvature of the upper lip was considered the most attractive by general and laypersons. This finding is in contrast with finding reported by Geron and Atalia et al. (2005), who examined the influence of the gingival presentation on the perception of the aesthetics of smile in lay people and reported that the most attractive smile images were those with a coverage of the upper lip of the central incisors by 0-2 mm (Geron and Atalia 2005; Rozhkova et al. 2019; Valverde-Montalva et al. 2021).

However, it was observed that the shape of downward curvature of lip had a negative influence on aesthetic assessment of the smile in all assessment groups with gingival exposures of 3 mm or more. This finding agrees with that of Valverde-Montalva et al. (2005), the study carried out reported that downward curvature of lip had a negative effect in all groups, but at the same time laypeople had a more positive impact by high upper lip curvature with 5 mm gingival exposure (Valverde-Montalva et al. 2021).

Table 1. Represents the values of one-way ANOVA.

	Mean sum of squares	df	Sig.
0 mm gingival exposure with high lip curvature	0.46	2	0.47
3 mm gingival exposure with high lip curvature	7.20	2	0.032
5 mm gingival exposure with high lip curvature	2.06	2	0.23
0 mm gingival exposure with low lip curvature	0.20	2	0.78
3 mm gingival exposure with low lip curvature	4.4	2	0.07
5 mm gingival exposure with low lip curvature	0.46	2	0.50
0 mm gingival exposure with straight lip curvature	1.86	2	0.53
3 mm gingival exposure with straight lip curvature	2.06	2	0.36
5 mm gingival exposure with straight lip curvature	1.40	2	0.54

Laypersons' views on the assessment of gingival display may differ from those of orthodontists. Other investigations using laypersons as evaluators seem necessary. If the aesthetics of the orthodontist does not match the perception of the patient, the results may be unacceptable to the patient. Therefore, it is important that patients must participate in the decision-making process when planning orthodontic treatment. Limitations of the current study include a small sample size (Rozhkova et al. 2019; Valverde-Montalva et al. 2021).

CONCLUSION

Within the limitations of the current study, it was observed that upward or straight upper lip curvature shapes have a positive effect on the perceived smile aesthetic, while the downward curvature shapes of the upper lip have a negative effect on perception when different degrees of gingival smiles are rated. Future scope suggests that along with the amount of gingival display, different factors of smile aesthetics have received attention: the presence of the smile arc and buccal corridor spaces and the same should also be evaluated in the Dravidian population.

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Conflict of Interest: There was no recorded conflict of interests.

Ethical Clearance Statement: The Current Research Work Was Ethically Approved by the Institutional Review Board (IRB) of Saveetha Dental College and Hospital, Chennai, India.

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Biotechnological Communication

Use of Sericulture Byproduct as Feed for Tilapia–*Oreochromis niloticus*

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ABSTRACT

Sericulture is a well-established rural agribusiness in India and produces about 40,000 metric tonnes per year of silkworm pupae (SWP) on dry weight basis. This SWP contains enormous amount of crude protein (34.40%) one of the major feed component, besides considerable amount of crude lipid (10.63%), carbohydrate (9.95%) etc. claiming as a promising ingredient of fish feed. Fingerlings of tilapia, *Oreochromis niloticus* (Linn.) with mean weight 5.0 ± 0.20g were randomly stocked at 15fingerlings per tank in three replicates (tank size, L x B x H; 1m x 1m x1m) for 90 days to evaluate the effect of sericulture byproduct on growth and flesh fatty acid profile of tilapia. Two isonitrogenous pelleted feeds viz. silk worm pupae meal (SWM) and market available feed (MAF) were prepared and fed to the fish twice daily. The feed (SWM) containing fermented silk worm pupae as principal ingredient was the most efficient in terms of final average weight gain, specific growth rate, protein efficiency ratio and fatty acid profile (Poly unsaturated fatty acid and n3/n6 ratio). Fish PUFA, especially the n3 fatty acids and n3/n6 ratio are affected positively when fed with SWM which is good for the quality of the fish produced with respect to the benefits of human health. The results showed that fermented silk worm pupae can be used in the diet for tilapia which enhances production as well as quality of cultured fish. This research has been conducted to develop scope for future studies on researching sericulture byproduct as a feed. As currently not many researchers are working on this topic, this report will help by acting as a suitable milestone and an updated reference whenever needed.

KEY WORDS: FISH PUFA, N3/N6 RATIO, SERICULTURE BYPRODUCT, TILAPIA.

INTRODUCTION

Several studies have been observed to make supplementary feeding of fish cost effective have been directed to substitute the high-cost fish meal with less expensive protein sources. This aspect of feed development research is centered on the search for inexpensive, locally available and nutritious protein sources that can supply all the nutritional needs of the fish. Sericulture is a well-established rural agribusiness in India and produces about 40,000 metric tonnes per year of silkworm pupae (SWP) on dry weight basis (Rangacharyulu et al. 2003). A small quantity of this SWP is sun-dried and used in animal feeds, while a considerable amount is discarded in open places though it contains enormous amount of crude protein (34.40%) one of the major feed component, besides considerable amount of crude lipid (10.63%), carbohydrate (9.95%) etc. claiming as a promising ingredient of fish feed (Table 1) (Gabriel et al. 2007; Bag et al. 2013).

An appropriate fermentation ensiling process has been developed to prepare a pathogen free. Annison (1993) reported that microbial fermentation and nutrient synthesis was typically important in organisms with a diet high in fibre. Fermentation is a simple and cheap process where there may be an increase in the nutrient level through xxmicrobial synthesis, apart from microbial degradation. Many studies have been carried out to evaluate the effects of nonconventional ingredients used in diets as FM substitutes on fish fatty acid composition. Surprisingly, such an important farm waste product for making nutritionally balanced fish feed formulation is remained unexplored. Research information on utilization of such alternative fish feed source is scanty (Yashoda et al. 2001; Bag et al. 2013).

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The beneficial fatty acid in fish body is synthesis from the feed materials they consumed (Horrobin et al. 1990). The beneficial effects of fish lipids on human health have already been well established. It is therefore of utmost importance to determine the influences of feed on growth as well as fat deposition in fish flesh (Cengiz et al. 2003; Mukhopadhyay, 2009). Keeping the above facts in view, the investigation was carried out with the main objective to study the growth performance of O. niloticus by using fermented silkworm pupae as alternative source of protein in fish feed and also to study the qualitative changes in fish flesh as human food (Bag et al. 2013).

MATERIAL AND METHODS

Twenty fingerlings in triplicate groups were used in two different treatments. Altogether one hundred and twenty (120) Nile tilapia (male and female ratio 1:1) fingerlings were utilised in this experiment. The fish fingerlings were treated with potassium permanganate solution (1 mg L^{-1}) to remove any external parasites and were acclimatized in a big tank for five days. Experiments were carried out at the tanks of aquacultural engineering section of IIT-Kharagpur, Paschim Medinipur, West Bengal, India from June to August 2018. Each group of fingerlings also were initially weighed to record the initial biomass content. They were stocked in six rectangular cemented tanks (1000 L). The water system was static in nature and the bottom of the tank was filled with local agricultural soil (pH 6.4 ± 0.05). The experiment was conducted for 90 days from June to July in the year 2019. Dechlorinated well water (temperature 26 ± 3 °C, pH 7.v0 ± 0.05, free CO_2 0.4 ±0.01 mg L^{-1}, available nitrogen 0.5 ±0.05 mg L^{-1} and dissolved oxygen (DO) 6± 0.5 mg L^{-1}) was used in the experiment. The principal feed ingredient (silkworm pupae) was collected from local sericulture farm after silk reeling at very low cost. These waste substances were economically cheap but contained significant amount (35– 37%) of crude protein.

Biochemical composition of silkworm pupae used in the feed for tilapia is shown in Table 1. Diets used for growth trial were prepared in such a manner, the feed formulations remain almost isonitrogenous (30 g 100 g^{-1}) and isoenergetic (4.0 Kcal g^{-1}) in nature. The choice of these nutrient levels, particularly protein, was intended to reflect the practical diets used in our country like India. Diet formulations are presented in Table 2. Mustard oil cake, wheat flour, rice bran and egg shell dust were the ingredient of Market available feed (MAF). In addition to these ingredients the chief ingredient of SWM feed was fermented silkworm pupae. These ingredients were used to compensate lipid, protein and ash deficiency in formulated feed. Wheat flour was selected as binder. Prepared feed was fortified with egg shell dust which is available free of cost for calcium supplement. This was added keeping in mind that the developing fish needs huge quantity of calcium for its bone development. The different ingredients were thoroughly mixed using a food mixer (A200 Hobart Ltd). The proportion of different feed ingredients was determined by using Pearson's square method.

The mixture was given the shape of pellets using a Pellet Mill (Model CL2) with a 12 mm die. The resulting pellets were dried in a hot air oven for 48 h at 50 °C, packed in polythene bags and kept in dry and cool place. The feed was given ad libitum in a feeding bag hung from an iron rod in four locations in each tank. Unconsumed feed was removed after 1hour from the beginning of feed administration and dried in a hot air oven at 55°C. Growth and nutrient utilization were determined in terms of feed intake (FI), specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER), and hepatosomatic index (HSI) as follows : FI (g fish-1 day-1) = Total feed intake per fish/number of days SGR (% day-1) = 100 × (ln[final body weight]- ln[initial body weight])/no. of days FCR = feed intake/live weight gain PER = live weight gain/crude protein intake HSI (%) = 100 × (liver weight/total body weight) GSI (%) =100 × (weight of gonad /total body weight).

Feed and carcass samples were analyzed following standard procedures (AOAC, 2002), dry matter (DM) after drying in a hot air oven (Gallenkamp, UK) at 105°C for 24 h; crude protein (CP) by Kjeldahl method (N × 6.25) after acid hydrolysis, crude lipid (CL) after extraction with petroleum ether for 7-8 h by Soxhlet method (40–60°C boiling range), total ash by igniting at 550°C for 3 h in muffle furnace (Size 2, Gallenkamp, UK). Organic matter (OM) was calculated by subtracting total ash from DM (Giri et al. 2000). Crude fibre was determined using a moisture free defatted sample which was digested by a weak acid HCl (0.1N) followed by a weak base NaOH (0.1N) using the Fibertec System 2021(FOSS, Denmark). Nitrogen-free extract was determined by subtracting the sum of Crude protein, crude lipid, crude fibre and ash from DM (Giri et al. 2000). Gross energy was determined using a Bomb Calorimeter Model-DFU 24 following the process as described below. The sample was combusted in a chamber pressurized with pure oxygen and resulting heat measured by increase in the temperature of the water around the bomb (Giri et al. 2000).

The total lipids were extracted from all the samples, (fish flesh-2, feed-2) following the method of using methanol-chloroform (2:1, v/v), methanol-chloroform-water (2:1:0.8, v/v/v), and then again with the first solvent system viz., methanol-chloroform (2:1, v/v). Sample was dust with the solvent methanol-chloroform (2:1, v/v), filtered through Whatman no. 1 filter paper and residue was extracted with the next solvent system, consisting of methanol-chloroform water (2:1:0.8, v/v/v). The process was repeated once again with methanol chloroform (2:1, v/v) (Bligh et al., 1959; Giri et al. 2000). Finally, the three extracts were pooled, diluted with three volumes of water (100-200 ml, depending on the volume of pooled extracts) and layer was allowed to separate in a separatory funnel made by Pyrex glass Co. The chloroform layer at the bottom of the separatory funnel was withdrawn and dried over anhydrous sodium sulphate in glass stoppered conical flasks, by Pyrex. Total lipid of various (fish flesh-2, feed-2) samples was dissolved in anhydrous methanol containing concentrated Sulfuric acid (1.0%, v/v) and the mixture was refluxed for 2 hours.

Methanol was evaporated to a small volume (1-3 ml) and cooled to 4°C, in a freezer.

Distilled water 10–15 ml was added to the cooled mixture (1-3 ml) in hard glass test tubes by Pyrex and the methyl esters of fatty acids were extracted 3 times with aliquots (5-10 ml) of diethyl ether, vortexed in a Vortex mixer. The ethereal extracts were taken out by Pasteur pipettes, pooled and dried over anhydrous sodium sulphate, (1-2 gm) in conical flasks (25-50 ml capacity) with glass stopper, filtered through Whatman no. 1 filter paper, vacuum dried, redissolved in n-hexane (1-2 ml volume) and kept in a freezer at 4 °C for future use. Fatty acid methyl esters were purified by TLC using a solvent system of n-hexanediethyl ether (90:10, v/v) (Mangold 1969; Christie1982). A standard methyl ester was also run on the same plate in a separate lane, for identification of the methyl ester bands in the samples. The location of methyl ester bands was indicated by placing the TLC plate in an iodine vapour chamber by Pyrex glass co. The methyl ester bands corresponding to the standard were marked and then scrapped off the plate with a sharp rajor blade. GLC of fatty acid methyl esters were done on a Chemito 1000 instrument, equipped with Flame Ionization Detector (FID). Quantifications were done by computer using specific Clarity Lite software. GLC of FAME was done on a BPX- 70 mega bore capillary column of 30 mt length and 0.53 mm internal diameter obtained from SGE, Australia (Christie1982).

Oven temperature was programmed from 150 °C – 240 °C with a rate of 8 °C/min.Initial and final temperatures were kept isothermal for 1 minute and 20 minutes respectively. Injection port and detector temperatures were 250 °C and 300 °C respectively. Nitrogen gas was used as carrier gas and its flowing rate was 6.18ml/min. Data from each treatment were subjected to one-way Analysis of Variance (ANOVA). The data are presented as mean±SE of three replicate groups; statistical analysis was performed using the SPSS 11.0 for windows. Duncan multiple range test was used to compare the mean values between individual treatments (Duncan, 1955).

Table 1. Biochemical composition of silkworm pupae used as feed for tilapia

Ingredient (%)	Silkworm pupae
Dry matter	90.72
Crude protein	34.54
Crude lipid	10.52
Carbohydrate	9.59
Ash	13.14
Nitrogen free extrac	13.93
Crude fibre	9.76
Gross energy (Kcal g-1)	3.71

RESULTS AND DISCUSSION

The highest weight gain (75.80g) was obtained in the SWM applied feed. The growth rate was always faster in SWM fed fish than fish fed with MAF (Figure 1).

This indicates that fish can assimilate the SWM feed well. This was possibly due to their higher palatability and preference of the fish to take it as their potential food. Amount of feed intake was highest (2.21g) in SWM fed fish.

The feed conversion ratio (FCR) was differed significantly and lowest value (2.29) was recorded from SWM fed fish indicating an encouraging effect on economic involvement in fish farming.

The specific growth rate (0.91) and protein efficiency ratio (1.66) were highest in SWM fed treatment. This indicates the better quality of protein in the feed produced from fermented silkworm pupae (SWM). The hepatosomatic index (HSI) and gonadosomatic index (GSI) were not differed significantly between two treatments (Table 3).

In two feeds, SFA was less and MUFA was high in amount. But in other fed fish the situation was just opposite indicating that fish may be converted MUFA of feed into the SFA of its flesh. The amount of 22:1 ω11 fatty acid was reduced absolutely in fed fish which is the main reason for such reduction.

Amount of DUFA was reduced nearly 3 times in fish than in feed (table 4). The value of linoleic acid (18:2ω6) of formulated feed was nearly 15% which has been found to be much lower (nearly half) in both the fed fishes. The mechanisms behind this conversion depend on the efficiency of the experimental fish by adopting desaturation and chain elongation process. This result indicates that fish may convert DUFA to SFA. The amount of PUFA and n3 fatty acids (EPA and DHA) was less in feed and more in fed fish. But n6 PUFA was reduced significantly in all fed fish in comparison to the feed offered. The n3/n6 ratio showed an increasing tendency in all fed fish and SWM fed fish exhibited significant enhancement of n3/n6 ratio (1.45).

Because amount of n6 fatty acid was lowest in SWM fed fish indicating transformation of n6 fatty acid to n3. The most interesting aspect of the present experiment is that the fishes provided with high level of n6 fatty acids in feeds offered however, the fishes were able to covert n6 fatty acids to n3 fatty acids efficiently to a very low n3/n6 ratio to comparative higher value of n3/n6. The mechanism of this conversion could not be explained at present. However, it proves that these fishes have the ability for such a conversion for the maintenance of physiological homeostasis (Ackman et al. 2002).

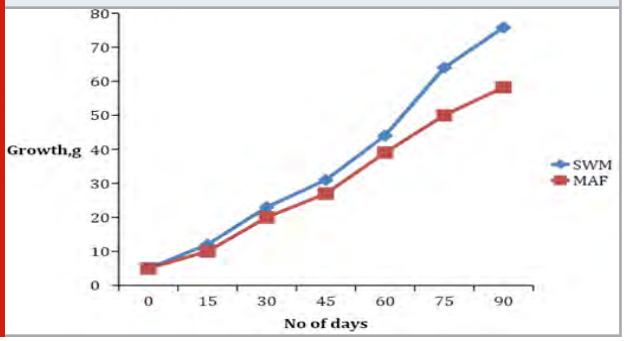
Ackman (2000) stated that only 14 fatty acids are really needed to describe the fatty acids of fish. However, Ackman (2000) Enlisted 64 fatty acids from 5 fresh water fishes of West Bengal, India. The fish under discussion recorded 28 fatty acids of the total lipid (TL) and the result is more or less similar to those reported from other tropical and certain temperate zone fresh water

fishes. According to Ackman et al (2002) dominant fatty acids in lipids of all the fishes were myristic (14:0), palmitic (16:0), stearic (18:0), palmitoleic (16:1ω7), oleic (18:1ω9), linoleic (18:2ω6), linolenic(18:3ω3), arachidonic (20:4ω6), eicosapentaenoic (20:5ω3) and docosahexaenoic (22:6ω3) acids. The present results ascertain with the above findings. The total SFA of the experimental fish was nearly double than the amount reported by (Ackman et al. 2002).

Table 2. Detailed calculations of SWM and MAF diet

S l. No.	Name of feed	Ingredients	% of CP in ingredient	% of ingredient in formulated feed	% of crude protein in feed	% of lipid in feed	% of carbohydrate in feed	Calorific value of feed (kcal/g)
1	SWM	Silk Warm	22.25	40.0	25.46	8.1	10.4	4.0
		Pupae MOC	34.65	30.0				
		Wheat flour	9.08	28.0				
		Egg shell dust	1.8	2.0				
2	MAF	Fish meal	40.54	36.0	30.4	8.2	10.5	4.0
		MOC	33.52	34.0				
		Wheat flour	8.80	28.5				
		Egg shell dust	1.7	1.5				

Figure 1: Growth rate of *O. niloticusfed* with SWM and MAF



Fatty acid deficiency in fish species is indicated by the presence of eicosatrienoic acid (20:3ω9) (Watanabe, 1982). Thus, the absence of eicosatrienoic acid in these fish indicates that these fish are not suffering from any fatty acid deficiency and the formulated feeds fulfill the requirement of fatty acids for tilapia. This observation corroborates that for hybrid striped bass in the USA (Nematipour,1993). The n-3 PUFA is the chief group of components through which the beneficial effects of fish are mediated. The principal effects of n-3 PUFA are antithrombogenic and antiarrhythmic, whereas that of n-6 PUFA is antiatherogenic (Watanabe, 1982; Nematipour, 1993; Ackman et al. 2002). Ackman et al. (2002) stated that n3/n6 ratio should range 1–2 for fresh water fish.

The n3/n6 ratio of SWM fed fish was within the same range. The fish fed with SWM stores more n3 fatty acids than n-6 fatty acids which increase n3/n6 ratio (1.45) in return. Fish oil, lipids and their constituent fatty acids

Table 3. Growth performance and nutrient utilization of *O. niloticusfed* with SWM and MAF

Particulars	SWM	MAF
Initial weight (g)	5.10 ±0.01a	5.10 ±0.02a
Final weight (g)	75.80 ±0.13a	58.25 ±0.24b
Initial length (cm)	4.50 ±0.03a	4.50 ±0.01a
Final length (cm)	14.50 ±0.12a	11.50 ±0.10b
Feed intake (g fish-1 day-1)	2.15 ±0.05a	1.79 ±0.02b
Specific growth rate (% day-1)	0.90 ±0.19a	0.72 ±0.05b
Feed conversion ratio	2.28 ±0.23a	2.65 ±0.05b
Protein efficiency ratio	1.66 ±0.12a	1.26 ±0.04b
Hepatosomatic index	1.55 ±0.06a	1.17 ±0.07b
Gonado somatic index	1.62 ±0.07a	1.12 ±0.06b

have high digestibility and energy value. Fish lipids contain high levels of n3 PUFA which may be essential for worm blooded animals like human. Fish is more beneficial than fish oil but for CAD patients prescribed amount of n3 PUFA (EPA and DHA) is required as the best insurance against sudden death24. Low fat and easy

digestibility of the two batches of experimental fed fishes under investigation together with its EFA (n3 fatty acid) resource and other attributes discussed above can be recommended as a better diet on par with the recognized fish diet (Ackman et al. 2002).

Table 4. FA profiles of *O. niloticususfed* with SWM and MAF feeds (% w/w of each component in total fatty acids)

Components	MAF feed	SWM feed	MAF fed fish	SWM fed fish
Saturated				
14:0	0.7	0.6	5.0	4.4
15:0	0.2	0.5	0.9	1.3
16:0	5.3	5.4	30.5	27.9
17:0	0.4	0.2	2.8	2.5
18:0	2.0	2.31.8	7.8	8.0
20:0	0.7	0.7	0.5	0.3
22:0	0.7	0.1	3.9	4.0
24:0	1.1	2.0	0.9	1.8
∑SFA	11.1	11.3	52.3	50.2
Monoene				
14:1	0.0	0.0	1.0	0.3
15:1	0.0	0.0	0.3	0.2
16:1	1.2	1.4	7.5	7.6
17:1	0.0	0.0	0.4	0.7
18:1ω9	22.6	21.9	14.6	12.0
20:1ω9	7.0	8.1	1.6	2.1
22:1ω11	27.1	27.7	1.6	0.9
24:1	1.3	1.7	1.7	1.6
∑MUFA	59.2	60.8	28.7	25.4
Diene				
16:2	0.0	0.0	0.2	0.5
18:2ω6	16.2	14.6	8.7	7.7
20:2	0.0	0.0	0.0	0.0
∑DUFA	16.2	14.6	8.9	8.2
Polyene				
18:3ω6	0.5	0.4	0.3	0.4
18:3ω3	2.6	2.9	2.3	3.0
20:3ω6	1.1	1.1	1.4	1.0
20:3ω3	0.00	0.02	0.07	0.02
20:4ω6	0.9	1.0	1.1	1.0
20:5ω3	1.2	1.7	1.1	1.6
21:5ω3	0.5	0.5	0.5	0.4
22:5ω6	0.2	0.5	0.4	0.6
22:5ω3	2.0	2.6	2.1	2.8
22:6ω3	4.5	5.6	4.0	5.6
∑PUFA	13.5	13.32	10.17	16.22
Total -ω3	10.8	9.82	12.57	13.42
Total -ω6	17.8	17.6	11.9	9.2
n3/n6	0.60	0.75	1.05	1.45

CONCLUSION

The feed prepared from fermented silkworm pupae enhances growth and thereby yield of O. niloticus. It improves quality of fish by accumulating more n3 PUFA in the flesh of the fish as well as increasing the n3/n6 ratio which is beneficial for human health in relation to cardiovascular diseases. Moreover, the feed can be formulated by sericulture byproduct at local level leading to control pollution, lowering feed cost and employment generation in rural areas of India.

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Dental
Communication

Impact of Student-Generated Videos on Self-Reported Engagement, Critical Thinking and Learning of Saudi Dental Students

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ABSTRACT

The impact of student-generated videos on students' learning and understanding of specific topics has not been fully examined. Therefore, this study aimed to evaluate the effectiveness of student-generated videos as an educational collaborative learning tool on a group of students' self-reported perceptions and satisfaction levels in a preclinical dental course. Third-year students enrolled in a biomaterials course—having already taken their prerequisites—participated in this study. They were each asked to produce an educational video on one of the many subjects in their dental course materials. Three years later, these students' perceptions were assessed with a 26-item, self-administered questionnaire. Data were analyzed using the Statistical Package for the Social Sciences (SPSS). The response rate in this study was 85.7%. The students believed that preparing the video scripts motivated them to read about the topics (52.5%) and to work with a team (55%). Education, Approximately 73% of the students agreed that preparing the videos enhanced their understanding of dental materials and their applications in both the lab and the clinic. About 76% of the participants learned that the video medium can be a powerful communication tool. In addition, the videos improved their understanding of teamwork, as well as their communication, problem-solving, and organizational skills. These videos also improved their responsibility and professional behavior toward their dental teams. Almost all of the students perceived the student-generated video as a contemporary, essential educational tool that enhances student collaborative learning and problem solving, promotes mastery of learning, clarifies various topics, and provides authentic learning opportunities.

KEY WORDS: EDUCATION, VIDEOS, LEARNING, THINKING, DENTAL, GPA, COMMUNICATION.

INTRODUCTION

Lectures have been widely criticized as a learning strategy because they are often associated with frustration, lethargy, or somnolence (McLaughlin and Mandin, 2001). In health professions education, one of the roles of educators is to continuously endorse students' abilities to practically apply theoretical lessons. During the transition from the preclinical to the clinical phases, a "theory practice gap" may occur due to a lack of linking classroom learning to clinical application (Baxter, 2007). Applying

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knowledge in practical situations is essential in preparing proficient, skillful practitioners (Mahmoud, 2014).

Thus, educators are challenged to integrate new teaching methods, adapting their approaches to enrich the educational process and satisfy the varying learning needs of their students (Logan, 2012). Pre-recorded videos developed by the course director and student-submitted videos were among the methods recently adopted as a hybrid format that would allow for the development of the fine psychomotor skills necessary in dental hygiene during COVID-19 crisis (Horne et al., 2021). Dentistry is considered a challenging profession since students must acquire complex cognitive and practical skills and be able to apply these to clinical situations. Beyond this, dental students must continuously improve their clinical judgment and practical skills through ongoing education—including lectures, conferences, and reading assignments—to become competent dental practitioners (Reissmann et al., 2015).

In a recent study, learning dental treatment procedures through e-learning on a smartphone was found to be more effective in developing participants' understanding of dental treatment procedures and four-handed techniques (Takenouchi et al., 2020).There are many difficulties facing dental students in getting a clear view of the operating field during clinical demonstrations. These include limited operating spaces, the small sizes of oral cavities, and the precise detailed nature of dental procedures. However, these difficulties can be overcome by using video-based learning (Fakhry et al., 2007). To this end, videos can present knowledge in a clear, structured way, producing better understanding of concepts that are hard to describe through text. In addition, using videos in healthcare is a better way to learn clinical and procedural skills, as well as problem-solving (Gao et al., 2015). More recently videos were reported to be more effective in understanding the pharmacology concepts as measured by the students' performance in exams (Sumanasekera et al., 2020). Both medical and dental instructors have had to overcome certain limitations to meet educational needs, including educational staff and instructor shortages, increasing class sizes, fewer patients, and treatment complexities (Walker et al., 2008). Video learning could be useful in helping medical and dental instructors by helping to promote self-directed learning, in which students could view previously recorded clinical procedures before directly performing them on patients (Amer et al., 2011).

For instance, the undergraduate Chinese dental students at the University of Hong Kong considered videos to be essential learning tools because they can access them whenever they need, they can easily control the videos' functions, and they can use these videos in certain situations missing from textbooks or lectures (Botelho et al., 2019). Crean et al. (2001) described how videos generated by university students can be used as one type of learning tool (Crean, 2001). Among the previously reported benefits of using these videos as learning tools, they are enhancing learners' motivations and engagement in the learning process. In addition, they are strengthening students' abilities to communicate effectively and collaboratively with their colleagues (Hanan Omar and Khan, Ajuwon et al., 2016, Shewbridge and Berge, 2004). From the perspectives of the students, they preferred watching video to using conventional learning methods. The application of the enhanced video demonstration resulted in a better theoretical knowledge retention but not practical performance. (Abd-Shukor et al., 2020).

Video-based learning is a popular tool, although its production requires significant time and other resources (Basu Roy and McMahon, 2012). Limited studies have examined the impact of the use of student-generated videos on students' understanding of the applicable aspects of the preclinical dental courses. Therefore, the aim of this study was to assess dental students' perceptions and learning experiences, as well as the long-term effects of student-generated videos as educational, collaborative learning tools during a preclinical dental course.

MATERIAL AND METHODS

Ethical Approval: This interventional study was conducted at the Faculty of Dentistry at King Abdulaziz University between September 2015 and 2019. The study was approved by the Research Ethics Committee at King Abdulaziz University, Faculty of Dentistry.

Study Design: A total of 189 third-year dental students (94 female, 68 male) were enrolled in this study after taking their prerequisite classes for the preclinical biomaterials course. After finishing the required lectures, video development was taught to the students as a learning activity. The students were first divided into eight groups. Each group were assigned to create and produce a video on a specific assigned topic of the dental material, and each group was to explain their topic's composition, properties, uses, advantages, and disadvantages. In addition, they were to demonstrate how to use their subject in the dental clinic or lab. Beyond this, each group had a faculty advisor with experience lecturing on the same or a related topic. Finally, students were given three weeks to prepare their videos.

After producing their videos, students were requested to upload their videos on YouTube and to present them to their classmates in their biomaterials sessions. Meanwhile, a special committee of eight faculty members in different specialties evaluated the videos according to a given rubric and gave feedback to the students. Finally, the students presented their movies to all of the faculty members on a special "Biomaterials movie day." Next, the students were evaluated by two independent committees, which included eight faculty members from eight different departments. The committees evaluated the content and preparation of the videos, as well as the students' presentation skills, using a structured rubric prepared for this purpose. After this, they gave the students feedback on their performances. Finalists from

both committees were declared, and the winners were awarded certificates of recognition.

Three years later, in the same students' final year of dental school, the students' perceptions of their learning experiences during the video creation and their satisfaction with its impact on their skills and knowledge throughout the program were assessed through a questionnaire. This 26-item, self-administered questionnaire was constructed and validated (Cronbach's alpha 0.951). This questionnaire was distributed anonymously to all the students who participated in the creation of the videos (n = 189).The results were analyzed by using the Statistical Package for the Social Sciences (SPSS), Version 16. The correlation between the rank variables was assessed using Kendall's test. At last, the percentages were compared for significance using the qi-square test. P values less than 0.05 were considered significant.

RESULTS AND DISCUSSION

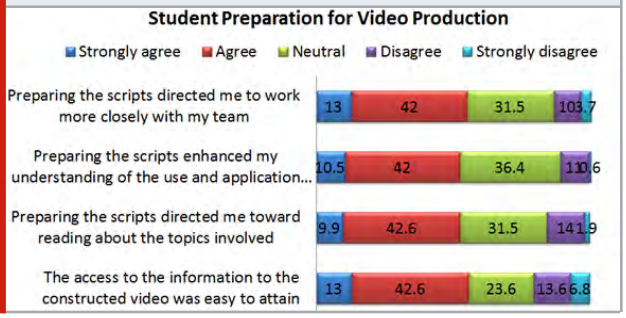
The response rate to this study (162 out of 189 students). It was observed that 58% of the students who participated in this study were female, and more than 70% of the participants were between 24 and 25 years. Fifty percent of the students had a GPA between 4 and 4.5 (on a scale from 0 to 5). Regarding the time it took the students to create the videos, approximately 54% of the students took more than one week to complete their videos, whereas about 40% of them needed only one week, as shown in Table 1.

Table 1. Demographics of the participants

VARIABLE	NUMBER	PERCENTAGE
AGE		
- 22-<23	3	1.9
- 23-<24	40	24.7
- 24-<25	115	71
- 25-<26	4	2.5
GENDER		
- MALE	68	42
- FEMALE	94	58
GPA		
- <3	2	1.2
- 3-3.5	7	4.3
- 3.5-4	61	37.7
- 4-4.5	81	50
- 4.5-5	11	6.8
HOW LONG DOES IT TAKE TO PERFORM THE VIDEO		
- LESS THAN A WEEK	10	6.2
- A WEEK	65	40.1
- MORE THAN A WEEK	87	53.7

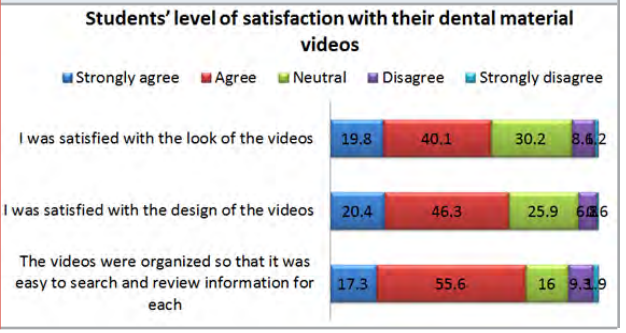
As for the students' video preparation processes, approximately 55% of them believed that access to the information in their video was easy to attain. They added that preparing the scripts made them read more on their topics (52.5%), which enhanced their understanding of these topics (52.5%) and encouraged them to work in teams (55%). Beyond this, there was no significant difference in students' satisfaction with their benefits from the video preparation process, as shown in Figure 1.

Figure 1: Students perception regarding the process of video preparation.



The issue that most satisfied the students concerning the videos was that the latter were organized in such a way that it was easy to search and review information for each instrument (72.9%). Furthermore, approximately 67% were satisfied with the design of their videos and 60% with the look, with no significant difference in students' satisfaction with any of the video parameters, as shown in Figure (2).

Figure 2: Students' level of satisfaction with their dental material videos



Overall, the students' were satisfied with their video-based learning experience. In fact, a considerable percentage of the students thought that video creation improved their language and communication (69.8%), organizational (60.5%), problem-solving (55.5%), and project management skills (54.9%). However, there was no significant difference between these skills, as shown in Figure (3).

Among the benefits reported by the students about watching videos were that they improved their understanding of the composition of dental material (72.8%), the properties of this material (71%) and the safety precautions in their preclinical labs (63.6%). On the other hand, no significant difference was found between these benefits, as shown in Figure (4). Similarly,

there was no significant correlation between the GPAs and overall satisfaction of the students who generated the videos (r = 0.056, p = 0.43) or their willingness to participate in a similar activity in the future (r = 0.042, p = 0.55), as shown in Table (2).

Figure 3: Students' perception about skills developed during participation in video creation.

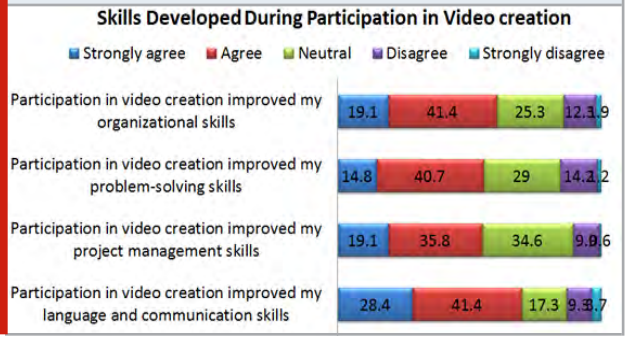
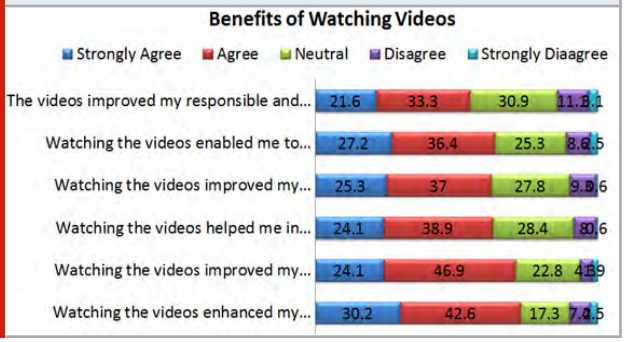


Figure 4: Students' perception about benefits of watching videos.



Approximately 33% of the students participating in the video creation activity thought it was worthwhile, and 47% of them noted that they would like to participate in video creation again and would recommend this activity to other students in the future. Moreover, a significantly ($p < 0.05$) higher percent, 76%, of the participants learned that the video medium can be a powerful communication tool, as shown in Figure (5).

Transferring practical skills obtained through didactic sessions into clinical practice is considered a challenging process for undergraduate dental students. In order to accomplish this aim, these students need particular training on procedures and demonstrations in hands-on sessions (Wong et al., 2019a). E-learning has been reported to effectively support medical and clinical education, as people learn efficiently through multimedia, which is thus significant in medical education (Jang and Kim, 2014).

Transferring practical skills during teaching sessions could be enhanced by using visual aids, including images and audio recordings. Current evidences suggest that the use of videos in teaching practical skills and techniques has many benefits in pedagogy. It has been reported that videos attract attention and involve students in practicing necessary skills, as such teaching methods allow them to communicate facts and demonstrate procedures (Wong et al., 2019a). On the other hand, a recent study was conducted to investigate the effectiveness of an in-class and on-demand enhanced video to support learning on removable partial dentures in terms of knowledge acquisition, perception and clinical skill performance. They reported that enhanced video demonstration improved the students' short-term knowledge acquisition compared to the non-enhanced group. The practical performance did not differ between the two groups. The students were more likely accept the enhanced video as a replacement of the existing teaching method rather than a teaching supplement (Abd-Shukor et al., 2020)

In this study, a large percentage of the participants reported that student-created videos enhanced their understanding of the topics of the biomaterials course—e.g., the composition and properties of dental materials, as well as the safety precautions required in the preclinical lab. These findings were in line with those of (Wong et al., 2019a). They reported that the content provided through visual aids could establish a connection between handling and recalling of knowledge by controlling functionality.

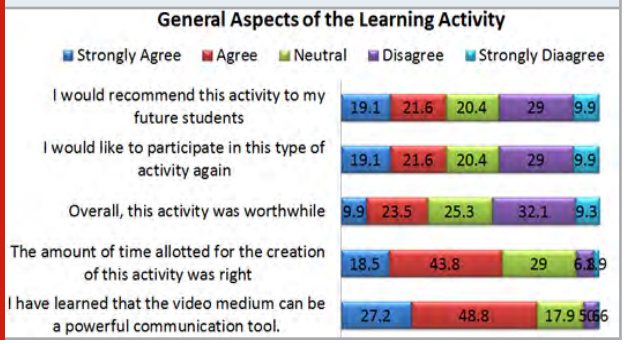
In this way, students can review components of complex techniques to achieve better processing of clinical procedures. In addition, this format motivates students, stimulating deep learning and improving the learning process in general. Their study reported that using videos to supplement traditional class instruction could help students develop cognitive skills, enhancing their performance and increasing the knowledge they retained (Cooper and Higgins, 2015, Wong et al., 2019b). This was evident in this study as well.

Table 2. Correlation between GPA and overall satisfaction with the video creation

	Overall, this activity was worthwhile.		I would like to participate in this type of activity again	
	Correlation	P value	Correlation coefficient	P value
GPA	0.056	0.43	0.042	0.55

Confirming this conclusion, the fourth- and fifth-year dental students reported that video learning materials allowed them to clarify knowledge, improve cognitive thinking, and enhance revisions of theoretical concepts and clinical skills (Botelho et al., 2019). On the same note, such videos supply audiovisual stimuli and can enhance different teaching conceptions (Farooq and Al-Jandan, 2015). Therefore, using video has been endorsed in dental education (Kalwitzki et al., 2011). In a recent study, using instructional video showing the light-curing technique was comparable to individual verbal instruction and an effective tool for teaching the light-curing technique per the students' ability to deliver sufficient amounts of irradiance and radiant exposure to simulated cavities (Al-Zain and Al-Osaimi, 2021).

Figure 5: Students' perception about the general aspects of learning through video generation. *Significant ($p<0.05$).



In the dental field, video education has shown numerous benefits in both teaching and training (Edrees et al., 2015). Although using videos to deliver content has been practiced for many years, videos have yet to be implemented in all dental schools. In a previous study, communication and treatment procedures were among the aspects perceived as beneficial by dental students at Karolinska Dental Institute in Stockholm, Sweden, after patient demonstration videos were given in predoctoral endodontic education (Edrees et al., 2015). These findings were in agreement with this study. The students reported a positive experience with the video creation because it enhanced their language, communication, organizational, problem-solving, and project management skills and required them to work in a team.

Such reactions were consistent with another study, which found that the student-generated video activity was used to introduce an early appreciation of the clinical role and responsibilities of a dentist in the dental program (Haron et al., 2012). In addition, it increased students' autonomy, promoted active learning, and provided the opportunity for group learning, as well as language development (Haron et al., 2012). A recent Saudi study was performed to evaluate using videos, either watched or student-generated, for teaching stronger interpersonal communication skills to dental students. They found that using videos in teaching communication skills was effective and added that producing videos had more benefits than simply watching videos (Al-Khalifa and Gaffar, 2021).

Although the dental students reported in more than one study that they preferred video-based teaching to traditional teaching, there have been mixed findings on the effect of video-based teaching on students' grades and performance (Chi et al., 2014, Kyaw et al., 2019). In a previous study, video cases used by dental students in an introductory public health dentistry course were reported to be associated with significantly higher mean scores and were effective in helping students achieve cognitive and affective objectives, in contrast with paper cases (Chi et al., 2014). On the other hand, the meta-analysis conducted by Kyaw et al. (Kyaw et al., 2019) revealed that there was no statistically significant difference in post-intervention skills scores between digital education and traditional learning.

In addition, it was concluded that there were no statistically significant differences in post-intervention skills or knowledge scores between the blended traditional and online or offline digital education and traditional learning alone (Kyaw et al., 2019). These studies supported the findings of this study, as there was no significant correlation here between the academic performance scores of the students and their overall satisfaction with the generated videos.

The students were asked to upload their videos on YouTube, which is one of the most popular media services and is available to anyone with internet access. The use of YouTube in dental education has been shown to sometimes play a complementary role in dental teaching, but it should not be used without validated instructional material (Aldallal et al., 2019). Students independently wrote the scripts and directed their videos, and most of the videos were of acceptable quality.YouTube can be used as a tool to supplement dental education due to its easy accessibility. It provides many sources of information that can be used by those working in the dental field or preparing to instruct dental students (Mukhopadhyay and Suryadevara, 2014). One unexpected finding in this study was that students weren't interested in participating again in this kind of activity, which could be due to the condensed content of the third-year dental curriculum, which gave them little availability.

Recall bias given the three-year lag between when the students participated in the video creation and when the students were surveyed could be one of the limitations of this study. Among the limitations as well, was the inability to measure the students' retention of knowledge and applications of skills learned from the student-generated videos. Therefore, there is a need for further research assessing the long-term efficiency of these videos in the areas of knowledge or skills retention. Another limitation was the activity evaluation, which was based mainly on students' perceptions. Thus, more thorough evaluation of students' performance should be considered.

CONCLUSION

To summarize, the results of this study showed that almost all students positively perceived the video-based learning and teaching. More specifically, they perceived the student-generated videos as relevant, vital educational tools that enhance student collaborative learning, promote mastery of learning, and increase understanding of topics in the field. Students' own video recording can be used for further research discussion and stimulate critical thinking.

Declarations

Ethics approval and consent to participate: The study was approved by the research ethics committee at King Abdulaziz University, Faculty of Dentistry". Accepting to fill the questionnaire was considered as a consent to participate.

Consent for publication: Not applicable

Availability of data and materials: The data will be made available upon request

Conflict of Interests: The authors have no conflict of interest with this study

Funding: No funding was provided for this study

Authors' contributions: G.H. N, H.Y.E have designed the study and collected that data. S.M.A., S.M.B performed the statistical analysis and interpreted the data. H.A.M, MTH wrote the initial manuscript. All the authors have read and approved the final manuscript.

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Technical Communication

Predictive Validity of Cognitive Load Patterns in Mathematical Problem-Solving Stereotypical Thinking in the Inferential Statistics Course Among Psychology Department Students

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ABSTRACT

The main objective of this study was to investigate the contribution of the cognitive load patterns in predicting the stereotypical problem-solving thinking in the inferential statistics course prescribed to 2nd year Psychology Department students, Ismailia College of Education, Suez Canal University. The correlational approach was adopted to test the study hypotheses. The sample consisted of seventy-six students (70 females, M_{age} = 19.81, SD = 0,43) who were chosen intentionally. The researcher prepared the cognitive load and the stereotypical thinking scales. Results revealed that germane cognitive load predicted stereotypical thinking with inferential statistics problem-solving.

KEY WORDS: STEREOTYPICAL THINKING, PROBLEM-SOLVING IN MATHEMATICS, COGNITIVE LOAD.

INTRODUCTION

Statistics courses are prescribed by the Department of Educational Psychology in the colleges of education in the Arab Republic of Egypt. They are derived from the branch of Pure Mathematics. These courses serve the field of education by converting psychological phenomena into figures describing such psychological phenomena and determine their existence as predictors of decision-making in terms of modifying psychological, personal and cognitive phenomena.Psychological statistics is a course based on the combination of mathematical problems requiring deductive calculations and verbal issues requiring the learner's mastery of logical thinking, abstraction and representation skills (Hsin & Paas, 2015). The psycho – deductive statistical course is a course based on the linkage between meaningful real-life problems that require meta-cognitive skills and mathematical thinking skills to analyze and study given tasks (Leahy & Sweller, 2019). (Redifer et al., 2019) noted the cognitive load in essence is a mental effort that refers to the cognitive sources needed to perform certain tasks. Sweller founded the theory of cognitive load as he criticizes the traditional methods of solving mathematical problems, because they load working memory with new cognitive processes, and thus do not transfer new information to long-term memory (Leahy & Sweller, 2019). He tried to study other possibilities to embody problems related to practical examples and open tasks so that processing processes improve better during Problem Solving to reach the Psychological statistics is a course based on the combination of mathematical problems requiring deductive calculations and verbal issues requiring the learner's mastery of logical thinking, abstraction and representation skills (Hsin & Paas, 2015).

The psycho – deductive statistical course is a course based on the linkage between meaningful real-life problems that require beyond-knowledge skills and mathematical thinking skills to analyze and study given tasks (Leahy & Sweller, 2019). (Redifer et al., 2019) noted the cognitive load in essence is a mental effort that refers to the cognitive sources needed to perform certain tasks. Sweller founded the theory of cognitive load as he criticizes the traditional methods of solving mathematical problems as they exhaust working memory with new cognitive processes, and thus do not transfer new information to

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long-term memory (Leahy & Sweller, 2019). He tried to study other possibilities to embody problems related to practical examples and open tasks so that treatment processes improve better during Problem Solving to attain the target goals. instead of routinely transferring knowledge and classifying it into routine patterns of solution ideas. Accordingly, this study attempted to pose mathematical problems via the psychological statistics decision based on open tasks establishing new ideas instead of traditional mathematical problems with familiar images as reported by Treffers (2019).

The current study proposes mathematical problems via the psycho-deductive statistical course that provides open tasks allowing the learner to employ new ideas rather than relying on familiar images of traditional mathematical problems (Treffers, 2019). Open tasks in mathematics aim to incorporate cognitive, linguistic, social, and emotional processes into problem-solving processes (Fuchs et al.,2019). The inclusion of those processes is limited by the ability of the working memory and is influenced by many other characteristics of the learners and current knowledge, as well as the nature of the new information available during the tasks analyzed by learners. Thus, learners with effective working memory undergo a less cognitive load (Redifer et al., 2019).

The Cognitive Load Theory: The cognitive load theory is an educational theory based on human knowledge structure. Mathematical problem-solving are a complex cognitive and perceptual task that imposes a large disruption on working memory. It involves combination problems of words, mental schemas, and information not related to the problem and the selection of the most suitable solving strategies and applying mathematical processes principles to expose the required response (Fuchs et al., 2019; Hsin & Paas, 2015). These principles as the following (Chen, Castro-Alonso, Paas & Sweller, 2017; Leahy & Sweller, 2016; Sweller, 2011): Mathematical operations and problem solving are complex cognitive and cognitive tasks that impose a significant cognitive load on the working memory as they involve problems combining words, mental representations, information unrelated to the assigned problems, selecting the most appropriate strategies for the solutions and applying the principles of mathematical operations to reach the desired response (Fuchs et al., 2019; Hsin & Paas, 2015). They can be summarized in the following principles (Chen, Castro-Alonso, Paas & Sweller, 2017; Leahy & Sweller, 2016; Sweller, 2011):

1. Long-term memory is a storehouse for keeping information, wherein one keeps a huge amount of information stored in their long-term memory.
2. Integration of Schema Theory with the principle of recall and reorganization. Most information is stored in long-term memory in the form of cognitive charts. Schemes are called upon by imitating, reading, viewing material or listening to others.
3. Problem-solving, and random start-up occurs if information is not available in long-term memory. Problem solving necessitates examining the cognitive schemata and processing information for the current situation sequentially. An example of this happens when a teacher is asked to explain an incomprehensible mathematical issue to a student outside class. Redifer et al. (2019) state that problem solving depends on differences between learners in cognitive ability and the nature of the tasks they perform, and the shortcoming increases as the complexity of the content of those tasks is enhanced.
4. Working memory and its limited capacity for change. The process of organizing and generating the cognitive structure requires the ability to produce unlimited sets of new information while excluding unnecessary information that prevents working memory from processing the information as a limited processing source in terms of capacity and time.
5. Long-term working memory and the principle of interconnection and environmental organization to form the cognitive structure of the information being processed. In the light of this principle, it is possible to transfer unlimited amounts of information from long-term memory to working memory so as to generate new procedures suitable for accommodation with the environment and then built out a sequential organization in the form of cognitive and, personal and social schema.

According to these principles, the main goal of learning is to create cognitive schemes, process them in the working memory and then keep them in long term memory. Learning becomes effective if schemes fail to disrupt the information structures previously stored in long-term memory (Leahy & Sweller, 2016, 2019). Thinking processes require cognitive structures formation based on the datum of the presented statistical problems. This information requires secondary information that demands recall data from long-term memory and should be organized to benefit from the instructions available in the statistical problem and help the learner acquire new knowledge (Chen et al., 2017). Datum analysis requires a series of interactions of separate elements that must be processed simultaneously in the working memory (Leahy & Sweller, 2019; Sweller, 2015). Thinking processes entail the formation of cognitive structures from the initial information (data) of the statistical problems at hand and this information entails secondary information recalled from long-term memory and organized to take advantage of the instructions available in the statistical problem to help one acquire new knowledge (Chen et al., 2017).

Data analysis requires a chain reaction of discrete elements to be processed simultaneously in the working memory (Leahy & Sweller, 2019; Sweller, 2015). The interaction of the element varies due to the internal cognitive load by changing what to learn or by changing the experience of the learners. Once learning occurs, information is encoded and stored as interactive elements (cognitive schema) in long-term memory (Leahy & Sweller, 2019; Sweller, Kirschner & Clark, 2007). The knowledge of working memory is addressed by adopting the principle

of organization and environmental connectivity as one element so as not to exhaust the working memory.

For example, a statistical problem can be read easily and quickly to find out its variables and methods of solution already written in long-term memory. Information and equations are retrieved to the working memory as solution equations whose variables are changed according to the nature of the mathematical problem. Thus, the interaction of information is automated, i.e. thinking becomes stereotyped. If mathematical problems are open-ended, i.e., when the learner himself raises research questions in the light of the piece of reading that presents the statistical problem, the methods of solution are numerous, the learner is free from stereotypical thinking and the cases of internal and external load are reduced, and the predominant load in this case is the extraneous load (Tricot & Sweller, 2014). The cognitive load is concerned with producing educational techniques that rely on the efficiency of limited information processing ability of learners to apply the knowledge and skills acquired in new situations. Cognitive load depends on the cognitive structure treated in the working memory as partially independent processing units for visual and auditory information, which interacts with unlimited long-term memory (Paas, Tuovinen, Tabbers & Van Gerven, 2003).

Cognitive load and mathematically complex tasks: The cognitive load refers to the mental effort or cognitive resources needed to perform mathematics tasks. Tasks' nature imposes the working memory load during learning processes. The higher the mental effort on complex tasks, reducing the learner's performance on these mathematical tasks (Orru & Longo, 2018; Peck, Doan, Bourne & Good, 2018).Whenever the mathematical problem requires an innovative intellectual product, this internal cognitive load depends on the successive mathematical task difficulty. The external cognitive load may be related to the learner or the task to be accomplished. Given that creative thinking requires some characteristics of complex cognitive tasks it is expected that a more complex implicit nature enhances the cognitive structure efficiency, which has negative influences on those with stereotypical thinking in statistical problem-solving (Redifer, Bae & DeBusk-Lane, 2019; Seufert, 2018).

Complex tasks require intervention during problem-solving to develop evaluations, especially in multi-step problems, which increases the learner's personal experience in dealing with similar tasks in content a different context later (Chen, Retnowati & Kalyuga, 2019). Training on mathematical and statistical problem solving reduces stereotypical thinking and improves monitoring accuracy and individual judgment with increasing age (Baars, Van Gog, de Bruin & Paas, 2017).

Factors affecting cognitive load

Task switching: Most math-related problems can be classified as variable problems consisting of an initial state and a goal to solve problems in a simple manner. These problems can be solved using the means-ends analysis method which involves trying to minimize differences between problem elements, identifying solution sub-strings deducing what is required to prove and then building the proof in the commonly accepted ways. The learner then proceeds to analyze new sub-elements, which are not included in his statistical cognitive charts, on his mind, (Sweller, 1988). Analyzing the problem into its sub-elements also decreases the mental effort involved in solving problems.

The time needed to process a task in the working memory: The learner's ability to control attention helps to predict a successful solution to problems that require certain stages of conclusion and reconstruction of the cognitive structure to reach the goal of problem-solving (Goel & Schnusenberg, 2019; Wieth & Burns, 2014).

Common errors: Common errors result from random retrieval of information and subsequent failure to read and process all elements of statistical issues in light of insufficient time for the central port to work in synergy with the visual component. Common errors usually range from 35% to 37% in the resolutions of issues with similarities between elements of prior cognitive schemes (Schaper & Grundgeiger, 2019). In addition, common errors may occur as a result of the learner's cognitive beliefs, which relate to the nature of knowledge use and become inputs to the metacognitive processes. Implicit beliefs influence higher problem-solving skills such as effective observation of understanding, application of strategies, and perseverance (Redifer et al., 2019).

Dynamic Critiquing: It occurs when a learner departs from the usual framework of thinking, providing proofs or evidences, employing steps to attain a critical solution to a statistical problem without being constrained by the evaluator in a particular way in the solution. The external or intrusive cognitive load occurs as a result of inverse thinking that is supposed to determine the nature of the cognitive scheme and lead the learner to choose the dynamic construction of the solution to frame the cognitive structure of the solution (Sweller, 1988; Wieth & Burns, 2014).

Cognitive Inhibition: Cognitive inhibition refers to the mental effort involved in processing information needed to perform a task that appears to form stereotypes as key considerations for solving statistical problems, constraining the working memory and limiting its paths. If learners encounter a problem that requires creative solutions, this leads to limitations related to factors within and outside the person associated with the tasks that do not compromise with their thinking patterns (Redifer, Bae & DeBusk-Lane, 2019).

Issues with Hybrid text problem methods: Problem solving occurs through three stages: searching for an initial representation of problems, reaching a predicament, restructuring the representation of the problem using alternative methods for a particular stage of mathematical proof formation (Wieth & Burns, 2014). The more creative the learner is in perceiving

the relations between data issues, the more he or she becomes able to adopt flexible statistical methods that enable him to formulate the research questions, test the hypotheses leading to marked improvement in the speed of processing information in the working memory. Learner's assimilation of a large number of ideas addressed before, is the central controller of information processing that turns deep encryption to automatic encryption during information processing (Goel & Schnusenberg, 2019; Norouzi, Vaezmosavi, Gerber, Pühse & Brand, 2019). In the absence of identical elements in the statistical problem, which the learner solves with the prior knowledge schemes, leads to heterogeneous cognitive processes resulting in a load that reduces the efficiency of the working memory in processing the available information (Neumann & Russell, 2019).

Cognitive load patterns: Cognitive load theory summarizes the structure of information as chains reducing difficulty by focusing cognitive activity on schema acquisition. Cognitive load theory deals with learning difficulties and artificial problem solving where they can be addressed by educational design (Sweller, 1994). This theory includes guidelines for reducing cognitive activity that hinders learning and estimating the multifaceted relationship between learning and assessment. Key issues around cognitive load types include the context wherein learning occurs, the continued use of single-component mental effort assessments, the timing of cognitive load and measurements of learning outcomes. The types of cognitive load can be presented as follows (Leppink, 2017; Orru & Longo, 2018; Sweller, 2011).

1. Internal cognitive load is the difficulty related to the content being processed. It is necessary to realize that the cognitive load that results from the interactive elements of information that need to be addressed simultaneously to achieve the goal of education. It is often concerned with the implicit or explicit meaning of information.
2. External cognitive load refers to the way information is presented to learners under the control of the educational designer. It is produced by the requirements assighned by the teacher to learners, or the instructions that they are asked to follow by integrating a set of information before being able to examine the paths of possible solutions (Chinnappan, 2010). This load increases by enhancing ineffective learning methods that inadvertently distract learners who have distracting information or make a task more complicated than it should be. The internal cognitive load occurs during creative thinking due to the difficulty of the mathematical task itself while the external load can be caused by factors within learners themselves, encouraging them complete the task (Orru & Longo, 2018; Redifer et al., 2019). Practical examples reduce the external load as they are effective ways to teach complex problem-solving skills (Paas & Van Gog, 2006).

The intrusive cognitive is defined by Sweller (2010) as a purely function of the working memory resources allocated to the interaction of specific elements of the internal cognitive load. The load is assumed to enter the learner's high motivation. The intruder load is also inversely proportional to the external load provided the learner's cognitive abilities are high. It is produced by building schemes and is considered desirable, helping to learn new skills and other information (Paas & Van Gog, 2006; Seufert, 2018). The scheme is conceived as a notion or a specific object that tells us what to expect when we encounter it in the future. Sprinkle Sweller (2010) state that the interaction between the elements in this load is associated with characteristics of the learner. A less experienced learner may process a range of information within the working memory, this multi-information is one structure possessed by more experienced learners regardless of the nature of the subject they study. In other words, the intruder load is independent of the information provided. For example, assuming that there is a learner with stable levels motivation, the learner has no ability to control the extraneous cognitive load. If the internal cognitive load increases and the external cognitive load decreases, the intrusive cognitive load becomes very high because the learner has to allocate a large proportion of the working memory to deal with the scientific material.

Modular thinking in solving inferential statistics problems: Stereotyped thinking is an approach to thinking based on a prosaic fringe of aspects of the world around us. It aims to integrate multiple and diverse perspectives to provide a comprehensive understanding of insights beyond those provided by each perspective separately. It focuses on studying the whole rather than understanding its parts. This allows a better understanding and perception of human, social, biological, and engineering systems, especially when they are characterized by high levels of complexity. Stereotypical thinking is receiving more attention in different areas as a necessary skill for dealing with contemporary problems. Notable areas include management (Salado, Chowdhury & Norton, 2019).

Stereotyped thinking is based on finding routine mathematical operations studed by the learner at school. It refers to learning clues and similarities to solve problems successfully (Foong & Koay, 1997). While solving mathematical problems with creative ideas helps to master a skill the learner has not been trained on, and then leads him or her to creativity rather than stereotypes of thinking (Salado et al., 2019).However, when the learner begins to learn mathematics by merely acquiring traditional mathematical procedures, this results in the development of defensive strategies that usually end with poor performance, and some negative effects of using stereotyping when writing mathematical proof (Leahy & Sweller, 2019).

Stereotyped thinking is defined as the processes of self-classification and initial modeling of the expression of behaviors in situations of personal uncertainty and non-integration with the compulsive context that decreases perception and motivation. Uncertainties in understanding the context are reduced whenever

learning is collaborative and in the light of actions of an association in which self-motivation and self-evaluation merge and the mathematically desired derivation occurs (Hogg, 2000). The researcher defines it procedurally as the score the learner obtains on the scale of stereotypical thinking in solving mathematical problems. The nature of statistical problems and stereotypical thinking: Statistical problem-solving needs mental representations that differ in accessibility and work within goal systems. The implicit nature of goal activation and its effectiveness can be illustrated not only in the heart of the influence of stereotypical thinking on how one responds to members of stereotypical groups, but also in the implicit control of stereotypical activation in the first place (Moskowitz & Ignarri, 2009). Stereotyped thinking is divided into two types: explicit stereotyped ideas and implicit stereotyped ideas. Implicit stereotypes are characterized by bias, but these bias decreases when evaluating these actions explicitly among learners (Park, Felix & Lee, 2007).

Stereotyped threats theory: There is evidence that stereotype threat interferes with performance and achievement (Carr & Steele, 2009). Osborne (2001) concluded that many emotional and cognitive – anxiety mediators such as the increase in the level of arousal, changing performance expectations, working-memory interference, and cognitive load are influential variables in stereotypical thinking.According to Schmader, Johns & Forbes (2008), the stereotypical activation of trivial mathematical ideas and the working memory undermines impulse, emotional, and cognitive processes, causing poor performance in a cognitive context characterized by intellectual stereotyping. When the learner is faced with a new statistical-oriented research problem, he or she undergoes a state of imbalance that disrupts cognitive monitoring and interpretive processes, causing cognitive load.

The more statistical problems are associated with previously acquired cognitive schemas, one exhibits a combination of high performance and low mental effort (Seufert, 2018; Van Gog & Paas, 2008), since the information to be processed does not exceed a template already stored in the form of a cognitive schema in long-term memory, even if the schema is in the form of texts and graphs (Chandler & Sweller, 1991). Stereotypical thinking also carries a hidden self-threat to its over-reliance on the framework of traditional solutions (Seitchik, Jamieson & Harkins, 2014).

Reasons for doing the study: The study is justified by the fact that stereotypical expectations are different for both genders in Math and Science tests. Stereotypical thinking deficits increase when girls work alone compared to mixed-gender groups (Huguet & Régner, 2007). The researcher also proceeded from studies (Baars et al., 2017; Redifer et al., 2019) that confirmed that problem solving helps reduces stereotypical thinking, improve observation accuracy, and enhance personal judgments by increasing age. The study variables were related to secondary school students, while stereotypical thinking was found that females more than males in this regard. Therefore, hypotheses of the study were formulated for female students in the Department of Psychology, Faculty of education in Ismailia. .

Research Motivation: The study is based on the premise that stereotypes are different for both genders in math and science tests. Stereotypical thinking deficits increase when girls work alone compared to mixed groups of both genders (Huguet & Régner, 2007). The researcher also started from a study that confirmed (Baars et al., 2017; Redifer et al., 2019) that problem-solving helps to reduce stereotypical thinking and improve the accuracy of observation and the growth of individual judgment by increasing age. The stereotyping has found that females are higher than males in this regard. Then the hypothesis study on females from the Psychology Department students was conducted.

Statement of the Problem: Being a lecturer in the Department of educational psychology and teaching the psychological statistics course for students of the second division of psychology for two years, the researcher noticed that in the academic year 2017/2018 the tests were essay type and students were trained to solve mathematical and statistical problems in this course with high levels of thinking and that students ,who tried especially in open-ended problems that require the learner to impose questions and assumptions and test them using the six test steps, got high score. As for the 2018/2019 year to which the results were applied, the change in the general policy of the tests was observed, due to the nature of the course of psychometric statistics requiring higher levels of thinking. The researcher tried to diversify the test questions to keep balance between the objective questions and the methodological problems requiring the use of the six test steps in which the questions are different from those familiar to the learner. Specifically, the researcher noticed the following:

1. Some students replaced some variables in the research statistical questions that were given to him with other variables not included in the question and provided the solution based on the new variables.
2. Some students used the same variables given in questions while ignoring the level of measurement needed to test the difference statistically and complete solution of the problem in statistics.
3. Some students that replaced the variables in the first case adopted a certain cut-off point and then crossed out the variables they replaced and changed the results they provided in the first case and completed the solution.

It is noted that the different nature of the research questions to be answered from the learner brought about an internal and external cognitive load that resulted in the implicit or explicit stereotypes shown in the previous three cases.

Objectives of the study: The ultimate objective of the current study is to assess the relationship between types of cognitive load and stereotypical thinking in solving

problems in the course of Inferential Statistics among the students of the psychology department at the Faculty of education in Ismailia.

Significance of the study: The study was hoped to guide the faculty members to identify the patterns of cognitive load causing stereotypical thinking during the solution of mathematical problems during Inferential Statistics course among students of the Department of psychology at the Faculty of education in Ismailia. It would help modify the teaching and learning methods and guide students to interact with rich activities and different types of creative problem-solving techniques so as to reduce cognitive load they undergo on solving mathematical problems.

Hypotheses: Two hypotheses were formulated and tested:

- There is a correlation between patterns of cognitive load and stereotypical thinking in problem-solving in the Inferential Statistics Course among Psychology Department students at the Ismailia College of Education.
- The contribution of cognitive load patterns varies in predicting stereotypical thinking in problem-solving in the Inferential Statistics course.

MATERIAL AND METHODS

Participants: Seventy-six 2nd year students (70 females, M_{age} = 19.81, SD = 0,43), enrolled in Psychology department, College of Education, were chosen intentionally.

Table 1. Factor loading and attribution of each item based on factor analysis.

	Items	Factor loading		
		Intrinsic load	Extraneous load	Germane load
1)	I feel confused in determining the appropriate statistical test for the answer			0.48
2)	I am solving the vocabulary of the statistic test	0.33		
3)	I look for complementary approaches when choosing the best solution	-	-	-
4)	Offer different solutions to the same statistical questions	0.59		
5)	Use the properties of each statistical techniques to determine the most appropriate method of solution	0.58		
6)	Start solving problems of statistical testing, link each issue with mathematical blocks familiar to me	0.58		
7)	I need to study solved problems to understand different solutions to problems			0.49
8)	I rearrange questions by test			0.32
9)	I am afraid of frying pans issues on which all the statistic exams are presented		0.83	
10)	I am not afraid of statistical methods			0.69
11)	I am powerless when asked to suggest methods and numbers for solving statistical problems		0.70	
12)	I prefer to choose statistical methods to solve than my restriction to specific methods	-	-	-
13)	I feel that every statistical method has its distinctive matters	-	-	-
14)	I imagined in a way that enables me to develop renewed solutions to statistical issues	0.79		
	Eigenvalue Variance explained	2.20 10.69%	1.89 13.48%	1 4.42%

Instruments: To collect the target data the researcher prepared the following

The instruments were used: The cognitive load scale: It is a 14-item scale assessing the mental effort exerted on solving statistical problems in the working memory. The researcher developed a scale considering Sweller's (2011) modified cognitive load theory. The scale was a five-Likert point scale (1= never, 2= rarely, 3= sometimes, 4= often, 5= always). stereotypical thinking scale: It is a 7-items scale assessing the stereotypes way of thinking while problem-solving in inferential statistics. The

researcher developed the scale based on the studies of Foong & Koay, (1997); Salado, Chowdhury & Norton, (2019) .

Scale Development: Cognitive load scale: A total of 76 students were recruited in testing validation and reliability assessment. Exploratory factor analysis resulted in a three-factor solution. Bartlett's Sphericity test and Keiser-Meyer-Olkin (KMO) were calculated as measures of the suitability of data for structure detection. For data to be considered suitable, Bartlett's test should be significant and the KMO value should be over .80 (Bartlett, 1954; Kaiser & Rice, 1974). The data were suitable for factoring as Bartlett's test was significant ($p < .001$) and the KMO value was .65 showing accepted values for factorial analyses. EFA was conducted for the categorical data using a Principal axis factoring approach. Promax rotation method was used to obtain factor loading as (table 1).

The factor loading ranged from 0.33 to 0.79 for the intrinsic load, 0.73 to 0.80 for the extraneous load, and 0.32 to 0.69 for the germane load. Items 3, 12, and 13 were not attributed to any of the factors. The main explanation for this finding is that items related to statistical techniques could be used in problem-solving. These items reflected an overlap between the three factors of the cognitive load. The internal consistency of the cognitive load scale was adequate with a Cronbach's alpha of 0.71 for intrinsic load, 0.79 for the extraneous load, and 0.55 for the germane load.

Table 2. Factor loading, and attribution of each item based on factor analysis.

	Items	Factor loading	Communalities
1)	I fail when I try to improve my image of others about my performance	0.37	0.16
2)	I worry about my learning path due to my poor performance in statistics	0.85	0.73
3)	I am afraid of the effect of the statistic on my future performance in college	0.89	0.80
4)	I am afraid of confirming the stereotype of my performance to prevent me from achieving my dream	0.77	0.59
5)	I feel powerless if the operative question differs from what I studied	0.73	0.55
6)	I am looking to compile statistical issues into ideas before I study them	0.71	0.50
7)	I use a distinct sign for every statistical test that characterizes my problem	--	0.06
	Eigenvalue Variance explained	2.77 39.50%	

The stereotypical thinking scale: EFA resulted in 1st order general factor solution. Bartlett's Sphericity test was of statistical significance ($p < .001$) and the KMO was .80, showing meritorious value for factor analysis. For the categorical and ordinal data, EFA was performed by a principal axis factoring approach. Factor loading was obtained by using Promax rotation solution as (table 2). The factor loading ranged from 0.37 to 0.89. Item 7 was excluded because it might cause the shyness of the learner to respond to it, even if it is true that there are indicators that distinguish each problem from the other. The internal consistency of the stereotypical thinking scale agrees with a Cronbach's alpha of 0.78. Alpha if item deleted ranged from 0.68 to 0.84.

Design: The study relied on the correlational approach to verify the relative contribution of the cognitive load in predicting typical thinking among students of the 2nd year, Department of Psychology, Ismailia College of Education, in the Inferential Psychological Statistics Course.

Procedures and Data Analysis: Data analysis was performed by IBM SPSS V.20 statistics for windows. Exploratory factor analysis was used to obtain factor loading by the Promax rotation method. Internal consistency was assessed. Statistical significance was set at 0.05. The relationships between cognitive load patterns and stereotypical thinking were evaluated using the Pearson correlation matrix. Multiple regression was used for testing hypotheses.

Table 3. Pearson's correlations between Cognitive load and Stereotypical thinking.

Variables	Stereotypical thinking	
	r	p
Intrinsic cognitive load (ICL)	0.026	>0.05
Extraneous cognitive load (ECL)	0.394	<0.05
germane cognitive load (GCL)	0.653	<0.05

RESULTS AND DISCUSSION

Correlation between cognitive load patterns, and stereotypical thinking: The relationship was moderate and strongest statistically significant correlations for extraneous cognitive load (r = 0.349, P = 0.000) and germane load (r = 0.653, P = 0.000). No correlation was found between intrinsic load and stereotypical thinking (table 3).

The correlation between the extraneous load and stereotypical thinking is attributed to the fact that when students read inferential statistics problems, they compare them with the previously stored templates of problems and issues in the working memory. Some parts of this led them to think stereotypically as Park, Felix & Lee (2007) argued.The study assumed that extraneous load is the result of other factors that relate to individuals or factors related the assigned task (as in the circumstances of the second-semester test). It also confirmed that the complex implicit nature of cognitive structures had negative effects on those with stereotypical thinking in statistical problem-solving, as indicated by Redifer et al. (2019).

Table 4. Pearson's correlations between Cognitive load and Stereotypical thinking.

Dependent variable: stereotypical thinking Independent variables	coefficient	(95% CI)	P-Value
intrinsic cognitive load (ICL)	-0.005	-0.23-0.22	0.958
Extraneous cognitive load (ECL)	0.253	0.21-1.40	0.008
germane cognitive load (GCL)	0.577	0.74-1.45	0.007

Figure 1.1: scatter plot for the intrinsic load and stereotypical thinking.

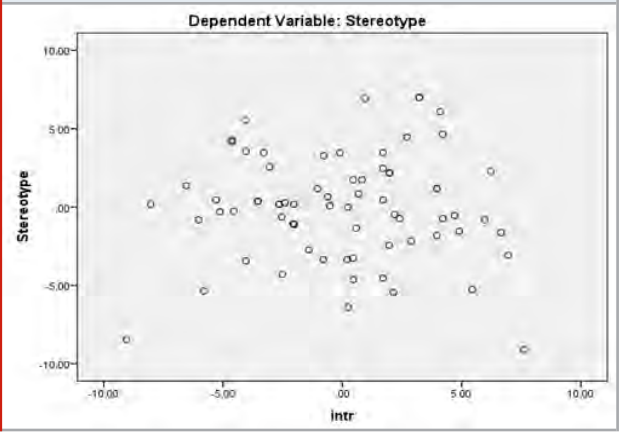
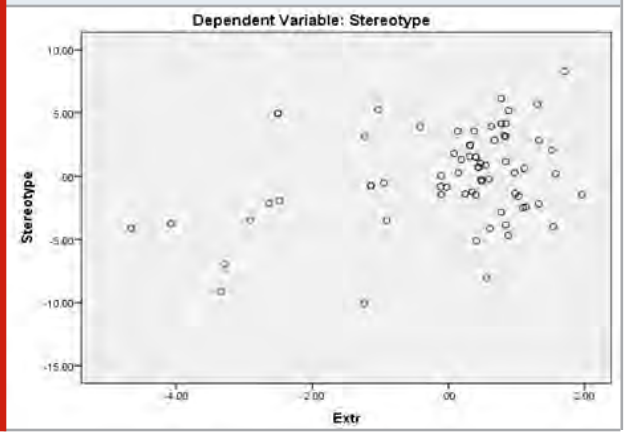


Figure 1.2: scatter plot for the extraneous load and stereotypical thinking.

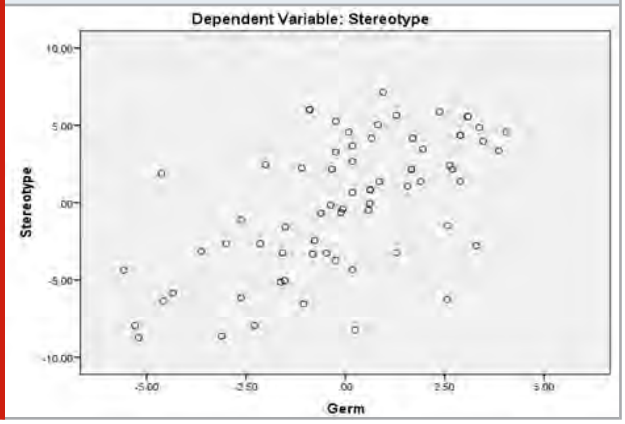


Regression Analysis: The multiple regression coefficients were 0.253 ($p < 0.05$) and 0.577 ($p < 0.05$) respectively (Table 4). Fig. 1 shows the relationship in the scatter plot. A positive, statistically significant relationship between extraneous and germane cognitive load patterns and stereotypical thinking (Table 4). The relationships between intrinsic load and stereotypical thinking were not significant in the multiple regression model.

The results indicate that cognitive load patterns can contribute to the prediction of stereotyped thinking in solving mathematical problems in the students of the psychology department at the Faculty of Education, Suez Canal University in the course of psychological statistics. The study found that the intrusive load as a predictor of stereotypical thinking in solving mathematical problems in the Psychological Inferential Statistics Course resulted from the lack of information in long-term memory that satisfies the process of creating and processing information in a sequential manner needed for the formation of proofs and the solution of mathematical problems which is supported by the study of Leahy & Sweller (2016). Results also revealed that the apparent stereotyping of the formation of unwanted magmatic schemes in the learner was a result of the seriousness and novelty of ideas of mathematical matters which helped to find future solutions to problems that seemed different in their being. This result agrees with the conclusions of Leppink, (2017) and Sweller (2011). In addition, the nature of the problems posed by the researcher in the test of Psycho-deductive statistics was clearly prolonged by

employing a certain method that develop in the familiar series of solutions provided by the students.

Figure 1.3: Scatter plot for the germane load and stereotypical thinking.



In addition, the researcher presented the data of the issue in new ways that helped to solve mathematical problems and justified the inability of the internal load as a predictor of stereotypical thinking. This prevented the interaction between the elements required in the solution which were provided in advance to the students. This result agrees with the conclusions of Sweller, (2011). The study also confirmed that that stereotyped thinking resulted of the failure to create and process charts within the working memory, the failure to make changes in the information structure previously stored in the working memory, regardless of the constraints of working memory, as concluded by Leahy & Sweller (2016, 2019). Deficiencies in thinking processes were characterized by weak formations of cognitive structures that rely on the initial information included in the statistical problems. This information requires secondary types of information retrieved from the long-term memory to be organized with with primary information (data) to solve the assigned problems (Chen et al., 2017).

Also, the external cognitive load was limited and did not require the interactions of the elements during the solution as the researcher administered the test of Psychological Inferential Statistics in a way which enabled students to deal with the statistical problems winch were different from those studied before, i.e. the effect of the external cognitive load was adjusted and this justifies the lack of statistical significance. Problem-solving processes did not entail the development of a link and infrastructure of the new issue and therefore was not affected by the working memory capacity and the necessary address information. This result is consistent with that of Chen et al., (20170 and Sweller, (2011). The study also led to the conclusion that the interactions between the discrete elements in the new formulations to solve the problem were fictitious as the researcher gave the solution method so as not to confuse learners while answering the test. This was another reason why the external cognitive load was not indicative, as revealed by Leppink, (2017) and Tricot & Sweller,(2014).

This result may be attributed to the fact that the researcher tried to reduce the size of the differences that appear in the elements of the problem in order to decrease the number of sub-series of the solution inferred in the accepted methods of solution, which reduced the number of common errors. Results also indicate that the internal and external cognitive load was not inhibited by stereotypical thinking and this is consistent with (Orru & Longo, 2018; Redifer et al., 2019). The increase in the internal cognitive load with more creative thinking, may mean that mathematical problems in the statistics course were familiar to students.These results agree with the findings of Paas & Van Gog (2006) that the decrease in the external cognitive load may due to training on complex mathematical practical examples. They also agree with Sweller (2010) that the intruder load increases twice more than the external load. Moreover, results are consistent with the results of Foong & Koay (1997) in that the cognitive load decreases using similarities and clues in solving mathematical problems.

The results are also consistent with the conclusions of Orru & Longo (2018) and Peck et al., (2018) in that the intrusive load depends primarily on the learners abilities such as motivation regardless of the nature of the learning material. The greater the internal cognitive load, the more thinking processes become critical, deductive and creative. Mathematical problems posed by the researcher during Inferential Statistics course entailed creative intellectual products. This load required internal knowledge depend on the difficulty of the mathematical tasks and its value was not significant which means that the tasks needed only stereotypes of thinking. This result is consistent with the results of Redifer et al., (2019) and Seufert (2018). The study have some limitations including the fact that its results cannot be generalized to males studying the Inferential Statistics Course because the deficit in stereotypical thinking increases among females than males. Caution should be taken on applying the results to graduate students at different levels (educational diplomas, master's, doctorate) where stereotypical thinking and personal judgment decrease with increasing age.

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Biomedical Communication

Phenolic-Fractions of *Kedrostis foetidissima* Leaves to Ameliorate Lysosomal Damage and Inflammation of Isoproterenol-Induced Myocardial Infarction in Rats

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ABSTRACT

Cardiovascular diseases (CVDs) are a significant health burden with an ever-increasing prevalence. They remain the leading causes of morbidity and mortality worldwide. Contemporary medicine has been used to take care of myocardial infarction (MI), a subset of CVDs, and have been relatively successful but not without adverse effects. As a result, this question has stimulated interest in the use of natural products, which may be equally effective and better tolerated. Therefore, this study aims to analyze the cardioprotective effect of partially purified phenolic fraction derived from *Kedrostis foetidissima* leaves (PFK) on isoproterenol (ISO) induced MI in male Wistar rats. Animals pretreated with different doses of PFK (50 and 100 mg/kg body weight) and α-tocopherol for 45 days were subjected to subcutaneous ISO injection (20 mg/kg body weight) for two consecutive days to induce MI. Heart tissue and serum of the sacrificed rats were used for assay of hs-CRP, homocysteine, NF-kB, IL-6, TNF-α levels, and lysosomal enzymatic activities (β-glucuronidase, β-N-acetylglucosaminidase, cathepsin-B & D, and β-galactosidase) to evaluate cardiac damage. The results obtained from this study confirms the severe ISO-induced cardiac cell damage in terms of rapidly increased lysosomal enzyme concentration (β-glucuronidase, β-N-acetylglucosaminidase, β-galactosidase), raised hsCRP, homocysteine, NF-kB, IL-6, and TNF-α levels, and reduced activity of the β-glucuronidase and cathepsin-D lysosomal enzymes versus the normal rats. Contrarily, the experimental rats pretreated with PFK had a significantly altered lysosomal enzyme activity, reduced hsCRP, homocysteine, NF-kB, IL-6, and TNF-α level when compared to MI control rats. In conclusion, this study revealed that the PFK play a cardio protective role by regenerating the cardiac defence system against ISO induced cardiac damage.

KEY WORDS: LYSOSOMAL ENZYMES, MEDICINAL PLANTS, MYOCARDIAL INFARCTION, PHENOLS.

INTRODUCTION

Cardiovascular disease (CVD) is an important cause of mortality and morbidity both in developed and developing countries. A major form of CVD is myocardial infarction (MI), which is commonly encountered as a silent infarct owing to its detection well late in the disease progression. When the homeostasis between the blood supply to the cardiac vessels and the requirement of the cardiac tissue is disturbed, the cardiomyocytes are subjected to a prolonged ischemic insult resulting in necrosis, commonly referred to as acute MI (Boarescu et al., 2019).

With a recent change in lifestyle and predisposition to co-morbidities, the incidence of MI has been reported in the young and increasing with age in the middle and older age groups, affecting both men and women (Sangeethadevi et al., 2020). The reduced blood flow to the heart results in a shortage of oxygen to the cardiac muscles, which if left untreated results in irreversible necrotic damage to the myocardium. In most cases, the heart attack is silent, no obvious symptoms of pain and

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usually has a sudden acute onset (Hoffman and Buckberg 2014; Virani et al., 2020).

Oxidative stress is the most important of all the causative factors in inducing MI by generating highly toxic free-radicals that cause a wide array of metabolic changes and myocardium dysfunction (Sammeturi et al., 2020). Additionally, it destroys the bio molecules (carbohydrates, lipid, proteins, and nucleic acids) and interrupts their interaction with each other leading to cell death (Jansy et al., 2021). Conventional synthetic drugs have been quite successful in the treatment of CVD, but the side effects of prolonged use pose a challenge, necessitating the need for alternative sources like drugs of natural origin (Shaito et al., 2020). Isoproterenol (ISO) is an artificially synthesized catecholamine and a β-adrenergic agonist, if used in large doses can induce cellular damage to the myocardial membrane (Procaccini et al., 2019; Shaito et al., 2020).

Experimental rats when injected with ISO underwent hypoxia, hypertension, increased formation of free radicals and calcium overload (Pavithra et al., 2020; Sangeethadevi et al., 2020). A probable reported mechanism of this ISO-induced cardiac damage is the production of highly toxic free-radicals by auto-oxidation of catecholamines. This excess oxidative stress damages the structural integrity of the cardiac tissue, besides a progressive decline in the function of several antioxidants and an increase in the lipid peroxidation products. Henceforth, irreversible damage of the heart tissues sets in (Alam et al., 2018; Sangeethadevi et al., 2021). Medicinal plants are comparatively cost effective and are since considered as a better alternative to synthetic drugs owing to their cardioprotective properties. They provide nutritional substances, mainly phytochemicals, that are potentially restorative and help maintain a balanced body system. Antioxidant compounds derived from plant sources act as free radical scavengers, protecting the body by delaying or inhibiting the free radical induced cellular damage (Swapna et al., 2020). A variety of free radical neutralizing antioxidants are found in various dietary sources like fruits, vegetables, and fresh leaf vegetables, etc (Uddandrao et al., 2019; Kalaivani et al., 2020).

Many researchers have also reported the biological role of phytoconstituents in ameliorating cardiotoxicity (Alam et al., 2018; Parim et al., 2019). Besides, plant derived phenolic compounds are reported to inhibit the auto oxidation of lipid molecules which limits the formation of low density oxidized lipoproteins, thereby impeding further cardiovascular damage (Cosme et al., 2020). Recently, a group of phenolic compounds, known as polyphenols, have come to light because of their biological activity, especially the antioxidant and free radical scavenging properties (Khan et al., 2019). *Kedrostis foetidissima*, an annual herb of the Cucurbitaceae family, is traditionally used by inhabitants of the Asian and African countries to prevent and cure diseases, besides serving as a dietary component.

Previously, reported that *K. foetidissima* has a high amount of antioxidant properties and cardioprotective efficacy (Pavithra et al., 2020). However, to the best of our knowledge, the effect of *K. foetidissima* as a cardioprotective agent on hsCRP, membrane damage, and inflammatory mechanism in MI is yet to be studied. Hence, the present research aims to study this effect of the partially-purified phenolic fraction of *K. foetidissima* leaves (PFK) on cellular and serum indicators of cardiac tissue damage in male Wistar rats with ISO-induced MI.

MATERIAL AND METHODS

For the preparation of PFK, the ethyl acetate fraction of methanolic leaf extract of *K. foetidissima* leaves were subjected to fractionation *in silica* gel column chromatography using ethyl-acetate and methanol as solvent (the procedure has been described in an earlier report by Pavithra et al., 2020). Male albino Wistar rats (weight – 150-180g) were used in the study obtained from the Nandha College of Pharmacy, Erode, Tamilnadu, India. All experimental animals were kept under standard laboratory conditions and fed with permitted commercial food and water adlibitum. The experiments of this study was carried out as per the procedures of animal ethical committee constituted by the Nandha College of Pharmacy, Erode, Tamilnadu, India and the study approval number is NCP/IAEC/2018-19/14. For the experimental design, the procured 30 male Wistar rats were divided into five groups of six rats each.

Group I: Normal control Group II: MI untreated control Group III: Albino Wistar rats treated with oral dose-I of PFK (50mg/kg BW) (Pavithra et al. 2020) for 45 days and isoproterenol (20 mg/100g BW) (Saravanan et al., 2013) administered subcutaneously twice at an interval of 24hours. Group IV: Albino Wistar rats treated with oral dose-II of PFK (100mg/kg BW) (Pavithra et al. 2020) for 45 days and isoproterenol (20 mg/100g BW) administered subcutaneously twice at an interval of 24 hours.Group V: α-tocopherol as standard for 45 days (60 mg/kg b.w) (Saravanan et al., 2013) and isoproterenol (20 mg/100g BW) administered subcutaneously twice at an interval of 24 hours. After 45 days of treatment, all the animals were sacrificed under light ether anaesthesia by cervical decapitation. Heart tissues were dissected, and blood was collected for plasma separation. For the estimation of hsCRP and homocysteine, the plasma was used to assay the hsCRP. The standard chemiluminescence immunoassay kit (Roche Diagnostics, Switzerland) was used for assaying plasma hsCRP levels. While the Microtiter Plate Assay package (Diazyme Laboratories) was employed for estimating the plasma homocysteine concentration.

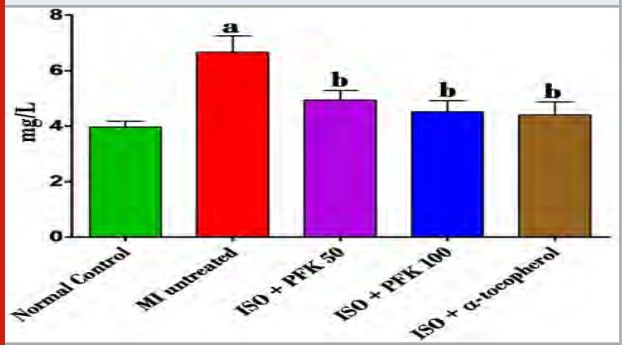
For the evaluation of serum inflammatory cytokines, the serum levels of inflammatory cytokines such as nuclear factor kappa B (NF-kB), tumor necrosis factor alpha (TNF-α), and interleukin-6 (IL-6) were measured as per standard protocols (Signosis Inc., Santa Clara, CA, USA). For the assay of lysosomal enzyme, the lysosomal

enzyme activity, namely β-glucuronidase, β-N-acetyl glucosaminidase, and β-galactosidase, were estimated using the methods described by Kawai and Anno (1971), Moore and Morris (1982) and Conchie et al (1959) respectively. One contrary, as per the procedure by Barret (1980) and Sapolsky et al., (1973) the Cathepsin-B and D lysosomal enzymes were assessed. For the statistical analysis, all data were analyzed using SPSS version 10.0. Besides the descriptive analysis, the five groups were compared for hypothesis testing using a one-way analysis of variance (ANOVA), followed by the least significance test (LSD) in case of significant results. $p < 0.05$ was considered significant for all data ($\alpha = 5\%$).

RESULTS AND DISCUSSION

In this study, we made an attempt to observe the effect of the medicinal plant extract, PFK, as a cardio protective agent against ISO-induced cardio toxicity leading to MI. CRP, known as hsCRP, is an acute-phase protein and an important inflammatory marker, often increased in case of tissue damage and infection contributing to the major process of coagulation. The association between the size of the infarct in an acute MI and the level of circulating hsCRP is a well-established fact (Saravanan and Ponmurugan 2012; Zhong et al., 2013), suggesting hsCRP to be a reliable indicator for diagnosing underlying coronary artery damage in addition to the myocardial necrosis (Saravanan and Ponmurugan 2012; Carrero et al., 2019). In this study, we found that a significant ($p<0.05$) increase in the plasma hsCRP levels in the rats injected with ISO to induce MI when compared to normal control rats (Fig 1).

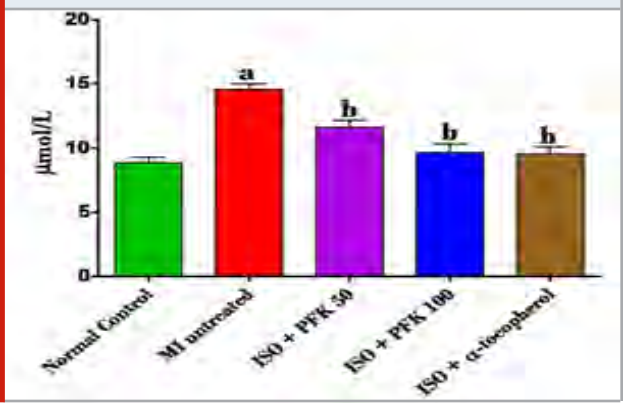
Figure 1: Effect of PFK on hs-CRP levels of control and ISO treated rats. Values are mean ± SD, n = 6, [a] Significantly ($p < 0.05$) different from normal control, [b]Significantly ($p<0.05$) different from MI untreated.



Whereas, on comparing groups with the ISO-induced MI, near normal levels of plasma hsCRP were obtained in rats pretreated with PFK than the rats receiving ISO injections only. These findings are further reinforced by studies that point to a direct correlation between hsCRP and acute MI in humans (Badiger et al., 2014; Carrero et al., 2019). Furthermore, serum hsCRP level is not merely an indicator of the extent of tissue damage following an MI, but also responsible for the development of the irreversible necrosis of the myocardium (Reindl et al. 2020). This also suggests that elevated serum levels may be influenced by the size of the myocardial infarct (Zhong et al. 2013; Lucci et al. 2020). The induction of MI by subcutaneous ISO injection used in this study caused injury to the cardiomyocytes, initiating an inflammatory response. This tissue damage produced intramyocardial haemorrhage and pericarditis because of the gradual leakage of blood into the pericardial space. The local inflammation in the heart increases the production of proinflammatory mediators leading to a continuous marked rise in CRP levels in the blood (Sammeturi et al. 2020).

However, the results of the present study show that the orally administered PFK significantly reduced these plasma hsCRP levels in the rats with ISO-induced cardiotoxicity. A possible explanation is the inhibition of the platelet mediated inflammation leading to a reduction in the release of proinflammatory mediators (Chen et al., 2020). Hyper-homocysteinemia, a phenomenon associated with the early development of heart and blood vessel disease (Babu et al., 2015). The key features include the production of free radicals, lipid peroxidation of the polyunsaturated fatty acids bound to the cell membrane, monocytic production of interleukins, and up-regulation of the vascular cell adhesion molecules, which encourage the development of atherosclerosis (Shahzad et al., 2019; Jansy et al., 2021). The changes in homocysteine levels due to PFK administration has been described in figure 2.

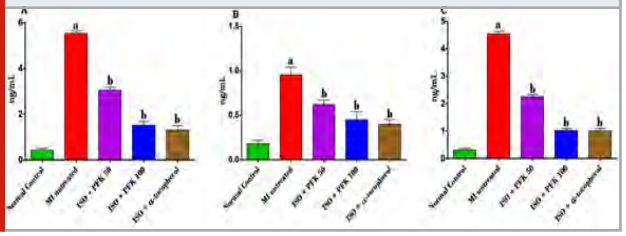
Figure 2: Effect of PFK on homocysteine levels of control and ISO treated rats. Values are mean ± SD, n = 6,[a] Significantly ($p<0.05$) different from normal control, [b]Significantly ($p < 0.05$) different from MI untreated.



In the experimental rats, the introduction of ISO caused a significant increase in plasma levels of homocysteine compared with control rats ($p<0.05$). Alternately, these changes in ISO-injected rats were reversed as a result of the prior administration of PFK and α-tocopherol. In the present study, the elevated homocysteine is due to an imbalance in the dietary methionine that contributed to the development of atherosclerosis. The available literature supports the use of highly lipotropic molecules (cerivastatin and fluvastatin) to reduce the risk of CVD

and the likelihood of formation of atherosclerotic plaque, the proposed mechanism for which are non-lipid pathways like a reduced expression of interleukins (Boren et al., 2020; Jansy et al., 2021).

Figure 3: Effect of PFK on the protein levels of (A) NF-kB, (B) IL-6 and (C) TNF-α levels in serum of control and ISO treated rats. Values are mean ± SD, n = 6, [a]Significantly ($p<0.05$) different from normal control, [b]Significantly ($p<0.05$) different from MI untreated.



PFK contained more phenolic compounds with high lipotropic properties, which can easily pass through the smooth muscle cell membrane of the vessels, thus limiting the production of both homocysteine and interleukin. The recent evidence supports the association between lipid metabolism in the body and the inflammatory process (Uddandrao et al., 2020). Strikingly, hyperlipidemia which is the major pathogenic process underlying the development of atherosclerotic plaque in the blood vessels has been found to inhibit the acute inflammatory response. Additionally, adipose cells produce inflammatory cytokines which are known contributors to a host of metabolic disorders including MI. These findings led to the use of circulating inflammatory proteins CRP, TNF-α and IL-6 as markers to predict an impending cardiovascular adverse event (Carrero et al. 2019; Sammeturi et al., 2020; Hamad et al., 2020). The effect of PFK administration on the inflammatory markers (NF-kB, IL-6, and TNF-α) has been illustrated in figure 3A-C.

Table 1. Effect of PFK on the activities of lysosomal hydrolases in serum of experimentally induced myocardial infarcted rats

Groups	β-glucuronidase (µmol of p-nitrophenol liberated/h/ mg protein)	β-N-acetyl glucosaminidase (µmol of p-nitrophenol liberated/h /mg protein)	β-galactosidase (µmol of p-nitrophenol liberated/h/mg protein)	Cathepsin-B (µmol of p-nitrophenol liberated/h/100 mg protein)	Cathepsin-D (µmol of tyrosine liberated/h/ 100 mg protein)
Normal control	12.60±0.78	8.567±0.6	10.6±0.81	15.64±0.47	15.98±0.77
MI untreated	19.65±+2.38	20.88±1.48a	17.87±0.81[a]	17.81±1.41a	27.89±1.69
ISO + PFK 50	16.65±0.96b	12.45±0.73b	13.58±0.88[b]	16.23±0.52b	19.67±0.7
ISO + PFK 100	15.66±1.1b	11.65±0.45b	13.52±1.02[b]	16.19±0.39b	19.31±1.08
ISO+ α-tocopherol	15.6±0.73[b]	11.42±0.55[b]	13.28±0.41[b]	16.11±0.32[b]	17.68±1.13

Values are mean ± SD, n = 6.
[a]Significantly ($P<0.05$) different from normal control
[b]Significantly ($P<0.05$) different from MI untreated

A statistically significant increase ($p<0.05$) in the NF-kB (Fig. 3A), IL-6 (Fig. 3B), and TNF-α (Fig. 3C) was seen in the rats injected with ISO versus the control rats. However, these levels were significantly low ($p<0.05$) in the PFK pre-treated rats when compared to those who were administered only ISO (Sammeturi et al., 2020; Hamad et al., 2020). IL-6 is a fundamental inflammatory mediator that induces the production of hsCRP in the hepatic tissue. Many metabolic disorders with an inflammatory component, including acute MI, are often precipitated by hyperlipidemia leading to greater expression of pro-inflammatory genes, such as IL-6 and TNF-α (Sammeturi et al., 2020). The results of the present study concur with these findings, evident from the changes in transcription factors such as NF-kB and a boost in the production of the pro-inflammatory factors IL-6 and TNF- α, which is closely linked with the steatotic and inflammatory responses found in coronary heart disease. Usually, in a dormant cell, the cytoplasmic protein, inhibitory kappa B (IkB), checks the expression of the NF-kB factor (Chen et al., 2014; Albensi 2019).

Whereas, upon activation, NF-kB translocates into the nucleus up regulate the gene expression of various pro-inflammatory cytokines (Uddandrao et al. 2019; Jansy et al. 2021). Our results add to this knowledge as the serum IL-6 and TNF levels increased in the ISO-intoxicated rats, signifying an acute inflammatory process; this is in line with results reported by Sangeethadevi et al. (2021). Whereas, the rats treated with the experimental drugs, PFK had lower levels of IL-6 TNF-α and NF-kB, reinforcing the role of PFK in limiting the inflammatory process (Sangeethadevi et al., 2021). A great emphasis has been laid on the lysosomal alterations occurring concurrently with ischemic or hypoxic damage to muscle cells. Lysosomes are the cell organelles in the animal cell responsible for intracellular digestion of cell components by the processes of autophagy,

heterophagy, and endocytosis. The typical sequences of vacuole formation, lysosomal disruption, and spread of the lysosomal enzymes in the cell have reportedly been observed in the case of cardiac ischemia (Nirmala and Pandian 2015; Chi et al., 2020).

Table 2. Effect of PFK on the activities of lysosomal hydrolases in heart of experimentally induced myocardial infarcted rats

Groups	β-glucuronidase (µmol of p-nitrophenol liberated/h/ mg protein)	β-N-acetyl glucosaminidase (µmol of p-nitrophenol liberated/h /mg protein)	β-galactosidase (µmol of p-nitrophenol liberated/h/mg protein)	Cathepsin-B (µmol of p-nitrophenol liberated/h/100 mg protein)	Cathepsin-D (µmol of tyrosine liberated/h/ 100 mg protein)
Normal control	16.24±1.57	14.37±0.81	46.75±4.63	11.37±0.51	10.61±1.15
MI untreated	28.73±3.3[a]	26.55±2.24[a]	81.29±3.19[a]	20.23±0.69[a]	19.38±1.31[a]
ISO + PFK 50	20.31±1.42[b]	16.17±0.69[b]	59.74±7.39[b]	14.63±0.67[b]	12.90±1.15[b]
ISO + PFK 100	18.96±3.07[b]	15.71±1.06[b]	58.46±5.69[b]	14.14±0.61b	12.16±0.85[b]
ISO+ α-tocopherol	19.80±0.82[b]	15.51±0.78[b]	56.79±6.18[b]	14.03±0.7[b]	11.54±0.66[b]

Values are mean ± SD, n = 6.
[a]Significantly (P<0.05) different from Normal control
[b]Significantly (P<0.05) different from MI untreated

Table 3. Effect of PFK on the activity of β-glucuronidase and Cathepsin-D in lysosomal and cytosolic fractions of the heart in normal and experimentally induced myocardial infarcted rats

Groups		β-glucuronidase (µmol of p-nitrophenol liberated/h/mg protein)	Cathepsin-D (µmol of tyrosine liberated/h/100 mg protein)
Normal control	Lysosomal	15.23±2.7	27.87±2.56
	Cytosol	20.18±1.58	21.93±1.71
MI untreated	Lysosomal	9.05±0.46[a]	10.45±1.51[a]
	Cytosol	30.43±3.3[a]	32.50±3.26[a]
ISO + PFK 50	Lysosomal	11.30±0.87[b]	19.03±2.86[b]
	Cytosol	23.75±1.33[b]	20.14±1.14[b]
ISO + PFK 100	Lysosomal	12.82±1.54[b]	20.80±1.71[b]
	Cytosol	23.57±1.82[b]	19.79±2.71[b]
ISO+ α-tocopherol	Lysosomal	13.20±1.57[b]	23.42±2.59[b]
	Cytosol	22.35±1.45[b]	19.44±0.79[b]

Values are mean ± SD, n = 6.
[a]Significantly (P<0.05) different from normal control
[b]Significantly (P<0.05) different from MI untreated

Besides this, lysosomes also aid in the cellular secretion and transport process (Bonam et al., 2019). On the other hand, the activity of various lysosomal enzymes was significantly increased (p<0.05) as evident from the serum and cardiac tissue assays of rats treated with ISO versus the normal control rats. Conversely, the experimental drugs (PFK and α-tocopherol) when orally given, significantly reduced (p<0.05) the serum (Table 1) and cardiac tissue (Table 2) activity of these enzymes in ISO-injected rats pretreated with PFK versus only ISO-injected rats (Bonam et al., 2019). Acid hydrolases located in the myocardial cells, along with the lysosomal and cytosolic enzymes are responsible for the myocardial injury and cell death in the state of ischemia (Prince and Hemalatha 2018; Mishra et al., 2019). Similarly, in this study, enhanced activity of the lysosomal enzymes, both in serum and heart tissue was observed in the ISO-intoxicated rats. The lysosomal membrane is a potential site of an attack by free radicals because of its lipid moiety. This phospholipid rich membrane

loses its stability leading to the release of lysosomal enzymes within the cell, that are capable of progressively damaging the cardiac cells, progressing from a reversible ischemic injury to irreversible cardiomyocyte necrosis (Akila et al., 2017; Chi et al., 2020).

On the other hand, β-glucuronidase and cathepsin-D activity significantly reduced ($p<0.05$) in the lysosomal fraction of the heart of rats with ISO-induced cardiotoxicity when compared to normal control rats. Also, the cytosolic fraction of these enzymes increased considerably ($p<0.05$) in rats with ISO-induced cardiotoxicity versus normal control rats. Conversely, pretreatment with PFK and α-tocopherol to the rats subjected to ISO-induced MI showed a significant increase in the lysosomal fraction ($p<0.05$) and a decrease in the cytosolic fraction ($p<0.05$) of the heart (Table 3) (Akila et al., 2017; Chi et al., 2020).

We observed significantly lower levels of β-glucuronidase and cathepsin-D in the lysosomal fractions of the ISO-intoxicated rats as compared to normal control rats. This reduced activity confirms the aforementioned mechanism of abnormal release of lysosomal enzymes into the cell leading to cell death in the myocardium (Akila et al., 2017; Trivedi et al., 2020). Administration of PFK significantly altered the concentration of these enzymes in the cardiac tissues of the ISO-intoxicated rats. A probable explanation could be the inhibition of cellular peroxidation or preventing the iron catalyzed oxidative reactions, which lead to peroxidation of cellular membranes. This leads to a stabilization of the lysosomal membrane, thus avoiding the leakage of lysosomal contents (Prince and Hemalatha 2018; Lopez et al., 2020).

CONCLUSION

The findings of the present study reveal that pretreatment of rats with PFK had a significant protective role against the ISO-induced MI by maintaining the levels of hsCRP, TNF-α, IL-6, NF-kB and lysosomal enzymes activities in both serum and heart tissues. This effect may be attributed to the anti-lipoperoxidative and antioxidant properties of phenolic compounds in the partially purified fraction. Based on these results, it can be concluded that PFK may be used as potential source in the management of CVD.

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Biomedical Communication

Assessment of Progressive Resistance Exercise Training on CD4 Count and Weight of People with HIV/AIDS in A University Teaching Hospital, Ebonyi State Nigeria

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ABSTRACT

This study aimed at assessing the effect of progressive resistance exercise training on CD4 count and weight of people living with HIV/AIDs in Alex-Ekwueme Federal University Teaching Hospital, Ebonyi State Nigeria . The study adopted experimental research design. The population of the study was 40 HIV/AIDS patients that attended HIV clinics at AE-FUTHA. The sample size for the study was 38 due to drop out of two subjects in the control group. Simple random sampling technique by balloting was adopted for the study. Flow cytometry (FACS) and Omron BF 400 were the instruments used for data collection of CD4 counts and weight respectively. Mean, standard deviation and ANCOVA were used to analyze the data obtained. The instruments were not validated because they are standard. The reliability coefficient score obtained from the pilot study were 0.848 and 0.994 representing CD4 counts and Weight of the participants respectively. The major findings revealed that PRE had positive effect on CD4 counts and weight. The hypotheses result showed that PRE had statistical significant effects on CD4 counts but did not have effects on weight. Based on these findings, recommendation were made among others that awareness should be created to government by Physiotherapists, Exercise Physiologists, Physicians and Health Educationists to adjunct PRE which boost immune system by improving CD4 counts and in the management of PLWHA.

KEY WORDS: PROGRESSIVE RESISTANCE EXERCISE, CD4 COUNTS, WEIGHT AND HIV/AIDS.

INTRODUCTION

Progressive resistance exercise (PRE) is a system of dynamic resistance training in which a constant external load is applied to the contracting muscles by some mechanical means (usually a free weight or weight machine) and gradually increased using repetitive maximum as a basis for progression. Similarly, PRE is a style of strength training exercise that involves the utilization of resistance with the overload principle via activities such as isotonic or isometric exercises. The overload principle defines a solution to the problem of our bodies adapting to one maximal dynamic concurrent exercise due to constant repetition of moderate enduring training (O'Brien et al., 2004, Kisner and Colby, 2012).

The principle states that in order to see ongoing training benefits, the load placed on our bodies via exercise must continue to be increased until our bodies adapt to the

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current load. More specifically, PRE basically involves activities like chest press, overhead shoulder press, upright rowing, biceps muscle curls, triceps muscle extension, knee extension with weight, knee flexion with weight, leg press, calf raises and dumbbell lifting. Moreover, PRE as a therapeutic tool in patient's management; is considered safe and effective in improving immune system especially $CD4^+$ T helper cells (Roubenoff et al. 1999, Guadalupe-Grau et al., 2009, Chitu-Tisu et al., 2016, Baker, 2020, Petre et al., 2021).

Anxiety and depression are some of the most common symptoms experienced by HIV population and indicators of low quality of life with concomitant stress (Ma et al. 2013). Stress reduces CD4 cell counts, while exercise enhances CD4 cell counts by reducing negative emotional states and modulating levels of endogenous opiates and stress hormones (Beck et al. 2016). This remarkable increase in CD4 cell counts could also be attributed to the fact that exercise training stimulates the formation of certain antibodies that prevent some potent HIV protein molecules (glycoprotein 120) from attaching to receptor sites of CD4 cells, by this process, the damaged immune system is reconstituted, and HIV disease progression to AIDS is slowed down, similar to the role played by ART/HAART (Battalora et al. 2014, Matovu et al., 2016).

Surprisingly, despite the recorded psychological, immunological and physiological benefits of progressive resistance exercises, some related (empirical) studies which were reviewed disclosed inconsistent experimental research findings concerning the effects of different therapeutic exercises especially PRE with respect to CD4 counts (Shojaa et al., 2020). A previous study reported a stable CD4+ cell count with resistance exercise during a 12-week intervention (45–60 min, 3times/week) study, unlike the control who recorded a significant decrease. Similarly, another study reported that structured resistance exercise also triggers a specific immune response in PLWHA (Multanen et al., 2015). Another previous study demonstrated that resistance exercise for 12weeks (3 times/week) is effective in boosting the CD4+ and CD8+ cell counts with consequent improvement in the integrity of the immune system (Harada and, Rodan, 2003).

Equally, another study documented a significant effect of PRE in three outcome measures including CD4 count (Seeman and Delmas, 2006). In related studies PRE improved the CD4 count of elderly HIV people as well (Glenn, 2009). Contrarily, Dolan, Fronter, Librizzi, Liungquist and Juan, (2006) stated that PRE does not have positive effect on CD4 cell count, also, Terry, Sprinz and Ribiero, (2006) reported that PRE did not have positive effect on immunologic makers (CD-4 and CD-8). Similarly, O'Brien, Nixon, Tynan and Glazier (2015) equally reported insignificant effects in CD4 count and viral load. More consistently, PRE brought about increase in the weight of HIV/AIDs individuals, hence, PRE is proven to be safe and could be beneficial in improving the weight of adults living with HIV/AIDS. Hence the need for this study to add to knowledge and close off the gap created by inconsistency (O'Brien et al., 2004, Souza et al., 2008).

The main purpose of this study is to evaluate the effects of progressive resistance exercises on CD4 counts and weight of people living with HIV/AIDS (PLWHA) who are on either anti-retroviral therapy (ART) or highly active anti-retroviral therapy (HAART) in Alex-Ekwueme Federal University Teaching Hospital, Abakaliki, Ebonyi State (AE-FUTHA). Specifically, this study sought to determine: the effects of 6weeks progressive resistance exercises on CD4 count of people living with HIV/AIDS, also, to ascertain the effects of 6 weeks progressive resistance exercises on weight of people living with HIV/AIDS.

MATERIAL AND METHODS

Research Design: This study adopted an experimental research design with an equivalent (randomized) pre-test and post-test data, utilized to observe the response of the dependent variables (CD4 count and Weight) of the treatment group (progressive resistance exercise).

Area of Study: The setting for this study was at Physiotherapy department of AE-FUTHA in Ebonyi State. This health facility is a teaching hospital in Abakaliki. It has about 2000 beds, and up to 20 departments. The hospital was chosen based on the information gotten from health record that they diagnose up to 20 HIV/AIDS patients weekly and has up to 25% of 0.8% population of those living with HIV/AIDS in Ebonyi State. Participants These include all HIV/AIDS patients that attend HIV clinics at AE-FUTHA between December, 2019 to February, 2020. Forty volunteers who were on ART/HAART (highly active anti-retroviral treatment) for not less than 24months were randomly grouped into 2 groups (A= the exercising group and B = control group).

Sample and Sampling Technique: Forty (40) participants on ART/HAART who were willing to participate and met the inclusion criteria were assigned to the two groups (A: PRE and B: control), using balloting by replacement [13]. However, only 38 subjects completed the study due to drop out by 2 persons from the control group. Thus, the sample size became 38 for the study.

Selection Criteria: Inclusion Criteria: Only HIV/AIDS patients within the age range of 18 to 60 years and female between 18 to 50 years. Only HIV/AIDS patients that have started taking their ART/HAART for the duration of 24 months and above, prior to the study, and attend HIV/AIDS clinics.

Exclusion Criteria: HIV/AIDS patients below 18 years and above 60years old. Patients that were not on ART/HAART up to duration of 24 months and above prior to the study, also, all subjects with previous history of cardiac and diabetic complications. However, all pregnant subjects were excluded to avoid interference on CD4 count and weight.

Instrument for Data Collection: The following instruments were used for data collection in this study: Flowcytometery (Partec Cyflow counter), Germany; and Omron BF400 weighing scale. The instruments (i.e. Flowcytometery [Partec Cyflow counter] and Omron BF400 weighing scale) are standard and used worldwide. Hence they do not need to be validated. The obtained data during trial testing was subjected to Pearson Product Moment Correlation Co-efficient and the results were 0.848 and 0.994 for CD4 count and weight respectively. These are said to be reliable because they are greater or equal to 0.8 (Glenn, 2009).

Experimental Procedure: The procedure for data collection in this research was assisted by 4 field assistants including; a Physiotherapist, a Radiologist, a Nurse, a laboratory Scientist and a medical doctor. The subjects were recruited at AE-FUTHA and informed consent was issued explaining the purpose, procedure, and relevance of the study before the onset of the intervention. The intervention was supervised by Research and Ethics Committee (REC) of AE-FUTHA. All the willing participants were assessed for baseline data, which included age, weight, blood pressure (BP) and heart rate. Randomized control trial technique by balloting was used to divide the willing participants who met the inclusion criteria into two groups (A: PRE and B: control). None of the groups were blinded. The control group (B) was not allowed to participate in the exercises and they were also asked not do any active exercise program for the period of six (6) weeks of the study.

The activities of daily living of the participants in the control group were monitored through a checklist and it was confirmed that none of them participated in any form of active exercise program. While the progressive resistance exercise group (A) used 10 repetitive maximum of 10 percent of their body weight in progression, using 1/3 of their 10% body weight in the first two weeks, 1/2 of their 10% body weight in their 2nd two weeks and finally 10% of their body weight in their last two weeks. The weights were gradually/progressively increased during the 6 weeks of the exercise with consideration of bone adaptation and fracture prevention. Heart rate and blood pressure were monitored before and during each exercise bout. All subjects participated three times a week for the period of six weeks. Post intervention/treatment data of CD4 T-cells and Weight were then collected through Flowcytometery (Partec Cyflow counter) and Omron BF400 weighing scale respectively.

Method of Data Collection: Blood samples were drawn venopunctually for the CD4 count using syringe into a test-tube. Reagents used were brought to room temperature, 850µl of the count check bead green analyzed to make sure that the cyflow machine was working effectively. The needed numbers of Rohren test tubes were labelled appropriately and placed in a test tube rack. 20µl of CD4 easy count kits (CD4 Mab-PE) were pipetted into them for the assay. Thereafter, 20µl of blood samples were also pipetted into each test tube and incubated in the dark for 15 minutes at room temperature after mixing properly followed by the addition of 850µl easy count. Lyse buffer was not added to each test tube. In order to avoid air bubbles, this was mixed properly and analyzed on the Partec Cyflow. The outcome was displayed and copied from the screen.

Ethical Consideration: Ethical approval was sought and obtained from the ethical committee of AE-FUTHA. Participants' privacy and confidentiality was maintained using code numbers instead of names, and ensuring that records were destroyed at the end of the study. Subjects' informed consent were obtained from the subjects before commencing the study and the principle of the Helsink and Ninemberg declarations on the protection of the right of subjects while conducting experimental human research was strictly observed.

Method of Data Analysis: The data obtained (i.e. CD4 counts and weight) in the main study were analyzed using mean, standard deviation and analysis of covariance (ANCOVA).

RESULTS AND DISCUSSION

Table 1. Effects of 6 (six) weeks progressive resistance exercise (PRE) on CD4 counts of people living with HIV/AIDS

Group	N	Pre-test Mean	Post-test Mean	Differences in Mean (Post-Pre)	Pre-test Standard Deviation	Post-test Standard Deviation	Differences in STD (Post-Pre)
PRE	20	414.20	442.15	27.95	197.45	202.37	4.92
Control	18	461.28	384.61	-76.67	237.40	199.20	-38.2
Difference in Mean Effect		-47.08	57.54		-39.95	3.17	

The result in the table 1 above revealed that the participants in experimental group who took part in the PRE had higher pretest observed mean in CD4 counts compared to their counterparts in the control group. Those who were exposed to treatment had mean of 414.20 while those in control group had 461.28 for the pre-test. This shows that there is a difference of -47.08 in favour of subjects in the control group before the intervention. Participants in PRE got a mean value of 442.15 while subjects in control group got 384.61 in the post-test. The mean difference is 57.54 in favour of subjects in the intervention group (i.e. those who

participated in PRE). The difference in observed mean after 6 weeks' intervention of the PRE is 27.95 while that of control is -76.67, showing positive therapeutic benefits in favour of the PRE group. However, the difference in standard deviation values between pretest and posttest is heterogeneous for both groups (i.e. no similarity) as the STD (i.e. 4.92 and -38.2) are above 0.9.

The result in the table 2 above revealed that the participants in experimental group who took part in the PRE had higher pretest observed mean in weight compared to their counterparts in control group. Those who were exposed to treatment had mean of 69.02 while those in control group had 67.08for the pre-test. This shows that there is a difference of 1.45 in favour of subjects in the experimental group before the intervention. Participants in PRE got mean value of 71.09 while subjects in control group got 67.57 in the post-test. The mean difference is 4.01 in favour of subjects in the intervention group (i.e. those who participated in PRE). The difference in observed mean after 6 weeks' intervention of the PRE is 2.07 while that of control is 0.49, showing a weight gain of subjects in PRE group and a slight weight gain in the control group. However, the difference in standard deviation values between pretest and posttest is homogeneous for both groups (i.e. similarity) as the STD (i.e. 0.46 and -0.69) are less than 1.

Table 2. Effects of 6 (six) weeks progressive resistance exercise on weight of people living with HIV/AIDS

Group	N	Pre-test Mean	Post-test Mean	Differences in Mean (Post-Pre)	Pre-test Standard Deviation	Post-test Standard Deviation	Differences in STD (Post-Pre)
PRE	20	69.02	71.09	2.07	12.58	13.04	0.46
Control	18	67.08	67.57	0.49	17.84	17.15	-0.69
Difference in Mean Effect		1.45	4.01		-5.26	-4.11	

Table 3. There was no significant difference after 6 weeks' progressive resistance exercise (PRE) on CD4 count of people living with HIV/AIDS

Source	Type III Sum of Squares	Df	Mean Square	F	P-value	Effect Size
Corrected Model	1.104E6	2	552153.827	50.886	.000	
Intercept	30837.785	1	30837.785	2.842	.101	
Group(PRE/Control)	84369.691	1	84369.691	7.775	.009	3.84
Pretest CD4 Count	1072942.903	1	11072942.903	98.881	.000	
Error	379777.925	35	10850.798			
Total	8025316.000	38				
Corrected Total	1484085.579	37				

a. R Squared = .759 (Adjusted R Squared = .745)
b. Significant level-*P < 0.05, Ns Not significant: (P > 0.05)
c. Effect size: d = 0.2 (small effect); d = 0.5 (medium effect); d = 0.8 (large effect)

The table above compared the post test of PRE Group and Control Group on CD4 count. The result is on the effect of progressive resistance exercise (PRE) on CD4 count of people living with HIV/AIDS. The table shows a probability value (significant value) of 0.009 for progressive resistance exercise and control groups. The significant value in the table above for groups is less than the alpha level of 0.05. The decision rule is that if the probability value (significant value) is less than the alpha level of 0.05, then the earlier stated null hypothesis will not be accepted. This means that the hypothesis earlier stated will not be accepted. Thus, there is a significant difference after 6 weeks' progressive resistance exercise on CD4 count of people living with HIV/AIDS. On the aspect of effect size, the table shows that the mean difference between pre-test and post-test from the study when experimental and control groups are compared is high. This shows that there is a large effect size of PRE on CD4 counts and it implies that the mean difference is important.

The result in the above table is on the effect of progressive resistance exercise (PRE) on weight of people living with HIV/AIDS. The table shows a probability value (significant value) of 0.134 for progressive resistance exercise (PRE) and control group. The significant value in the table above for groups is greater than the alpha level of 0.05. The decision rule is that if the probability value (significant value) is greater than the alpha level

of 0.05, then the earlier stated null hypothesis will be accepted. This means that the hypothesis earlier stated will be accepted. Thus, there is no significant difference after 6 weeks' progressive resistance exercise on weight of people living with HIV/AIDS. The result in the table shows that there is a large effect size of PRE on Weight. This large effect size indicates an increase in the mean value after comparing the pre-test and post-test of the two groups in the study.

Table 4. There was no significant difference after 6 weeks' progressive resistance exercise (PRE) on Weight of people living with HIV/AIDS

Source	Type III Sum of Squares	Df	Mean Square	F	P-value	Effect Size
Corrected Model	7394.064a	2	3697.032	130.372	.000	
Intercept	56.657	1	56.657	1.998	.166	
Group(PRE/Control)	66.854	1	66.854	2.358	.134	2.68
Pretest WEIGHT	7241.895	1	7241.895	255.379	.000	
Error	992.512	35	28.357			
Total	190310.610	38				
Corrected Total	8386.576	37				

a. R Squared = .882 (Adjusted R Squared = .875)
b. Significant level-*P < 0.05, Ns Not significant: (P > 0.05)
c. Effect size: d = 0.2 (small effect); d = 0.5 (medium effect); d = 0.8 (large effect)

In table 1, the findings revealed that the mean difference is 57.54 in favour of subjects in the intervention group (i.e. those who participated in PRE). The difference in observed mean after 6 weeks' intervention of the PRE is 27.95 while that of control is -76.67, showing a positive therapeutic benefit in favour of the PRE group. However, the difference in standard deviation values between pretest and posttest is heterogeneous for both groups (i.e. no similarity) as they are above 0.9. This implies that after 6 weeks of intervention, PRE had positive effect on CD4 counts of people living with HIV/AIDs. This finding is in support of the findings of Souza, Jacob-Filho, Santarém, Silva, Li and Burattini, (2008) that PRE improved the CD4 count of elderly HIV people. On the contrary, this finding disagrees with that of Dolan, Fronter, Librizzi, Liungquist and Juan, (2006) that PRE does not have positive effect on CD4 cell count.

In table 2, the findings revealed that the mean difference is 4.01 in favour of subjects in the intervention group (i.e. those who participated in PRE). The difference in observed mean after 6 weeks' intervention of the PRE is 2.07 while that of control is 0.49, showing a Weight gain of subjects in PRE group and a slight weight gain in the control group. However, the difference in standard deviation values between pretest and posttest is homogeneous for both groups (i.e. similarity) as they are less than 1. This result shows that people living with HIV/AIDs had increase in weight after 6 weeks of PRE. This finding supported that of Souza, Jacob-Filho, Santarém, Silva, Li and Burattini, (2008) that PRE brought about increase in the weight of HIV/AIDs individuals. In addition, it was also supported that PRE is proven to be safe and could be beneficial for adults living with HIV/AIDS in their weight increment (O'Brien et al., 2004).

The finding in table 4 shows that there is a significant difference after 6 weeks' progressive resistance exercise (PRE) on CD4 count of people living with HIV/AIDS. The finding shows that there is a significant increase in CD4 count in posttest compared to pretest. Thus, the result is in line with that of Souza et al. (2008) who reported that PRE had significant increase in CD4 count of elderly HIV people. On the contrary, a previous study reported that there was no significant difference in mean changes of CD4 count of participant in PRE for PLWHA which is not in support of the present findings (O'Brien et al. 2017). There is no significant difference after 6 weeks' progressive resistance exercise on weight of people living with HIV/AIDS. Though, PRE had only a very slight increase (i.e. 69.02 for pretest and 71.09 for posttest) in weight of people living with HIV. Thus, the finding agrees with previous studies that reported that PRE never had any significant effect on weight of elderly HIV people (Crothers et al. 2014; Chaparro et al. 2018). This did not concur with the present study findings because they found in their study that there exists a statistically significant improvement in weight of participants in PRE living with HIV/AIDS (O'Brien et al., 2017; Dianatinasab et al., 2018).

The observed mean increase in CD4 counts of PRE group compared to subjects in the control group shows that PRE has positive therapeutic response on immune system of PLWHA when combined with ARV drugs. This implies that CD4 T-cells' production and proliferations are stimulated by PRE (Aboodarda et al. 2012; Yarasheski et al. 2011). The PRE group equally revealed a higher increase in Weight compared to subjects in the control group. This is very understandable because PRE is a weight bearing exercise that builds the muscle mass

through repetitive maximum and definitely can lead to weights gain of the participants over time (Gresele et al., 2012; Ibeneme et al., 2019; Kovacs and Hoffman 2011).

The revealed significant difference after 6 weeks' progressive resistance exercise on CD4 count suggested that PRE introduction to the management of PLWHA improved the immune system of PLWHA which is associated with the production of CD4 T-cells. Though, this production maybe as a result of proliferation of the T-cells from organs to the blood stream (MacArthur et al., 2012; Maduagwu et al. 2015; Marques et al., 2012). Thus, this may explain why the reports are always said to be noted with caution. The result revealed no significant difference on weight of PLWHA who participated in PRE compared to subjects in the control group maybe as a result short duration of the exercise (Nall, 2005, Ntsekhe and Hakim, 2005; Tiozzo et al., 2013).

CONCLUSION

The major findings of this study have revealed that PRE had positive effect on CD4 counts and weight. The hypotheses result showed that PRE had statistical significant effects on CD4 counts but did not have effects on weight. Based on these findings, recommendation are made among others that awareness should be created by Physiotherapists, Exercise Physiologists, Physicians and Health Educationists to adjunct PRE which boost immune system by improving CD4 counts and in the management of PLWHA.

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Biomedical Communication

Next-Generation Sequencing (NGS) in the Population of Indian Tropical Tasar Silkworm–*Antheraea mylitta*

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ABSTRACT

Antheraea mylitta Drury silkworm, a polyphagous, Sericigenous, lepidopteran insect commonly known as tasar silkworm is a species, found in form of 44 ecoraces, feeding on a number of food plants. In India, it covers more than twenty states as ecoraces, with variations in phenotypic traits like fecundity, voltinism, cocoon weight, silk ratio etc. The distribution of the species has encountered diverse geographic and climatic variations of the distinct areas, leading to marked differences in not only phenotypical and physiological traits but also in the commercial and technological aspects. A. mylitta Drury (Andhra Local ecorace), which is an exclusive ecorace of the states of Andhra Pradesh and Telangana, is well known for its superior commercial characters, but, is on the verge of extinction due to its weaknesses in voltinism, emergence, hatching, low yield etc. The ecorace conservation is essential to utilize their valuable genes in enhancing productivity and to build variation in new population through hybridization. Modern sequencing methods like Next-Generation Sequencing technologies and *in silico* analysis are used in population genetic studies to investigate the evolutionary forces affecting genetic variation. In the present studies, the genomic DNA of parental ecoraces – Andhra local and Daba TV of A. mylitta and their hybrid populations were sequenced independently using the Illumina NextSeq500 in order to analyze their genetic relationship. The sequencing library revealed that the fragment size ranged between 200bp to 700bp and identified 35877 sites in 8 samples. Further, the phylogenetic tree showed closely and distantly related taxa among the populations.

KEY WORDS: ANTHERAEA MYLITTA, ECORACES, NEXT-GENERATION SEQUENCING, PHYLOGENETIC TREE, VARIATIONS.

INTRODUCTION

Antheraea mylitta, a wild sericigenous insect is a species widely distributed in the form of 43 ecoraces from West Bengal in the East to Karnataka in the South with its natural inhabitation in the forest areas of Andhra Pradesh, Bihar, Orissa, Madhya Pradesh, Maharashtra, and Telangana. It is a polyphagous insect feeding on a number of food plants primarily on *Terminalia arjuna* and *T. tomentosa*, and a host of secondary food plants like *Ziziphus, Tectona, Bauhinia, Lagerstroemia*, etc. A wide range of distribution of the species has encountered diverse geographic and climatic variations of the distinct areas, leading to marked differences in not only phenotypical and physiological traits but also in the commercial and technological aspects.

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Among fifty races of sericigenous insects, twenty-five races of *A. mylitta* widely available (Jolly et al. 1974). *A. mylitta* Drury (Andhra Local ecorace), is an exclusive ecorace of the state of Andhra Pradesh, Telangana and is well known for its superior commercial characters. However, it is on the verge of extinction due to its weaknesses in voltinism, emergence, hatching, low yield etc. (Thangavel, 1992; Srivastava et al. 2003; Kavane, 2009). The ecorace conservation is essential for utilizing their valuable genes in enhancing productivity and to build variation in new population through hybridization (Kumaresan et al. 2004; Mirhoseini et al. 2004; Kavane, 2009).

Andhra local ecorace of *A. mylitta* D. possesses superior commercial cocoon traits, but its commercialization could not be undertaken due to weak voltinism, asynchronized moth emergence (35-40%), poor fecundity (165-205), less amenable to human handling and heavy crop loss

during late age silkworm rearing stages resulting in low productivity (10-12 cocoons/dfl). However, this genetic resource material is bestowed with superior commercial characters like low denier (7%), high reliability (66%) and higher silk ratio (16.8%) (Vestergaard et al. 2021). Daba is an adapted ecorace of *A. mylitta* D. with commercial exploitation under the tasar seed sector. This ecorace shows sustenance in cultivating breeding pocket with moderate fecundity (200-250), S.R. % (13.50-14.50%) and good survival (50-60 cocoons/dfl). Hence, the breeding programme has been formulated involving two ecoraces viz., Daba TV and Andhra local ecoraces. This work aims at the improvement of Andhra local ecorace with bestowed with superior characters i.e., survivability and fecundity of Daba TV (Vestergaard et al. 2021).

The breeding of Tasar Silkworm *A. mylitta* D (Andhra Local and Daba-TV) has a rich genetic resource as 44 ecoraces, however, the Tasar culture, an important co-discipline of applied forest biology, needs special understanding and addressing towards breeding perspective to promote the sustainable utilization of this precious natural resource (Manohar et al. 2010). Earlier reports revealed that the genetically pathetic characters of this ecorace could be overcome if methodical breeding activities are undertaken probing into the binding capacities and combining abilities of *A. mylitta*. Identification of several potential markers that contribute to develop genetic characteristics of silkworm population and reveal genetic divergence within low and high yielding strains could have potential practical utility in prospective silkworm breeding program (Vestergaard et al. 2021).

This present work undertaken characterizes the ecoraces of Tasar silkworm, *A. mylitta*, from different parts of tropical forest zones, as a basis for identification and genetic diversity among the tasar populations. Based on these reports, a comprehensive breeding programme could be evolved to conserve the dwindling population of Tasar silkworm, *A. mylitta*, Andhra local ecorace. The application of next-generation sequencing (NGS) technology has led to remarkable advances in whole genome sequencing, which provides ultra-throughput sequences to revolutionize plant genotyping and breeding (Jiangfeng et al. 2014). NGS analysis are in constant demand, thus alignment tools and variant calling tools are still improving and are an active area of research (Vestergaard et al. 2021). Further, NGS usage has also been extended to large crop plants, like maize and wheat, to sequence multiplexed samples that combine molecular marker discovery and genotyping. GBS is a novel application of NGS protocol for discovering Single Nucleotide Polymorphism in plant crop populations.

MATERIAL AND METHODS

For the collection of Tasar Silkworm *A. mylitta* D (Andhra Local and Daba-TV) the parental stocks of ecoraces viz., Andhra Local and Daba TV of Tasar Silkworm *A. mylitta* raised during the seed crop rearing season, July-August. The Andhra local and Daba TV seed cocoons were collected from RTRS (Warangal district) and Telangana State Silk Board (Chennur Mandal, Adilabad district and Mahadevpur, Karimnagar districts) respectively (Fig. 1.a, b). They arranged in the form of garlands in grange chambers. The disinfection of room is using with 2% formaldehyde, prevented by arranging suitable nylon net, ventilated grainage chambers in the laboratory. The date of emergence of each of the ecorace (male/female) noted. The male and female moths emerged out of Non – diapause cocoon stocks of the above divergent geographic ecoraces used for the study.

Figure 1: A. Collection of seed cocoons Telangana State Silk Board; B. Daba- TV Seed cocoons.

The moth emergence was studied during the first grainage season (June-July). The emergence of moths starts late in the evening mostly, between 18.00 to 22.00 hr. Coupling of moths just after emergence is not allowed. By keeping male and female moths separately in the cages for about 2-3 hr so as to from 19.00 hr and continued u to 23.00 hr. The number of moths emerged and male-female synchronization was recorded in Andhra local and Daba-TV parental ecoraces. The male moths start emerging 1- 2 days earlier than female moths. Immediately after emergence male-female moths were selected from different batches as per the requirement, to prepare backcrosses among Andhra local and Daba-TV ecoraces (Fig 2).

Figure 2: Moth emergence of (Andhra local and Daba TV) Tasar Silkworm, *A. mylitta* D.

For the backcross methodology, the backcross breeding of silkworm using parents with preferred traits and selection in subsequent generations offer superior varieties. The outcrossing or repeated backcrossing with exotic races can improve targeted character(s) and induction of polyvoltine traits into uni/ bivoltine enhances resistance in breeds the retention of 50% of superior economic traits of in-situ grown wild ecorace in F1 generation and subsequent attainment of 75%, 87.5% and so on of domesticated blood in BC1, BC2 and so on progenies makes their stock maintenance easy, and to backcross

with amenable Daba ecorace than choosing a wild ecorace (in-situ or ex-situ) as donor (recurring parent) for backcrossing (Aruga, 2001).

Breeding of silkworm means the improvement of silkworm strains, which is an extremely important issue for the silk industry. Methods adopted for the improvement of silkworm strains are more or less the same as those in other animals and plants. Three methods are followed viz., (1) selection of pure lines, (2) cross-breeding, and (3) induction of mutations. At present, out of the three methods, cross-breeding is the most popular and widely applied to develop a large number of strains. In cross-breeding, in most cases, an objective is set and decided upon and strains suitable for the purpose are carefully selected. By producing hybrids of the two parent strains, various combinations of characters manifested in the filial generations are observed and the excellent ones are selected and fixed (Aruga, 2001). In the present study, the breeding of tasar ecoraces was done as follows: The preparation of F1 hybrids was done by crossing Daba TV (donor/female) and Andhra local (recipient/ male) which is aimed at the introgression of survivability traits.

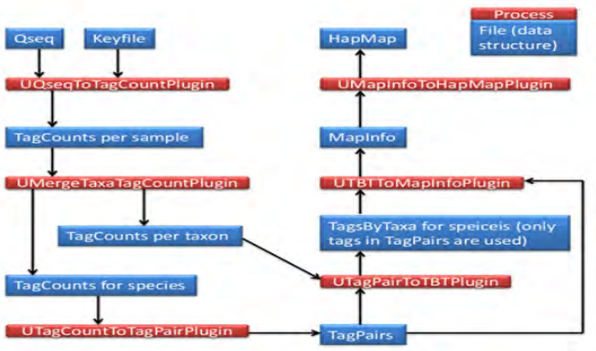
Crosses were made by releasing male and female moths in the ratio of 2:4 under in situ conservation model The dfls of F_1 was prepared and incubated in the laboratory. The F_1 progeny was brushed and reared under ex-situ conditions during the first crop season.Parental stocks of Daba and Andhra local were maintained in separate locations to prevent chances of intermixing of genetic characters. The F_1 hybrid male (Daba TV X Andhra local) was backcrossed with the female of Andhra local to produce the back-cross dfls in F_2. Incubation of back-cross dfls was carried out in the laboratory. The F_2 progeny was brushed and reared under ex-situ conditions during the second crop season.Observations and interpretations were noted down.

Production of F_1 and F_2 Hybrids: During hybridization, Daba TV (female) was crossed with Andhra local (male) to produce F_1 hybrids, in which Daba TV (female) will be donor parent whereas male counterpart of Andhra local acts as recipient parent. F_1 (male) was crossed with Andhra local (female) to produce F_2 hybrids. Rearing performance of different backcrosses was observed for different parameters and recorded as follows. During hybridization, in the next crop, i.e., during September, F_1 (male) was crossed with Andhra local (female) to produce F_2 hybrids, in which F_1 (male) will be donor parent whereas male counterpart of Andhra local acts as recipient parent. This method is aimed at introgression of survivability traits of Andhra local ecorace. Genomic DNA isolation: The Genomic DNA isolation and the quantification of the 1-8 samples of Tasar Silkworm, *A. mylitta* D Parental ecoraces (Andhra local, Daba TV, F_1 and F_2) of each line by using HiPurATM Insect DNA Purification Kit.

DNA was dissolved in TE buffer (Tris-EDTA, pH 8.0), revealed an ideal concentration of DNA ng / µl of the genomic sample i.e., between 1.8 – 1.9 in 260 / 280 ratio) checked against 1 kb standard DNA ladder was obtained. It been observed that DNA has been isolated without any protein or any other contamination and was used for further studies in PCR and NGS analysis. Genetic characterization of 4 populations viz., parental ecoraces (Andhra local, Daba TV), F_1 and F_2 were done (Suzuki et al. 1972; Nagaraja and Nagaraju 1995; Aruga, 2001). Database & Tools used for analysis of sequence: The genomic DNA of *A. mylitta* was sequenced independently using the Illumina NextSeq500; 75 paired-end and data analysis were done by FastQC1, bowtie2, and Tassel-33 respectively. Total no of sample sequences is 8 and PstI-HF – MluC1 enzymes were used. Later, the sequence was Converted using tagCount, this plugin derives a tagCount list for each sample. It keeps only good reads having a barcode and a cut site. Trims off the barcodes and truncates sequences that have a second cut site, or read into the common adapter. In the current studies, Tassel3 UNEAK pipeline, a denovo GBS analysis approach was opted due to the lack of reference genome. The entire protocol (Fig 3 and 4) is explained step by step in the following sections.

Figure 3: Experimental Work flow for Genotyping by Sequencing Library Preparation.

Figure 4: Schematic Overview of denovo GBS analysis workflow.



Library Preparation and Sequencing: GBS Library Preparation was performed at Genotypic Technology's Genomics facility. 300 ng of Qubit quantified genomic DNA was used for double digestion by Pst-HF and MluCl restriction enzyme at 37°C for 4 hours. The resulting digested fragments were directly ligated to pair of restriction site-specific adapters at 20°C for 1hour using T4 DNA ligase. The adapter ligated fragments were subjected to PCR amplification (10 cycles) to amplify the adapter-ligated fragments and to add sample specific dual index barcodes (Nextera XT v2 index kit, Illumina, U.S.A.). The amplified Illumina-compatible sequencing library was quantified by Qubit fluorimeter (Thermo Fisher Scientific, MA, USA) and its fragment size distribution was analyzed on Agilent 2200 TapeStation (Table1).

Table 1. Genomic DNA samples of parental and hybrid ecoraces of Tasar Silkworm *A. mylitta* D and their indexes.

#	Sample ID	Index1	Index1 Sequence	Index2	Index2 Sequence
1	SO_7874_Repl_S1	N701	TAAGGCGA	S507	AAGGAGTA
2	SO_7874_Repl_S5	N702	TAAGGCGA	S507	AAGGAGTA
3	SO_7874_Repl_S7	N703	TAAGGCGA	S507	AAGGAGTA
4	SO_7874_Repl_S8	N704	TAAGGCGA	S507	AAGGAGTA
5	SO_7874_Repl_S6	N705	TAAGGCGA	S508	CTAAGCCT
6	SO_7874_S2	N706	TAAGGCGA	S508	CTAAGCCT
7	SO_7874_S3	N707	TAAGGCGA	S508	CTAAGCCT
8	SO_7874_S4	N710	TAAGGCGA	S508	CTAAGCCT

Table 2. Complete summary of the Taxa of parental and hybrid ecoraces of Tasar Silkworm *A. mylitta* D.

Stat Type	Value
Number of Taxa	8
Number of Sites	35877
Sites x Taxa	287016
Number Not Missing	245187
Proportion Not Missing	0.85426
Number Missing	41829
Proportion Missing	0.14574
Number Gametes	574032
Gametes Not Missing	490374
Proportion Gametes Not Missing	0.85426
Gametes Missing	83658
Proportion Gametes Missing	0.14574
Number Heterozygous	75236
Proportion Heterozygous	0.26213

Table 3. Summary of Alleles of parental and hybrid ecoraces of Tasar Silkworm *A. mylitta* D.

	Alleles	Number	Proportion
C	45181	0.15742	0.18427
G	43408	0.15124	0.17704
N	41829	0.14574	0.1706
T	41490	0.14456	0.16922
A	39872	0.13892	0.16262
Y	24637	0.08584	0.10048
R	23574	0.08213	0.09615
W	9054	0.03155	0.03693
M	6716	0.0234	0.02739
K	6112	0.02129	0.02493
S	5143	0.01792	0.02098

RESULTS AND DISCUSSION

The sequencing library for all the samples are suitable for Illumina sequencing studies and found that the fragment size ranged between 200bp to 700bp. As the combined adapter size is approx. 120bp, the effective user- defined insert size is 80bp to 580bp (Table1). The GBS analysis of 8 samples revealed the related information of taxa; gametes, Number of Gametes, Gametes not missing, Proportion Gametes not missing, Gametes missing; heterozygotes- number and proportion. The results are shown in (Table 2). The GBS analysis of 8 samples revealed the related information of taxa; gametes, Number of Gametes, Gametes not missing, Proportion Gametes not missing, Gametes missing; heterozygotes-number and proportion. The results are shown in (Table 2). The GBS analysis of 8 samples revealed the related information of taxa; gametes, Number of Gametes, Gametes not missing, Proportion Gametes not missing, Gametes missing; heterozygotes- number and proportion. The results are shown in (Table 2). GBS sequencing analysis showing no. of alleles with their proportions are identified with respective nucleotides and amino acids (Table 3).

The sequenced 8 samples and identified their populations with related taxons. Moreover, the identified SNPs at the same location in all the sequences were found. When cluster analysis was performed, all the eight samples were same in length and clustered together at

identical in sequence length i.e., the range of 4 – 9. The eight sequences have shown unique GC rich regions (Table 4). GC content (guanine-cytosine content) was found to be high in S1, S5 and S6, compared to S2, S3, S4 and S7, However, a species with an extremely low GC–content was S8 (Fig.5 and 6). The figure 7 shows the comparative analysis of 8 sequences. From the sequence analysis it was found that some of the amino acid regions were aligned together as shown in green colour. The Phylogenetic tree has shown formation of Three clusters. F_1 and F_2 parents have formed individual clusters. Parental ecoraces and populations formed clusters (Fig 8).

Table 4. Information for the eight benchmark sequences and sequences Statistical analysis data of parental and hybrid ecoraces of Tasar Silkworm *A. mylitta*

sample method	S1	S2	S3	S4	S5	S6	S7	S8
Sequence length	75	75	75	75	75	75	75	75
GC%	36	35	36	36	36	35	35	33
Phrad Score	34	34	34	34	34	34	34	34
Clustered	6-8	4-9	4-9	3-9	4-9	4-9	5-9	4-8
Kmer	1-5	1-5	1-5	1-5	1-5	1-5	1-5	1-5
Total Sequence	2644277	2877078	3365729	2380003	3330948	3948642	2984491	1376395

Figure 5: Distribution of GC percentage.

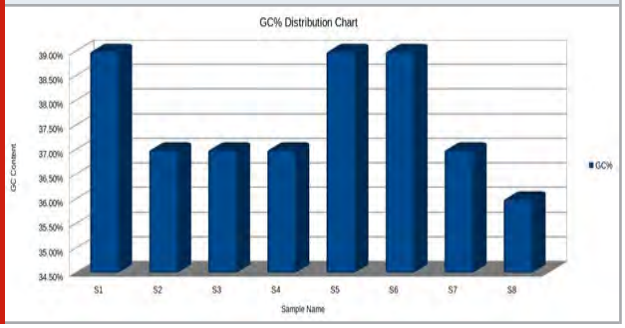


Figure 6: Sequences Read distribution.

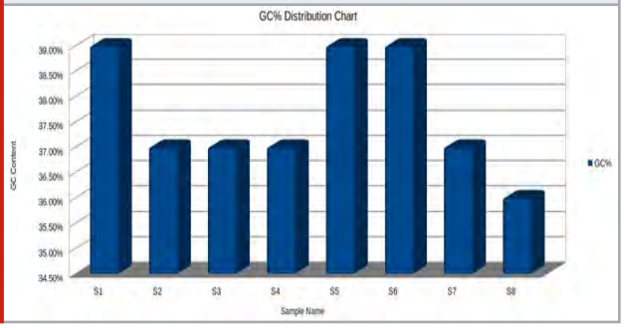
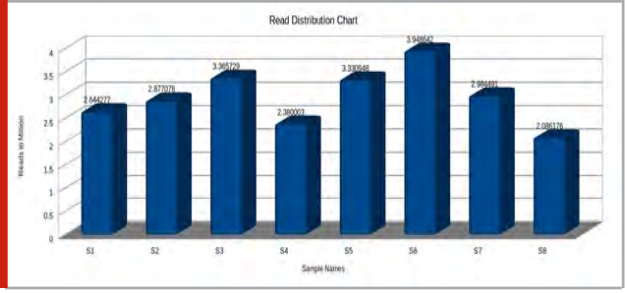


Figure 7: Eight samples DNA sequence as highlighted basis on their amino acid function.



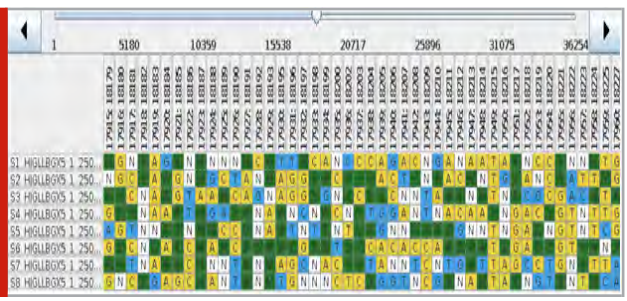
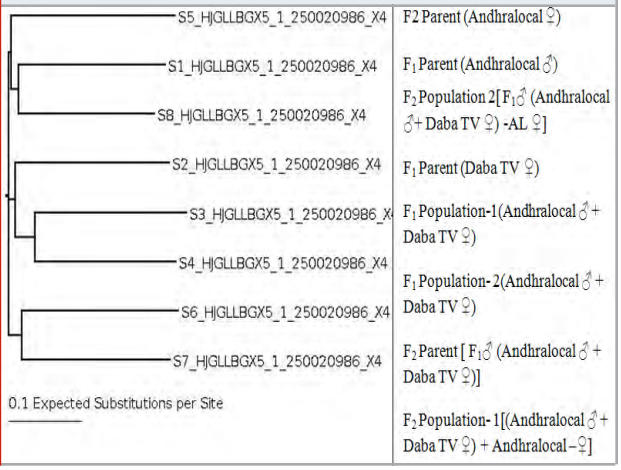


Figure 8: Phylogenetic analysis of Tasar Silkworm, *A. mylitta* D as obtained by MEGA.



F_1 parent & F_2 population formed one cluster, F_1 parent & F1population formed one cluster, F_2 parent & F_2 population formed one cluster. The molecular characterization and phylogenetic analysis revealed parental ecoraces and populations formed one cluster,

while F_2 Parent and F_1 Parent formed individual clusters. It may be noted that the F_2 population is genetically closer to the parental cluster (AL male-DTV female) than that of F_1. It is also interesting to note that F_2 population is closer to F_1 parent, that is Andhra local male.

NGS protocols for discovering and genotyping SNPs for crop improvement. The low cost of GBS makes it an attractive approach to saturate the mapping and breeding populations with a high density of SNP markers. Successive improvements of the sequencing chemistries and base-calling software will allow NGS technologies to deliver higher sequencing throughputs per run, which in turn enables deeper multiplexing for a fixed average sequencing depth per sample (Elshire et al. 2011; Poland et al. 2012). As the sequence-based genotyping is available for a whole range of genomic studies, GBS will stand to be one of the major components in silkworm genetics and breeding. Even though the availability of reference genomes is very useful to eliminate repetitive sequences, GBS can be used without a reference genome, by either using consensus sequences of reads as the reference or using the tags simply as dominant markers (Elshire et al. 2011). In addition, GBS markers can be done with extremely high accuracy, with a reference genome in biparental mapping populations, allowing for low coverage of the offspring at an even lower cost, after genotyping parents and their offsprings at high coverage (Elshire et al. 2011; Poland et al. 2012).

The sequencing of 8 samples in the present investigation has identified their populations with related to taxons and identified the SNPs at the same location in all the sequences. The cluster analysis of the eight samples has shown that they were of same length and clustered together at i.e., the range is 4 – 9. All such sequences have also shown unique GC rich regions (Poland et al. 2012). GC content (guanine-cytosine content) is found to be high in S1, S5, and S6 compared to S2, S3, S4, and S7. However, a species with an extremely low GC–content is S8. Most NGS platforms are able to generate reliable sequences and display near perfect coverage behavior on GC-rich, neutral and moderately AT-rich genomes. However, there are key differences between the quality of that data and the applications it will support. Illumina is less predisposed to homopolymer errors, it shows an overall accuracy greater than 99.5%, but sometimes can provide under-representation of regions (i.e., AT/GC-rich) and nucleotide substitution errors (Bentley et al. 2008; Dohm et al. 2008; Harismendy et al. 2009; Nakamura et al. 2011; Quail et al. 2012).

We applied the nine trimming algorithms on four different datasets (see Materials and Methods). The quality of these datasets was assessed with FastQC (see File S1 and Figure S1 for Q distribution plots) and measured by different metrics, such as the average PHRED error score, GC content biases and position-specific quality variations. The datasets vary conspicuously, possessing almost perfect quality parameters for the Yeast DNA-Seq dataset and somehow average-to-high for Lovell raw reads (Figure S1).

CONCLUSION

The sequencing of 8 samples in the present investigation has identified their populations related to taxons and identified the SNPs at the same location in all the sequences. The cluster analysis of the eight samples has shown that they were of same length clustered together at i.e., the range is 4 – 9. All such sequences have also shown unique GC rich regions. GC content (guanine-cytosine content) is found to be high in S1 (Andhralocal (male) –F1 Parent), S5(Andhralocal (Female) – F2 Parent), and S6(F1 (male) – – F_2 Parent) compared to S2(Daba TV (Female) – (F_1 Parent), S3 (F_1 Population1), S4 (F_1 Population 2) and S7(F2 Population 1). However, species with an extremely low GC-content is S8. The conservation of Tasar silkworm, A. mylitta D, Andhra's local ecorace needs further improvement on breeding practices, to enable the viability of F_1 and F_2 generations. The present investigation on PCR based Phylogenetic analysis using Mega Software in 4 tasar populations viz., parental ecoraces [(Andhra local, Daba TV), F_1 and F_2], derived from hybridization between two contrasting genetically variable ecoraces viz., of Andhra local and Daba TV was successful for two successive commercial crops from commercial viewpoint.

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Conflict of Interests: The authors declare no competing interests among themselves while conducting the research and preparing the report.

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Medical
Communication

Status of Various Clinical Attributes and Electrolytes in Oral Cancer Patients from Pakistan

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ABSTRACT

Cancer is a lethal disease and its incidences has been increasing day by day. The survival rates of the cancer patients are lesser due to lack of its awareness, available health facilities and other many socioeconomic conditions to treat it. Various abnormal metabolic and ionic changes occur in throat and mouth cancer patients than healthy persons. In this experiment, with average 36-42 years aged 21 throat and 24 mouth cancer patients were selected to analyze the changes in complete blood counts (CBC), liver functional tests (LFT) and electrolytes. Among the patients, Hb (g/dL) decreased and PLT (10^8/µL) increased significantly in both typed cancer patients than normal healthy persons. Higher ALP and bilirubin levels were observed high ($p \leq 0.05$), while ALT and urea concentration remained unchanged among the patients to normals. Serum electrolytes i.e. Mg^{2+} was observed higher in patients ($p \leq 0.05$), while Cl^- and Ca^{2+} were decreased significantly in both throat and mouth cancer patients. The K^+ mildly increased non-significantly remained within the reference ranges. Changes in biosynthesis of metabolites induces electrolyte imbalanced condition as well as the alterations in HB and PLT may also be caused by imbalanced nutrition-based factors. By early careful monitoring of such indicators in the cancer patients could play a preventive role in cancer disease prognoses.

KEY WORDS: CANCER PATIENTS, ELECTROLYTES, SERUM, CBC, LFTS, PLATELETS.

INTRODUCTION

In the world, the 2nd largest human killer disease is cancer with 25 % of total deaths per year including Pakistan and it has been elevating in numbers continuously (Jemal et al., 2010; Simard et al., 2012; Moore, 2013; Torre et al., 2016; Murray et al., 2020). New cancer incidences are increasing in Asian Pacific regions, while survival rates remained lesser due to lack of cancer awareness, lake of health facilities and other many socioeconomic conditions (Hanif et al., 2009; Begum et al., 2012; Cao et al., 2017; Ashaq et al., 2021). The cancer is the multi-factorial condition of organs or bodies, which have lost the control on their growth with excessive cell proliferation (Weinberg, 1996; Schmelzle and Hall, 2000; Chan and Steven, 2021). The causative cancer agents are originated with mutual sharing of multiple environment and genetic factors (Knox, 2010; Parsa, 2012; Yadav and Khodke, 2015; Caruso et al., 2021; Singh et al., 2021).

The environmental exposure on inters or intra-cellular are developing cancer gradually. Available preventions may be adopted against the accomplished factors, which are actively involved in disruption of cellular signaling pathways (Yadav and Khodke, 2015; Huang and Ping-Kun, 2020). Major priority is still prevention

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of carcinogenic exposures (Anetor et al., 2008; Rudel et al., 2014; Felter et al., 2021), while particular genetic pre-dispositions are more susceptible than others. Like as people with genetic alterations in BRCA1, BRCA2 and p53 locus have lost the abilities to suppress irregular cell growth (Bièche and Lidereau, 1995; Ingvarsson, 1999; Hilton et al., 2002; Narod and Foulkes, 2004; Colonna and Amanda, 2016; Ababou, 2021).

Meanwhile, a number of factors including gene locus, drugs, environment, industrial pollution, chemicals, radiation, food additives, diet, changed life style could be source to contribute in cancer disease process. Even individuals are living in the same environment and in some individual the cancer develop while not in others. The phenomena could be inherited genetic susceptibility for cancer (Douglas and Wildavsky, 1983; Lichtenstein et al., 2000; Hu et al., 2021). Natural chemicals of human diet appear to be major cause of DNA damage (Ames, 1979). In spite of that many studies are emphasizing that cumulative effects of chemicals among the individuals are acting with different path-ways. These chemicals and their related systems among the cells, tissues and organs could be plausibly conspire in the process to induce tumorigenesis and then ultimately into malignancies (Casey et al., 2015; Hu et al., 2015; Narayanan et al., 2015; Mwila et al., 2021).

The electrolyte disturbance might be involved in the mediation of cancerous micro-environment for the pro-carcinogenic outcomes. The role of dysregulation of electrolytes-homeostasis is the most recognized feature, which enable the induction, maintenance and progression of cancer (Woolf et al., 2016; Kamanga and Zhou, 2017; Robey et al., 2017; Diala, 2020). The electrolytes are involved in the regulation of the many intra-cellular systems like as protein synthesis, mutagenesis and oncogenesis. These regulatory intra-cellular metallic Mg, Na, K, Cl, H and Ca ions have specific correlations with physiological and growth rates of the cells. Among the turmeric cells, the elevated concentrations of Na and Cl are observed but Mg and K ions remain normal.

The regulation of Na and Cl concentrations have got prime importance to control their concentrations in both normal and tumor cells (Kopec and Groeger, 1988; Cameron and Smith, 1989; Varghese et al., 2021). It has been considered that many metabolic changes are occurring in cancer diseased organs, while an imbalanced electrolyte character is one of them. The present study is aimed for the careful monitoring of various electrolytes in the serum of cancer patients. As it plays destructive role in cancer prognosis.

MATERIAL AND METHODS

Subjects in the study: In present study, the women participated from Nursing Civil Hospital, Mirpur Khas District are arranged into two groups. The total numbers of subjects were confirmed 45 patients and first group of 21 throat and 24 mouth cancer patients (each group divided into 4 sub-groups with 6-indviduals = 1 replicate). Each individual patient was subjected for clinical as well as biochemical analysis. Here in this study, the confirmed replicates of each category were compared for the subjects reported, ethical clearance for the present study was obtained vide letter Ethical letter No Physiol. / ERI /60 / 08.05.19.

Collection of blood samples: The fresh ten milliliters blood serum samples of selected cancer patients in fasting were collected into standard Vacutainer® plain vials [anti-coagulated with EDTA-K_2 (ethylene di-amine tetra-acetic acid-dipotassium] from each participant in the blood diagnostic laboratory. The half of each blood sample was transferred for hematological analysis and other half allowed stand at room temperature to clot. The clotted blood was centrifuged for 15 min at 3,000 rpm for 15 min and plasma/serum used for electrolyte estimation and other different biochemical analysis (Alghamdi et al., 2012; Kumar and Gill, 2018).

Hematological Analysis of Samples: A number of hematological attributes were analyzed in collected blood samples of the patients. The parameters including CBC (complete blood count), hematocrit (HCT), Hb level, mean cell Hb (MCH), MCH concentration (MCHC), mean cell volume (MCV) and red blood cell distribution width were determined with Hematology Analyzer (Model-Advia 2120, Bayer Diagnostics, USA) and the liver marker i.e. alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin and creatinine and red cell distribution width (RDW) were determined with Beckman Coulter Automatic Biochemical Analyzer by following their procedure given in the manuals (Harthoorn-Lasthuizen et al., 1999; Bereket et al., 2011; Koster, 2015; Wang et al., 2017; Haq et al., 2018).

Determination of Electrolytes: Before absorption, the samples were digested by taking 0.5 ml sample in glass tubes than 3 ml crucibles (20 ml conc. HNO_3 + 40 ml H_2O_2) added. The mixture was placed at hot plate (90°C) for 2 hrs. When sample color appears a pale-yellow allow it to cool-down and 10 ml 0.2 M HNO_3 added. Its filtrate is used to run on atomic absorption spectrophotometer [Hitachi-Ltd 180-50 (Sr. #. 5721-2) with graphite furnace G-03] and ion selective electrode (ISE) methods (Transasia Bio-Medicals Ltd) for analysis of electrolytes including sodium, magnesium, calcium and chloride (Kamei et al., 1998; Kazi et al., 2006; Yadav and Khodke, 2015).

Statistical Analysis: The present study was comprised on 04 replicates per cancer patient's category. The collected data expressed as mean with standard error (St. Err.) and these analyzed with CoStat (version 3.03) CoHort software (Berkeley, USA). The significant ($p \leq 0.05$) mean values further analyzed with Duncan Multiple Range Test (DMRT) at 5 % (d Steel and Torrie, 1986; Behrens, 1997; Johnson and Bhattacharyya, 2019).

RESULTS AND DISCUSSION

The cancer prevalence has been increasing day by day, leading to acute need of care for growing population

of patients. It is essential to adopt patient-centered approach to achieve good quality care. There step-care approach (i.e. correct and timely detection) for these patients is crucial to manage the increasing severity of the disease. In present study, clinical samples of 21 throat and 24 mouth cancer patients (36-42 years aged) were subjected for various biochemical assessments including complete blood counts (CBC), liver functional tests (LFT) and electrolytes (Table 1).

Table 1. Comparative analysis of clinical attributesincluding electrolytes in patients of mouth and throat cancers to healthy persons.

#s.	Characters	Reference values	Healthy persons	Cancer patients		F-signif-icance
				Mouth	Throat	
A. Complete blood count (CBC) analysis						
01.	Hb (g/dL)	13.0-18.0	^{a}13.93±0.281	^{b}11.20±0.667	^{b}11.53±0.599	7.527^{ns}
02.	WBC (109/L)	3.50-10.0	^{b}8.100±0.158	^{a}10.55±0.790	^{b}12.300±0.579	13.87**
03.	RBC (1012/L)	3.50-5.50	^{b}4.855±0.197	^{a}5.248±0.449	^{b}3.800±0.171	6.235^{ns}
04.	HCT (%)	35.0-55.0	^{a}36.75±1.461	^{b}34.00±1.046	^{bc}32.38±0.892	3.645^{ns}
05.	MCV (fl)	75.0-100	^{a}85.90±0.938	^{ab}83.73±2.939	^{b}73.40±0.660	13.44**
06.	MCH (pg)	25.0-35.0	^{a}27.15±0.504	^{b}23.63±1.250	^{ab}26.13±0.774	4.083^{ns}
07.	MCHC (g/dl)	33.0-38.0	^{a}35.25±0.841	^{ab}34.00±0.471	^{b}33.13±0.507	2.884^{ns}
08.	RDW (%)	11.5-15.5	^{a}13.21±0.121	^{b}16.45±0.074	^{ab}15.42±0.147	3.224^{ns}
09.	PLT (108/µL)	150-400	^{a}253.8±11.24	^{b}342.0±14.14	^{c}410.3±9.801	43.74***
10.	NEU (%)	35.0-80.0	^{b}62.25±2.428	^{a}79.75±2.496	^{a}79.75±2.955	14.67**
11.	LYM (%)	15.0-50.0	^{a}31.00±1.472	^{b}48.25±1.887	^{b}52.50±2.102	28.95***
12.	MO (%)	2.00-10.0	^{a}3.250±0.479	^{b}2.250±0.250	^{b}2.000±0.000	4.500^{ns}
13.	EOS (%)	1.00-6.00	^{a}3.000±1.472	^{a}2.250±1.887	^{a}2.250±2.102	1.929^{ns}
14.	BASO (%)	0.00-0.20	^{a}0.119±0.013	^{b}0.093±0.001	^{b}0.087±0.004	5.148^{ns}
B. Liver functional tests (LFT)						
01.	ALP (UL^{-1})	36.0-141	^{a}120.5±4.444	^{b}96.75±5.360	^{c}81.50±4.330	17.24**
02.	ALT	4.0-38	^{a}20.75±1.887	^{b}12.75±1.797	^{b}15.00±1.225	6.158^{ns}
03.	BR (mg dl^{-1})	0.20-1.2	^{a}0.950±0.065	^{a}0.825±0.144	^{b}0.425±0.048	8.331^{ns}
04.	Urea (mg dl^{-1})	10.0-40.0	^{b}17.63±1.253	^{a}56.30±2.464	^{a}50.00±3.536	64.14***
05.	Crt (mg dl^{-1})	0.4-1.40	^{a}0.880±0.149	^{a}2.175±0.858	^{a}1.075±0.111	1.899^{ns}
C. Electrolyte analysis						
01.	Mg^{2+} (mg/dL)	1.50-2.10	^{b}1.761±0.085	^{a}2.188±0.035	^{a}2.228±0.049	18.55**
02.	K^{+} (mmol/L)	3.50-5.0	^{a}4.165±0.070	^{b}4.425±0.078	^{b}4.458±0.077	4.561^{ns}
03.	Cl^{-} (mmol/L)	95-108	^{a}101.8±1.071	^{b}97.70±0.615	^{b}97.00±0.221	12.84**
04.	Ca^{2+} (mg/dL)	8.10-10.2	^{a}9.248±0.129	^{b}8.638±0.098	^{b}8.633±0.097	10.56*

CBC: Complete blood count, RBC: Red blood cells, Hb: Hemoglobin, MCV: Mean corpuscular volume, HCT: Hematocrit, MCH: Mean carpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, RDW: Red cell distribution width; NEU: Neutrophillus, MO: Monocytes, EOS: Eosinophillus, LYM: Lymphocytes, BASO: Basophillus, WBC: White blood cells, PLT: Platelets, ALP: Alkaline phosphatase, ALT: Alanine aminotransferase, BR: Bilirubin, Crt: Creatinine, Mg+2: Magnesium, K+: Potassium, Cl-: Chloride, Ca+2: Calcium.

Note: The data shown in table is mean of four replicates expressed as mean ± SE. The Duncan's Multiple Range (DMR) test calculated at 0.05 % significance ($p \leq 0.05$).

As in table 1, results showed that means of red cell indices in both mouth and throat cancer patients likely to MCV (83.73±2.939 fl and 73.40±0.660 fl; $p \leq 0.05$), MCH (23.63±1.250 pg and 26.13±0.774 pg; non-significant) (34.00±0.471 pg and 33.13±0.507 pg; non-significant) and MCHC (34.00±0.471 g/dl and 33.13±0.507 g/dl; non-significant) observed lowered respectively than healthy control person. The mean values of red cell distribution width (RDW) observed higher in the patients than healthy persons but non significantly. The hematological tumors have observed in close relation with malnutrition, changes in microenvironment and disturbances in erythropoiesis, while RDW levels symbolize the functional survival of abnormal RBC (red blood cell) (Mantovani et al., 2008; Lee et al., 2014; Huang et al., 2016; Matsui et al., 2021).

From other CBC parameters, the WBCs, lymphocytes and neutrophil showed higher concentrations in both typed cancer patients than healthy controls. Due to a fact that neoplasms are associated neutrophilia, so demarginating neutrophils are normally tumor cells which are occupying the vascular spaces. These are killed by $CD8^+$ cytotoxic T-lymphocyte without prior sensitization, while for evading immunity, there is downregulation of class 1 MHC (major histocompatibility complex) molecule's expression (Warren et al., 1994; Swigut et al., 2004; Mwimanzi et al., 2017). There the lymphocyte count observable either elevated or depressed. The platelet counts also found higher in cancer patients than healthy control (Table 1).

From the results of liver function tests, the blood urea is a sensitive indicator of abnormal renal functions. Its levels from control to patients were increased (Chauhan, Yadav, Kaushal, & Beniwal, 2016). The serum creatinine (Crt) level is further considered as more sensitive over urea for kidney function and it is observed increased ($p > 0.05$) in patients (Devi, 2015). Meanwhile, increased levels of serum uric acid among the cancer patients (Iseki et al., 2001; Zhu and Cao, 2012; Nincevic et al., 2019) associated renal insufficiencies (Hunsicker et al., 1997; Iseki et al., 2001; Rapa et al., 2020). Activities of serum alkaline phosphatase (ALP) observed significantly lower than healthy persons.

In other studies, the observed parameter was found between the normal ranges (Stieber et al., 1992; Van Hoof et al., 1992; Ray et al., 2017). Similar trend in data also observed total bilirubin levels (Liu et al., 2014). For alanine aminotransferase (ALT), data showed lower ALT in both typed cancer patients than healthier ones but between the normal ranges. The ALT is a sensitive indicator but lack in specificity for hepatocellular injury as it is also in muscles and kidneys (Söderberg et al., 2010; Weber et al., 2019).

he data about the serum electrolytes, the Mg^{2+} levels observed significantly higher from the healthy persons (1.761 ± 0.085 mg/dL) in the mouth (2.188 ± 0.035 mg/dL) and throat (2.228 ± 0.049 mg/dL) cancer patients, while non-significant increase in K^+ level found among the patients. The serum chloride levels in normal healthy controls were 101.8 ± 1.071 mmol/L, whereas 97.70 ± 0.615 mmol/L and 97.00 ± 0.221 mmol/L in mouth and throat cancer patients respectively. The statistically significant low chloride (Cl) levels were observed in serum of cancer patients from healthy controls but values remained within the reference ranges (McAndrew et al., 2021). Alea et al., (2017) group found that serum levels of Ca^{2+} was significantly decreased in patients in comparison to control group (Thompson, 2010; Doshi et al., 2012; Ephraim et al., 2014). The electrolyte imbalance causes additional risk factor in cancer patients. The prior detection of electrolyte levels night be helpful for the clinicians to manage the cancer prognosis and their proper monitoring (Berghmans et al., 2000; Capdevila et al., 2018; Fassnacht et al., 2018).

CONCLUSION

We have concluded in present study that the imbalanced attributes of CBC, LFTs and electrolyte contents impose additional health risk factors in cancer patients. Their prior detection among the patients might be helpful for the clinicians to manage the cancer prognosis and their proper monitoring. This study of bio-content's analysis may be a helpful diagnostic tool in terms of minimizing the imbalanced characters in patients with different supplements.

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Pathological
Communication

Enzyme Alterations in Haemolymph of the Silkworm, *Bombyx mori* During Grasserie Infection

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ABSTRACT

The silkworm *Bombyx mori* is a domesticated tiny insect having a remarkable economic significance. Occurrence of diseases in silkworm *Bombyx mori* is fairly common and inflict serious losses. The major disease affecting mulberry silkworms is Grasserie, which is a viral infection causing reduced production of silk in India and other countries primarily by the *Bombyx mori*, *Nuclear Polyhedrosis Virus* (BmNPV). In India, >50 % of silk cocoon crop losses are attributed to BmNPV infections. Presently, there are no specific preventive measures to treat the spread of BmNPV infection other than sanitized rearing methods, where the only commercial practice today is to discard large stocks of worms during the infection. Once infected, the disease pathogen in silkworm becomes highly efficient at manipulating the physiological and endocrinological responses of the body. In order to provide references for further study on the infection and pathogenesis of BmNPV, this research was explored for the changes in the haemolymph enzyme activities. For the study, healthy and Grasserie infected mulberry silkworms were collected from the local sericulture units and reared in the laboratory to analyse the intensity of enzyme activity. The enzyme profile of both healthy and infected worms was recorded by using Clinical Analyser. The study revealed, significant decrease in enzyme activity of alkaline phosphatase, acid phosphatase, alanine amino transferase, aspartate amino transferase, in the infected larvae as compared with control healthy silkworms. Enzyme alterations reported in the present study, can be used as a marker, for indication of Grasserie disease in local mulberry silkworm colonies.

KEY WORDS: ACID PHOSPHATASE, ALANINE AMINO TRANSFERASE, ALKALINE PHOSPHATASE, ASPARTATE AMINO TRANSFERASE, BOMBYX MORI.

INTRODUCTION

he silkworm *Bombyx mori* is a domesticated tiny insect having considerable economic significance. It is a well-known and economically important insect as it is a producer of valuable silk. Over 85% of the global silk production comes from the mulberry silkworm, *B. mori*. In India, the silk industry has made significant progress during the past two decades where it occupies the second position in global silk production next to China. As per the statistics, India has 82 lakh farmers in 62,000 villages engaged in sericulture and Indian raw silk production is 35,000 metric tonnes, (Statista Research Department, 2021).

Diseases in silkworm *Bombyx mori* are fairly common in occurrence and are serious in inflicting losses. The country has produced 35,820 MT of silk against the target of 38,530 MT during 2019-20 achieving 93.0% of the target. This is the progress during the past decades of silkworm industry but, this progress stops or reduces production when disease occurs. Grasserie is a viral infection, in silkworms which is the major cause for damage and reduced production of silk in India and other countries. The disease is caused primarily by *Bombyx mori Nuclear Polyhedrosis Virus* (BmNPV). The disease occurrence is a common phenomenon during all stages in silkworm rearing (Joshi and Raja 2013, Annual Report 2020).

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The investigation revealed by Joshi (2015), these pathogenic diseases form major factors that greatly contribute to the crop failure and instability in cocoon production in all the rearing seasons. Once infected the disease pathogen in silkworm becomes highly efficient at manipulating the physiological and endocrinological responses of the body. In the present study, by doing investigation the oxidizing enzymes activity in the hemolymph of healthy or control and infected or diseased silkworms was recorded. Aspartate amino Transferase (AST), Alanine amino Transferase (ALT), Alkaline Phosphatase (ALP), Acid Phosphatase (ACP) are the parameters widely used to understand the effect of diseases or infection, as these could give indications of progressive toxic effects long before the actual manifestation of the disease. The mode of viral infection and the activity of enzyme metabolic alterations involvement in silkworm immunity was studied by Babu et al. (2009). This study reveals, to understand the molecular and metabolic changes in diseased and control silkworms, it may form the potential basis and can be used in other fields such as medicine and virology (Babu et al. 2009; Joshi 2015 Gau et al 2020).

Rajitha and Savitri (2014) reviewed that among the many constraints that influence the success of cocoon production, the menace of disease is the prime one. The major disease affecting mulberry silkworm is Grasserie. The Grasserie disease occurs when the silkworms feed on mulberry leaves, contaminated with BmNPV (Rajitha and Savitri 2014; Jiang and Xia 2014 Gao et al 2020). All these biomolecules play very important role in many pathways that operate within the living cells as they are also the intermediates of many physiological reactions The enzyme levels in haemolymph especially Alkaline phosphatase (ALP), Acid phosphatase (ACP), Aspartate amino Transferase (AST) and Alanine amino Transferase (ALT) are the parameters broadly used in the disease diagnosis in Silkworms and it can be used as a model organism in virological research (Jiang and Xia 2014, Gao et al 2020).

The present study reveals that the enzyme alterations observed could prove a good model for studying the interaction between insect and virus. Alterations in the enzyme parameters, on exposure to disease causing pathogens, in the hemolymph could be used as an indicator for the health status of Silkworm *Bombyx mori*, as well as can be taken as target system to measure physiological and biomolecular stress induced alterations in the body during the occurrence of diseases.

MATERIAL AND METHODS

In the present study healthy and Grasserie infected mulberry silkworm larvae were collected from the local sericulture units, carried and reared in the laboratory. The haemolymph of reared silkworms were collected at early (Day 3) and late (Day 6) stages of Vth instar larva. Haemolymph was collected everyday into pre-chilled centrifuge tubes with a pinch of phenyl thiourea by clipping third pair of abdominal legs of silkworm larvae and the haemolymph was taken for the enzymatic studies. At the end the statistical analysis of the recorded data was done and recorded in the Table I and graphically given in Fig I. The analysis for the intensity of enzyme activity in both controlled and Grasserie infected larval samples were done by using Clinical Analyser. The Chemical kits used for enzyme assay were from Excel Diagnostics Ltd. The following method was used to estimate the enzyme alterations.

Enzyme ALT and AST Activity (U/L) = Change in (Absorbance /min) x 1746 factor

$$\text{Enzyme AKP and ACP in } \frac{U}{L} = \frac{(\text{Absorbance of Test} - \text{Absorbance of control})}{(\text{Absorbance of Standard} - \text{Absorbance of Blank})} \times \text{Std conc.} \times 7.1$$

1 KA units/dl =7.1 U/Lit

Enzyme Activity (U/L) = Change in (Absorbance /min) x 1746 factor
1 KA units/dl =7.1 U/Lit

RESULTS AND DISCUSSION

In the present study, the enzyme profile of healthy and Grasserie infected silkworms was recorded by the method described above. All the recorded data has been summarized in the Table I. The levels of Alanine aminotransferase (ALT) in the haemolymph of healthy and Grasserie infected Silkworms are recorded in the Table I and Fig I. On the basis of unit volume of haemolymph, the ALT levels were low at the beginning of the larval development both in the healthy and Grasserie infected silkworms.

At early stage it was recorded 445.67 U/Lit. as compared to the healthy control 375.31 U/Lit. At late stage of infection, the changes in the levels of the healthy and infected larvae were 645.37 U/Lit and 241.36 U/Lit respectively, which showed decreased trend in infected larvae as compare to non-infected healthy worms. Accordingly, Aspartate aminotransferase (AST) activity in the haemolymph during both early and late infection with Grasserie were disturbed a little. At early infection the enzyme activity in the haemolymph showed significantly reducing effect 283.70 U/L in infection than the control level of the enzyme in haemolymph 240.21 U/Lit.

On the other hand, the enhancing effect on AST activity in the haemolymph was exhibited in late infection was 242.10 U/Lit as compared to the healthy control 281.80 U/Lit. From the values depicted in Table I and Fig. I, the Alkaline phosphatase (ALP) activity enhanced initially 0.286 U/L during early infection with Grasserie in comparison to the control non infected larva 0.037 U/Lit at early stage. However, in late stage the haemolymph in the diseased worm undergoes rapid increase in Alkaline phosphatase (ALP) activity 0.609 U/L, when compared to the control 0.125 U/Lit. Results. Recorded in Table I and Fig. I, explored the effect of Grasserie on the Acid phosphatase (ACP) activity in haemolymph of silkworm at early and late infection.
Acid phosphatase activity (0.183 U/L) enhanced initially during early infection with Grasserie in comparison to the control non infected larva 0.162 U/Lit, on day two of 5th instar larva. However, on 5th day the haemolymph in the diseased worm undergoes a rapid rise in Acid

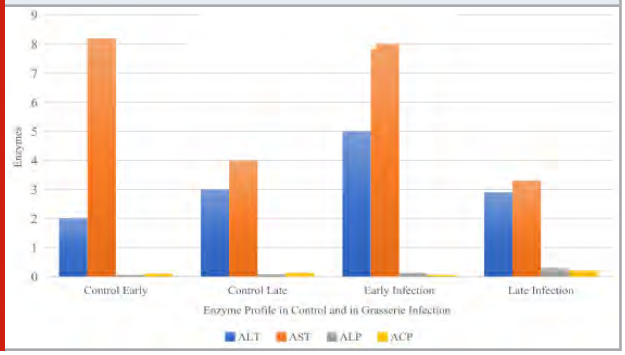
phosphatase activity 0.354 U/L, when compared with the control 0.195 U/Lit. Enzymes provide the energy needed for metabolic reactions essential to immune health. Enzymes have been shown to stimulate the body's natural defenses while breaking down offending pathogenic immune complexes. This helps relieve stress on the body and strengthen the immune system, which can speed the healing process. Changes in enzymatic action along with metabolic modulations during the progress of pathogen play an important role in understanding the interaction between the host and pathogen as a part of a survival strategy.

Table 1. The Enzyme Profile of the silkworm haemolymph during Grasserie infection

Enzyme	Healthy Control		Grasserie Infection	
	Early	Late	Early	Late
Alanine Amino Transferase (ALT) U/Lit	375.31 ±2.00	645.33 ±3.00	445.67 ±5.00 a	241.36 ±2.90 b
Aspartate Amino Transferase (AST) U/Lit	240.21 ±8.2	281.8 ±4.00	283.7 ±8.00 a	242.1 ±3.30 a
Alkaline Phosphatase (ALP) U/Lit	0.037 ±0.06	0.125 ±0.08	0.286 ±0.13b	0.609 ±0.39 b
Acid Phosphatase (ACP) U/Lit	0.162 ±0.10	0.195 ±0.133	0.183 ±0.06b	0.354 ±0.21a

Conc.: Concentration, mean ± SE followed with the same letter (a): is not significantly different (P>0.05), (b): significantly different (P<0.05), (c): highly significantly different (P<0.01), (d): very highly significantly different (P<0.001).

Figure 1: Showing significant differences in enzyme activities in haemolymph in the silkworm during infection with Grasserie



In view of this, the present study has been carried out to understand the dynamics of enzyme during the progress of BmNPV in silkworm *Bombyx mori*. Many workers through their studies have emphasized the importance of diseases to explain the effects on bimolecular and physiological mechanisms of the infected silkworm especially as they relate to, the composition of body fluids, enzyme systems, development of immunity, and predisposition to diseases and parasites (Gillespie et al. 1997; Doreswamy et al. 2004). In the present study we found the significant fluctuations in enzyme activity levels in the haemolymph. In insects, ALPs are involved in several biological processes and respond to stress, including pathogenesis, or infection Miao (2002). Senthil et al., (2005) reported a decrease in the activity level of the Acid phosphatase in *Spodoptera litura* after exposure to pathogenic infections with virus. The effects of NPV on midgut enzymes of *Spodoptera litura* were investigated by Nathan et al. (2005) and it was demonstrated that alkaline phosphatase is decreased after infection by virus.

Conversely, Matindoost (2006) showed that BmNPV had caused a considerable decrease in activity of this enzyme in silkworm after infection of a cell line established from silkworm embryo (Bm-EK1). It was noted that infection with microbial pathogens is capable of activating, inactivating or neutralizing enzyme production and subsequent release to the system. These types of alterations in enzyme profile could possibly be resulting due to mobilization and detoxification of microbial toxins and explains the sudden alterations in enzyme activity as observed in the present study (Doreswamy 2004; Etebari 2007; Nirupama 2015). Many workers, reported the significant fluctuations in enzyme activity levels in the in haemolymph of healthy and Grasserie infected silkworms. Nirupama (2015) reported the same alterations in enzymes at healthy and infected silkworms as we recorded in the present study. Enzymes are important biological molecules responsible for the thousands of biochemical interconversions that sustain life.

Enzymes serve a wide variety of functions, act as catalysts and help in complex reactions occur everywhere in life. Without enzymes, metabolism would neither progress through the same steps, nor be fast enough to serve the needs of the cell. In the absence of enzymes, this occurs so slowly or insignificantly (Reddy 2004; Mahesha et al. 2009; Mahalingam et al. 2010; Mahesha et al. 2013; Nirupama 2015). The present investigation reveals that the haemolymph levels of Acid Phosphatase non-significantly changes during Grasserie. The present study reported similar alterations during infection (Gao et al. 2020). He reviewed that, the expression levels of (ALP) alkaline phosphatase activity were all increases first and then decreases sharply in the midgut, haemolymph and fat body of silkworm after infection with BmNPV. The gene expression was basically consistent with the change of enzyme activity, which was closely related to

the physiological metabolic process of silkworm after infection with BmNPV. It suggested that the alkaline phosphatase played an important role in the antiviral process of silkworm (Gao et al. 2020).

CONCLUSION

Enzyme alterations in the studied parameters, on exposure to disease causing pathogens, in the hemolymph, suggest that these parameters could be used as indicators of the health status of the silkworm, *Bombyx mori*, as well as can be taken as target system to measure physiological and biomolecular stress induced alterations in the body during the occurrence of diseases. This can assist in better monitoring and effective health management of silkworms which is an economically important silk producing bio-machine. The present information may provide the underlying mechanisms in altering the metabolic modulations in pre-spinning silkworm larvae during Grasserie infection and to protect the commercial characteristics of cocoon yield in addition to suggest suitable measures in regulating the disease.

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Agricultural Communication

The Impact of Various Planting Timelines on the Makings of Harvested Wheat Grains *Triticum aestivum* of Cultivars in Lorestan Province of Iran

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ABSTRACT

A research was taken place to examine the impact of planting timelines on growth and the makings of harvested grains of various wheat cultivars in Iran. The examination was organized in Randomized Complete Block Design (RCBD) with a split-plot arrangement comprising of three replicas in December 2011 in Boroujerd, Iran. Five planting timelines (i.e. October 31, November 15 and 30, December 15 and 30) were observed in main plots, while five wheat cultivars (Pishgam, Parsi, Bahar, Sivand, and Pishtaz) were cultivated in subplots. The findings of this study indicate that the impact of planting timeline was highly pronounced within all of the factors with the exception of HI. The impact of cultivars was especially observed within all factors with the exception of the weight of1000 grain. The highest number of grains per spikelet was observed with regard to Pishtaz cultivar. Nonetheless, the highest number of HI was observed in Pishgam and Bahar cultivars. Parsi cultivar exhibited the highest rate of grain produce (10.23 ton/ha) and the Pishtaz cultivar exhibited the least multitude of grain produce (8.59 ton/ha). The highest rate of grain produces when it comes to planting timeline (10.15 ton/ha) November 15th and the least multitude of grain produce (6.1 ton/ha) was observed on December 30th. The Parsi cultivar exhibited the highest rate of grain produce when it comes to planting timelines but Pishgam cultivar exhibited a better growth ratewhen it was sowed on November 15th and Bahar cultivar exhibited a better growth rate when it was sowed on December15th.

KEY WORDS: WHEAT, PLANTING TIMELINE, HARVEST TIMELINE.

INTRODUCTION

Wheat *(Triticum aestivum* L.) is a significant edible grain which is normally in winters. The value of wheat as an edible grain can be realized while considering that it consists of 42% of gross sown area and 32% of the gross sown rice (*Oryza sativa* L.) area in the rice-wheat cropping pattern in South Asia (Karasakal et al., 2020b, Jia et al., 2020, Aletor, 2021). When it comes to wheat crops, uniform stand establishment and early seeding vigor are two of the most important underlying factors of crop performance (Alayi et al., 2020). The parameters which might deter the uniform stand establishment consist of poor seed quality (Arjaghi et al., 2021), inadequate seedbed preparation (Khayatnezhad and Nasehi, 2021), poor irrigation methods (Harris, 1996), outdated cropping methods (Esmaeilzadeh et al., 2020), delayed planting, and sub-optimal temperature at the time of cropping,(Farhadi et al., 2020, Hewitt, 2021).

Delayed planting negatively impacts the growth, yield, and quality of wheat, because timely planting cultivates a greater quantity of grains than delayed planting because of the much longer period of time that the crops have in order to grow. Temperatures rates that vary from the standard temperature lead to changes in crop performance and yield. In delayed sown wheat, lower levels of temperature which exist at the time of germination considerably impact the sprouting of seeds and seedling emergence. Germination is a substantial step in the growth of the seeds and temperature rates below 12°C caused eficient and asymmetrical seedling emergence (Gholamin and Khayatnezhad, 2021, Si et al., 2020). As

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a result, the seedling emergence and final development percentage are determinant parameters in establishing the crop performance in different temperature rates in wheat cultivation.

During delayed planting season, the soil temperature is anticipated to be below 10°C, which negatively impacts the sprouting of the seed and stand establishment. Normally wheat along with most the cold season crops is planted ahead of time in order to have the highest amount of time for growth and full development before the likely heat stress. Nonetheless, planting of winter wheat in the middle of the season is normally more desirable for any environment, while delayed planted wheat endures more distress during the winter, which results in the development of fewer tillers and ripening in lower grain weight which in turn affects the number of grains produced by each plant (Gholamin and Khayatnezhad, 2020a, Sun et al., 2021). The genotypic response of wheat to planting time lines is different for each parameter involved in crop development because of their varying genetic capacities. A downtrend in cultivation is observed in the cultivars which indicate that they need more time to growth an the time they are cultivated within the usual planting conditions. Higher temperature can lead to the minimization of the heading stage (Khayatnezhad and Gholamin, 2020b, Huma et al., 2021).

Correspondingly, cultivars developed much faster after delayed planting, which demonstrates that higher rates of temperature shall pressure crops into faster development. Upon providing ideal condition by the wheat cultivar, the grain filling period was reported to be much longer in contrast with delayed planting conditions under high-temperature stress which results in faster development of the crops. As a result, the current research was carried out in order to analyze the impact of varying planting timelines on the makings of the harvested grain in wheat cultivars.

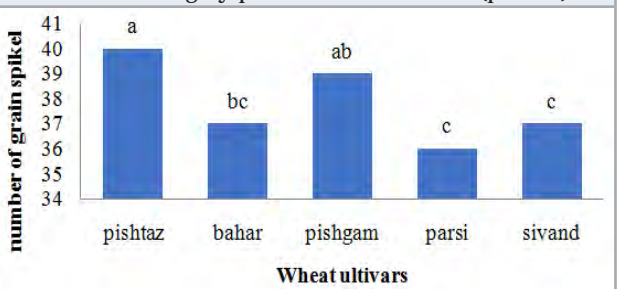
MATERIAL AND METHODS

As an attempt to examine the impact of planting timelines on the makings of the harvested grain in common wheat (*Triticum aestivum* L.) cultivars, a trial was carried out under a specific thermal state at an agricultural farm in Lorest an province (Boroujerd station) of Iran. The soil texture was designated as clay loam with apH of 7.9 and EC = 0.40 d s m_1. The area of Boroujerd enjoys a constant semi-arid climate and it rains up to 369 mm there every year. Approximately 50% of it relates to the rains in the time of wheat and barley growth. The trial scheme was a split-plot comprised of three replicas. Twelve rows were created in each plot; with a length of 1 m and having 0.2 m space within them.

Table 1. The Estimationof Population Variance (Mean Squares) for the Makings of the Harvested Wheat Cultivars within Various Planting Timelines

Source	df	number of grain spike	1000 grain weight	seed yield	Biomass yield	HI
R	2	0.04	0.04	0.01	0.08	0.03
date of sowing (A)	4	1.43*	0.29**	3.11*	0.72*	0.13
Error a	8	0.32	0.01	0.05	0.22	0.03
cultivar (B)	4	0.27*	0.09	3.12*	0.87*	0.32*
A*B	16	0.24	0.17	2.52*	0.56*	0.14
Error b	40	0.12	0.01	0.07	0.23	0.09
CV	8.87	5.6	2.12	8.87	7.2	6.65

Figure 1: Simple Mean Comparisons for the Multitude of Grains per Spikelet in Wheat Cultivars The mean values exhibited by the uncommon characters in each column demonstrate a highly pronounced difference (p<0.05).

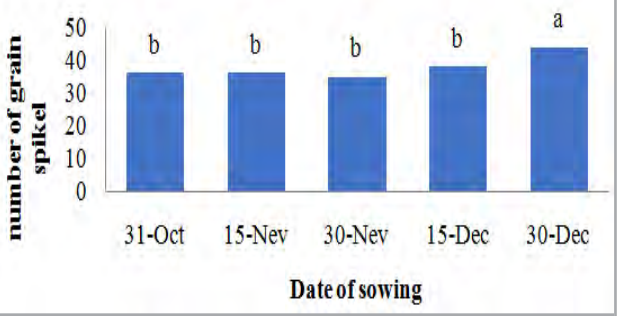


In the trials taking place in main plots consisted of five planting time lines including October 31 (A1), November 15 (A2), November 30 (A3), December15 (A4), and December 30 (A5). The experiments taking place in subplots that consisted of five cultivars included Pishtaz (B1), Bahar (B2), Pishgam (B3), Parsi (B4), and Sivand (B5). Upon full development, two of the external rows of each plot, 25 cm away from all the edges of the plots, were left as borders, and the crops planted in the 1 m2 central space of the two middle rows were gathered. Every single one of the representative grains was heated in the oven at 80 . C degrees and the harvested wheat rate was estimated, and following that the makings of the harvested grain of wheat cultivars were examined. The SAS Proc GLM procedure was chosen as the preferred data analysis framework for this study.

RESULTS AND DISCUSSION

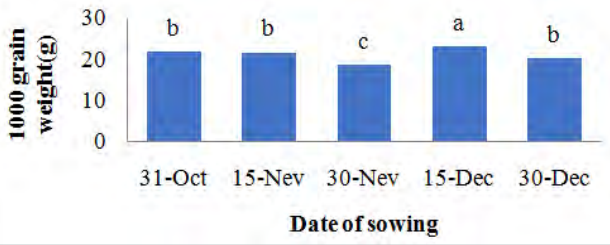
The Number of Seed Spikelet: The findings of this study exhibited that the impact of planting date and cultivars on the number of grains produced per spikelet was highly pronounced (Table 1). The evaluation of the mean values of the number of grains produced per spikelet for cultivars demonstrated that the Pishtaz cultivar produced the most (40) and the Parsi cultivar produced the least multitude of pods per plant (33) (Figure1). The estimation of the mean values of the multitude of grains per spikelet for the planting time line demonstrated that planting on December 30th produced the most grains (40) (Figure2).

Figure 2: Simple Mean Comparisons for Multitude of Grains per Spikelet in Wheat Cultivars within Various Planting Timelines



The mean values exhibited by the uncommon characters in each column demonstrate a highly pronounced difference ($p<0.05$).1000 Grain Weight. Results indicate that the impact of planting date on the 1000 grain weight was highly pronounced (Table 1). The estimation of the mean values of the 1000 grain weight for planting time lines demonstrated that December 15thplanting time line produced the most (23g)and the November 30th planting timeline produced the least amount of grains (19g) (Figure3).

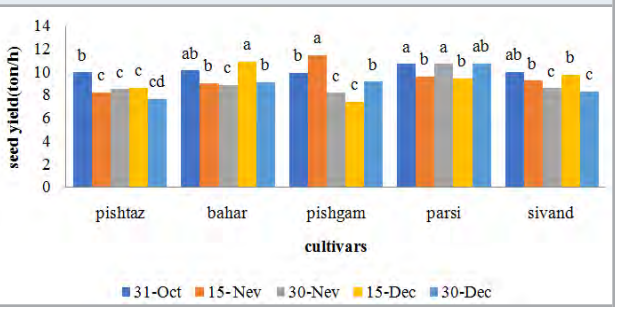
Figure 3: Simple Mean Comparisons for the Amount of 1000 Grain Weight of Wheat Cultivars within Various Planting Timelines



The mean values exhibited by the uncommon characters in each column demonstrate a highly pronounced difference ($p<0.05$).

Grain yield: Different wheat cultivars within various planting dates demonstrated dissimilarities amid the harvested grains of wheat (Table 1). The comparison of the mean values for grain production exhibits that the Parsi cultivar produced the highest number of harvested grains (10.23 ton/ha) and the Pishtaz cultivar produced the least amount of harvested grains (8.59 ton/ha) which indicates a highly pronounced difference (Fig4). The Parsi cultivar was the most productive amid all the cultivars when it comes to the production of harvested grains. The findings of this study were in other researches (Kabir et al., 2021, Radmanesh, 2021, Rodríguez, 2021,).

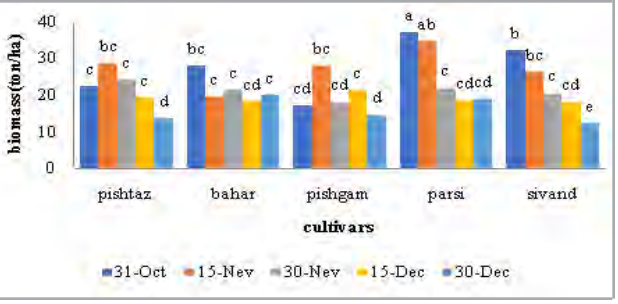
Figure 4: The Interaction Effect of Planting Timelines and Cultivars on the Harvested Grains of Wheat



The mean values exhibited by the uncommon characters in each column demonstrate a highly pronounced difference ($p<0.05$).

Biomass Production: Different wheat cultivars within various planting dates demonstrated dissimilarities when it comes to the biomass production of wheat (Table 1). The comparison of the mean values for biomass production exhibits that Parsi cultivar produced the highest number of harvested grains (34.3 ton/ha) on October 31st and the Sivand cultivar produced the least amount of biomass production (11.2 ton/ha) on October 31stwhich indicated a highly pronounced difference (Fig5).

Figure 5: The Interaction Effect of Planting Timelines and Cultivars on Biomass Production of Wheat

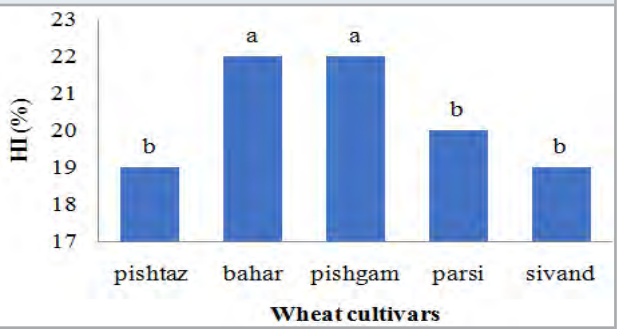


The mean values exhibited by the uncommon characters in each column demonstrate a highly pronounced difference ($p<0.05$).

The Ratio of Grains to Total Shoot Dry Matter: Results indicated that the impact of cultivars on HI was highly pronounced (Table 1). The comparison of the mean values of the HI for cultivars demonstrated that Pishgam

and Bahar cultivars exhibited the most (22%) and the Sivand cultivar exhibited the least amount of HI(18%) (Figure6).

Figure 6: Simple Mean Comparisons for HI of Wheat Cultivars



Simple mean comparisons for the harvested grains of wheat within various planting timelines demonstrate that the most amount of produced grains (10.15 ton/ha) was reported on November 15th planting timeline and the least amount of produced grains (6.1 ton/ha) was reported on December 30th planting timeline which indicated a highly pronounced difference. The decline in the amount of the grains produced was sharply affiliated with a lower amount of 1000- grain weight observed in delayed planted crops, based on the account, (Gholamin and Khayatnezhad, 2020 d, Khayatnezhad and Gholamin, 2021b, Huang et al., 2021).

The period of time from planting to the time when the crops are fully grown was more outstretched within the delayed planted crop, as opposed to the timely planted crops, probably because of the comparably lower temperature rates in the period of time when the crops are fully grown when it comes to the crops that were planted much later. Green et al. (1985) asserted that crops planted within various timelines move forward within each growth stage under varying ecological conditions (Green et al., 1985). Therefore, the delayed planted crops within this research moved forward within lower temperature rates and were affiliated with delayed blossoming. Ishag and Mohamed (1995) stated that different developmental phases of wheat are impacted by genetic and ecological parameters. The planting timeline exhibited a significant impact on the time that it takes for the grain to become fully grown. Delayed planted crops (early and mid-August) were gravely impacted by extremely cold weather strikes within the second and third weeks of November in both seasons and at the two locations (Ishag and Mohamed, 1995).

The estimation of differences within the study indicates that the impact of the planting timeline multiplied by the cultivar was highly pronounced at a 5% rate (Table 1). The interaction effect of planting timeline and cultivars on the harvested grains of wheat indicates that the most amount of the grains produced (11.43 ton/ha) was observed on November 15th planting timeline for the Pishgam cultivar and this cultivar demonstrated a highly pronounced difference within other planting timelines. The least amount of the grains produced (7.44 ton/ha) was reported on December 15th planting timeline for the Pishgam cultivar and this cultivar demonstrated a highly pronounced difference within other planting timelines (Fig3). Nonetheless, for the Pishtaz cultivar the highest amount of grains produced (9.97 ton/ha) was reported on October 31st planting timeline and the least amount of grains produced (7.66 ton/ha) was observed on December 30th planting timeline. In the Bahar cultivar,the most amount of grains produced (10.89 ton/ha) was observed on the December 15th planting timeline and the least amount of grains produced (8.64 ton/ha) was reported on November 30th planting timeline. Parsi cultivar produced the highest amount of grain son December 30th, October 31st, and November 30th respectively and the least amount of grains produced was reported on December 15th and November 15th respectively. Sivand cultivar produced the most and the number of grain son October 31stand December 30th respectively (Fig 3).

The findings of this study indicate that cultivars must be chosen in concurrence with the seasonal break, which might change from October to December. Under delayed planting, early sprouting and seedling development are highly significant for more effective stand establishment of the wheat crop, which is caused by the capacity to survive within lower temperature ratesin the period of germination. Benjamin (1990) and Stewart et al. (1990) stated that lower temperature rates within the period of germination or sprouting and within the earlier stages of seedling growth exhibits harmful impacts on the crop establishment and productivity (Benjamin, 1990, Stewart et al., 1990, Gholamin and Khayatnezhad, 2020b).

Tillering stage initiates after the finalization of the sprouting stage and approaches its fully developed state at the end of the vegetative phase which is the period of growth between germination and flowering. The highest number of productive tillers results in the most amounts of grains produced. Nonetheless, when it comes to delayed planted crops, Bahar and Pishgam cultivars resulted in more productive tillers because of their finer germination stage and stand establishment in contrast with other cultivars which had ineffective stand establishment. Poor emergence and ineffective stand establishment causeless productive tillers (Karasakal et al., 2020a, Gholamin and Khayatnezhad, 2020c, Li et al., 2021). The method of tillering is impacted by the planting timelines because of the variations in temperature rates and the fact that the effect of tillers in the production of grains is mostly observed within the timely planting of crops which declines with delayed sowing.

The timely planting brought about more desirable growth of the grains because of the more time that the crop had to develop. The early sown wheat had more time for the dry matter accumulation to bring about the higher amounts of grains produced (Khayatnezhad and Gholamin, 2020a,). The findings of this study indicate that timely planting in November provides the best results when it comes to the production of the grains of

wheat cultivars. Nonetheless, in order to get the very best results in the amount of grains produced, the planting timeline can be expanded to the last week of November. Parsi cultivar produced the highest number of harvested grains for planting timelines but Pishgam cultivar exhibited higher grain production rates when planted on November15th and Bahar cultivar exhibited higher grain production rates when planted on December15th. Therefore, in order to achieve the highest amount of the grains produced these cultivars should be planted within this specific timeline.

Conflict of Interest: The authors declare that there is no conflict of interest in this study.

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Biomedical
Communication

Relationship Between Nutritional Status and Academic Performance of Primary School Children in Rural Bankura Region of West Bengal, India

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ABSTRACT

The period of school age is an active phase for both the physical growth and mental development. Hence, proper nutrition during this period is very important as it lays the foundation of life time health, strength and intellectual capacity. However, malnutrition, especially undernutrition during primary school age is one of the important causes of poor school enrolment, high absence from school, unsatisfactory educational performance and early dropout. So, to ascertain the relationship between nutritional status and academic performance of the primary school children, this study was performed in rural Bankura district of West Bengal in India. A total of 269 primary school children aged 6-10 years were selected as participants of this study and a structured schedule was used for data collection. Nutritional status of the children was evaluated from three indices of undernutrition – underweight, stunting and wasting. For the estimation of overall magnitude of undernutrition, Composite Index of Anthropometric Failure (CIAF) was used. Moreover, academic performance of the primary school children was evaluated using seven-point grading system. Statistical analysis was performed using χ^2 test and one way ANOVA. In this study, the prevalence of underweight, stunting and wasting was 27.88%, 17.10% and 15.24% respectively. The overall prevalence of undernutrition was 39.03% as determined by CIAF. Conclusively, it was observed that there was positive association of academic grades with underweight ($P<0.001$), stunting ($P<0.05$), wasting ($P<0.001$), and CIAF ($P<0.001$). Moreover, the academic grades were positively associated with BMI (boys $P<0.05$; girls $P<0.001$ and sex combined $P<0.001$). This study elicits high prevalence of undernutrition among rural primary school going children and also shows positive relationship between nutritional status and academic performance of the children. These findings will not only help to design efficient measures to abate the burden of childhood undernutrition but also serve as a guideline for the development of better future generation.

KEY WORDS: ACADEMIC PERFORMANCE, CHILDREN, STUNTING, UNDERNUTRITION, WASTING.

INTRODUCTION

The children between the age of 6 and 10 years are termed as primary school children. The period of primary school age is nutritionally significant because this is the prime period to build up body stores of nutrients for the utilization during rapid growth of adolescence. Moreover, proper nutrition during primary school age is important as this period lays the foundation of life time health, strength and intellectual capacity (Chadha and Mathur 2015; Chandramohan et al., 2015; Sharma et al., 2017). The period of school age is the active phase of physical growth as well as mental development of the children. In this period, nutrients play a critical role in the development of the brain (Dey and Nath 2017; Karavida et al., 2019). Overall, children's quality of growth and development, status of health and the quality of life are indicated by their nutritional status (Eze et al., 2017; Marwat et al. 2019; Sathiadas et al., 2021). The nutritional imbalance at school age period can result in critical health problems throughout the life of children (Srivastava et al., 2012)

Long-term undernutrition during the childhood is associated with delayed development in cognition and

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grave health impairments at the later part of life that affects the quality of life in adulthood (Srivastava et al., 2012). Improved nutrition is linked with optimal brain function and nutritional deficiencies can significantly affect brain development (Nyaradi et al., 2013). It was evident from previous studies undernutrition during primary school age is an important cause of poor school enrolment, higher absence from school, unsatisfactory educational performance and early dropout (Subhaprada 2015). In addition to these factors, recent findings have established poor nutritional status of school children as one major reason of their poor academic performance (Agarwal et al., 2018; Khan et al., 2020).

The children, especially of rural areas, are at risk of undernutrition because of inadequate diet, improper care, repeated infection and uneven food distribution in the family (De and Chattopadhyay 2019). In spite of economic growth, malnutrition especially undernutrition is a major health-related issue of developing countries till today (Mohseni et al., 2019). For the development of better future generation and development of the nation, an understanding of the impact of nutritional status on academic performance of primary school children is immense important. But there is no sufficient information related to it. So, to find out the relationship between nutritional status and academic performance of primary school children, the present study was performed in different villages of Bankura district of West Bengal in India.

MATERIAL AND METHODS

A community based cross-sectional study was carried out among the primary school children aged between 6 and 10 years residing at rural Bankura district of West Bengal. A total of 269 children were selected from six villages by multistage random sampling. To carry out the survey, written permission was taken from District School Authority (Primary Education) and Local Bodies (Gram Panchayats). Informed consent was also taken from the parents/guardians of the children. Data was collected using a structured schedule. Nutritional status of the children was assessed using different indices based on two anthropometric parameters weight and height. Weight was measured by digital weighing machine and height was measured by anthropometer. Body mass index (BMI) was calculated as dividing weight (kg) by height^2 (meters). Date of birth of the children was recorded from birth certificate issued by the Department of Health and Family Welfare.

Table 1. Age and sex wise distribution of the children

Age group (years)	Boys		Girls		Total	
	Number	%	Number	%	Number	%
6	17	11.72	11	8.87	28	10.41
7	38	26.21	31	25.00	69	25.65
8	35	24.14	31	25.00	66	24.54
9	30	20.69	30	24.19	60	22.30
10	25	17.24	21	16.94	46	17.10
Total	145	100.00	124	100.00	269	100.00

Three types of Z scores – weight-for-age Z-score (WAZ), height-for-age Z-score (HAZ) and weight-for-height Z-score (WHZ) – were calculated from reference values of National Centre for Health Statistics (NCHS). Using these three Z-scores, three indices of undernutrition – underweight (WAZ < – 2SD), stunting (HAZ < – 2SD) and wasting (WHZ < – 2SD) were defined. For estimation of overall magnitude of undernutrition Composite Index of Anthropometric Failure (CIAF) was used (Nandy et al., 2005; Khanra et al., 2019). Academic performance of the children was evaluated from their 'academic grades' which were computed by the school authorities from overall percentage of marks obtained in three summative examinations.

A seven point grading system A+ (90-100%), A(80-89%), B+ (70-79%), B (60-69%), C+ (45-59%), C (25-44%) and D (< 25%), was used in schools for academic grading. As no examination was held for the children of class one, a questionnaire containing ten questions on basic knowledge was used and same grading system was followed to evaluate academic performance. In the present investigation, the SPSS for Windows statistical software package (Version 16.0) was used to perform data processing and analysis. To find out the association among different groups, χ^2 test was carried out. One way ANOVA was done for group comparison. The p value of <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

The present study was performed on 269 primary school children, out of which 145 (53.9%) were boys and 124 (46.1%) were girls. Age and sex wise distribution of the children is presented in table 1. The three anthropometric parameters (weight, height and BMI) of the children were expressed as mean and SD, and these are presented in table 2. Mean (with SD) weight, height and BMI of boys were 20.93±3.96 kg, 121.10±8.18 cm and 14.16±1.40 kg/m2 respectively; whereas those of girls were 21.28±4.46 kg, 121.90±7.67 cm and 14.17±1.64 kg/m2 respectively. In sex combined, mean (with SD) weight, height and

BMI were 21.09 ± 4.19 kg, 121.47 ± 7.95 cm and 14.17 ± 1.51 kg/m2 respectively. It was observed that the mean weight, height and BMI of girls were higher than boys. The possible reason behind higher weight in girls than boys may be of lower level of activity in girls than boys. In similar studies the mean weight and height of girls were found higher than boys (Dey and Nath 2017; Firdos et al. 2018; Yankanchi et al., 2018).

In present study, the nutritional status of the participants was judged from three indices of undernutrition – underweight, stunting and wasting. The overall prevalence of undernutrition was determined by CIAF. Prevalence of different forms of undernutrition is presented in table 3. In this study, the prevalence of underweight, stunting and wasting was 27.88%, 17.10% and 15.24% respectively. In boys, the prevalence of these indices was 30.34%, 19.31% and 14.48%; whereas that of girls was 25.00%, 14.52% and 16.13% respectively. The overall prevalence of undernutrition was 39.03% as determined by CIAF.

Table 2. Anthropometric parameters of the children

Anthropometric parameters	Boys (N=145) Mean ± SD	Girls (N=124) Mean ± SD	Sex combined (N=269) Mean ± SD
Weight (kg)	20.93 ± 3.96	21.28 ± 4.46	21.09 ± 4.19
Height (cm)	121.10 ± 8.18	121.90 ± 7.67	121.47 ± 7.95
BMI (kg/m^2)	14.16 ± 1.40	14.17 ± 1.64	14.17 ± 1.51

Table 3. Prevalence of undernutrition among children

Nutrition status	Boys		Girls		Total	
	N	%	N	%	N	%
Underweight	44	30.34	31	25.00	75	27.88
Stunting	28	19.31	18	14.52	46	17.10
Wasting	21	14.48	20	16.13	41	15.24
CIAF	61	42.07	44	35.48	105	39.03

Table 4. Relationship of academic achievement and nutritional status

Academic Grade	N	Underweight N (%)	Stunting N (%)	Wasting N (%)	CIAF N (%)
A+	42	2 (4.76)	2 (4.76)	1 (2.38)	3 (7.14)
A	60	8 (13.33)	5 (8.33)	6 (10.0)	17 (28.33)
B+	77	26 (33.76)	16 (20.78)	7 (9.09)	34 (44.15)
B	45	20 (44.44)	10 (22.22)	9 (20.00)	22 (48.89)
C+	22	8 (36.36)	5 (22.73)	7 (31.81)	14 (63.63)
C	23	11 (47.82)	8 (34.78)	11 (47.82)	15 (65.21)
χ^2 test		χ^2=30.283 P<0.001	χ^2=14.895 P<0.05	χ^2=33.280 P<0.001	χ^2=35.744 P<0.001

This prevalence is similar to another study performed in the state of West Bengal in which the overall prevalence of undernutrition was found 38.1% (Mondal et al., 2015). In sex wise consideration, the prevalence of undernutrition in boys (42.47%) was found higher than girls (35.48%). In a similar study the undernutrition was found more common in boys than girls. Some recent studies also revealed high prevalence of undernutrition in different district of West Bengal (Sharma et al. 2017; Khanra et al., 2019; Pramanik 2020).

Academic performance of the primary school children was evaluated using seven-point (A+, A, B+, B, C+, C and D) grading system. In the present study only 15.61% children obtained the grade A+. Grade A was obtained by 22.30% children. Grade B+, B, C+ and C were obtained by 28.62%, 16.73%, 8.18% and 8.56% children respectively where nobody obtained grade D. To ascertain the relationship between nutritional status and academic performance of the children, χ^2 test was carried out and this relationship is presented in table 4.

Table 5. Association of academic achievement and BMI

Academic Grade	BMI					
	Boys		Girls		Sex combined	
	N	Mean±SD	N	Mean±SD	N	Mean±SD
A+	17	14.90±1.49	25	15.36±1.45	42	15.17±1.47
A	33	14.63±1.24	27	14.60±1.87	60	14.61±1.54
B+	46	13.91±0.96	31	14.14±1.43	77	14.00±1.17
B	19	13.58±1.31	26	13.41±1.00	45	13.48±1.13
C+	14	14.06±1.45	8	13.26±1.38	22	13.77±1.45
C	16	13.93±2.17	7	12.38±1.01	23	13.45±2.01
ANOVA	F=2.927; P<0.05		F=8.067; P<0.001		F=9.328; P<0.001	

It was observed that there was positive association of different academic grades with underweight (P<0.001), stunting (P<0.05), wasting (P<0.001) and CIAF (P<0.001). As CIAF is the indicator of overall state of undernutrition, it was clear that there was a positive association of nutritional status and academic performance. A similar study conducted at rural areas of Karnataka established a positive association between different indices of nutritional status and academic performance of students, apart from establishing a high incidence of malnutrition among school children (Rashmi et al., 2015). Similar findings were also observed in other studies conducted at different areas (Verma et al. 2019; Ayalew et al. 2020).

As BMI is another indicator of nutritional status, we tried to find out relationship between academic performance and BMI. For this purpose, ANOVA was performed and this relationship is presented in table 5. From this study it was clearly observed that the academic grades were positively associated with sex wise and sex combined BMI of the children (boys P<0.05; girls P<0.001 and sex combined P<0.001). This finding also established positive association of nutritional status and academic performance. Similar result was obtained from a cross sectional study conducted at urban Meerut, Uttar Pradesh (Agarwal et al. 2018). Significant relationship between nutritional status and academic achievement of the children were observed in the studies conducted at Indonesia and Soudi Arabia (Rahmatillah and Mulyono 2019; Khan et al., 2020).

From previous researches, it was established that the children who are well-nourished are commonly more prepared in learning, more interested in attending school and able in taking advantage of educational opportunities (Naik et al., 2015). So, nutritional status acts as an important determinant of the academic performance of the children. Poor nutrition status adversely affects the cognitive development of children (Opoola et al., 2016; Dey and Nath 2017). Poor academic performances as a consequence of undernutrition can cause debarring a child from a promising future (Agarwal et al. 2018). Abatement of undernutrition can help to improve children's cognitive achievement which in turn potentially improves their grade progression. A child with good nutrition will focus better and thereby perform better academically (Acharya et al. 2019; Okafor et al. 2020).

CONCLUSION

This study elicits high prevalence of undernutrition among rural primary school children and it also shows positive relationship between nutritional status and academic performance of the children. The findings of this study indicate the cause of poor academic performance of the rural primary school children. So, this study will not only help to design efficient measures to abate the burden of childhood undernutrition but also serve as a guideline for the development of better future generation.

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Conflict of Interests:The authors declare no conflict among their interests in this study.

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Virological
Communication

The Combined and Isolated Effect of Spinosad and Nuclear Polyhedrosis Virus on the Mediterranean Brocade *Spodoptera littoralis* in laboratory Conditions

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ABSTRACT

The contaminative effect of the two organic pesticides, Spinosad and NPVs, on the newborn worms of Spodoptera littoralis (Bosiduval) (Lepidoptera: Noctuidae) was examined within lab examination circumstances to discover their competitive capacity. The capability of Spinosad to guard the Split NPV against Ultra Violet impacts under manufactured examination room circumstances was demonstrated, and various biological features of both organic pesticides and their combination were examined. In an attempt to discover whether or not there is a coordinated impact when the two of these organic insecticides are mixed with each other, six particular Spinosad composites (1, 2, 5, 10, 15 and 30 ppm) in isolation and in connection with a dangerous mixture of split NPV (1×10^3) were examined. As the Ultra Violet impact was recognized, the LC_{90} of NPVs was combined with LC_{10} of Spinosad in an attempt to inspect the capacity of Spinosad in extending the virus life. A study was taken place in the Department of Entomology (Virology Unit) Faculty of Agriculture, Cairo University, between July 2012 and May 2013. The mortality rate multiplied, as it was 11.66, 19.33, 33.33, 55.00, 71.66, and 85.00 % in comparison with 11.66, 13.33, 15.00, 26.66, 36.66, and 63.33 % in isolated Spinosad and 20.11% in isolated NPVs analysis. A virtually unmitigated protection was observed after 30 minutes of exposure to manufactured ultraviolet light and showed 47 % mortality rate 5 hours after the procedure, in contrast with the 2.8 % mortality rate which is exhibited when NPVs is employed in isolation. The larval stage was only impacted by Spinosad; pupal stage and adult lifespan were not impacted by the entire examination conditions. The sum of eggs laid by each female and their fertility rate were impacted in Spinosad and Spinosad NPVs coordinative functions in comparison with of the number that was reported by the control group. The discoveries of this study signify that Spinosad and NPVs, the organic pesticides, can be employed in affiliation with each other, which in turn lead to propitious results when it comes to annihilating the Mediterranean Brocade. The results of the research suggest that Spinosad and NPVs introduce a significant option to be employed in combined efforts in insect tackling in which *Spodoptera littoralis* is the primary insect.

KEY WORDS: SPODOPTERA LITTORALIS, SPINOSAD, NUCLEAR POLYHYDROSIS VIRUSES, COORDINATION, BIOLOGICAL TRAITS.

INTRODUCTION

Spodoptera littoralis (Boisduval) (Lepidoptera: Noctuidae) is one of the most hostile insects in Africa, Asia, and Europe (Khayatnezhad and Gholamin, 2021a, Gholamin and Khayatnezhad, 2020d, Karasakal et al., 2020a, Si et al., 2020, Aletor, 2021). Its negative impact on the vegetables and decorative plants undermines their marketability. The extensive use of various insecticides to annihilate this insect has led to obstruction of many approved insecticides (Alayi et al., 2020, Esmaeilzadeh et al., 2020). The makeup of entomopathogens could highly impact their effectiveness as organic nanoparticle pesticides (Arjaghi et al., 2021). The makeup in specific can affect the immutability of the pathogen in when it comes to its storing ability and the effectiveness of its utilization on the crop. Furthermore, specific makeup assistants can improve the function of the pathogen and enhance its ecological resistance (Khayatnezhad and Nasehi, 2021, Gholamin and Khayatnezhad, 2020c, Sun et al., 2021).

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In an attempt to enhance the function of the pathogen, one can mix them with limited amounts of cooperative components such as visual enhancers (Hewitt, 2021), mineral acids (Fataei, 2017, Ghomi Avili and Makaremi, 2020) or moderately deadly combinations of chemical pesticides (Gholamin and Khayatnezhad, 2020b, Huang et al., 2021). Nonetheless, the cooperation between a pathogen and other composites might as well bring about adverse results because of the cutback in nourishment or the alteration of pH in the intestines (Karasakal et al., 2020b, Li et al., 2021), or the autonomous function of each substance which may cause an increase in the mortality rate (Fataei et al., 2018). Spinosad is a combination of spinosyns A and D generated while the soil actinomycete *Saccharopolyspora spinosa* is being dissolved. Spinosad is fundamentally an intestine toxin which exhibits various external activities and is specifically poisonous when it comes to Lepidoptera and Diptera.

Nonetheless, contamination examinations suggest that Spinosad possesses literally no contamination risks to birds and mammals and possesses approximately low contamination risks to specific bug assailants in the nature (Omrani and Fataei, 2018), while a number of insect hunters and freeloaders seem to be vulnerable to Spinosad contamination (Jia et al., 2020, Gholamin and Khayatnezhad, 2020a, Huma et al., 2021). It is rather significant to examine the contamination of Spinosad and NPVs as organic pesticides and the effects of their combination on the new born larvae of *Spodoptera littoralis*, alongside the impact of such procedures on specific biological features in an attempt to understand whether Spinosad has an impact on guarding the NPVs or not. The purpose of this study is to improve the effectiveness of NPVs by mixing them with moderately deadly composites of Spinosad in an attempt to achieve a higher level of command over *Spinosad littoralis*.

Empirical Data

Insect Colony: A colony of the Mediterranean Brocade, *Spodoptera littoralis* (Boisduval), was set up as the trial insect group on a somewhat unnatural daily food intake of Shorey and Hale (1965) under the pursuing circumstances in the laboratory: Thermal reading of 25 ± 2°C and 65 ± 5%R.H. and the physiological response to the light and dark periods of 16:8 (L: D).

Virus Inoculation: A separate group of *Spodoptera littoralis* combined with another separate group of Nuclear Polyhedrosis Virus (Split MNPV) which was previously secluded in Egypt was employed within the study.

Chemical tested: The insecticide upon which the examination was carried was Tracer (24% Spinosad, liquid formulation containing solid pesticide active components that need to be mixed with water before use; Dow Agro Sciences, Alexandria, Egypt).

The Biological Assessment: The effect of sunlight UV rays (SUV) was replicated by a set of four UV lamps (Ultra-Vitalux, OSRAM, Germany), which were vertically placed 160 cm away from the susceptible virus samples, and there was a 60-cm space in the middle of the two lamps. The organic impact is estimated nearly 6-7 times more than the actual sun-light when the space between unnatural sunlight, lamp, and moisture less solid is determined exactly 50 cm (Huber and Ludcke, 1996). A surfactant (Teepol 2.5%) was intermixed with the virus composite, and 50 µl of it was diffused within a Petri dish (10 cm in diameter) through a fine pipette . When the moisture from the surfaces was removed using air, the dishes containing the virus films were subjected to the lightening source of the experiment which entailed the subjection of virus combination to UV rays (2000 fold LC_{90} PIB's).

The polyhedra solids in the Petri-dish were ejected into in 10 ml distilled water with 2.5% Teepol for the second time in order to achieve the desired state for the application in biological assessments. The virus infected Split NPV was diffused in distilled purified water and the virus composite was altered to be composed of 108 OBs/ml (=LC 90-95 %). Virus endurance was estimated by % OAR (percentage of Original Activity Remaining) according to 100% destruction rate at '0' day after the examination was taken place. Analytical assessments were once more taken place in three simulated experiment groups with 10 larvae used for each examination (Shapiro et al., 2008).

Biological Features: The juvenile worms that withstood all conditions within the experiment were gathered and some biological features of them were documented in order for them to be compared with the larvae (control) that were not employed within the experiment. These biological features involved larval stage, pupal stage, adult life-span, the number of the eggs each female larva laid and their fertility rate.

Numerical Assessment: Concentration-dependent mortality lapse were estimated in order to discover the impact of plant-based substances, which might act as Split MNP UV shielding supplements. Slopes and LC50s were evaluated based on the design introduced by Finney (Finney, 1971). Initial motion remaining percentages were designated for each demonstrated experiment by the application of both factors to guarantee the substances' capacity to extend the virus endurance as detailed by Muro and Paul (Martignoni and Iwai, 1985, Kabir et al., 2021).

RESULTS AND DISCUSSION

The United Impact: In a study on organic insecticides that was taken place recently researchers located in the countries with less developed industrial base demonstrated that composition was the most significant problem in the production of organic pesticides (Radmanesh, 2021). Because of the fact that spinosyns are developed through fermentation of an actinomycete, Spinosad has been categorized as an organic insecticide (Huang et al., 2021), despite the fact that it evidently

possesses features that affords it to be used as a pesticide which can be distinguished from most of entomopathogen-based organic insecticides (Gholamin and Khayatnezhad, 2021, Rodríguez, 2021).

Table 1. The Impact of Spinosad and NPVs in Isolation and Their Combination on the New Born Larvae of *Spodoptera littoralis* under Examination Room Circumstances.

Spinosad concentrations	Mortality percent											
	Spinosad				NPVs 1×103				Spinosad+ NPVs 1×103			
	R1	R2	R3	Mean	R1	R2	R3	Mean	R1	R2	R3	Mean
30 ppm	60	60	70	63.33					90	80	85	85
15 ppm	40	30	40	36.66					75	70	70	71.66
10 ppm	20	40	20	26.66					55	60	50	55
5 ppm	15	25	10	15.00					35	35	30	33.33
2 ppm	10	15	15	13.33					20	15	20	19.33
1 ppm	10	10	15	11.66	18.66	19.46	22	20.11	10	15	10	11.66

Table 2. The Impact of LC_{10} Spinosad Additive on the Endurance of Spodoptera littoralis NPV Split NPV Subjected to Unnatural UV Light Rays and Biological Assessment on *S. littoralis* New Born Larvae.

Irradiation period (min)	Mortality (%) due to Spli NPV alone	Mortality (%) due to virus + Spinosad at LC_{10}
Control (D. W.)	0	0
300 min	2.8	47
240 min	4	50
180 min	11.6	56.5
120 min	14.28	66
60 min	38	96
30 min	70	99
Zero time	89.4	90

The cooperation of Spinosad with entomopathogens has not been studied before, and the attempt of doing scientic research on SlMNPV–Spinosad intermixtures was thought to be worthwhile since Spinosad exhibits no antimycotic, antimicrobial or antiviral capacity (Khayatnezhad and Gholamin, 2021b, Wan, 2021). The LC_{50} rate which was estimated for newborn larvae of S. littoralis subjected to Spinosad under the diet of Shorey and Hale through surface intoxication method was literally entirely dissimilar to the 3 ppm rate (95% C.L.: 1.10–6.60) of spinosyn A documented for *S. littoralis* larvae in the undetermined stages which were submerged in water..The rate was documented while employing composites ranging from 1-100 ppm and it was reported to be 27.23 ppm.

The destruction rate of larvae subjected to SlMNPV 1×10^3 combined with the smallest amassing of Spinosad (1ppm) proved to be less than the anticipated rate in isolation (i.e., a level of hostility was noticed between these items). A small level of cooperation was noticed in the larvae subjected to SLMNPV+ 30 ppm Spinosad, while the biological reason for such interplay is not known at the very moment. The sequence of insect destruction during different periods of time was considered significant because of the notable dissimilarities in the mortality rate of SlMNPV and Spinosad (Gholamin and Khayatnezhad, 2020b). The normally approved quantile function or the logarithm which are used in biological assessments do not provide us with the appropriate assessment mediums when it comes to the analysis of the concentration–dependent destruction rate in virus–pesticide combinations; first, since the binomial distribution of reactions does not normally abide from logistic or Gaussian distributions because of the dissimilarities in the activity patterns and/or interplay among the virus and pesticide, and second, because of the fact that the sum of reactions of entities involved in an examined group are associated with the elements of time (Khayatnezhad and Gholamin, 2020a,).

Nevertheless, the combination of Spinosad exhibited a significant impact on the mortality rate which in turn entailed a notable reaction in less than 48 hours which was then trailed by virus-caused destruction of the larvae that withstood the Spinosad examination a few days later. The control rate of S. littoralis larvae was estimated due to the decline in larval rehabilitation within the plants that were used in the trial (probably because of the Spinosad-caused destruction) and the destruction rate that was noticed in the examination room caused by the virus. In comparison to the virus in isolation, the level of insect control was increased to a great degree by adding the 30 ppm Spinosad to the virus combination (Khayatnezhad and Gholamin, 2020b).

The Preservative Impact: Spinosad was examined as a preservative supplement in order to discover whether the intermixture of these two organic insecticides have improved their effects as preservative substances from the ultraviolet light, which might be taken into account as one of the most mitigating forces in employment of the virus. The results exhibit (Table 2) that moderately deadly quantities of Spinosad LC_{10} provided total preservation

effect 30 min after being employed in the trial and in 5 hours exhibites 47% destruction rate in contrast with 2.8 in the examination of the virus in isolation. No sufficient data is at hand on the effect of Spinosad and NPVs on *Spodoptera littoralis* within this study; it was been discovered that examining different kinds of pesticides that destruct or mitigate the biological procedures of the examined insect provide valuable insight on the various options on the combined insect control methods.

Table 3. The Impact of NPVs, Spinosad in Isolation and NPVs Spinosad Intermixture on various biological

Aspects / Pesticides	Larval Duration/ Day	Pupal Stage/ Day	Adult longevity/ Day	No. of Laid Egg/Female	Hatchability percentage
NPVs	15.2	9.0	13.1	373.7	60.2
Spinosad	15.3	9.0	12.9	294	49.2
NPVs Spinosad Mixture	15.1	9.3	12.8	301	45,2
Control	15.4	9.1	13.2	375.8	61.3

The Impact on the Biological Features of the Insect: The larval stage, pupal stage, and adult life-span were not impacted in the tested group as opposed to the control group (Table 3). No dissimilarities were observed within all the tested groups (Table 3) in larval stage, pupal stage, and adult life-span. The number of eggs laid by each female was impacted in all the examined groups in comparison with the control group, specifically while spinosad and SpLiNPV were employed as a mixture. The fertility rate was declined in all the tested groups, and specifically within the Split NPV Spinosad combination (45.2%) as opposed to the control group (60.2%). Wang et al. (2009) discovered that Spinosad in moderately deadly combinations notably prolonged the growth age of Helicoverpa armigera and reduced the development rate, fertility, and life-span of the adults. Pesticides that were incorporated together present a significant area to explore in *S. littoralis* management for the public health purposes.

Diverse effects of such mixtures go hand in hand with that of conventional pesticides, which entails that endurance is definitely anticipated to develop when the pesticide is employed during a long period of time and one cannot exactly determine to what extent combinations such as this one are going to remain effective since the worms have developed resistance to other combinations before, and continue to develop in spite of them. However, pesticide mixtures with varying roles could contribute to a great deal in *S. littoralis* management methods, specifically in areas where *S. littoralis* already exhibits high levels of endurance to normally used pesticides. The accessibility of new groups of pesticides has been limited with the past 10 years and depending upon the emergence of new pesticides is not a viable alternative for the management of enduring insects within the upcoming days or even years. However, the choice of incorporating different insecticides with various functions is within reach at this very moment.

CONCLUSION

The contamination rate of the two organic pesticides, Spinosad and NPVs, on the new born larvae of *Spodoptera littoralis* (Bosiduval) (Lepidoptera: Noctuidae) was examined within the examination room circumstances with an attempt to examine their antagonistic features. The capacity of Spinosad to guard the Split NPV against Ultra Violet light rays within cooperative examination room circumstances was discovered, and various biological features of the two organic pesticides and their combination was examined. The results actively suggest that organic pesticides Spinosad and NPVs can be employed as a mixture which in turn exhibit potential impact on the destruction of Mediterranean Brocade. The findings of this study indicate that Spinosad and NPVs provide a significant option for application in combined insect control methods when *S. littoralis* is the insect of interest.

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Biomedical Communication

A Descriptive Analysis on Gender Conformity and Deteriorating Mental Health Among Men in Kerala, India

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ABSTRACT

Far from the celebrations of long grown beards and no shave November lie crucial matters of importance to be addressed regarding vital issues, like deteriorating male mental health conditions, hard effects of toxic masculinity models, and a hike in the number of male suicides, which all demands utmost attention of researchers, sociologists, psychologists and doctors. Sociological studies on male gender roles have attributed an invariable connection between the decreasing life expectancy of men and the traditional notions associated with male roles and masculinity. There is an existing undertone of gendered perseverance in the analysis and further aid provision of mental health care in our society. This study purports to systematically evaluate the current findings thereby estimating the extent of psychological conditions faced by men, their implications and solutions. Studies were identified using PubMed, PubMed Central, MeSH, Google Scholar and NCBI databases. A GAD-7 questionnaire helped in providing the anxiety severity scale among the study population. Pooled prevalence rate of the physiological and psychological symptoms was recorded. GAD self-administered screening survey was filled by the 100 research subjects under study. Among the participants 25% of the subjects were identified as having minimal or zero anxiety, 52% with mild anxiety, 12% with moderate anxiety, and 11% with severe anxiety. The quotients of the survey were calculated by evaluating the rate of occurrence and percentage. Further the study recorded the range of difficulty faced by study subjects due to the prevalence of anxiety episodes. The study shows 30% subjects finding it 'not difficult at all', while 60% found it 'somewhat difficult'. 4% found these conditions 'very difficult' to handle and 1% found it 'extremely difficult'. The results showed that a proper remolding of male gender expressions is vital, one that accommodates gender-neutral behavioral patterns, which vouches for a more psychologically robust and socially healthy individual.

KEY WORDS: ANXIETY, DEPRESSION, GENDER ROLES, MASCULINITY, MENTAL HEALTH.

INTRODUCTION

There is an existing undertone of gendered perseverance in the analysis and further aid-provision of mental health care in our society. These undertones are now analyzed within the disciplines of psychiatry and public health. Depression and anxiety disorders are now being recognized as the fourth most cause of dysfunction around the world. Mental illnesses and psychotic diseases are often associated with women. This stereotype prevails primarily due to the lack of propriety in the documentation of male mental illnesses. Women have been diagnosed with varied psychological conditions from time immemorial (Keles et al., 2019).

Illnesses like hysteria and melancholy have been repeatedly represented as standard female temperaments in popular discourses (Kornstein and Clayton 2019). However, this situation must have derived from men's lack of interest in concurring to the mental conditions, reaching out for professional help, or sometimes simply lack of self-awareness. These ignored conditions may arise from their obstinate adherence to the tenets of the gender they follow in the name of masculinity (McKenzie et al. 2018; Rippon, 2019). In most cases, depression and anxiety disorders in men surface mostly as aggressive tendency, substance abuse, and hostile nature. Where, experts recognize these symptoms as the masculine

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variants of markedly feminine expressions of crying, fearfulness and other recognized temperaments (Cochran and Rabinowitz 2003; O'Brien et al., 2005). Moreover, psychiatrists identify these symptoms as cover-up mechanisms men adopt to conceal their breakdown.

However, a thorough analysis of the collected patient information from various psychiatric centres reveals fewer male patients than women. Experts assume that men overtly rely on self-screening methods and self-management techniques to cure their conditions (Ahmed, 2015; Struik et al., 2019). Often these psychic difficulties in men are recognized by others through their ebbed performances at workplaces or by assessing their difficulty in executing their day-to-day activities (Barry et al., 2019; Stiawa et al., 2020). Male mental health conditions are often acted out in alcoholism, substance abuse, and elaborate zoning out from daily chores (Mahalik et al. 2003). Psychological conditions like Schizophrenia are among the significant illnesses that men confess to, while anxiety and depression are unrevealed psychological conditions they face. Rage, bad temper, and seclusion are temperaments that men regularly display, however, these conditions are often not recognized as psychic issues that need to be rectified. Sociological studies elucidate the health care stratifications based on gender, where men are mostly ignored compared to women (Clarke and Amerom 2008).

Therefore, it is evident that the limited institutional mental healthcare provided for or accessed by men can contribute to the categorization of these distresses. This leads to an obscuring of masculine identity and male subjectivity. The concealed facets of their emotionality ought to be identified by the subjects themselves (Messerschmidt, 2018; Koutsimani et al., 2019). The cultural appropriation of gender-specific emotional expressions facilitates through various cultural and subcultural entities (Stiawa et al., 2020). In the article, Mednick and Hochschild (1985), Arlie Hochschild talks about the importance of regulating feelings by individuals in different situations which can be regarded as the cultural shaping of a body's sentiments. Regulation and managing of feelings as well as emotions are expected from a competent adult, and anything other than that has been frowned upon (Mednick and Hochschild 1985; Yafı and Yafı 2019). As a replacement for these vulnerable expressions, they are conditioned to act self-sufficient, spirited, emotionally suppressed, and self-restrained. Moreover, these factors influence standard male actions, experiences, and health care seeking attitudes.

Where, issues related to health, employment, finances, and relationships can trigger anxiety and depressions in men. Psychologists are now analyzing the widening scope of male anxiety and depression (Amin et al., 2018; Patton et al., 2018). The lack of willingness in men's acknowledgement of their aggressive tendencies and hostility, as indicators of poor mental health leads to the limited studies in this arena. They tend to display more resistance towards such symptoms (Herreen et al., 2021). This study empirically evaluates the sociological aspects involved in this issue rather than solely concentrating on the psychological aspects.

MATERIAL AND METHODS

A comprehensive narrative analysis of anxiety disorders and psychic conditions in men was conducted to assess the practical importance and magnitude of the subject under study. The evaluation included an inclusive revision of cognitive issues faced by men, the reasons behind these illnesses, and the possible aftermath of these conditions when left unattended. Men go through different physiological and psychological changes once they reach puberty. Unlike the mark of menstruation in females, males hardly identify the changes. Significant signs of male depression include feelings of hopelessness, irritability, substance abuse, insomnia, and panic (Stein and Vythilingum 2015; Addis and Hoffman 2019). In order to proficiently study the broadening span of anxiety disorders in men, 100 research subjects were approached to participate in a self-assessment scale called GAD-7 having seven questions (Spitzer et al., 2006). GAD-7 scale is numbered by setting points from 0 to 3, where the points denote the prevalence of anxiety as 'not at all', 'several days', 'over half the days' and 'nearly every day' respectively. Further, this study assessed the extent to which these conditions affected the participants and their day-to-day activities.

A significant evaluation in this area of literature was made. Several quantifiable papers and the medical literature were methodically examined through PubMed, PubMed Central, MeSH, and NCBI databases. Surface statistics prove men in better mental health positions than women, nevertheless, several cases go unidentified and undetected, leaving these men at acute risks. A clear binary is detected even in the studies about male mental health, which is chiefly dominated by studies on female mental health issues (McNeish et al., 2020). Evaluations on mental health issues dominantly focus on female emotions and distress (Sediri et al., 2020). An objective method of analysis would provide extensive scrutiny of the actual repercussions of these ignored conditions. A qualitative methodological approach was adopted for the study conducted among a self-assigned group of men. After the necessary approval obtained from the Institutional Human Ethics Committee, 100 male subjects were approached using the purposive sampling technique.

The ages of the participants ranged from 18-60. This non-probability sampling technique proved helpful in assessing the stress levels among a wide array of men. The GAD-7 questionnaire was concurrently sent to 100 research subjects who had proper access to internet facilities. The research subjects were verified to be cognizant in English, as all the items were in English. The participants were well informed about the nature and intention of the study. The research subjects were recommended to read and understand the questions correctly and enter responses.

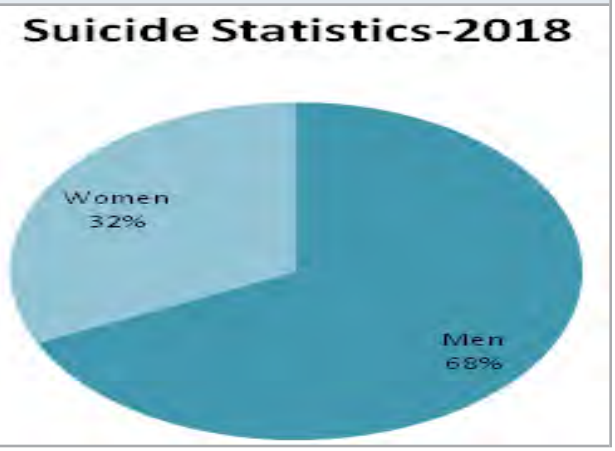
As the study adopted an explanatory approach, the sampling techniques were avoided. The discretion of responses recorded was guaranteed to all the participants. This questionnaire helped to analyze one's awareness of his conditions of anxiety. This aided in assessing the altercations in conduct, cognitive, psychic aspects and the constructive self-awareness regarding the unnecessary concerns and pessimistic thought patterns. GAD-7 comprises of seven questions to calculate the extent and seriousness of anxiety disorders. Each question was numbered and calculated using a Likert scale (0-3) having minimum and maximum scoring ranged from 0 to 21 respectively. The scores were categorized into four categories namely minimal, mild, moderate, and severe with cut off scores for each of the categories were 5, 10 and 15. Further assessment was recommended for those with a score above 10. The survey did not render a medical analysis but recommended seeking further medical assistance for high score subjects.

RESULTS AND DISCUSSION

Academicians and clinical researchers are now analyzing the variant expressions of depression in men. Unlike the symptoms of broodiness and melancholy in women, anxiety disorders in men surface through tiredness, bad temper, and insomnia (Keddie, 2006). Moreover, psychic conditions, if unattended, could affect relationships, financial conditions, and personal health. Some individuals choose to self-assuredly involve in specific cognitive exercises to deal with traumatic or taxing situations. This process, termed 'coping' assist people in regulating their psyche in certain demanding situations. Proper awareness on the types of depression and their predicaments would reveal the seriousness of these conditions. Empirical studies on depression and anxiety disorders provide the true origin, symptoms, and variety as available. Seeking professional help reduces the intensity of these conditions in 80% to 90% of men (Martin et al., 2013; Seidler et al., 2016; Radez et al., 2020).

Men while treatment, are often supported by medications, cognitive exercises, and psychotherapy. The majority of these conditions go undetected and ignored, pertaining to the social taboos on mental illnesses. Recent analytical studies on the productivity and efficiency of individuals at job, prove a lack of output in male employees due to an increasing number of depression and anxiety cases. Lack of knowledge on these psychic conditions, their origin and indicators, and the accessible remedies or assistance is at its crest when these conditions affect men (Radez et al., 2020; Salari et al., 2020). Studies show that often depression and anxiety disorders in men culminate in suicides and substance abuse. Suicide mortality rates for men are four times higher compared to that of women. Extensive studies show an alarming difference in suicides among men and women, wherein countries like Ireland and Finland, the ratio remains 10:1 and 11:1, respectively. Figure 1 elucidates the gender-wise suicide statistics in India in the year 2018. Studies show half the suicide cases in India to be impulsive. The alarming hike in the number of male suicides triggers doubts regarding men's mental health care attention (Young, 2019).

Figure 1: Pie chart showing distribution of suicide cases by gender in the year 2018.

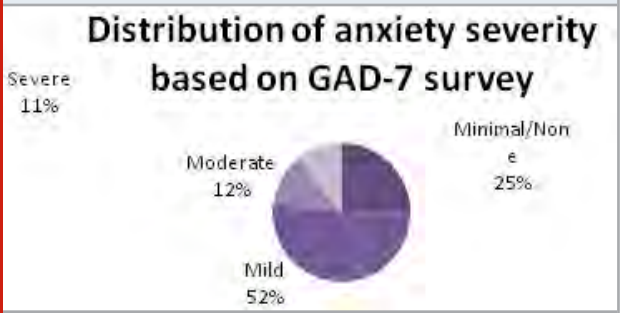


Unlike the fatal means of coping with these conditions, most men rely on averting through diversion, rejection, isolation, detached demeanour, substance abuse, gambling, or indulging in excessive and physically strenuous activities. According to Hanson et al. (2020), an affliction to violent tendencies is the most fundamental expression of high inebriation. Alcoholism and drug abuse are the two significant issues directly connected to these psychic conditions (Hanson et al., 2020). In men, violence and aggressive tendencies due to depression are more, compared to women. In matter of aggression, men are known to be more violent than women. The addiction to violence is the simplest and direct form of elevated intoxication, for the reason that most often, men behave in a dominating way by tormenting those around them (Singh, 2020). GAD self-administered screening survey was filled by the 100 research subjects under study. As specified in figure II, 25% of the subjects were identified as having minimal or zero anxiety, 52% with mild anxiety, 12% with moderate anxiety, and 11% with severe anxiety.

The quotients of the survey were calculated by evaluating the rate of occurrence and percentage. The results were categorized into different tables and pie charts. Figure III records the range of difficulty faced by study subjects due to the prevalence of anxiety episodes. The study shows 30% subjects finding it 'not difficult at all', while 60% found it 'somewhat difficult'.4% found these conditions 'very difficult' to handle and 1% found it 'extremely difficult'. As research by Scheuermann and Zürn (2020), Talcott Parson in his work, Family, Socialization, and Interaction Process (1960) defined how men were identified to be undertakers of 'instrumental' functions and women of 'expressive' functions. Further, Helen Hacker's paper, The New Burdens of Masculinity, elated these men's emotional struggles to keep up to the social expectations; despite the emotional 'repression' they endured (Scheuermann and Zürn 2020). At the

same time, women were intentionally instilled with the feminine expectations endorsed by female sex role norms. However, with the coming of academic feminism, there was a political questioning of these regulatory systems set up for women (Qing, 2020).

Figure 2: IIPie chart showing distribution of anxiety severity based on GAD-7 survey.



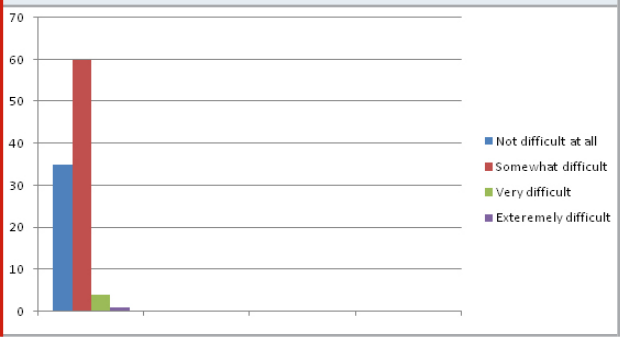
The ferment created by Feminism in the 1970s led to several men's liberation movements. They elucidated on the dangers of traditional male sex roles and their emotionally repressed positions. These aspects were generally titled as 'the inexpressive male: a tragedy of American society and 'Warning: the male sex role may be dangerous to your health' (Johnson and Smith 2020). Therefore, the sex-role theory presents the complementary positions constructed by the male and female sex role strata (Parsons-expressive/instrumental).

Thus, these normative models of sex roles restrict the scope for social transformation as discussed in The Myth of Masculinity (Pleck 1987) The concept of sex-role prevents individuals who violate the traditional role for their sex from challenging it; instead, they feel personally inadequate and insecure. As infant boys and girls cry in equal amounts, but when they mature, boys are trained to express lesser emotions than girls (Maccoby and Jacklin 1974; Pleck 1987). Therefore, this difference in child behavior primarily forms out of the gap in adult response towards children (Johnson and Smith 2020). A study by Fagot et al. (1985) on thirteen-month-old boys and girls in daycare, illustrated that the teachers responded to girls when they jabbered and gestured, and to boys when they screamed and whined. Eleven months later study showed that the girls talked more than the boys, who still screamed and whined. Although not all children get indulged in differing gender behaviors, adult responses definitely play a part in this dichotomizing trend.

Studies show that children learn to identify the societal amplifications of their gender by the age of three (Josephidou and Bolshaw 2020). Once these children leave their babyhood they are exposed to the differential meanings of gender. As they grow up children are not expected to be a good person but instead a "good boy" or a "good girl". These expectations set forth by the adults around them determine their desires and aspirations of the self-identity that they associate themselves with (Schalkwyk et al. 2020). Most discourses on masculinity depict hesitance in men to ask for aid, despite the consequences of their psychological conditions. In addition to this, men display an adamant resistance towards professional and personal aids in redeeming their psychic conditions (Möller-Leimkühler 2002). Studies show that most adolescent subjects, both men and women, opt for self-diagnosis of their psychic conditions. This often results in a settlement with the stigma accompanied by the accepting, regularizing, and refusing of psychological issues in men.

This could lead to complicated situations of crisis, including suicide, self-harm, and aggressive tendencies towards others. Men are generally brought up in a specific pattern, where they are often forced to contain their emotional planes and vulnerabilities to themselves. In most cases, men are forced to regulate themselves to the adherence of the hegemonic masculine ideals (Anand, 2020). A recent study on male mental health conditions (Sileo and Kershaw 2020) revealed the effectiveness of seeking help from peers, friends, or relatives. Rehabilitation centres and self-help groups have also been found effective. A proper remoulding of male gender expressions is vital. Men often evade seeking help bearing in mind it as a symbol of weakness (Rice et al. 2020). The proper altercations made in these attitudes and rendering of knowledge regarding these hegemonic gender notions could help reduce male anxiety and subsequent issues.

Figure 3: Bar diagram showing distribution of coping difficulties among study subjects.



A proper realization of these conditions and ensuing actions taken by the patients could reduce the physical and psychological difficulties that follow (Velasco et al., 2020). Recent online surveys illustrate the varying levels of psychological impacts exerted on men by the current situation of the pandemic. The uncertainties that came with the pandemic at various fronts have mentally and physically affected men in the worst ways (Huremovic, 2019). The conferred status of the 'sole breadwinner' role has taken a toll on the psychological well-being of many men (Huremovic, 2019; Velasco et al. 2020).

Unlike their pre-pandemic lifestyles, men are now mostly working from home and are obligated to various household chores previously undertaken by their partners alone (Vindegaard and Benros 2020). This

mode of division of labor is new to men and several men are finding it hard to amend their lives to the new modes of working along with the novel domestic roles bestowed upon them. Lack of job security and deteriorating financial stability are other reasons that are now driving men into the qualms of mental health illnesses. Similarly, there is a whole another group of individuals who are forced to stay away from their families due to lesser travel facilities, which has led to isolation and consequent cases of depression. Therefore, this study purports to acknowledge and understand the male psychological realities, mental illnesses, modalities, reasons, and possible amendments in this issue. While it is an extremely complex process to identify and address the constructional realities associated with the male psyche and masculinity, it is possible to initiate minor changes starting from balanced gendered socialization to wide-scale political altercations in bureaucratic amendments (Giorgi et al., 2020).

Establishing social roles is a way of linking a particular position within the social hierarchy to a normative cultural concept. However, the study shows that there are two ways used by the involved participants in materializing these sex roles in everyday life. One is by applying it into a specific situation and the other is by adhering to the general set of ideologies attributed to a particular sex, namely masculinity or femininity. This normalization is done through the process of socialization (Chatmon, 2020) However, once these sex roles are identified as part of a social process there is a scope for change. This can be done through effective alterations in gender expectations propagated by certain discourses (Binet et al., 2021). Following a comprehensive reading and analysis of men's mental health conditions, five assumptions specifying the reasons for this alarming situation have been made through the study; firstly, the strict imposition of male gender roles is perilous (Binet et al., 2021)

Male gender roles overtly encourage the suppressions of open emotionality among men where they are expected to possess an unsentimental emotional quotient; secondly, these rigid impositions hinder men from seeking help; thirdly, men often relate their psychic conditions to "stress" rather than grief or despair; fourthly, men often possess the inclination to refute their mental conditions and treatments and lastly, men often find refuge in risky rebounds rather than seeking of help.

CONCLUSION

With respect to future investigations in this area, researches probing the available social networks and support systems aiding in male mental conditions would be beneficial. More infrastructure and assistance in respect to the normalization of male psychological issues must be initiated. Forthcoming researches may evaluate the different facets of gender socializations and negotiations in respect to masculinity and related studies. Studies cross-examining the social relations maintained by men and their expressiveness in these relationships may also be vital subjects to study. This study concludes by calling for a gender-neutral mental health care approach encompassing intensive changes at individual and public planes.

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Ethical Clearance Statement: The Current Research Work Was Ethically Approved by the Institutional Review Board (IRB) of Amrita Viswa Vidyapeetham, Kochi Campus Kerla, India.

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Genetical
Communication

Investigation on the Genetic Variability of Soybean Seed Sucrose Content in Germplasm Accessions from Different Countries of Origin

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ABSTRACT

The palatability of soy-food products can be enhanced by increasing sucrose content in soybean grains which are used as raw material. Therefore, soybean genotypes with high sucrose content are desired for processing good quality soy-food products with higher organoleptic acceptance and sweetness. In this study, estimation of sucrose content was carried out through high performance liquid chromatography (HPLC) in 321 soybean accessions from 14 countries. Sucrose was resolved using a silica NH_2 column as stationary phase. The mobile phase (acetonitrile/water 75:25 v/v) was run isocratically at a flow rate of 1.0 ml/min. The elution was monitored by a refractive index detector. Wide genetic variability in sucrose content was observed, with a range of 1.2 -9.6 g/100g thereby exhibiting about 8-fold genetic variation. Twenty-six genotypes were identified which showed sucrose content >7.0 g/100g. However, nine genotypes were identified which showed sucrose content < 2.0 g/100g. The highest sucrose content was observed in two genotypes, namely, PP-45 (9.6 ± 0.84 g/100g) and P5-40-2 (9.4 ± 0.78 g/100g). Genotypes identified from diverse background and with contrasting levels of sucrose content may be used for developing mapping populations which can be used for tagging genomic regions underlying biosynthesis of this trait in soybean seeds. Further, these genotypes can be used for developing novel genotypes with sucrose content higher than observed in the germplasm lines evaluated in this study.

KEY WORDS: GENETIC VARIABILITY, HPLC (HIGH PERFORMANCE LIQUID CHROMATOGRAPHY), SUCROSE CONTENT, SOYBEAN, SOY-FOOD.

INTRODUCTION

Soy-based food products are fast gaining the sobriquet of 'functional food of the century' due to the presence of several nutraceutical components that stave off atherosclerosis, diabetes, breast cancer, osteoporosis at bay (Kumar et al., 2010a). Apart from basic nutrients like protein (40%), oil (21%), vitamins, soybean has tocopherols and isoflavones as major nutraceutical components. Despite its nutrients-rich profile, utilisation of soybean in food products is very meagre. Presently, only 7-10 % of the total soybean produced in the country is utilized in processing soy-food products. The quality of these food products depends upon the seed attributes used as initial raw material. Apart from the presence of antinutritional factors, its astringent/bland taste is also the main culprit for poor acceptance of food products processed from soybean seeds (Taira et al., 1990; Kumar et al., 2011; Salari et al., 2020).

This taste related deterrent can be overcome by increasing sucrose content of soybean seeds which imparts sweetness and enhances organoleptic acceptance of food-grade soybean (Taira et al., 1990; Kumar et al., 2011). Sucrose constitutes 41.3–67.5% of the total soluble sugars in soybean seed. Of the dried seed, sucrose content is about 2.5-5.0%. Globally, high sucrose content soybean genotypes are desired by soy food industry for processing soy milk, tofu, natto and other soy food products as sucrose contributes to favourable taste (Escamilla et al., 2019). In soybean meal, high sucrose content is desirable as it contributes positively to the potential metabolizable energy and thereby leading to the weight gain among animals as per their genetic potential (Bilyeu and Wiebold 2016; Salari et al., 2020).

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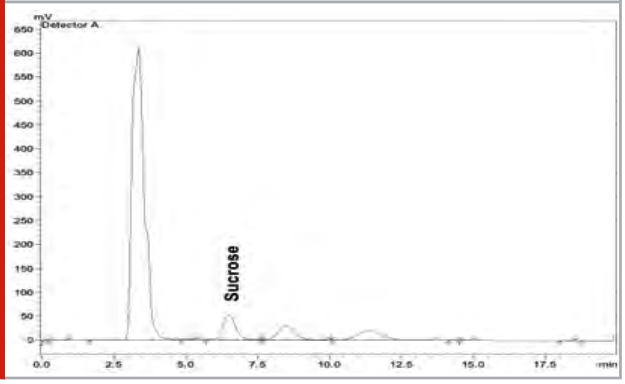
Therefore, development of high sucrose content soybean genotypes is important plant breeding objectives in soybean to meet the requirements of soy food and feed industry. The studies focusing on the screening of soybean germplasm for sucrose content are limited and were conducted only in selected fewer number of genotypes (Kumar et al., 2007; Kumar et al. 2010b). Hou et al., (2009) and Ficht (2018), investigated the genetic variability for sucrose content in 241 and 296 soybean genotypes. The high sucrose genotypes viz. PI 200508, V99-5089, PI 243545, and LD02-4485 mentioned in previous studies are not in the public domain due to the strict IPR regime (Skoneczka et al., 2009; Mozzoni et al., 2013; Zeng et al., 2014; Salari et al., 2020). Therefore, it is important to constantly screen large number of germplasm lines to identify and develop new genetic combinations with high sucrose content, along with focused crossing programme. In the present investigation, we screened 321 soybean germplasm accessions from different countries for the identification of genotypes with high sucrose content.

MATERIAL AND METHODS

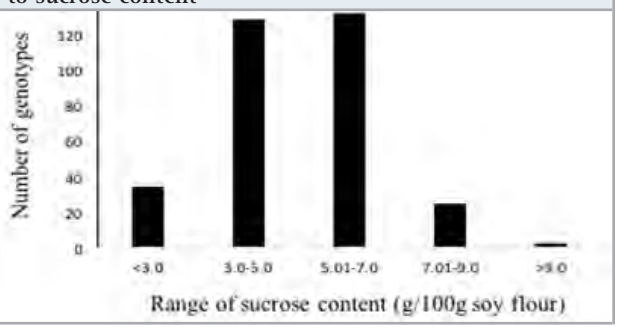
For the plant material, three hundred twenty-one soybean accessions comprising of exotic accessions, indigenous collections, advanced breeding lines developed in plant breeding programme for food grade characters were raised in the field of ICAR-Indian Institute of Soybean Research, Indore, India in single row plot of 3 m length with row-to-row spacing of 45 cm and plant-to-plant distance of 5 cm, in triplicate in the randomized block design. Standard agronomic practices recommended for soybean cultivation in Central India were followed from sowing to harvesting. Genotypes were harvested at maturity and freshly harvested seeds were subjected to sucrose estimation though HPLC.

Figure 1: Chromatogram showing the separation of sucrose standard (10mg/ml).



For the determination of sucrose content using HPLC, extraction of sucrose from mature seeds of soybean accessions from 14 countries viz. Australia (1), Brazil (3), China (2), Ghana (2), Hungary (1), India (221), Italy (1), Japan (1), Philippines (1), Russia (1), Shri lanka (2), Taiwan (11), USA (15) and 59 genotypes of unknown origin was carried out following the method of Liu and Markakis (1987). The extracted sugars were determined through HPLC as described elsewhere (Kumar et al. 2007). The extracted sugar sample obtained was filtered using syringe membrane filter (0.22µm, 13 mm diameter), and 20µl of this sample was injected in Shimadzu high performance liquid chromatography (LC 10 AT vp).

Figure 2: Distribution of 321 soybean genotypes according to sucrose content



Sucrose was resolved using a silica NH2 column (Phenomenex Luna 5µm, dimension 250mm×15mm), preceded by a guard column, maintained at 40 ^{0}C in Shimadzu CTO 10AT vp oven. The mobile phase, acetonitrile /water (75/25 v/v), was run isocratically at a flow rate of 1.0 ml/min and the elution was monitored by means of a refractive index detector (Shimadzu, RID10A). Peak of sucrose in the sample was identified using the retention time of the peak of the sucrose standard (10mg/ml), which was obtained at 6.5 mins (Figure 1). The concentration of sucrose (per gram of the flour) in the sample was computed by comparing its peak area with that of the known concentration of the standard, procured from Sigma Aldrich, using software CSW 1.7. Seeds of all the soybean accessions were analysed in triplicate samples for sucrose content. For the statistical analysis, phenotypic data and standard deviation was analysed and performed in Microsoft excel 2019.

RESULTS AND DISCUSSION

Three twenty-one soybean accessions were collected from 13 different countries. The concentration of major soluble sugar viz. sucrose was determined in seeds of the 321 soybean genotypes which exhibited a normal distribution ranging from 1.2 to 9.6 g/100g with the majority (260) of the genotypes containing 3.0 to 7.0 g/100g sucrose (Table 1, Figure 2), thereby exhibiting about 8-fold genetic variation. Twenty-six genotypes from four different origins had sucrose content above 7.0 g/100g (Table 2), whereas nine genotypes from Indian origin had sucrose content below 2.0 g/100g. The highest sucrose content was identified in PP-45 (9.6 g/100g) from unknown country of origin followed by P5-40-2 (9.4 g/100g), which was from India. The lowest sucrose content genotypes were P1-12-3, VP64-2, P1-11-2 and P4-5-3 (1.2, 1.21, 1.35 and 1.5 g/100g, respectively), and all these genotypes are from India.

Table 1. Average sucrose content (g/100g soy flour) in soybean genotypes from different countries.

Country of origin	Number of genotypes	Range of Sucrose content	Average Sucrose content
Australia	1	5.0	5.0 ± 0.40
Brazil	3	4.8-6.9	5.6 ± 1.14
China	2	5.4-5.8	5.6 ± 0.63
Ghana	2	5.9-6.1	6.0 ± 0.83
Hungary	1	3.7	3.7 ± 0.35
India	221	0.89-9.4	4.7 ± 0.84
Italy	1	3.9	3.9 ± 0.00
Japan	1	7.5	7.5 ± 0.64
Philippines	1	4.9	4.9 ± 0.00
Russia	1	6.0	6.0 ± 0.46
Shri Lanka	2	4.2-6.4	5.3 ± 0.22
Taiwan	11	3.6-7.6	5.4 ± 0.75
USA	15	3.8-8.1	5.9 ± 1.06
Unknown	59	2.5-9.6	5.4 ± 0.75
	321	4.43-7.48	5.31 ± 0.84

Table 2. Soybean genotypes exhibiting sucrose content higher than 7 g/100g soy flour or lower than 10 g/100g soy flour.

Genotype	Sucrose content(g/100g)	Country of origin
PP-45	9.60 ± 0.84	Unknown
P 5-40-2	9.40 ± 0.78	India
IC 567316	8.88 ± 0.62	India
P 2-19-3	8.13 ± 0.50	India
EC 457201	8.12 ± 0.58	USA
P 3-8	8.10 ± 0.52	India
EC 457286	8.06 ± 0.43	Unknown
JS 20-82	8.03 ± 0.37	India
EC 685250	7.94 ± 0.29	Unknown
ECP-125-738	7.85 ± 0.27	Unknown
SKY/AK-1403	7.81 ± 0.21	India
IC 574378	7.71 ± 0.19	India
EC 170267	7.70 ± 0.22	Unknown
CAT-19	7.63 ± 0.23	Taiwan
JSM-226	7.63 ± 0.17	India
P 2-2-1	7.60 ± 0.19	India
EC 65772	7.56 ± 0.21	USA
CAT-842	7.50 ± 0.17	Japan
EC 963805	7.44 ± 0.12	Unknown
CAT-135A	7.40 ± 0.10	Taiwan
CAT-1099	7.35 ± 0.18	India
EC 95289	7.20 ± 0.16	USA
EC 458346	7.15 ± 0.10	Unknown
VP 96-2-2	7.10 ± 0.10	India
IC 263278	7.03 ± 0.11	India
NRC105	7.01 ± 0.01	India

Table 1 shows the country of origin of 321 soybean genotypes, their range and average of sucrose content investigated in this study. Genetic variability for sucrose content was the highest in soybean accessions from India (1.2-9.4 mg/g soy flour-7.8 fold) followed by USA (3.8-8.1 g/100g soy flour-2.2 fold) and Taiwan (3.6-7.6 g/100g soy flour-2.1fold), respectively. With regard to average sucrose content of soybean accessions from different country of origin, soybean accessions from Japan, Ghana and Russia exhibited average sucrose content of 7.5, 6.0 and 6.0 g/100g soy flour, respectively. Average sucrose content of soybean accessions from USA was 5.9 g/100g soy flour. Soybean accessions from Brazil and China both showed average sucrose content of 5.6 g/100g soy flour followed by Taiwan (5.4 g/100g flour), Sri Lanka (5.3 g/100g soy flour) and Australia (5.0 g/100g soy flour), respectively.

Maximum number of 221 soybean accessions were from India with average sucrose content of 4.7 g/100g soy flour. Hou et al., (2009) reported genetic variation of 59.6-fold for sucrose content in 241 plant introductions (PI). In an earlier study, Kumar et al., (2010b) investigated sucrose content in 148 soybean genotypes and reported 4.80-fold genetic variation. Moreover, Ficht (2018) investigated sucrose content of 296 soybean lines obtained from University of Guelph germplasm panel and reported only 2.3-fold of genetic variation. In comparison to the results by Hou et al., (2009), the genetic variation (8-fold) revealed in the present study for sucrose content was much lower but significantly higher than that reported in other related studies (Hou et al.,2009; Kumar et al., 2010b; Ficht 2018).

CONCLUSION

Wide genetic variability (8.0-fold) existed for sucrose

content in 321 soybean germplasm accessions of different country of origin screened in the present study. Germplasm accessions identified with high sucrose content were from India, USA and unknown country of origin, while accessions for low sucrose content were from India. The diverse genetic background of these high and low sucrose content genotypes can be exploited in developing mapping population to identify the genomic regions underlying sucrose biosynthesis. High sucrose content genotypes from diverse background can be crossed to develop soybean genotypes with higher sucrose content value than the maximum value observed for this trait in this investigation.

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Conflict of Interests: The authors declare no conflict of interests among themselves.

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Toxilogical
Communication

Analysis of An Indoor Amount of Environmental Tobacco Smoke (ETS) with Quantified Particulate Matter

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ABSTRACT

This research aimed was to establish an indoor amount of environmental tobacco smoke (ETS) by calculating particulate matter less than 2,5 µm (PM 2,5) as the predictor of the ETS in hospitalities across the environment of the college. . We established smoking conditions in business establishments and analyzed internal quantities of PM 2.5 at 20 internet cafes, 38 pubs, and 20 billiard rooms employing a sidepak am510 direct-reading handheld real-time display from October 2014 to December 2015. In 65% of internet coffee shops and 85% of billiard shelters in 2015, cigarettes were found. The incidence in pubs that had been officially banned from smoking declined from 33,3 percent in 2014 to 10 percent in 2015. The total 2015 concentration of PM 2.5 in Internet cafes, bars, and pool sheds was 98.6 µg/ m3, 29.6 µg/ m^3, and 135.4 µg/ m^3, respectively. PM 2.5 levels in online coffee shops and billiard rooms were 2 to 2.7 times higher than the 24-hour intake level (50 µg/m3) for outside PM 2.5 established by the Korean Ministry of Environment. While a prohibition on smoking was placed on internet cafes and bars, smoking was already occurring in those areas. For the effectiveness of law-based efforts to protect employers and employers' wellbeing from second-hand exposure to smoke, more strict compliance is essential. A cigarette prohibition should be enforced in billiards rooms as soon as possible.

KEY WORDS: ANALYSIS, SMOKING OF CIGARETTES, ENVIRONMENTAL TOBACCO.

INTRODUCTION

Smoking of cigarettes, cigars, tobacco piping, etc., which resulting in second-hand smoke-exposure is created by environmental tobacco smokers (ETS). It consists of mainstream smoke(s) and sidestream smoke (SS) emitted after an artificial tobacco product dissipated by cigar smoke. The empirical confirmation of passive smoking has contributed to many human illnesses, conditions, and fatalities. ETS is significant as it is one of the primary contaminants determining indoor environmental quality. It includes approximately 60 forms of compounds reported or believed to be human carcinogens, namely nitrosamines, benzopyrenes, beta naphthylamine, and polonium 210. Approximate 30% of deaths from cancer are induced by smoking, and second-hand smoke has led to several health consequences, including lung cancer, cardiac failure, and allergies (Muhibbu-din, 2020, Omrani and Fataei, 2018, Zaeimdar et al., 2019, Aletor, 2021).

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This is why ETS is listed as a category of (known) cancerous agents as recommended by the Environmental Protection Agency (EPA) and from the data of various studies conducted on plants as well. ,(Barth, 2021). ETS has since been classified as a carcinogenic material of the same classification as asbestos or benzene by the US National Institute of Occupational Health and Safety. Moreover, cigarette smoke can quickly disperse and is impossible to eliminate such that it is exposed to a vast number of individuals, regardless of the smoker's purpose. General banning on the smoking of crowded locations, including the workstations, restaurants, and pubs, is followed by using centers for disease prevention (CDCs) according to these threats as evidenced by several; studies like that of Khayatnezhad and Gholamin, (2020a).

Many experiments on indicator materials have been conducted (Jia et al., 2020) to quantify and test airborne ETS quantities, including around 6,000 chemical substances (Khayatnezhad and Gholamin, 2020b, Hewitt, 2021). A sense with optimal circumstances that physically and chemically alter due to the development of ETS is hard to characterize (Si et al., 2020, Huma et

al., 2021). The usage of nicotine by young people with a comparatively high prevalence of smoking under the auspice of the WHO-funded framework convention on tobacco control (FCTC) is to be minimized in a realistic regulative attempt. This bans smoking in some public indoor-atmosphere places, including schools, internet cafes, and restaurants, through national health enforcement acts throughout these places (Gholamin and Khayatnezhad, 2020a, Kabir et al., 2021).

A smoking ban is increasingly being extended in leisure areas, including billiards as small sporting venues. In Korea's smoking, 39.4% of male adults (age 19 and older) are estimated to decrease steadily, according to national health and nutrition surveys (2015). In comparison, this study recorded a smoking prevalence rate of 42.3% for males and 5.6% for females in the 10–19 age category, the third-highest average (Korea center for illness control and avoidance) (Fataei, 2017, Ghomi Avili and Makaremi, 2020, Radmanesh, 2021). Youngsters, i.e., people aged 19 to 24 years, were 2.45 (95% CI: 1.60, 3.73) times more possibly to smoking than those aged 60 years and over (Karasakal et al., 2020a, Gholamin and Khayatnezhad, 2021).

Excellent health behaviors between younger people are extremely significant since these patterns are sustained throughout the period and can significantly influence individual wellbeing. Besides, there is a need to maintain the smoking ban program's efficacy in the banquet centers. Throughout this report, we measured the extent of second-hand smoke emission by calculating PM 2.5 as an ETS measure in online cafes, pubs, and billiards, both places where younger people consume a substantial level of time in the 20s, in the vicinity of a university campus in Changwon, South Korea. We have also surveyed the rate of enforcement of the prohibition of smoking in online cafes and pubs that are listed as non-smoking places under the National Health Promotion Act and also examine the need to quit smoking in billiard rooms not yet set down for non-smoking.

MATERIAL AND METHODS

This research included 78 interior rooms, comprising 20 online cafes, 38 pubs (18 before and 20 after), and 20 billiard facilities throughout 3 km of the college campus for three months (among October and December) in 2014 (before regulation, 18 pubs) and the same three months in 2015 (following principles, n=60). In the context of the bar, the same locations were preferred before and following the rules.

Evaluation of Airborne: ETS sensitivity properties were studied through screening PM 2.5, a marker of airborne ETS volumes. The atmospheric PM 2.5 quantities in the internal air were calculated at a stream rate of 1.7 L/min employing a sidepak real-time aerosol sensor (version AM510, TSI Inc., Shoreview, mn 55126, USA), which is a light-emitting-type direct-reading instrument that measures mass level by spreading light with a wavelength of 670 nm. Because the light-emitting procedure calculates mass quantities as a function of particle size and refractive indicator, the second-hand smoking intensity was determined by adding a conversion factor of 0.295 to the specified values (Gholamin and Khayatnezhad, 2020d, Li et al., 2021, Sun et al., 2021).

To concentrate on particulate substance with a diameter of 2.5 μm or greater, a PM 2.5 impactor was mounted to the sidepak, and zero adjustment was conducted employing a HEPA (high-efficiency particulate air) detector until the screening. On the weekdays between 7:00 pm and 11:00 pm, and 5 minutes before and after the implementation of the internal inspections outside of the houses, the field investigator examined the hostel sites and assessed the concentration of PM 2.5. The concentration of the internal PM 2.5 was considered for around 40 minutes by putting the lateral pack on a table or seat was not influenced explicitly by PM 2.5 origins, including doors, windows, or ventilation. Over 1-min periods the samples were drawn. The amount of the interior room, the percentage of smokers, and the air-conditioning were also reported during the measurement, impacting the concentration dispersion.

Estimation and computation of smoking density (SD): During the measurement phase, smoking cigarettes and the number of fans mounted at the ventilation site was recorded. DLR130 assessed the internal venue volume (Robert bosch tool corp., mt. prospect, Malaysia)

RESULTS AND DISCUSSION

As seen in Table 1, the incidence of smoking in the three forms of acceptance areas across the university, namely cafes, bars, and billiards, was 65%, 15.8 %, and 85%. For bars and internet cafés, smoking was still present on the spot, while a national health promotion act appointed smoke-free areas as from 1 January 2015. The smoking incidence throughout pubs was lowered in 2015 by implementing the regulation from 33.3% (6/18) to 10% (2/20).

Kim et al. registered a 42 percent smoking rate in bars before the regulation on the smoking prohibition. Nevertheless, Billiards are not identified as smoke-free places, with three installations showing the maximum smoking incidence. Eight bars and thirteen online cafes have reported smoking, and the indoor prohibition must be strictly implemented to enhance indoor air characteristics. For the three forms of wellness centers, the range of the PM 2,5 levels in the interior air was the log-normal according to the W-test ($p<0,01$). The distribution of PM 2,5 concentrations across the three forms of installations was statistically significantly different ($p<0,001$). The PM 2.5 values were spread about 4.4 times greater in billiard facilities than the bars ($p<0.001$), and a gap between the maximum and minimum was about 48.9 fold. There was a gap in disseminating PM 2,5 both prior and following the smoking control legislation between online cafes and billiard rooms.

Correlation analyzes were conducted to evaluate the association between SD and PM 2.5 in accommodation locations. As seen in Fig. 2, the association rate was 0.58. Previous experiments have documented the connection between internal concentrations of PM 2.5 and SDs in guest rooms. In the United States, PM 2.5 tended to have an excellent relationship to SD in 10 accommodation sites, like restaurants and bars (Arjaghi et al., 2021, Esmaeilzadeh et al., 2020). The associations among SD and PM 2.5 amounts were found in the above analysis ($r=0.576$, $p<0.005$). The frequency of PM 2,5 is more associated than the number of tourists or the filtering method, which is most definitely attributed to the smoking's relation to internal PM 2.5 dissemination.

Table 1. Summary of smoking conditions and sampling results

Year	Smoking status	No. Venues	Average of fans	Average of volume	Average smoking	Arithemetic Mean	GM	Range
Internet Cafes		20	10.5	441.6	-	98.6	91.5	9.5
		13	10.5	539.4	0.036	127.8	100.9	233
		7	10.6	259.9	-	44.5	29.2	9.5
PUBS Total		38	4.4	352.4	-	30.8	26.8	6.1
		20	4.4	635.2	0.006	30.3	30.2	28.1
		2	8	313	-	29.5	24.1	6.1
		18	4	360.4	-	32.2	29.3	9.4
		18	4.3	404.9	0.005	41	39.9	25.7
		6	6	338.1	-	27.9	25.2	9.4
		12	3.5	451.2	-	135.4	121.8	10.9
Biliard Hals		20	5.9	437.7	0.021	150.8	143.1	90.1
		17	6.7	527.5	-	47.6	32.9	10.9

Figure 1: Evaluation of combined chance diagrams of PM 2.5levels for three forms of accommodation sites.

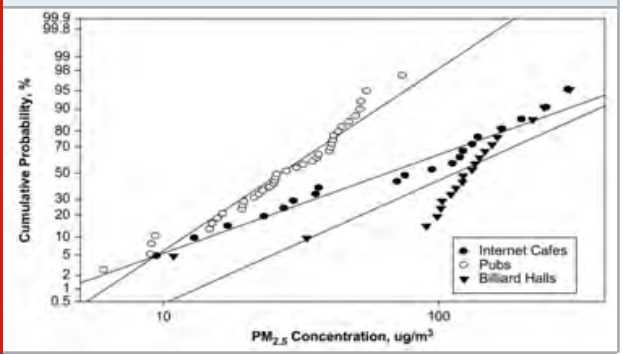
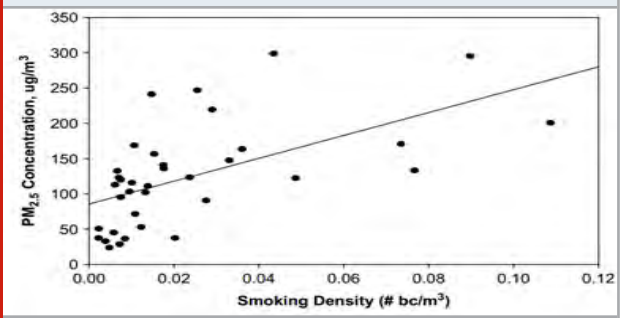
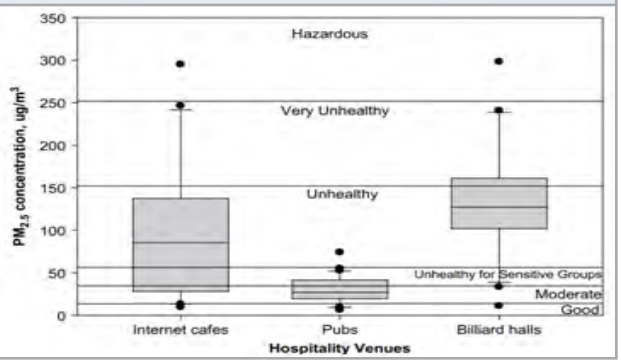


Figure 2: Regression evaluation of PM 2.5amounts and smoking intensities within smoking areas (y = 85.6 + 1,621.1x, r = 0.58).



According to earlier reports, passive smoking represents about 90% to 96% of breathable suspended matter in accommodation sites (casinos, pubs, billiard rooms) (Rodríguez, 2021). The PM 2.5 content was determined

Figure 3: Estimating indoor air quality relies on PM 2.5levels in online cafes, bars, and billiard facilities, utilizing six EPA AQI types. The columns' central rows are the average values; the columns' lower and higher limits are the 25th and 75th percentiles, respectively; the whiskers are the maximum data points inside the 10th and 90th percentiles, respectively; the actual data sets are outliers.



better to represent the smoking state of the replacement material. The median level of PM 2.5 throughout online cafes authorized to smoke (13 of 20) was 127.8 µg/ m^3, which was substantially greater ($p<0.05$) than online cafes where cigarettes were banned (44.5 µg/ m^3). The PM 2.5 levels were around three times greater in smoking spaces than non-smoking spaces in billiard facilities. The magnitude of the statistical disparity may, nevertheless, not be verified in the context of breweries.

According to WHO's FCTC (Huang et al., 2021), due to tightening the legislation on the banning of cigarettes, the

rates of smoking in Korea have been steadily declining. In South Korea, the Korea Centre for Disease Control and Prevention (Gholamin and Khayatnezhad, 2020b) announced that the incidence of sensitivity to second-hand smoking between non-smokers decreased to 57.9% in 2013, 52.1% in 2014 35.4% in 2015 following the public-sector anti-smoking legislation. Billard facilities, though, have been listed as entertainment rather than as public rooms, and the smoking ban has concentrated only on billard corridors that have greater than 1,000 capacities. In most billiard rooms, the number of people is much lower. Therefore, much of the billard corridors have not yet been regulated and display the maximum PM 2.5 level.

Inhalation of second-hand smoke is extremely probable. Thus, similar training and public access must be improved, and the smoking prohibition program expanded to Small Sports Centres to reduce the dangers posed by second-hand tobacco. The smoking ban law, if successful, is extremely effective in increasing the consistency of indoor air and alleviating quantities of the internal PM 2.5 , Wan, 2021). Throughout sustainable smoking prohibitions, stringent compliance and frequent surveillance are essential factors. One solution to minimize second-hand smoking is enforcing strict punishments for smokers in public areas.

Assessment of air modality employing AOI: To estimate the extent of environmental pollution, the US EPA has introduced an air quality index (AQI) utilizing the PM 2.5 levels (Alayi et al., 2020). This AQI has measured internal air competence for accommodation facilities. The mean unsafe values considering 24 hours of intake in PM 2.5 concentrations of indoor air have been calculated using billiard corridors (135.4 µg/ m^3) and online cafes (98.6 µg/ m^3). One online cafe and one billiard room were also rated as attaining the dangerous stage, which implied urgent upgrading of the internal air. In previous research, elevated PM 2.5 was also recorded when smoking was permitted in accommodation locations. The average of PM 2.5 (n = 62) values was 161µg/m (4.6 times greater than our air quality index) for smoking restaurants and Kentucky bars throughout the United States (Khayatnezhad and Gholamin, 2021a, Karasakal et al., 2020b).

The study's drawbacks are as follows: Initially, PM 2.5, the measure provided for ETS, could be influenced by a broad range of causes apart from cigarette smoke, like interior and exterior burning, indoor games, preparation, washing, etc. Second, only breweries were chosen for study settings surrounded by a college campus, so only those bars can be described. Therefore, the bars were chosen for the prevalence of smoking and other internal setting considerations without any preliminary details. Third, about the transmission of the particulates, the effectiveness of the ventilation system and the internal air-conditioning were not calculated (Fataei et al., 2018).

The form and theory of air ventilation system, the rate of air-conditioning, and the operational process are essential since ventilation rate is a determining factor of indoor air quality. Fourth, since the monitoring duration overlaps with the duration for end-term learners' tests, which decreases the number of consumers in the locations, a sampling outcome could be a downgrading. The sample calculation time and the number of specimens should be regarded to correctly evaluate and validate each accommodation site's reference value. The control of second-hand smoke exposure at these multi-use sites, the clarification of the compliance levels of the smoking truce program, and the improvement of the applicable legislation would in the future be crucial to increase public safety. Table 1. Overview of smoking practices and sample findings for PM 2.5 levels across three forms of accommodation facilities in 2014–2015. he columns' central rows are the average values; the columns' lower and higher limits are the 25th and 75th percentiles, respectively; the whiskers are the maximum data points inside the 10th and 90th percentiles, respectively; the actual data sets are outliers.

CONCLUSION

The research findings have been as follows. Firstly, throughout the billion rooms where the smoking ban legislation was not enforced, smoking was most substantial in specific accommodation locations near the university. Despite the enforced legislation (alcohol prohibition), smoking always took place in online cafes or bars. For both online cafes and bars, tighter smoking ban enforcement is mandatory. Secondly, in the range of billiard facilities, online cafes, and bars, the PM 2.5 as a marker used for cigarette ambient smoke was more incredible.

AQI assessments reveal that the standard of air in bars is reasonable; however, most online cafes and billiard shops are health dangers. Thirdly, the PM 2.5 levels of smoking locations were statistically more significant in online cafes and bars than in particular areas where smoking was banned ($p < 0.05$). The smoking ban incidence is rising according to mandatory standards, and the frequency of indoor air PM 2.5 is also diminishing. Fourth, the National Health Promotion Act's application must be expanded to the billiard facilities, where ETS quantities are most remarkable, to stop the guests and staff from passively smoking and preserve public health.

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Sports Science
Communication

Dynamics of Functional Indicators of Adolescents Against the Background of Regular Volleyball Trainings

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ABSTRACT

139 adolescent male volleyball players were examined. The impact of systematic volleyball training on the somatotype of adolescents was assessed. We found out the somatic parameters of volleyball players, taking into account their chronological age. Regular volleyball training lasting at least 1 year strengthened the body of athletes, increasing the prevalence of strong and medium build among them. This was accompanied by an increase in the proportionality of their physique. As the experience of volleyball training increased, an increase in endurance and functional reserves of the body was noted. Obviously, systematic volleyball practice in adolescents leads to an increase in strength capabilities and an increase in the tolerance of long-term cyclic loads.

KEY WORDS: ADOLESCENTS, VOLLEYBALL, PHYSIOLOGY, PHYSICAL TRAINING, STRENGTH TRAINING, MUSCLE ACTIVITY.

INTRODUCTION

Regular moderate muscle loads create conditions for the activation of most functions of the human body, form a consistently high level of resistance and increase the margin of safety of its functional systems. Long-term observations indicate a great functional benefit from physical training in people of any age and especially among young people (Zhvavy et al, 2001, Ereshko and Makhov, 2018; Makhov and Zakharov, 2018 Karpov et al., 2020).

This effect is promising to use for the widespread health improvement of young people, building up their labor potential and increasing the resistance of their body. For the most pronounced health-improving result from physical activity, obviously, young people should be more widely involved in the system of regular physical training, taking into account the existing preferences and biological characteristics. At the same time, it is the general physical status that should be considered as the basis for rational planning and dosage of muscle activity in the chosen sport (Galkina, 2008, Grechishkina, 2009; Kotova et al., 2017,Vorobyeva et al., 2018). Researchers recognize the presence of morphological and functional relationships that determine the physical individuality of a person, regardless of his attitude to sports. In this regard, the physical capabilities of a person should be strictly taken into account during the planning of muscular loads (Makurina et al, 2020; Kulikov et al, 2020).

At the same time, the process of individualization of training in any kind of sport should not be determined only by age, gender and the current general functional state (Skoryatina and Zavalishina, 2017). Modern researchers recommend that beginners take into account the peculiarities of constitutional characteristics, adaptive properties and typological status (Usha et al., 2019). Obviously, taking these indicators into account helps to establish the general level of viability and the severity of the adaptive potential of the organism (Vorobyeva et al., 2020).

The relationship between somatic parameters and physique features with the effectiveness of muscular loads of a different nature and achievements against their background of a certain degree of harmonization of the physical status of a person is known. Apparently, the athlete's somatotype strongly reflects the biological foundations of the body, which are significant for overcoming physical exertion. The presence of a number of morphofunctional differences inherent for

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certain constitutional types and associated different motor capabilities, adaptive and functional properties during systematic muscle training is recognized (Fayzullina et al., 2020).

It is believed that the type-specific method is very effective in helping to achieve a high individualization of sports training. This point of view is supported by the possibility of this method to optimize the functional state in the process of physical education. Positive somatic changes are based on physiologically beneficial changes in metabolic processes that ensure the functionality of the whole organism. Differences in somatotype are associated with differences in metabolic rate, motor skills, strength and speed abilities, and, ultimately, with the level of general performance (Zavalishina, 2020a). Regular physical activity, including in a playful way, is a proven way to correct the somatotype. An effective option for improving the level of physical fitness in adolescents is to regularly play volleyball. These trainings, carried out taking into account the current level of their physical development, are able to provide its correction. Purpose of the present work was to consider the changes in the morphological and functional characteristics of adolescents involved in volleyball for different periods of time.

MATERIAL AND METHODS

The performed studies were evaluated and approved by the local ethics committee established at the Russian State Social University on March 10, 2017 (Protocol No. 3). The work was carried out on the basis of the Russian State Social University, Moscow, Russia. 72 adolescent male volleyball players were examined. The volleyball players recruited underwent at least four workouts per week, with each workout lasting about 100 minutes. All surveyed volleyball players were 12-14 years old. According to the amount of volleyball training experience, all athletes were divided into 3 groups. The first group was considered the control group. It was formed by volleyball players with up to one year of sports experience (22 people). The second group of volleyball players consisted of athletes with sports experience from 1 to 2 years (26 people). The third group of athletes included volleyball players who have been training for 3 years and over three years (24 people).

In the course of the study, a number of indices were recorded to assess the somatic status of a person. The state of physical development of volleyball players was assessed taking into account the value of their body weight, the value of the size of the chest girth and the value of the linear body size. A method for assessing the strength of the physique was applied, which determines the somatic status, that is, the state of individual, functional and morphological characteristics with the registration of the value of the Pignet index. For this purpose, the following formula was used:

Pignet index = body length (cm) – [body weight (kg) + chest circumference at the moment of exhalation (cm)]

In thin asthenics, the value of the Pignet index > 30, with a dense physique (hypersthenics) the value of the Pignet index is <10, with a normal physique (normostenics) the Pigne index is 10-30. The state of harmony of the somatic development of adolescents was determined by the ratio of the shape of the chest and the linear size of the body. For this, the Erisman index was used, which was calculated using the following formula:

Erisman index = the size of the chest circumference in the pause of breathing (cm) -1/2 body length (cm)

If the value of the index was below +3.3 cm, it was said that a person had a narrow chest, in the case of an index value over +5.8, a wide chest was recorded.

Body mass index (Ketle index) was calculated using the formula:

Body mass index = body weight (kg) / linear body size (m^2)

Four levels of this index were recorded: with a body mass index <18.5, it was considered that there was energy deficiency (malnutrition) in the body, with a value of 19-25 it was considered that there was normotrophy, with a value of 26-31, the state was estimated as the presence of excess body weight (in other words, hypertrophy), in the case of its value over 31, obesity was revealed.In the examined subjects, the harmony of the somatic status was determined using the Rorera index, which makes it possible to determine the correspondence between the linear size of the human body and the value of his body weight. It was calculated according to the following formula:

Rorera index = body weight (kg) / body length (m^3)

In the case of a value of this index <10.3, it was said about the disharmony of development due to the small value of body weight. With an index value in the range of 10.4-13.7, they talked about the harmony of the physical status, in the case of the index value> 13.7, there was a disharmony of the somatic status due to the overweight of a person. The features of development were clarified taking into account the Pirke-Beduzi index, which made it possible to take into account the proportionality of standing height in relation to height in a sitting position. The following formula was applied:

Pirke-Beduzi index = [standing height (cm) – sitting height (cm)] / sitting height (cm) x 100%

The values obtained during the calculation of the Pirke-Beduzi index determined the relative size of the legs: values below 87% indicated a short leg length in relation to the height of a person, in the case of 87-92% they spoke of proportional physical development, in the case of a value of the indicator more than 92% the length of

the legs was considered large in relation to the height. The working properties of the respiratory system were determined by the level of the vital index, calculated as the ratio of the vital capacity of the lungs and body weight:

Vital index = vital capacity, lightness (ml) / body weight (kg)

The higher the level of the vital index, the more optimal the degree of development of the subject's chest breathing ability was considered. The average value of the vital index in adolescents is normally 55-60 ml / kg. The value of the strength index of the hand was determined. This indicator was considered as the ratio between body weight and the level of muscle strength in the main hand:

Arm strength index = [hand strength (kg) / body weight (kg)] x 100%

Low arm strength was noted in the case of the arm strength index <30%, the strength capabilities less than average were considered in the case of the index value from 31 to 41%, the average arm strength level was determined at the level from 42 to 64%, the strength capabilities above the average were found at the value from 65 up to 74%, high level of strength> 75%. The index of the back strength index allows to reveal the relationship between the size of the body weight and the level of strength capabilities of the back muscles:

Back Strength Index = [back dynamometry (kg) / body weight (kg)] x 100%

Low back strength was determined when the back strength index was below 101%, strength was assessed as less than average in the range from 102% to 119%, the average strength was indicated by the indicator in the range from 120% to 156%, the strength above the average level was from 157% to 174%, the strength that was in the range of more than 175% was considered a great strength. The type of biological reaction of the neuromuscular system of the surveyed was identified for their classification into "sprinters", "mixed" and "stayers" by calculating the Kaznacheev index using the formula below:

Kaznacheev index = maximum muscle strength / maximum muscle endurance

The value of the Kaznacheev index in the case of less than 1.0 was considered a sign of pronounced endurance (a person belongs to the "stayer" type), with a value above 2.0, it was considered a sign of prevalence strength characteristics (a person belongs to the "sprinter" type), with an index level between 1.0 and 2.0, there was a development of endurance and developed strength capabilities and the type was considered intermediate (a person belonged to the "mixed" type). Obtained in the course of the study, the indicators were statistically processed using the Microsoft Excel program with the subsequent calculation of the Student's criterion.

RESULTS AND DISCUSSION

The registration of the strength of the physique using the Pignet index showed the prevalence of this indicator in the athletes of the 2nd and 3rd groups in comparison with the individuals who formed the first group (Table 1). The surveyed volleyball players, included in the control group, had mostly weak physique (in about a third of cases they had a very weak physique). There were more athletes with an average physique in the 2nd group. In this group, there were fewer adolescents with a very weak physique compared to the control group. In the third group of athletes with a period of sports training for more than 3 years, the number of those with a weak and very weak physique was minimal. In this group, the bulk of the athletes had an average physique. In this regard, we can say that as the duration of the experience of sports training increases, the general physical status is strengthened, which indicates a beneficial effect on the physical development of regular volleyball loads.

The values of the Pignet index obtained in the study in the examined volleyball players of the first group said that they were mainly of hyposthenic body type. They rarely had a normosthenic type and rarely had a strong physique, assessed as a hypersthenic type. Regular volleyball training has shown the ability to strengthen adolescents' physique to medium to robust status. With regular exercise, chest size and mobility increased to meet the growing need for oxygen throughout the body. The value of the Erisman index among volleyball players turned out to be higher in the second and third groups in relation to the first group by 31.7% and 68.7%, respectively. A negative value of this index indicated the narrowness of the chest. The data obtained indicate that as the duration of volleyball sessions increased, the number of athletes of asthenic constitution decreased and therefore in the third group it turned out to be minimal.

The assessment of the state of physical development and adequacy of nutrition was allowed to carry out the body mass index. The value of this index was comparable for all observed volleyball players. Evaluation of individual indices of body mass index among volleyball players made it possible to find out that in the first observation group the main number of adolescents had normal body weight for their age and height. Disharmony of physical development as a result of weight loss in this group was rare. In the second group of volleyball players, even more athletes had a normal body mass index, and less than 10% had body mass deficit or excess. In the third group of volleyball players, almost all athletes had a normal body mass index and only in about 5% of cases there was some excess body weight. Apparently, this is due to the fact that athletes have a pronounced appetite against the background of frequent physical exertion, leading to significant energy consumption.

The mean value of the Rorera index was the highest in the first group (13.5±0.22 kg/m³), in the second and third groups they were lower and were comparable. Assessment of the harmony of the physique by the value of the Rorera index revealed some phenomena of disharmony in the first group in no more than 20% of cases due to low body weight. In the second observation group, this was noted in about 10.0% of cases. In the third group, developmental disharmony was present only in 5.0% of the surveyed. Basically, the volleyball players registered average, that is, quite harmonious development. In the third group, the number of athletes with a harmonious development of the somatic status was the largest in comparison with the rest of the observation groups.

Table 1. Values of somatic indices in the surveyed

No	Indicator	Examined groups, M±m		
		first group, n=22	second group, n=26	third group, n=24
1.	Pignet index, point	36.7±0.67	29.1±0.75 p<0.01	20.6±0.46 p1<0.01
2.	Erisman index, cm	-5.4±0.70	-4.1±0.62 p<0.01	-3.2±0.53p1<0.01
3.	Body mass index, kg/m²	17.9±0.32	18.7±0.16	19.6±0.31
4.	IndexRorera, kg/m³	13.5±0.22	11.5±0.18 p<0.05	11.0±0.25 p1<0.01
5.	Pirke-Beduzi index, %	93.0±0.83	95.9±0.72	93.8±0.96
6.	Life index, ml / kg	40.8±0.61	47.2±0.43 p<0.05	49.6±0.37 p1<0.01
7.	Kaznacheev index, point	0.4±0.06	0.6±0.07p<0.01	0.8±0.05 p1<0.01
8.	Leading hand strength index,%	36.2±0.68	41.7±0.75 p<0.01	53.4±0.61 p1<0.01
9.	Back strength index,%	59.7±0.73	85.2±0.60 p<0.01	89.8±0.83 p1<0.01

Note: p – is the significance of the differences between the first and second groups; p1– is the statistical difference between the first and third groups.

In the course of the study, the proportionality of the physique of volleyball players was determined using the Pirke-Beduzi index, which assesses the ratio of the linear dimensions of the legs and trunk, taking into account the ratio of growth in a standing position to the value of growth in a sitting position. The calculated values of the Pirke-Beduzi index allowed us to say that its average values in all observation groups did not differ significantly: in the first group, the index was 93.0 ± 0.83%, in the second group – 95.9 ± 0.72%, in the third it was 93.8 ± 0.96% in the group. Judging by the Pirke-Beduzi index, in the first group there were about 20% of people with short legs in relation to body length, in the second group there were less than 11.0% of such people, and in the third group it was less than 7.0%. Significant length of the lower extremities (Pirke-Beduzi index -> 90%) was more often observed among volleyball players who train for a long time: in the second group (over 70.0%) and in the third group (over 65.0%). In the control group with such parameters, it was about 60.0%.

The vital index assessment reflected the value of the vital capacity of the lungs in terms of per kilogram of body weight in all athletes. Its increase indicated a greater severity of general physical development (Zavalishina, 2020b). It was possible to trace the increase in this index with increasing experience of physical training. In the second group this indicator was 15.7% more than the control, and in the third group by 21.6%. It can be assumed that in the second and third groups of volleyball players, the respiratory characteristics of the chest prevail over those in the first group.

Monitoring of the type of functional characteristics of the neuromuscular system in volleyball players taken under observation revealed that in the first group the value of the Kaznacheev index was in the range from 0.3% to 0.6% (0.4±0.06%), in the second group it was in the range from 0.4% to 0.7% (0.6±0.07%) and in the third group it was in the range from 0.6% to 0.9% (0.8±0.05%). Apparently, all the volleyball players observed were "stayers", that is, they were able to withstand prolonged cyclic physical activity.

The examined volleyball players of the control group had low values of the strength of the leading hand. Volleyball players in group 3 (53.4±0.61%) showed great strength capabilities of the hand. This value was inferior to the values of the second group of athletes (41.7±0.75%), but exceeding the indicators of the first group by 15.2%. Differences between the values of the strength indices of the leading hand among volleyball players, when comparing groups 2 and 3, reached 28.0%. Registration of the back stance index made it possible to establish low strength characteristics of the back in all groups of athletes. The maximum value of this index was found among volleyball players who made up the third group (89.8 ± 0.83%). This value was inferior to the indicator of the second group by 5.4%, and the control group was inferior to 50.4%. In this regard, it can be assumed that as the duration of volleyball practice increases, the back muscles are trained.

CONCLUSION

In the course of increasing the length of service in

volleyball among adolescents, the morphofunctional status is strengthened. Regular volleyball training for more than one year leads to an increase in the prevalence of medium and strong physique among them and to a decrease in the incidence of a weak body type. At the same time, adolescent volleyball players experience an increase in the harmony of development and increase their endurance in relation to cyclic physical activity.

Conflict of interest: No conflict of interest is declared.

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Ethics Committee Resolution: The study was approved by the local ethics committee of the Russian State Social University on March 10, 2017 (Protocol No. 3).

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Biotechnological Communication

LYE-Peeling of Cassava Roots: Brush-Removal of LYE-Digested Peel from Cassava Roots

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ABSTRACT

Three process optimality options (optima-options) identified for the hydrolytic digestion of cassava peel into liquefied-digest-sludge or pulp were carried-over from paper-1 of the series and applied on the whole-unpeeled roots. The purpose was to validate the technical efficacy of each of the options for the complete removal of peel from cassava roots with zero or near-zero loss of the starchy-flesh-tissue followed by hand or mechanical scrub-brushing. A factorially designed experimental plan employing a cassava-peeling-efficiency-index (CPEI) factor linked generically with and analogous to the ICPSC (index of cassava peel structural collapse) value also generated from paper-1, was applied for the purpose. Our finding was that two of the process optima-options (a and b) were equally effective for complete peel-removal from cassava roots, viz:(a). 30% lye concentration at 32 °C and 50-minutes time-interval of immersion; and (b). 35% lye concentration at 50 °C and 7.5-minutes time-interval of immersion. The actual effectiveness of an option would depend on the technological circumstances of its application. While option (a) was the low-technology choice (no heating and no temperature control gadgetry), option (b) requires heating and temperature control with precision automatic handling devices attached to minimize the negative effects of heat-penetration into the peeled roots. Option (c) 25% lye-concentration at 103 °C (boiling point) and 4.5-minutes residence time-interval of immersion was dropped entirely as technically unfeasible.

KEY WORDS: LYE PEELING EFFICIENCY INDEX (LPEI), LYE-TREATMENT, PROCESS OPTIMA-OPTIONS, HYDROCYANIC ACID (HCN), STARCHY-FLESH-TISSUE OF PEELED CASSAVA ROOTS, HAND BRUSH-SCRUBBING, MECHANICAL BRUSH-SCRUBBING.

INTRODUCTION

This is a follow-up article in a series of two papers on mechanized lye-peeling of cassava roots using a 2-stage investigative approach. Paper-1 of the series by Tsekwi and Ngoddy (2018), defined three optimality-options for the hydrolytic digestion or disintegrative breakdown of cassava peel-specimens following their careful detachment by hand from the roots (otherwise known as hand-peeling), into liquified digest-sludge or pulp that conduced to easy disposal by wet brush-scrubbing by hand or mechanically in a machine. The optima-options defined in the paper for peel-breakdown in lye resulted from an optimization protocol used to investigate the combined effects of three process variables involved in the lye-treatment process, namely: lye concentration, temperature and residence time-interval of immersion. In the hierarchy of their potential effectiveness for successful peel hydrolysis, the optima-options fall into the following rank-order:(a) lye-concentration (30%), temperature (32 °C) and 50-minutes residence time-interval;(b) lye-concentration (35%), temperature (50 °C) and 7.5-minutes residence time-interval; and (c) lye-concentration (25%), temperature (103 °C) and 4.5-minutes residence time-interval of immersion, (Tsewki 2018, and Tsewki and Ngoddy 2018).

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The purpose of the present paper was to test, verify and validate the key findings of paper-1 in relation to the actualization of successful peel-removal from fresh whole-unpeeled cassava roots following withdrawal from lye-treatment, guided by the expeditious application of the pre-established process optima-options aforementioned. The approach employed to accomplish this task was to systematically test the 3 process options, one-by-one on a comparative basis, by applying each of them on sized whole-unpeeled roots in triplicate samples. On withdrawal from the lye-treatment bath, emergent cassava root samples were divided into two batches. One batch was subjected to wet manual (hand) scrub-brushing, while the second batch was channeled into wet mechanical scrub-brushing in a machine (Figure-1) developed for the purpose.

To determine the extent or degree of effectiveness of peel-removal achieved from each experimental cycle of lye-treatment followed by withdrawal and wet-brushing, an efficiency coefficient designated as lye-peeling efficiency index (LPEI) was defined quantitatively and applied using careful hand-peeling as the operating technical quality standard (control) for performance evaluation of competing mechanized cassava peeling techniques. In defining the LPEI-factor, earlier proposals by Wurdemann et al. (1976), Adetan et al. (2003) and Olukunle (2007) were taken into consideration in creating an efficiency index that facilitated the achievement of two complementary objectives, namely: (a) ensure total or complete peel-removal, while simultaneously, (b) engendering the minimization, if not outright elimination, of loss of the starchy-flesh-tissue of peeled cassava roots.

As defined, the generic-numerical character of the LPEI coefficient, as a quality performance criterion, straddled the figure 1.0 in such a manner that when:(a) LPEI = 1.0: peel-removal from root was complete and as good as hand-peeling;(b) LPEI < 1.0: peel-removal from root was incomplete compared to hand-peeling; and(c) LPEI > 1.0: peel-removal from root was excessive compared to hand-peeling, leading to proportional loss of starchy-flesh-tissue. Besides the necessity of satisfying the aforementioned criteria, two other factors loom-large within the cosmology of mechanized lye-peeling of cassava roots (Wurdemann et al., 1976; Igbeka, 1985; Deguchi et al., 2006) and the determination of cyanide content in three sweet cassava cultivars, (Ubwa et al 2015).

The following considerations are:(a) the necessity to avoid or minimize heat-penetration into the starchy-flesh-tissue of the peeled-root leading to its discolouration due to starch gelatinization; the occurrence of a gelatinized starch ring on the peeled-root imposes the need for additional costly abrasion-scrubbing to eliminate the ring, which is wasteful of otherwise useful starch; and (b) the need to ensure that lye-peeled roots satisfy food safety quality specifications of WHO/FAO relating to residual NaOH and residual HCN (hydrocyanic acid) levels.

MATERIAL AND METHODS

Materials used in the study were fresh cassava roots of TME-419 variety, harvested 12-months after planting at the University of Uyo farm, Nigeria. The chemicals used were sodium hydroxide pellets (NaOH), hydrochloric acid (HCl), phenolphthalein, sodium carbonate, and alkaline picrate; all of which were purchased from chemical supply stores in Uyo town, Akwa-Ibom State, Nigeria. Laboratory equipment used include standard items and wares such as wooden spatulas, plastic beakers, pipettes, conical flasks, measuring cylinders, fibre-brushes; thermometers (mercury in glass type), weighing balance, electronic calipers, spectrophotometer (UV/Vis:DO-83070-73); made in China), large metal cooking pots used as lye-baths seated on gas, kerosene or electric heating stoves; protective plastic hand-gloves, boots, coats and eye-goggles, stainless steel peeling knives; and a mechanical brushing machine as elaborated here-under as Figure-1.

Figure 1: Wet mechanical scrub-brushing machine (a) and vertical cross-section (b) showing the internal structure of its action-chamber

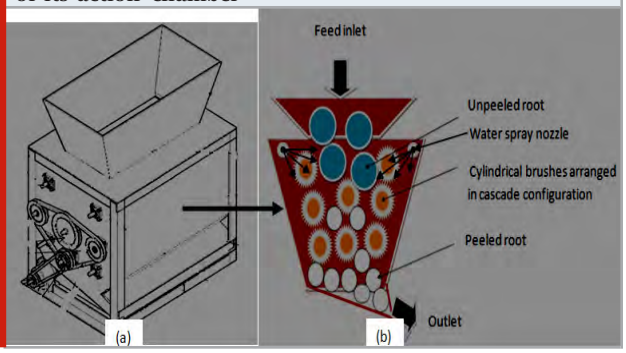


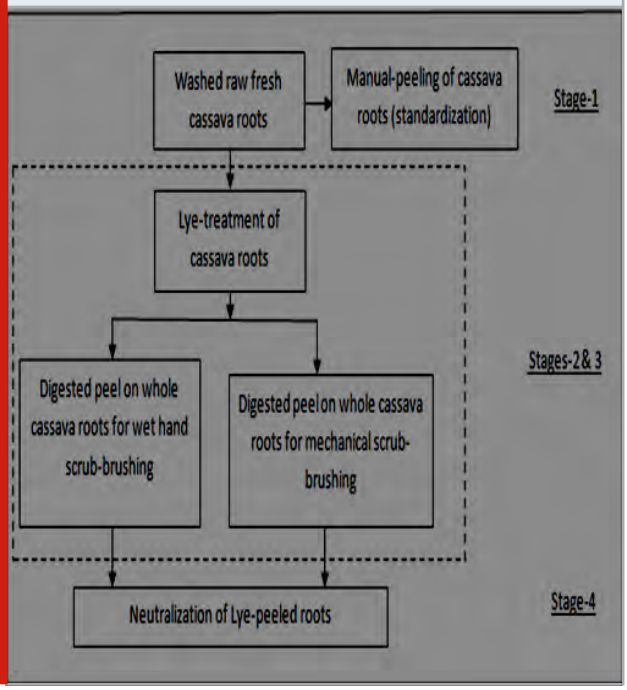
Figure-1 shows an exploded vertical cross-section of a wet mechanical brushing machine developed for the scrub-brushing of lye-treated cassava roots after withdrawal from the lye-bath. The equipment was fabricated in mild steel. Lye-treated roots were fed through the hopper on top. In the action-chamber, the roots cascade under a continuous spray of pressurized water, through three sequential tiers of triple counter-rotating differential-speed hollow-shafted cylindrical fibre-brushes. The scrubbed roots finally exit from a discharge chute at the bottom into lye neutralizing dilute solution of HCl in a receiving bowl.

Methodology: Figure-2 is a diagrammatic representation of the experimental protocol employed to execute the four stages of activities involved in the tests.

Stage-1: involved the manual-peeling of representative samples of cassava roots to establish a basis of standardization (or control) against which to compare the outcome of lye-peeling either scrub-brushed by hand or mechanically, following withdrawal from lye-treatment. 90 roots were selected for this purpose; 30 in each of the

three size-categories of small, medium and large roots, as specified in the following tabulation.

Figure 2: Flow diagramme of experimental protocol in 4-stages



Size category	Average diameter (mm)	Average length (mm)	Average weight (kg)
Small	40.0	200.0	0.52
Medium	55.0	360.0	0.65
Large	65.0	410.0	0.80

The percentage of cassava root loss (by weight) due to manual or hand-peeling was computed using the straight forward formula of Wurdemann et al. (1976) as expressed in equation-1:

Percentage root loss from hand-peeling [prlhp]=[mplhp/mrcr] x 100% (1)

Where: mplhp= mass of peel loss from hand-peeling [kg];mrcr= mass of raw cassava root [kg]; and prlhp= percentage (%) root loss (by weight) from hand-peeling.

Stages-2 & 3: involved the hydrolytic digestion (disintegration or degradation by liquifaction) of cassava peel (*in situ*) while on the roots into a wet scrub-brushable digest-sludge (or pulp) to determine the comparative effectiveness of lye digestion following immersion, withdrawal from lye and wet scrub-brushing either by hand or mechanically in the machine.180-roots were selected for this purpose; 90-roots per batch (x2-batches), grouped into 3 size- categories per batch of small, medium and large roots as earlier specified, with triplicate samples of 10-roots per size-category. Batch-1 roots were subjected to lye-treatment in accordance with process optima-option (a) (30% lye-concentration at 32 °C for residence time-interval of 50-minutes). Batch-2 roots were similarly subjected to lye-treatment by process optima-option (b) (35% lye-concentration at 50 °C temperature for residence time-interval of 7.5-minutes).

Process optima-option (c) (25% lye-concentration at 103 °C boiling point temperature of lye and 4.5-minutes residence time-interval of immersion), which could have constituted batch-3 of the experimental protocol was dropped for two reasons. First, 103 °C far exceeds the gelatinization temperature of cassava of 60 °C which, as paper-1 suggested, should be avoided because of contingent problems of heat-ring formation associated with heat-penetration. Secondly, boiling point temperature will induce such rapid heat transfer rates that would demand the use of high levels of automation and control devices for effective handling of the process to avoid heat-ring formation. For stages-2 & 3 tests, an index designated as the lye-peeling efficiency index (LPEI) was defined in accordance with earlier proposals by Wurdemann et al. (1976), Adetan et al. (2003) and Olukunle (2007) in the form of equation-2:

Percentage root loss from hand-peeling [prlhp]=[mplhp/mrcr] x 100% (2) Combining equation-2 with equation-1 resulted in equation-3:

$$\text{Lye-peeling Efficiency Index (LPEI)} = \left\{ \frac{\text{Percentage root loss from hand-peeling}}{\text{Percent root loss from lye-peeling}} \right\}$$

=[mphp/mrcr]/[mrcr/mpllp]=[mplhp/mpllp];
So that LPEI= [mplhp/mpllp]; dimensionless (3)

Where: mpllp= mass of peel loss from lye-peeling [kg];

As earlier explained in section-2 of this paper, the character of LPEI-factor was such that when:
(a) LPEI = 1.0: peel-removal from root was complete and as good as hand-peeling;
(b) LPEI < 1.0: peel-removal from root was incomplete and less than the loss from hand-peeling; and
(c) LPEI >1.0: peel-removal from root was excessive, leading to proportional loss of starchy-flesh-tissue.

LPEI-values calculated in this manner were applied to rank the comparative efficacy of the two optima-options, furnishing a rational basis of choice, if any.

Stage-4: involved the neutralization of lye-peeled roots following scrub-brushing in stages 2 and 3. Emanent lye-peeled roots were immersed in dilute HCl solution (of 0.1%) concentration to neutralize the residual lye in the roots to bring it down below the specifications of WHO/FAO safety quality standards for GRAS food

products. Prior to and after neutralization, lye-treated and scrub-brushed roots were tested to determine the residual NaOH in the roots. This was carried out by taking representative samples of the roots for titration against 0.01% HCl solution using phenolphthalein as indicator. Similarly, residual levels of HCN (hydrocyanic acid) in both the lye-treated and scrub-brushed roots and their correspondent digest-sludges were determined in accordance with the method of Wang and Field (1978).

RESULTS AND DISCUSSION

Table-1 presents the averaged summary of recorded values of the percentage of root loss (by weight) resulting from hand or manual-peeling of cassava roots. Hand-peeling was carried out carefully to provide a reliable standard for assessing or comparing competing root-peeling methods. In order to assist analysis, the table has combined in it, the body of available data on the physical characteristics of the cassava roots used in the study.

Table 1. Effect of cassava root size (Wt.) on percentage peeling loss (%)

1	2	3	4	5	6	7	8
Sample Diameter (mm)	Cassava Root Length (mm)	Sample Size	Sample Replicates	Wt of whole cassava Root (kg)	Wt of Peeled Cassava Root (kg)	Wt of Cassava Peel (kg)*	Percentage (wt) Peeling Loss (%)**
40.00	200.00	Small	Av. (10x3)	0.52	0.42	0.10	19.44
55.00	360.00	Medium	Av. (10x3)	0.65	0.52	0.130	20.50
65.50	410.00	Large	Av. (10x3)	0.80	0.63	0.173	21.80

*Column 7 was calculated as [column 5 – column 6]
**Column 8 was calculated as [(Column7/Column 5)] x100%

Figure 3: Raw whole-unpeeled cassava roots of mixed-sizes (left) side-by-side with hand-peeled roots (right)

The average root loss by weight (%) recorded in column 8 were 19.44, 20.50 and 21.80%, respectively for small, medium and large-size root-categories. Statistical analysis by ANOVA showed significant differences ($p<0.023$) among the size-categories investigated with respect to root loss (%) by weight when hand-peeling was conducted carefully by the incision of a sharp knife-edge followed by systematic unfolding of the root cortex and then by careful hand trimming of the proximal (head) and distal (tail) ends. Figure-3 is a photograph of raw cassava roots juxtaposed with hand-peeled roots. Figure-4 presents plots showing percentage root loss from hand peeling as a function of root size by weight.

When plotted (Figure-4) as a function of root size by weight, the percentage weight loss resulted in a positively sloped straight line which was regressed to equation-4 with the high coefficient of correlation of 0.9997 (Tsekwi, 2018);$\alpha h=8.4346\beta + 15.041$ (4) Where: αh=Percentage peeling loss (by weight) from hand-peeling [%]; and β =Size (weight) of cassava root [kg].

Figure 4: Percentage loss (% by weight) from hand-peeling of cassava roots as a function of root size (by weight)

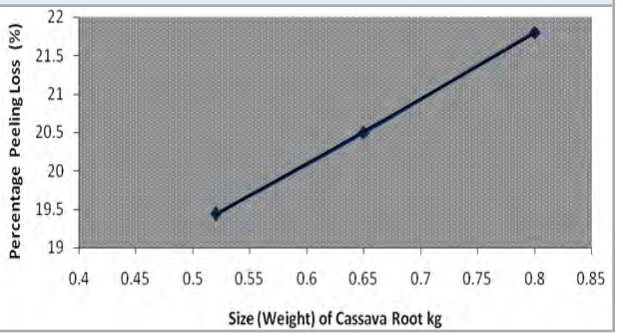


Table-2 presents the averaged summary of recorded values of measurements and calculations relating to the effect of applying, one at a time, the two optima-options of process variables carried-over from paper-1, on whole-unpeeled roots to determine the comparative efficacy of peel-removal from the roots that resulted following the sequence of lye-treatment, root withdrawal from lye and wet brush-scrubbing by hand of the roots. The lye-peeling efficiency index (LPEI) values computed from this set of tests designated as Batch-1 are recorded as column-10 on Table-2.

When LPEI-values were plotted as a function of time, Figures-5 and 6 resulted and were respectively regressed statistically to equation-5 at 30% lye-concentration for 32^{0}C temperature [process optima-option (a)]; and equation-6 at 35% lye-concentration for 50^{0}C [process optima-option (b)] with respective coefficients of correlation of 1.00 and 0.916;

$Ih_{32} = -0.002\theta 2 + 0.1700\theta - 2.425$ $Ih50 = 1.6247\theta - 0.2$ (6) Where:

Ih_{32} = LPEI- value for 30% lye-concentration at 32 ^{o}C after wet brush-scrubbing by hand;

Ih_{50} = LPEI- value for 35% lye-concentration at 50 ^{o}C after wet brush-scrubbing by hand; and

θ = residence time-interval of immersion (mins.).

Figures-7 and 8 are photographs of whole-unpeeled cassava roots taken following lye-treatment and wet brush-scrubbing by hand, respectively for 30% lye-concentration at 32 ^{o}C, and 35% lye-concentration at 50 ^{o}C.

Table-3 presents the averaged summary of recorded

values of measurements and calculations relating to the effect of the application, one-by-one, of the two process optima-options of parametric variables carried-over from paper-1, on whole-unpeeled cassava roots (*in situ*) to determine the comparative efficacy of peel-removal from the roots that resulted after wet mechanical brush-scrubbing of lye-treated roots. Lye-peeling efficiency index (LPEI) values computed from test data (Batch-2) appear in column-10 of the table and suggest a clear pattern of LPEI-value dependency on root-size by weight, irrespective of the lye-concentration and temperature, with LPEI-values decreasing in all cases with increase in root-size by weight.

Table-2: Summary tabulation of averages of the effect of lye-concentration, temperature and residence time-interval of immersion on the lye-peeling efficiency index (LPEI) in the case of cassava peel (*in situ*) while on whole cassava root after hand-brushing and scrubbing

1	2	3	4	5	6	7	8	9	10
Temp. (°C)	Residence Time (min)	Sample Size	Root Diameter (mm)	Wt. of whole roots (kg)	Wt. of peeled roots (kg)	Wt. of Peel (kg)*	Percentage Loss by Lye Peeling (%)	Percentage Loss by Hand Peeling (%)**	LPEI ***
32	40	Av. (30)	55.6	0.65	0.51	0.13	19.90	22.29	1.07
32	50	Av. (30)	55.6	0.66	0.52	0.14	21.07	22.29	1.05
32	60	Av. (30)	55.6	0.66	0.52	0.18	27.61	22.29	0.78
50	5.5	Av. (30)	55.6	0.65	0.48	0.14	21.72	22.29	1.03
50	6.5	Av. (30)	55.6	0.66	0.52	0.14	21.60	22.29	1.03
50	7.5	Av. (30)	55.6	0.69	0.53	0.15	22.14	22.29	1.00

*column 7 calculated as [column 5 – column 6], kg
**column 9 calculated as [column 7/ column 5] x 100%
***column 10 calculated as [(column 9/ column 8), dimensionless

Figure 5: Effect of residence-time of immersion on lye-peeling efficiency index (LPEI) for cassava root-peeling (in situ) while on the whole root at 30% lye-concentration

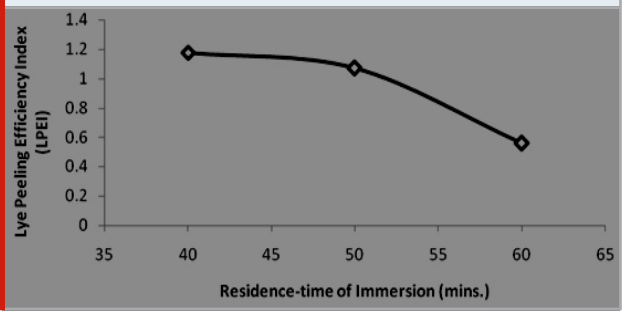
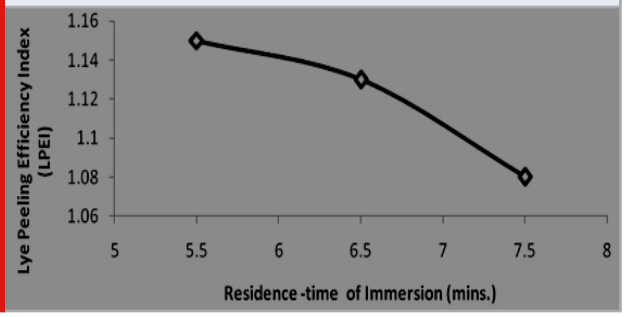


Figure 6: Effect of residence-time of immersion on lye peeling-efficiency index (LPEI) for cassava root-peeling (in situ) while on the whole root at 35% lye-concentration



In Figure-9, LPEI-values were plotted as a function of cassava root-size by weight and regressed statistically to equation-7 for 30% lye-concentration at 32 °C and 50-minutes residence time-interval of immersion; and equation-8 for 35% lye-concentration at 50 °C and 7.5-minutes residence time-interval of immersion with a coefficient of correlation of 1.00 for both plots leading respectively, to:

$Im_{50} = 0.275\beta 2 - 0.38040\beta + 0.908$ Where;
Im_{32} = LPEI for 30% lye-concentration at 32 °C after 50-minutes residence time-interval of immersion;
Im_{50} = LPEI for 35% lye-concentration at 50 °C after 7.5-minutes residence time-interval of immersion; and β = size (by weight) of whole-unpeeled cassava root [kg].

$$Im_{32} = 0.225\beta 2 - 0.4022\beta + 1.209 \quad (7)$$
$$Im_{50} = 0.275\beta 2 - 0.38040\beta + 0.908$$

Where;

Figure 7: Effect of 30% lye-concentration on whole cassava roots after hand brush-scrubbing of digested peel at 32 °C

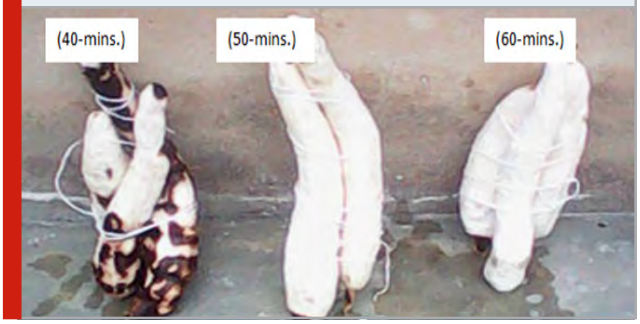
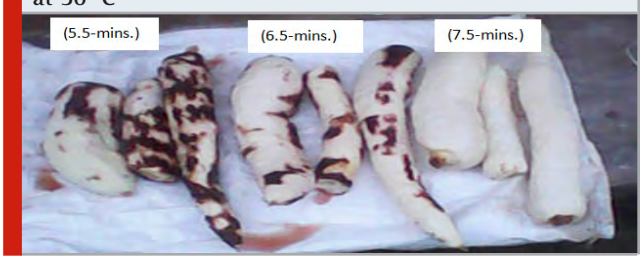


Figure 8: Effect of 35% lye-concentration on whole cassava root after hand brush-scrubbing of digested peel at 50 °C



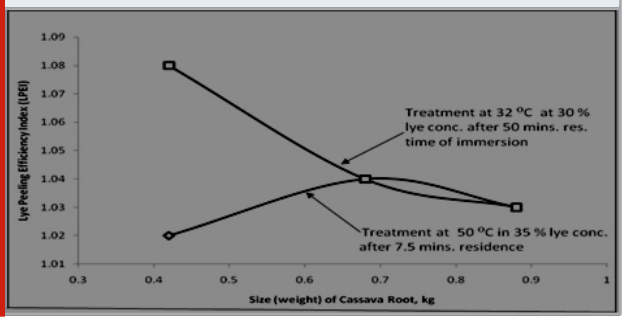
Figures-10 and 11 are photographs of peeled cassava roots taken following lye-treatment and wet mechanical brush-scrubbing, respectively for 30% lye-concentration at 32 °C after 50-minutes; and 35% lye-concentration at 50 °C after 7.5-minutes residence time-interval of immersion.

Table-3: Summary of averages of the effect of lye-concentration, temperature and residence time-interval of immersion on the lye-peeling efficiency index (LPEI) in the case of cassava peel *(in situ)* while on the whole cassava root after wet mechanical brushing and scrubbing

1	2	3	4	5	6	7	8	9	10
Temp. (°C)	Res. Time (mins.)	Sample Size	Sample Replicates	Av. Wt. of Whole Cassava Roots (kg)	Av. Wt. of Peeled Cassava Roots (kg)	Av. Wt. of Cassava Peel (kg)*	Percentage of Loss by Hand Brushing (%)**	Percentage Loss by Machine Brushing (%)	LPEI ***
32	50	Small	30	0.44	0.35	0.08	21.91	21.16	1.03
32	50	Medium	30	0.66	0.52	0.14	22.05	21.50	1.02
32	50	Large	30	0.81	0.62	0.18	22.91	22.83	1.00
50	7.5	Small	30	0.42	0.33	0.08	21.91	19.97	1.02
50	7.5	Medium	30	0.68	0.52	0.15	22.05	21.69	1.01
50	7.5	Large	30	0.88	0.69	0.19	22.91	22.64	1.01

*column 7 calculated as [column 5 – column 6], kg
**column 8 calculated as [column 7/ column 5] x 100 %
***column 10 calculated as [(column 9/ column 8), dimensionless

Figure 9: Lye-peeling efficiency index as a function of size (weight) of cassava roots following wet mechanical brushing and scrubbing of peel (in situ) while on whole cassava roots.



Residual NaOH in cassava roots recorded in the study after withdrawal from lye-treatment are as shown in the bar-chart (Figure-12) which compared these values with residual NaOH-values of raw fresh hand-peeled cassava roots and lye-peeled cassava roots following wet mechanical scrub-brushing from process optima-options [(a) 30% lye-concentration at 32 °C for 50-minutes and (b) 35% lye-concentration at 50 °C for 7.5-minutes]. The residual NaOH-values recorded were, respectively 2.00ppm (fresh hand-peeled roots), 96.90ppm (lye-peeled roots in 30% lye-concentration at 32 °C for 50-minutes time-interval of immersion); and 115.50 ppm (lye-peeled roots in 35% lye-concentration at 50 °C for 7.5-minutes time-interval of immersion). Therefore, the values of NaOH residue clearly increased at higher lye-concentration and temperature.

Values of the contents of HCN (hydrocyanic acid) in cassava roots recorded in the study before and following lye-peeling using process optima-options (a) and (b), respectively are as shown in the bar-chart (Figure-13) which compared the residual HCN-contents of fresh hand-peeled cassava roots and their detached peel; along with the residual HCN-contents of lye-peeled and mechanically scrub-brushed roots; and contrasted these values with their associated digest-sludges.

Figure 10: Effect of wet mechanical brushing and scrubbing of peel (*in situ*) while on whole cassava roots lye-treated in 30% lye-concentration at 32 oC for 50 minutes residence time-interval of immersion

The recorded HCN contents from the study were as follows: for fresh hand-peeled roots (50.34 ppm) and for the detached peel of cassava roots (86.17 ppm). For lye-peeled roots after mechanical scrub-brushing, the HCN contents were, respectively: (32.78 ppm) for the peeled roots and (49.80 ppm) for the digest-sludge (in 30% lye-concentration at 32 °C for 50-minutes), (26.00 ppm) for lye-peeled roots and (38.25 ppm) for the digest-sludge (in 35% lye-concentration at 50 °C for 7.5-minutes).

The recorded values of percentage loss of cassava root used as the basis of standardization of assessment in

this study were significantly lower than 27.8% reported by FAO (2012) for a pattern of hand-peeling in which the root cortex was removed by deep trimming which lumped-off excessive amounts of useful starchy-flesh-tissue adjacent to the cortex. They were, however, comparable to 20.0% reported by Salami et al. (2000) using a careful peeling technique such as applied in this study.

Figure 11: Effect of wet mechanical brushing and scrubbing of peel (in situ) while on the whole cassava root lye-treated in 35% lye-concentration at 50 oC) for 7.5 minutes residence time-interval of immersion

Figure 12: Sodium hydroxide (NaOH) residues in lye-peeled cassava roots compared with the raw fresh hand-peeled roots

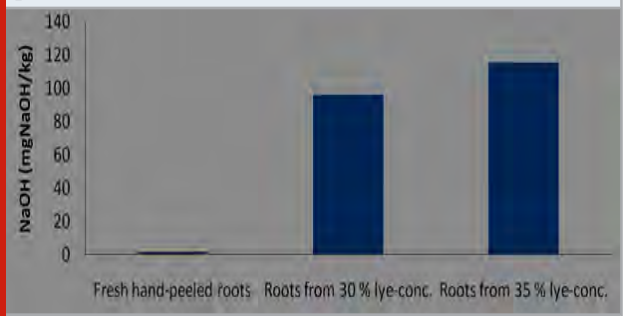
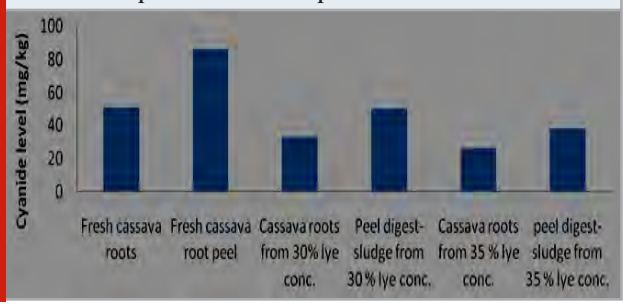


Figure 13: Hydrogen cyanide (HCN) content of lye-peeled cassava roots and the resultant sludges compared with raw fresh hand-peeled roots and peel



Our conclusion was that weight loss from hand-peeling was affected significantly not only by the root-size but also importantly by the technique of peel-removal by hand applied. The significance of technique in manual-peeling is brought into sharper focus by two considerations, namely: (a) the direct relationship between the manner (technique) and speed (residence time-interval) of hand-peeling which dictated the rate of output; and (b) wide variations in reported literature values of root peeling loss which, undoubtedly would depend on the disposition of the particular peeler, gender (women are reputed as better and more patient in peeling than children and men), age and, of course, the physical environment in which peeling is conducted. Figure-3 is a photograph taken of raw cassava roots juxtaposed with the hand-peeled roots.

The effect of lye-treatment using pre-established process optima-options followed by wet brush-scrubbing by hand on whole-unpeeled cassava roots to determine the efficacy of peel-removal from the roots suggested a clear pattern of LPEI-value dependency on residence time-interval of immersion in lye, irrespective of both the lye-concentration and temperature of lye-treatment, within the scope of the parametric variables involved in the tests. In all the cases, LPEI-values decreased as the time of lye-treatment increased. Close inspective scrutiny of Figure-7 showed a time progression pattern of incomplete (inadequate) peel-removal at 40-minutes, complete (adequate) peel-removal at 50-minutes and excessive peel-removal at 60-minutes and suggested 50-minutes as the best time-interval of immersion for 30% lye-concentration at 32 ^{o}C lye-treatment.

Figure-8 showed a pattern of inadequate (incomplete) peel-removal at 5.5 and 6.5-minutes and adequate (complete) peel-removal at 7.5-minutes, which although it portrayed that peel-removal was complete to the naked eye, may actually be excessive. The implication of these observations was that combining LPEI-values (Table-2) with photo-inspection (Figures-7 and 8) could provide a more reliable immersion-time prescription for lye-treatment.

For 30% lye-concentration at 32 ^{o}C, LPEI-values recorded were 40-minutes (1.07), 50-minutes (1.05) and 60-minutes (0.78). These figures confirmed, in conjunction with photo-inspection, that 50-minutes time-interval of immersion was optimal. For 35% lye-concentration at 50 ^{o}C, the LPEI-values recorded were at 5.5-minutes (1.03), 6.5-minutes (1.02) and 7.5-minutes (1.00). These figures suggest, in conjunction with photo-inspection, that a time period between 7.0 and 7.5-minutes would be the optimal time-interval of lye-immersion for the roots.

In the case of lye-treatment using pre-established process optima-options followed by wet mechanical brush-scrubbing on whole-unpeeled roots to determine the efficacy of peel-removal from the roots, LPEI-values recorded for 30% lye-concentration at 32 ^{o}C for 50-minutes residence time-interval of immersion were, respectively: small-size roots (1.08), medium-size roots (1.04) and large-size roots (1.03). Counterpart LPEI-values recorded for 35% lye-concentration at 50 ^{o}C for 7.5-minutes residence time-interval of immersion were, respectively: small-size roots (1.02), medium-size roots (1.01) and large-size root (1.01). These values clearly replicated and validated patterns observed for process optimality conditions defined for wet brush-scrubbing by hand as earlier discussed.

The percentage root loss by weight (%) recorded in this study from wet mechanical brush-scrubbing, averaged 22.0%. This value was comparable to 21.8% obtained for hand-peeling, but differed with values ranging from 25 to 42% and 12 to 41%, respectively reported in the literature by Ugwu and Ukpabi (2000) and Olukunle (2007) for mechanized abrasion-peeling of cassava roots. The LPEI-values recorded in this study were much higher than counterpart values of 0.11 and 0.15 reported by Bakare et al. (2011) for lye-peeled cassava roots. Generally, the literature values relate to circumstances of methodology, cassava root varieties and properties that often times were not properly defined by the investigators reporting them which, hither-to, had been a bane of reported data in the literature relating to cassava root-peeling (Odigbo, 1976a, 1976b; Adetan et al., 2003).

The recorded residual values of NaOH when compared with WHO/FAO guideline for GRAS food products (of 75-100 ppm of NaOH permissible in food products) appeared to be just within the acceptable levels for 30% lye-peeled roots at 32 °C for 50-minutes and not so for 35% lye-peeled roots at 50 °C for 7.5-minutes. The important conclusion from these observations is to underscore the necessity for adequate residual NaOH neutralization as an additional process step in the mechanized lye-peeling of cassava roots, thereby adding one more critical unit operation to the process.

The HCN-contents recorded in the study for the relevant entities of interest, showed that appreciable reduction of the poisonous chemical (HCN) occurred in lye-peeled roots and their associated sludges. At high lye-concentration and temperature of treatment, root samples and their sludges recorded considerably lower HCN contents than those of fresh hand-peeled cassava roots and the associated peel. Values reported for hand-peeled cassava roots and their associated peel by Ugwu and Ukpabi (2000) and Ubwa et al. (2015) vary respectively, from 41.60 to 54.34 ppm for peeled-roots and 63.41 to 108.90 ppm for the detached peel of cassava roots. Exactly, why such significant reductions occurred, although of great interest, was not investigated explicitly but should be the subject of future studies on the lye-peeling of cassava roots.

CONCLUSION

The peel of cassava roots can be efficaciously and profitably removed by lye-treatment with zero or near-zero loss of the starchy-flesh-tissue of the peeled roots and without heat-ring using two optimality-options of process variable-combinations identified in paper-1 of this series of two papers and validated empirically in this follow-up study that constitutes paper-2.The residual level of NaOH in lye-peeled roots was successfully neutralized using HCl solution to satisfy WHO/FAO food safety quality specifications for GRAS food products. A neutralization component is therefore a necessary unit operation for any lye-peeling hardware to be configured for the mechanized lye-peeling of cassava roots.

The contents of HCN (hydrocyanic acid) in lye-peeled cassava roots as well as in the digest-sludge from lye-peeling were found to be about half of the levels in the raw root and peel, suggesting significant reduction-effects of lye-treatment, which needs to be further investigated to establish the exact destination of HCN, because the mere dilution-effect of hydrolytically produced water in the treatment-solution cannot, ipso facto, explain the level of reduction observed. Because only one cassava variety (TME-419, at age 12-months only) was investigated in the study, further work is therefore necessary to test the findings on other promising varieties and ages-at-harvest of new cassava root lines that are mushrooming from biotech-assisted breeding efforts, which are on-going.

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Pharmacological Communication

Pharmacological Screening of *Amaranthus roxburghianus* Nevski Total Flavonoids for Anti-Arthritic Activity in Freund's Complete Adjuvant-Induced Arthritis Rat Model

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ABSTRACT

Amaranthus roxburghianus is a small-sized tree mustly used for iron tonic and inflammatory bowel disease.The aim of present investigation was to evaluate pharmacological screening for anti arthritic activity of total flavonoids of *Amaranthus roxburghianus* nevski in freund's complete adjuvant-induced arthritic rats model. The extraction of *A. roxburghianus* dried aerial partswas done using ethyl alcohol: water (70: 30) by the hot soxhlet method. Total flavonoids were separated from the extract and two doses 20 and 40 mg/kg of TFAR, were used against Freund's complete adjuvant-induced chronic immunological arthritis in Wistar rats. Arthritis study was carried out using morphological parameters, haematological studies, proinflammatory cytokines (TNF-alpha,IL-6) and histopathological findings to explore the mechanism of Antiarthritic potential. The results showed significant paw oedema inhibition for TFAR at a dose of 40mg/kg which was assisted by the results of paw volume and diameter. The TFAR also strongly reduced proinflammatory cytokines levels and depicts the histopathological alterations induced by Freund's complete adjuvant model.Finally it is concluded that TFAR protects synovial membrane by improving the health status exhibits promising anti-arthritic activity. This finding thus supports the traditional use of *A. roxburghianus* for arthritis.

KEY WORDS: A. ROXBURGHIANUS, HAEMATOLOGICAL, HISTOPATHOLOGICAL, INTERLEUKIN-6, ORGAN WEIGHT, TUMOR NECROSIS FACTOR-ALPHA.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic systemic, progressive autoimmune inflammatory disease that occurs in several joints as symmetrical polyarthritis associated with swelling and discomfort. RA is a complex multi-system disorder whose primary site of damage to inflammatory tissue is in the finger and foot joints. Destructive cartilage and bone changes and bone outgrowths limit joint mobility (Bihani et al., 2014). RA is a chronic inflammatory condition characterised by pain, inflammation of the synovial membrane, inflammation of the peripheral joints, morning stiffness, articular tissue destruction and reduced joint mobility (Choudhary et al., 2015; Uttra et al., 2017).

The anatomy and aetiology of RA is complex and unclear, can cause serious impairment, and eventually affects the capacity of a person to perform daily activities, reduces the quality of life, and causes premature death. RA is the most common inflammatory condition that affects about 1% of the global adult population, compared to males females are three times more prone to RA (Patil et al., 2012). Conventional treatment of RA with NSAIDS, corticosteroids, immunosuppressants and anti-

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rheumatic agents (TNF-alpha & monoclonal antibodies) has impediments (Yende et al., 2010; Kola et al., 2018). Chronic treatment with the aforementioned agents has significant adverse effects, such as haematological, cardiovascular, GIT and renal toxicity (Saleem et al., 2020).

Patients suffering from chronic autoimmune disorders are urged to take alternative symptomatic relief strategies (Kauthale et al., 2017; Zhang et al., 2018). *Amaranthus roxburghianus* Nevski (Family: Amaranthaceae) is a wild plant with tender leaves and edible shoots, widely used as a leafy plant (Mary et al., 2011). In Telugu, it is widely known as Chirikoora. It is used as an iron tonic (Nirmal et al., 2013) and its herbal formulations are rich in alkaloids mostly used in traditional Ayurvedic medicine (Oyeleke et al., 2018). *Amaranthus roxburghianus* is used as an abortifacient by tribes of Chittoor district, Andra Pradesh state of India(Alves de Almeida et al., 2017).

In conjunction with piperine, its root extract is used in the successful treatment of inflammatory bowel disease (Pamila et al., 2017) and its leaves have been reported for use in the treatment of sunstroke and urinary disorders(Bansod et al., 2010). Based on the above evidences for treatment of various diseases we made an attempt to analyse the anti-arthritis efficacy of *Amaranthus roxburghianus*. So we separeted the total flavonoids fraction using hydro-alcoholic solvent system and analysed its efficacy for treatment of RA using in chronic models of albino Wistar rats.

MATERIAL AND METHODS

A.roxburghianus leafy vegetables were acquired from near by Tirupati areas. The plant authentication was performed at Botany Department, Sree Venkateswara University, Andhra Pradesh, India with a plant voucher specimen (Ref. No. 1656). The aerial parts of the plant were washed with tap water and dried in the shade. The dried leaves were powdered in the grinder and defatted with petroleum ether and successive extraction was carried out with ethyl alcohol: water with (70: 30) ratio, by the hot soxhlet process.

The hydro-alcoholic extract was concentrated under reduced pressure in a rotary evaporator (Heidolph Instrument,Laborota 4000, Germany). The dried crude hydro-alcoholic *A.roxburghianus* extract was collected and preserved in an airtight glass container at 4-8 °C until final use(Ajayi et al., 2018). The total fraction of flavonoids (TFAR) (60g) was derived from an eluted fraction of H2O: ethanol (3:7) by standard Association of analytical chemists(AOAC) methods.

Animal: Male Wistar rats weighing between 180-200 g were used for experimental studies. The Institutional Animal Ethics Committee of the Sree Vidyanikethan College of Pharmacy, Tirupati, Chittoor Dist., A.P., India (Approval No. SVCP/IAEC/I-001/2018-19 dated 01/04/2019) approved all animal experiment protocols in compliance with the guidelines of the Committee for the Purpose of Control and Supervision of Animal Experiments (CPCSEA).

The animals were housed in Poly propylene cages and held in the light/dark cycle at 24 C ± 2 °C under 12 h and were fed with a regular pellet diet and had free access to water. A total of 30 male Wistar albino rats, 180-200 g in weight, were selected and assigned to 5 groups of 6 rats in each group (n=6). As a standard control, Group I was using. Group 2 was used as an Arthritic control group, Group 3 was used as a 10 mg/kg diclofenac, Group 4 was used as a 20 mg/kg TFAR group, and Group 5 was used as a 40 mg/kg TFAR group.

Acute toxicity studies: The toxicity effect of extract was recorded in previous studies(Oyeleke, et al., 2018) and found as safety of up to 200 mg/kg. Based on earlier studies, which recorded a better response, the desired doses of 20 and 40 mg/kg were selected (Hosseini, 2018).

Freund's complete adjuvant (FCA): All rats were injected intradermally with 0.1 mL of FCA (1mg/ml) into the left hind paw on day '0', with the exception of those in the normal control group. For arthritis to grow, an interval of 7 days was given. During this time, all the animals developed signs of arthritis, such as swelling, redness and restricted movement. The treatment finished on the 28th day (Alamgeer et al., 2015). Morphological studies including Paw volume and diameter, body weight, haematological studies, histopathologicaland the experimental animal blood was obtained and the serum was isolated by the process of centrifugation.

ELISA kits were used to test the protein concentration of serum proinflammatory cytokines such as TNF-alpha and IL-6, and the procedure was performed in accordance with standard instructions.The values were expressed as Mean ± SEM (n = 3).

Statistical analysis: Using one-way variance analysis (ANOVA) followed by the Dunnet test, the statistical significance was tested and $P<0.05$, $P<0.01$, and $P<0.001$ were considered statistically significant.

RESULTS AND DISCUSSION

Morphological studies: Paw volume and diameter s:The effect of TFAR on paw volume and diameter in arthritic rats induced by FCA was reflected in (Table 1). Challenge with CFA (0.1 mL) suggests paw edoema production that reached peak edoema on the 21st day of injection. The group treated with diclofenac demonstrated substantial inhibition of paw edoema on day 7th ($P<0.05$), day 14th ($P<0.01$), day 21st ($P<0.001$) and day 28 ($P<0.001$). TFAR (20mg/kg) demonstrates substantial paw edoema inhibition on day 21 and day 28 with ($P<0.01$).

Important paw edoema inhibition was also shown in rats treated with TFAR (40 mg/kg) on days 7th ($P<0.05$), 14th ($P<0.05$), 21st ($P<0.01$) and 28th ($P<0.01$). The paw diameter was raised and subsequently decreased

marginally until the 21st day of adjuvant induction. The group treated with diclofenac demonstrated substantial paw diameter inhibition on day 14th (P<0.01), day 21th (P<0.001) and day 28th (P<0.001). TFAR (20 mg/kg) demonstrates strong paw diameter inhibition on day 21th and day 28th with (P<0.01). Also rats treated with TFAR (40 mg/kg) shows significant inhibition of paw diameter on day 21st and day 28th (P<0.01).

Table1. Effect of TFAR on Paw Volume in FCA-induced arthritic rats

Groups	Paw volume (mL)				
	Day 0	Day 7	Day 14	Day 21	Day 28
Normal control	0.31±0.06	0.0.29±0.09	0.29±0.09	0.28±0.08	0.29±0.06
Arthritis control	0.83 ± 0.09	1.25 ± 0.19	2.12 ± 0.16	2.46 ± 0.13	2.59 ± 0.12
Diclofenac 10 mg/kg	0.47 ± 0.03	0.68± 0.21**	0.97±0.19**	1.13±0.21***	0.59±0.15***
TFAR 20 mg/kg	0.61 ± 0.02	0.88 ± 0.26	1.77 ± 0.16*	1.52 ± 0.12**	0.88 ± 0.12***
TFAR 40 mg/kg	0.59 ± 0.05	0.81± 0.17*	1.56 ± 0.20**	1.39 ± 0.21**	0.78 ± 0.23***

Values are expressed as mean ± SEM (n=6). *P<0.05,**P<0.01,***P<0.001 as compared with Arthritis control. (One-way ANOVA followed by Dunnet's test)

Table 2. Effect of TFAR on body wt. in FCA-induced arthritic rats

Groups	Mean Body wt. (gm)		Mean Difference in Body wt
	Before Induction	After Induction	
Normal control	179±1.23	179±1.23	--
Arthritis control	165 ± 3.13	186 ± 2.4	21 ± 1.26
Diclofenac 10 mg/kg	175 ± 2.24	206 ± 1.38	31 ± 1.11**
TFAR20 mg/kg	173 ± 1.12	202 ± 3.21	29 ± 2.03*
TFAR40 mg/kg	176 ± 3.65	207 ± 5.01	31 ± 1.67*

Values are expressed as mean ± SEM (n=6); *P<0.05, **P<0.01 as compared with control followed by Dunnet's test.

Figure 1: Effect of TFAR on hematological parameters in FCA-induced arthritic rats

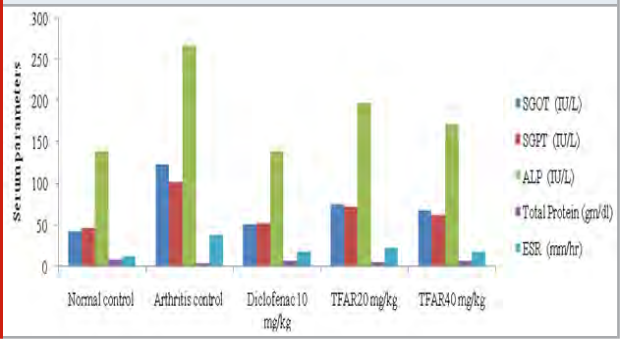
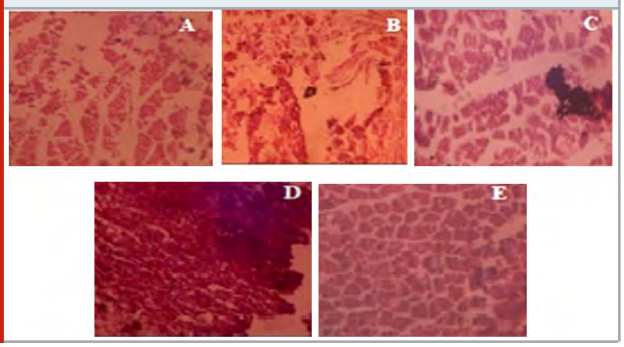


Figure 2: Histopathological observation of the rat ankle tissues (A) Normal control (B) Arthritis control(C) Diclofenac (10 mg/kg) (D) TFAR (20 mg/kg) (E) TFAR (40 mg/kg) treated rats. Magnification: x100; thickness: 5 µm.



Body weight studies: Effect of TFAR on body wt. in FCA-induced arthritic rats was tabulated in (Table 2) indicates the increased body wt. during treatment of standard drug and TFAR.

Hematological studies: The effect of TFAR on different serum and blood parameters in arthritic rats induced by FCA was tabulated in (Figure 1). The CFA (0.1 mL) study showed increase in the levels of SGOT, SGPT, ALP and decrease in the total protein levels in the control group. The group treated with Diclofenac reported a decrease in SGOT (P<0.01), SGPT (P<0.01), ALP (P<0.001) and Total Protein (P<0.01) levels. TFAR (20 mg/kg) suggested

a substantial decrease in SGPT (P<0.05), ALP (P<0.05) and total protein (P<0.05) levels respectively. The treated TFAR (40 mg/kg) community showed a decrease in SGOT (P<0.05), SGPT (P<0.05), ALP (P<0.01) and total protein (P<0.05) levels.

Table 3. Effect of TFAR on thymus and spleen with FCA-induced arthritic rats

Groups	Spleen wt. (mg/100 g b.wt.)	Thymus wt. (mg/100 g b.wt.)
Normal control	189.53±3.12	100.5±1.01
Arthritis control	259.34±3.61	71.18±2.34
Standard(10 mg/kg)	199.83±4.20**	91.00±1.46**
TFAR 20 mg/kg	224.50±2.36*	83.50±1.43
TFAR 40 mg/kg	210.00±2.34*	85.75±1.53*

Values are expressed as the mean ± SEM (n= 6); *P<0.05, **P<0.01 as compared with control (One-way ANOVA followed by Dunnet's test

Figure 3: Effect of TFAR on TNF-Alpha level in FCA-induced arthritis rats

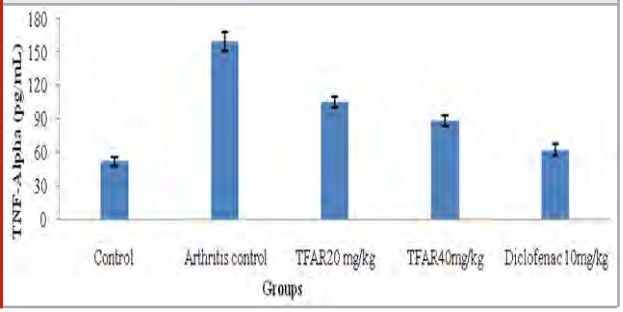
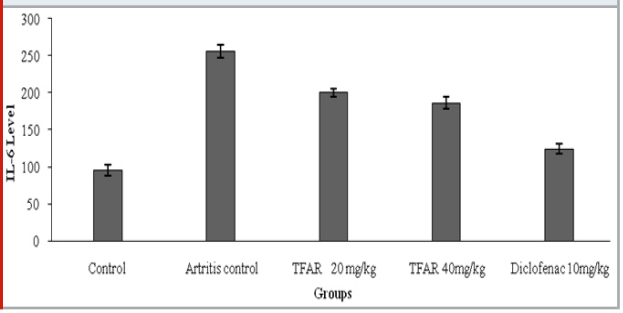


Figure 4: Effect of TFAR on IL6 level in FCA-induced arthritis rats



Histopathological studies: The histopathology of wistar rat ankle joint tissue are shown in Figure 2 and suggested damaging connective tissue lesions, joint space vascularity, and the development of granuloma in the arthritis control group. The normal control group showed the absence of necrosis in the ankle joint with normal connective tissue function. Treatment with standard showed normal ankle joint connective tissue with less oedema and no necrosis, compared to arthritis control group rats. TFAR (20mg/kg) treated rats showed oedema and necrosis with few inflammatory cells and granuloma formation. Rats treated with TFAR (40mg/kg) showed mild oedema necrosis, but there was no granuloma in the ankle joint.

Organs weight studies: A decrease was observed in thymus wt. in comparison with the control group, the mean spleen wt was increased in the FCA treated rats (Table 3). In rats treated with TFAR (20 and 40 mg/kg) and diclofenac (10 mg/kg) compared to FCA treated rats, the increase in spleen wt (P <0.01) was significantly inhibited. TFAR (40 mg/kg) and diclofenac (10mg/kg) therapy attenuated the decrease in wt. Significantly, of thymus (P <0.01).

Proinflammatory cytokines (TNF-alpha and IL-6): The proinflammatory cytokine analysis was performed and TFAR(40mg/kg) showed a substantial effect (P<0.5) relative to the regulation of arthritis. The results showed that proinflammatory cytokine inhibition was dose based. Compared to TFAR(40mg/kg) and athritis regulation, the regular diclofenac showed substantial (P<0.01) decreases in Proinflammatory cytokines. The TNF-Alpha and IL6 level results are shown in Figure 3 and 4. TNF-alpha and IL6 play a key role in inflammatory responses due to generation and propagation of inflammation. Various studies have been performed the effect of extract on inhibition of proinflamatory cytokines and reported dose-dependently reduced IL-6 in macrophages at both the gene and protein expression levels. It was also found that flavonoids inhibited the production of cytokines, TNF-α, macrophage inflammatory protein-1, and IL-6 via the activated monocytes (Farzaei et al. 2019).

CONCLUSION

The antiathritics activity of *A.roxburghianus* extract (TFAR) was performed by FCA induced athritis in rat model and it can be concluded from the findings that the total flavonoid fraction showed promising anti-arthritis activity by reducing the amount of proinflammatory cytokines and preserving the weight of the spleen and thymus. The TFAR treated rats showed oedema and necrosis with few inflammatory cells and granuloma formation were observed by histopathological study. Thus, *A.roxburghianus* plant could be a promising candidate for treatment of various antiinflammatory diseases.

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Conflicts of Interests: The Authors declare that there is no conflict of interest.

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Entomological Communication

Isolation and Characterization of *Pseudomonas* sp. Strain and its Role as Oviposition Attractant of the Filarial Vector *Culex quinquefasciatus*

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ABSTRACT

The coastal regions of Digha, West Bengal are endemic for lymphatic filariasis, especially *Culex quinquefasciatus* which is an established filarial vector there. Microorganisms in mosquito breeding sites is the key factor of either attraction or repellence of filarial vectors. The bacterial strains isolated from mosquito breeding sites were studied in this investigation. *Culex quinquefasciatus* plays a major role in transmitting lymphatic filariasis in the village areas of Digha, West Bengal. Water samples were collected from selected breeding habitats of *Culex quinquefasciatus* at the coastal villages of Digha. Five isolates (DX1, DX2, DX3, DX4 and DX5) were screened from the breeding habitats of *Cx. quinquifasciatus*, characterized and examined for oviposition bioassay. Test cup with the pure culture of *Pseudomanas* sp. DX5 was comparatively more attracted by gravid female *Culex* mosquitoes than other isolates in relation to oviposition. Oviposition Activity Index (OAI) was 0.8. Phenotypic, biochemical and molecular characterization of *Pseudomonas* sp. DX5 was done. *Pseudomanas* sp. DX5 showed negativity to Gram stain. Organisms looked like rods without having any spores. The colonies were spherical, opaque, yellowish in colour. The strain was positive for catalase, oxidase, urease, H2S production and negative for indole production, methyl red and voges-proskauer test, citrate utilization and nitrate reduction test. It was sensitive to some standard antibiotics like levofloxacin, ofloxacin, ciprofloxacin, streptomycin and doxycycline but was resistant against kanamycin, amoxycillin, nalidixic acid, ampicillin, chloramphenicol, gentamicin, tetracycline, vancomycin and erythromycin. Thus, elimination or control of the oviposition attractant bacterial strain of *Pseudomanas* sp. from breeding habitat water of filarial vector *Cx. quinquefasciatus* is an alternative strategy for filariasis management which is also a suitable topic of research in future.

KEY WORDS: CHARACTERIZATION, CULEX QUINQUEFASCIATUS, OVIPOSITION ACTIVITY INDEX,PSEUDOMONAS SP.

INTRODUCTION

Bancroftian filariasis is an important vector-borne disease transmitted by female *Culex quinquefasciatus* (Diptera: Culicidae) mosquitoes. The coastal belt of Digha in West Bengal. India is endemic for filariasis and *Culex quinquefasciatus* is the predominant mosquito vector at the coastal localities of Digha. The man-vector contact of *Cx. quinquefasciatus* is a key factor for high incidences of filarial infections in Digha (Azmi et al. 2015). The management of Culicidae is now difficult with due to the emergence of resistant vector mosquitoes to synthetic pesticides. Knowledge about the larval environments of *Cx. quinquefasciatus* is essential to control the mosquito vectors (Forattini, 1996). The selection of suitable oviposition sites has a major role on progeny fitness, larval diversity and population dynamics and overall maternal reproductive fitness and success (Spencer et al. 2002). The mosquito oviposition and subsequently their site selection are influenced by multiple physical, chemical and environmental factors. These are also attractants, repellents or stimulating factors (Bentley and Day, 1989; Azmi et al. 2015; Mondal et al. 2019).

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The oviposition attractants of mosquitoes include colour, odour, presence of semio-chemicals made by bacterial digestion or decomposition by bacteria of organic materials. The mosquito oviposition and subsequently their site selection are influenced by multiple physical, chemical and environmental factors. These are also attractants, repellents or stimulating factors (Bentley and Day, 1989; Mondal et al. 2019). It has been suggested by various workers that mosquito vectors have been influenced by bacterial semiochemicals (Leroy et al. 2011; Travanty et al. 2019).

Mosquitoes can interact with microbes in the breeding habitat water bodies in a variety of ways: as being the food items for the larvae, as being the symbionts, or as makers of kairomones (Merritt et al. 1992; Moreira et al. 2009; Shelomi 2019). Volatile compounds have been known to be associated with some specific bacterial strains that can enhance mosquito oviposition in the breeding sites of filarial vectors (Why and Walton 2020). Scanty literature is available in relation to the present theme of this study. Present study has been designed to isolate and characterize the oviposition attractant bacterial strains from the mosquito breeding habitats at the coastal regions of Digha, West Bengal.

MATERIAL AND METHODS

Samples were collected from varoius natural mosquito breeding habitat water bodies in Champabani village of Digha from March 2019 to February 2020. Isolation, enumeration and pure culture of mosquito breeding habitat water bacteria were done on Nutrient Agar (Holt et al, 1994). The isolate was plated and cultured on MacConkey Agar (Himedia, India) and Pseudomonas Isolation Agar (Himedia, India). Morphological characters of bacterial colony (shape, size, colour, margin and opacity) were recorded (Technic et al. 1957).

Gram-staining of the isolates were done and cell morphology was checked under a Phase-Contrast Microscope. The surface topology of the bacteria was studied under Scanning Electron Microscope at various magnifications following standard protocols (Lacey 1997). Smears of the pure culture of the isolates were done on cover glasses and heat fixed for 1-2 sec. The smears were then fixed for 45 min in 2.5% glutaraldehyde solution. hen at the beginning the slides were then dehydrated by passing through 50%, 70% and 90% ethanol and finally with absolute alcohol for 10 min each. The samples were gold coated, scanned and photographed under Scanning Electron Microscope (ZEISS). In order to study the biochemical properties of the isolate, catalase test, indole test, methyl red test, voges-proskauer test, nitrate reduction test, citrate utilization test, urease test, oxidase test was performed following standard methodologies (Smibert and Krieg 1995; Mc Clung 1985).

Sensitivity of the bacterial isolates to recommended doses of some commercially available standard antibiotics like kanamycin (30µg/disc), amoxycillin (10µg/disc), nalidixic acid (30µg/disc), ampicillin (10µg/disc), chloramphenicol (30 µg/disc), levofloxacin (5 µg/disc), gentamicin (50 µg/disc), ofloxacin (5 µg/disc), tetracycline (30 µg/disc), ciprofloxacin (5 µg/disc), vancomycin (30 µg/disc), rifampicin (5 µg/disc), erythromycin (15 µg/disc), streptomycin (10 µg/disc), and doxycycline (30 µg/disc) was recorded through disc diffusion technique following standard methodology (Brown and Izundu 2004). Pure culture of bacterial isolate (DX5) was inoculated in sterilized Nutrient Broth medium and incubated in a B.O.D. shaker incubator for 24h at 37 ± 2°C. Liquid bacterial culture of 1.8 mL of was centrifuged for 30 sec at 10000g to get the pellets and the genomic DNA was extracted by using DNeasy Ultra Clean Microbial Kit (Qiagen) following manufacturer`s instructions. Then amplification of nearly ~1.5 kb rDNA fragment of bacterial genomic DNA was extracted by using 27F forward and 1492R primer by polymerase chain reaction (PCR) technique (Brown and Izundu 2004).

Sequences of purified PCR products were obtained through universal bacterial primers in a DNA sequences. External service was taken for this purpose. Sequenced data were aligned in Clustal software. As per the research by Jukes et al. (1969), evolutionary distances were calculated. Phylogenetic tree was built following standard method (Tamura et al. 2007). Larvae of *Culex quinquefasciatus* were collected from the breeding habitats in the coastal areas of Digha and the mosquito colonies were maintained at the Microbiology and Parasitology Research Laboratory, The University of Burdwan. Mosquito larvae were reared in a water filled plastic bowls having sufficient yeast powder and dog biscuits in an acceptable ratio (1:1) in the laboratory conditions (29± 3 °C, 75-85% RH). Adult mosquitoes were released in wooden cages (30cm × 30cm × 30cm). Cotton soaked in 10 percent glucose solution was supplied to adult mosquitoes and adult female mosquitoes were regularly blood-fed to Wistar albino rat for egg development Tamura et al., 2007.

The pure cultured bacterial isolates were discretely added to 100 ml Nutrient Broth and incubated at 30±1°C for 72 hrs in a B.O.D shaker incubator to obtain a density of 10^5 cfu /ml. Sterilized Nutrient Broth media without-having any bacterial inoculum was taken as control. Ten adult gravid female *Culex quinquefasciatus* mosquitoes were released in each rearing cage (30cm x 30cm x 30cm). They were given two options for laying eggs. First cup was filled with 95 ml of sterile distilled water to which 5 ml (10^5 cfu/ml) of bacterial suspension was added. The second cup filled with 100 ml of sterile distilled water without having any bacterial suspension served as the control. Separate set of experiment were conducted for each and every bacterial isolate. Ten replications were made for each treatment, using a fresh female *Cx. quinquefasciatus* for each treatment. Oviposition Activity Index (OAI) was determined by the formula: OAI = (Nt – Nc) / (Nt + Nc) [Nt= number of eggs laid in test cups; Nc = number of eggs in control cups] (Kramer and Mulla

1979). The entire experimental set-up was maintained in an environmental chamber at a temperature of 28±2°C and relative humidity of 80±5% under 12:12h (light: dark) photoperiod (Kramer and Mulla 1979; Tamura et al. 2007).

Table 1. Number of eggs laid in test cups and control cups and Ovipositional Activity Index (OAI) in response to the suspensions of bacterial isolates.

Types of water provided for oviposition in dual choice bioassay	No. of eggs laid in test cup (Mean ± S.E)	No. of eggs laid in control cup (Mean ± S.E)	Oviposition Activity Index (OAI)
(95mL of sterile distilled water + 5 mL of DX1) vs. 100mL of sterile distilled water	103±1.76	37±2.02	0.47
(95mL of sterile distilled water + 5 mL of DX2) vs. 100mL of sterile distilled water	77 ± 3.21	37 ± 3.28	0.35
(95mL of sterile distilled water + 5 mL of DX3) vs. 100mL of sterile distilled water	125.0 ±1.52	68±2.08	0.29
(95mL of sterile distilled water + 5 mL of DX4) vs. 100mL of sterile distilled water	150.0±2.33	50±1.7	0.5
(95mL of sterile distilled water + 5 mL of DX5) vs. 100mL of sterile distilled water	383 ± 3.21	42.33 ± 2.33	0.8
(95mL of sterile distilled water + 5 mL of sterile Nutrient Broth vs. 100mL of sterile distilled water)	63±2.64	38±1.7	0.24

RESULTS AND DISCUSSION

Five morphologically distinct bacterial colonies (DX1, DX2, DX3, DX4 and DX5) were screened from the breeding habitats of filarial vector *Culex quinquefasciatus*. Number of egg rafts in treated and control test cups has been shown in Table 1.

Gravid mosquitoes showed comparatively higher oviposition attractancy to the test cup having Pseudomanas sp. DX5 than other isolates. Oviposition Activity Index (OAI) was 0.8. The bacterial colonies of the isolate DX5 were spherical, opaque, yellowish in colour in Nutrient Agar. Bluish green coloured colony was developed in *Pseudomonas* Isolation Agar. The bacterial isolate DX5 was rod shaped without having any spore (Table 2).

The Scanning Electron Micrograph of the bacterial isolate DX5 has been shown in Plate 1. The bacterial isolate (DX5) showed positive results for catalase, oxidase, urease, H_2S production test and negative results for indole production, methyl red and voges-proskauer test, nitrate reduction test and citrate utilization (Table 2). The bacterial strain was sensitive to the recommended doses of levofloxacin, ofloxacin, ciprofloxacin, streptomycin and doxycycline but resistant against kanamycin, amoxycillin, nalidixic acid, ampicillin, chloramphenicol, gentamicin, tetracycline, vancomycin and erythromycin (Table 2). The 16S rRNA partial gene sequence of the bacterial isolate DX5 has been submitted in the NCBI GenBank with an accession number assigned as MW513479. Nucleotide base composition (mol%) has been depicted in (Fig 1).

The values of AT content and GC content of the bacterial isolate DX5 were 46.92% and 53.08% respectively (Fig 1). Fingerprint of 16S rRNA partial gene sequence of *Pseudomanas* sp. DX5 has been depicted in (Fig 2). The Phylogenetic tree revealed that *Pseudomonas* sp. DX5 branched with *Pseudomonas fluorescens* (FJ972536) and *Pseudomonas fluorescens* (JF706525) having 58% bootstrap value (Fig 3).

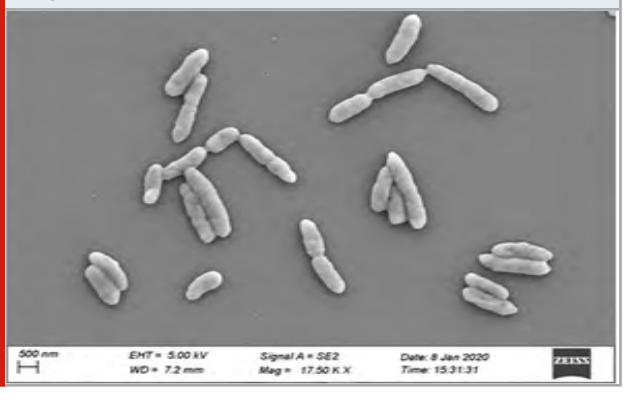
Coastal regions of Digha, West Bengal, India has been considered as filaria endemic region (Chandra et al. 2007; Azmi et al. 2015). *Culex quinquefasciatus*, the established filarial vector was the leading house-frequenting mosquito species in the coastal regions of Digha, West Bengal. The anthropophilic nature of *Cx quinquefasciatus* is was dependable factor for increasing intensity of filarial transmission in coastal areas of Digha (Azmi et al. 2015). Recent observations reported by various workers have revealed that some bacterial strains could act as larval food, midgut flora, and its metabolites as important oviposition attractants and/or stimulants in mosquitoes. There are two key reasons for oviposition behaviour of a female mosquitoes. Gravid

female mosquitoes must be induced for oviposition-by-oviposition media having specific bacterial suspension and also by some chemical inducers (Dethier et al. 1960). Oviposition attractancy depends on the composition and concentration of the microbial organisms found in breeding habitat water (Godwin et al. 2021).

Table 2. Cultural, phenotypic and biochemical properties of the bacterial isolate DX5

Character	Observation
Colony character	Spherical, 2.5 mm, opaque, elevated, non- consistent, yellowish green
Vegetative cell	Rod shaped
Spore	Non spore forming
Gram stain	Negative
Biochemical tests	
Catalase	+
Indole test	-
Methyl red test	-
Voges–Proskauer test	-
Nitrate reduction test	-
Urease production test	+
Citrate test	-
Oxidase test	+
H2S production test	+
Antibiotic sensitivity	
Sensitive (lg/disc)	Doxycycline, streptomycin, levofloxacin, ofloxacin, ciprofloxacin, rifampicin
Resistant	Ampicillin, tetracycline, kanamycin, gentamycin, vancomycin, nalidixic acid, chloramphenicol

Plate 1: Scanning Electron Micrograph of Bacterial isolate DX5



Some strains of *Acetobacter, Pseudomonas, Klebsiella, Gluconobacter*, and *Enterobacter* spp. isolated from the insect orders Isoptera, Homoptera, Heteroptera, Coleoptera, Hymenoptera, and Diptera had important roles in larval development Trexler et al. 2003; Roy et al. 2010. Microorganisms are responsible for the decomposition of detritus and release of volatile secondary metabolites. Gravid female *Cx. quinquefasciatus* mosquitoes were attracted to such habitats and were stimulated to lay eggs and *Pseudomonas* spp. are considered as resident bacterial flora of different mosquito breeding habitats (Kennedy 1942; Gerhardt1959; Ikeshoji 1968; Hosokawa et al. 2006, Rajagopal 2009). The oviposition bioassay clearly indicated that bacterial isolate DX5 (*Pseudomonas* sp.) served as an oviposition attractant for the filarial vector *Culex quinquefasciatus*. Now, the control of this oviposition attractant bacterial strains by some eco-friendly biocontrol agents like plant extracts and plant derived oils would certainly illuminate a new and alternative strategy of vector control.

Figure 1: Nucleotide composition of 16S ribosomal RNA gene sequence of bacterial isolate DX5

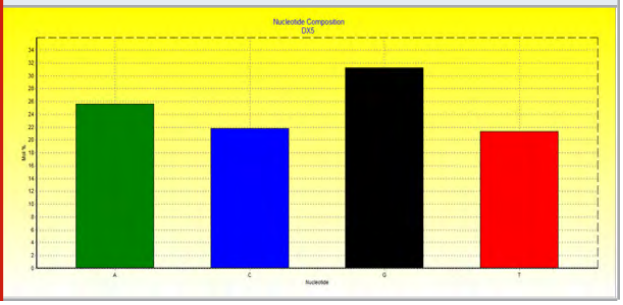


Figure 2: Fingerprint of 16S rRNA gene sequences of bacterial isolate DX5

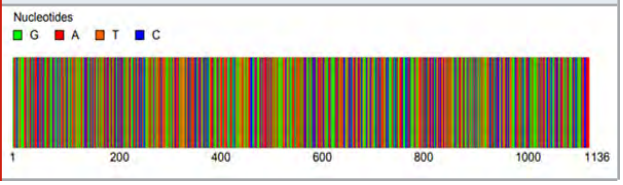
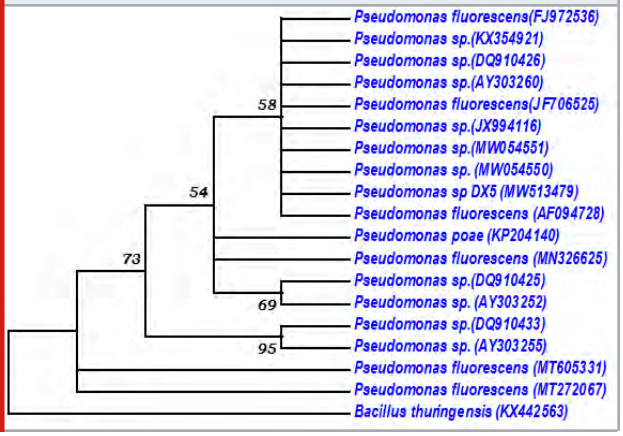


Figure 3: Neighbour joining tree based on 16S rRNA gene sequence of *Pseudomonas* sp. DX5 with similar 16S rRNA gene sequences retrieved from NCBI and RDP database.



CONCLUSION

The current study clearly showed that the strain of

Pseudomonas sp. had a high oviposition attractancy index in relation to the oviposition of filarial vectors in the breeding habitats occurring at the coastal areas of Digha. Biocontrol or environmental management of the oviposition attractant bacterial strains in the mosquito breeding habitats of the coastal areas of Digha, West Bengal would be considered as an alternative strategy of vector control.

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Pathological
Communication

Monitoring of Infectious Cattle Diseases in Tyumen Region

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ABSTRACT

The epizootic situation remains unfavorable for many animal diseases in the Russian Federation. The diseases of infectious etiology are regularly recorded among cattle, causing enormous economic damage to beef and dairy cattle breeding. Hence, this article makes an attempt to present an analysis of the epizootic situation concerning the infectious ethologydiseases in the Tyumen region. To meet the aim of the article, research within the framework of scientific work was carried out on the basis of the Veterinary Directorate of the Tyumen Region, at the Department of Non-communicable Diseases of Farm Animals, the Federal State Budgetary Educational Institution of Higher Education SAU of Northern Trans-Urals, the acarology laboratory of the All-Russian Research Institute of Veterinary Entomology and Arachnology - the branch of the Tyumen Scientific Center of the SB RAS and subordinate state veterinary institutions during the period from 2017 to 2019. Among the infectious diseases of cattle, they record anthrax, epizootic aphthae, infectious nodular dermatitis, tuberculosis, brucellosis, and leukemia. The monitoring of diseases of infectious etiology in the Tyumen region enables us to state only isolated cases of infectious nodular dermatitis - 11 cases in 2019. Based on the results obtained from the experiment, itcan be concluded that only isolated cases of infectious nodular dermatitis were recorded - 11 cases, and bovine leukemia - 620 cases in the Tyumen region (2019). Based on the results of the data obtained and a more detailed analysis of measures to treat and prevent infectious diseases, it is advisable to introduce additional and systemic preventive measures.

KEY WORDS: CATTLE, EPIZOOTIC APHTHAE,INFECTION, INFECTIOUS NODULAR DERMATITIS, THE DISEASES OF INFECTIOUS ETIOLOGY.

INTRODUCTION

These are sporadic outbreaks of anthrax, which were registered in the Republic of Altai, Dagestan (2017), the outbreaks of epizootic aphthae recorded in the Republic of Bashkortostan (2017), and in the Trans-Baikal Territory (2018) (Glazunov and Glazunova, 2015). This is the disease of large cattle with contagious nodular dermatitis, which has spread from the southern borders of the country to more than 30 constituent entities of the Russian Federation since 2015 (Kononov et al., 2017). Over the past 3 years, 27 outbreaks of cattle tuberculosis have been recorded in various regions of the country (Stolbova et al., 2014; Stolbova et al., 2016; Stolbova, 2019; Stolbova & Skosyrskikh, 2020).

Bovine leukemia virus breaks out to dominant positions and occupies one of the first places in the structure of infectious pathology among cattle (Sheikhshoaie et al, 2018; Sajjadi and Moosavi, 2019) Leukemia is widespread throughout the Russian Federation.The economic damage from infectious diseases of cattle consists primarily of disease elimination costs, carrying out restrictive measures, in some cases of compensation to animal owners for the removal of animals when especially dangerous diseases are detected, deaths, forced slaughter and culling of sick animals, reduced productivity, economic activity

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restriction, the cost of health-improving activities (Pavlik et al., 2020; Dittrich et al., 2021).

Russia in general and the Tyumen region in particular are aimed at domestic beef production increase in the long term and meat self-sufficiency reaching declared in the Doctrine of Food Security of the Russian Federation, the level of which must be 85% at least (Stolbova et al., 2014; Glazunov & Glazunova, 2015; Stolbova et al., 2016; Stolbova, 2019; Stolbova, 2020; Stolbova & Skosyrskikh, 2020). As the part of the Tyumen region strategy for the development of export potential, the task was set to increase the number of cattle, as well as to increase the production of livestock products - milk, meat, to obtain veterinary safe livestock products, and in some cases the products that meet the more stringent requirements of those countries where export of these products is planned (Stolbova, 2020).

All this is impossible without analyzing the incidence of various diseases among animals, primarily infectious, including especially dangerous ones, causing much more significant economic damage, studying the measures to combat and prevent these diseases, as well as identifying new, previously unexplored, factors in the spread of infectious diseases (Huffaker and Hartmann, 2021). As mentioned earlier, the main objective of the study is to monitor and assess the impact of factors stipulating the spread of infectious diseases among cattle in the Tyumen region. Due to an increase in the outbreak of these kind of diseases among castles in Russia, over the past years, it seems vital to investigate these infectious diseases closely and figure a way to tackle them properly.

MATERIAL AND METHODS

To monitor infectious diseases, the transmission of which is possible through sexual contact, we used the data of veterinary reporting in the context of municipalities and the Tyumen region on the registration of facts and ongoing antiepizootic measures of animal diseases according to the form No. 1-BET. In the course of the study, they analyzed the data of veterinary reporting generated by the State Autonomous Institutions of the Tyumen Region, by interdistrict veterinary centers, the city station for animal disease prevention, the regional veterinary laboratory and the regional antiepizootic detachment, including the data on the number of animals in the context of animal species, sex and age groups, the data on artificial insemination of cattle, the laboratory data, the data accompanying the collection of blood samples and pathological material, the acts of epizootic examination of disease foci when they are detected, etc. (Kononov et al., 2017; Shevchenko et al., 2019; Dittrich et al., 2021).

RESULTS AND DISCUSSION

The epizootic situation on the territory of any subject largely depends on the organization quality of veterinary preventive measures. In accordance with the Order of the Ministry of Agriculture of the Russian Federation No. 189 "On the Regulations for Submitting Information to the System of State Information Support in the Sphere of Agriculture" (02.04.2008), the state bodies of the constituent entities of the Russian Federation draw up and send information on infectious animal diseases to the Ministry of Agriculture of Russia quarterly. According to the results of the veterinary reporting analysis formed and directed by the Veterinary Directorate of the Tyumen Region to the Ministry of Agriculture of Russia, leukemia and infectious nodular dermatitis were recorded in the region from infectious diseases of cattle during the period from 2017 to 2019, the transmission of which is possible through sexual contact (Table 1).

Table 1. The number of cases of cattle with infectious diseases, the transmission of which is possible sexually

Item No	Disease name	2017	2018	2019
1	Infectious Dermatitis Nodosa (cattle)	0	0	11
2	Leukemia (cattle)	1031	827	620
3	Anthrax	Not recorded		
4	Epizootic aphthae			
5	Tuberculosis			
6	Brucellosis			

Taking into account the actually recorded cases of disease among cattle, a more detailed analysis of leukemia and infectious nodular dermatitis incidence was carried out among cattle within the framework of this study in order to study in detail the causes and spread of these diseases, as well as to assess the influence of their sexual transmission factor in the epizootic chain (Huffaker and Hartmann, 2021; Dittrich et al., 2021).The disease of leukemia among cattle is regularly recorded in almost all areas of the Tyumen region. For more detailed monitoring, the analysis of the incidence has been carried out since 2000, when the veterinary service of the Tyumen region began to apply the current Rules for the prevention and control of leukemia among cattle, approved by the order of the Ministry of Agriculture of the Russian Federation No. 359 (05/11/1999).

Figure 1: Dynamics of incidence and infection with leukemia among cattle in the Tyumen region

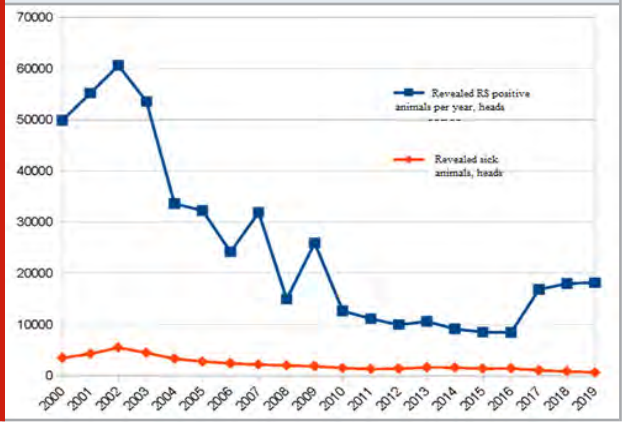
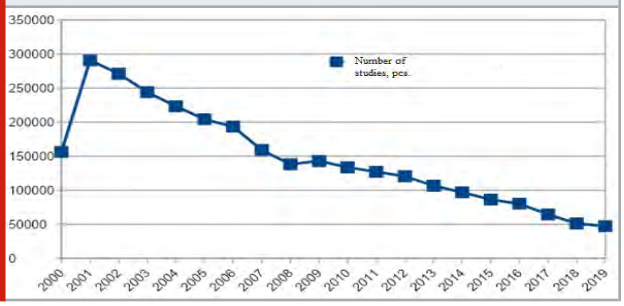


Figure 2: Dynamics of conducted hematological studies for leukemia among the cattle in the Tyumen region



The dynamics of leukemia disease among cattle, as well as the data on the identified virus carriers. Information on the number of studies carried out by the hematological method, as indicated above, the animals previously studied by the serological method and recognized as virus carriers are subsequently examined only by the hematological method (Pavlik et al., 2020) (Figure 1).It should be borne in mind that, depending on the infection of the farms, animals that are not infected with the bovine leukemia virus may also enter the statistics of hematological studies. If more than 30% of cows and heifers are found infected with the bovine leukemia virus on some farm, and it is not possible to carry out health-improving measures by separating healthy and infected animals, all adult animals, including healthy ones, are examined only by the hematological method every 6 months (Stolbova, 2020; Dittrich et al., 2021).

Table 2. Dynamics of conditional cattle leukemia infection in the context of municipal districts (group No. 1 with the highest infection rate)

District	Coverage by hematological examinations, % (conditional infection) of cattle for leukemia by years		
	2017	2018	2019
Aromashevsky	43,0	18,5	12,2
Vikulovsky	35,9	33,0	18,0
Zavodoukovsky	28,9	19,1	11,2
Isetsky	29,2	24,6	29,2
Sladkovsky	25,5	26,0	25,0
Uporovsky	54,8	28,5	22,6
Yurginsky	25,9	23,1	23,3
Average for group No. 1	34,4	25,7	21,6
Regional average	19,9	14,9	14,9

The largest number of cattle with leukemia was identified in 2002 - 5 434 heads. The smallest number was recorded in 2019 - 620 heads, which is 8.8 times less. The overall dynamics of leukemia decrease over the indicated period made 4.9% per year on the average. Most of the animal virus carriers were also detected in 2002 - 60,602 heads. By 2019, the number of animal virus carriers detected annually decreased to 18,166 heads, which is 3.3 times less. At the same time, the number of hematological examinations conducted over the specified period decreased by more than 5.7 times (271,241 examinations in 2002, 47,135 examinations in 2019) (Figure 2). The increase of detected infected animals during the period from 2017 to the present, as compared with lower rates in 2010-2016, is explained by the fact that the serological method was also used to study farm animals, the infectivity of which is more than 30%, and which were previously studied only by the hematological method and accordingly, they were not included in the statistics of virus carriers detected during the reporting year (Stolbova, 2020; Pavlik et al., 2020).

In order to analyze the causes of leukemia incidence among cattle, they studied the acts of the epizootic survey of farms. Given the long incubation period, the etiology of the disease does not contain the data indicating a possible sexual route of leukemia infection in the veterinary documents. To identify the influence of leukemia sexual transmission, an additional comparative analysis of cattle infection dynamics with leukemia in personal subsidiary plots was carried out in conjunction with the cattle population coverage by artificial

insemination in the context of the municipal districts of the Tyumen region. Studies have established that in case of free mating, there may be violations of the mucous membrane of the cervix, which contributes to the penetration of leukocytes affected by the virus into the animal's body and, accordingly, the infection of the animal with the bovine leukemia virus occurs.

Table 3. The dynamics of conditional cattle leukemia infection in the context of municipal districts (group No. 2 with the lowest infection rate)

Region	Coverage by hematological examinations, % (conditional infection) of cattle for leukemia by years		
	2017	2018	2019
Vagaysky	0,4	0	0,3
Kazansky	6,8	4,4	3,8
Nizhnetavdinsky	6,1	5,9	7,7
Tobolsky	0	0	0,7
Uvatsky	0,3	0,3	3,7
Yalutorovsky	9,8	5,6	18,6
Average for group No. 2	5,5	3,7	7,5
Regional average	19,9	14,9	14,9

Table 4. Dynamics of newly detected cattle leukemia virus carriers in the context of municipal districts (group No. 1)

District	The share of newly positive detected RID animals, %		
	2017	2018	2019
Aromashevsky	16,0	13,6	9,7
Vikulovsky	12,3	11,7	13,0
Zavodoukovsky	15,0	13,8	7,0
Isetsky	10,6	12,1	12,3
Sladkovsky	22,0	20,5	16,2
Uporovsky	14,0	13,4	11,3
Yurginsky	22,9	30,6	30,9
Average for group No. 1	15,7	15,5	12,9
Regional average	13,2	12,8	11,6

This is almost impossible during artificial insemination (Huffaker and Hartmann, 2021). Considering that different degrees of animal infection directly affect the degree of re-infection of animals with leukemia, the analysis was carried out taking into account this circumstance. The municipal districts in which the conditional infection rate (the coverage by hematological studies) was more than 20% (above the regional average according to the data of 2017) were grouped into one group - these are Aromashevsky, Vikulovsky, Zavodoukovsky, Isetsky, Sladkovsky, Uporovsky and Yurginsky districts (Table 2). During the study, a significant and stable decrease in conditional infection was noted in Aromashevsky, Vikulovsky, Zavodoukovsky and Uporovsky districts. The conditional infection rate in the Isetsky, Sladkovsky and Yurginsky districts has not changed almost.

At the same time, the municipal districts, in which the conditional infection rate (the coverage by hematological examinations) was the lowest and amounted to less than 10%, were grouped into the second group. This group includes Vagaysky, Kazansky, Nizhnetavdinsky, Tobolsky, Uvatsky and Yalutorovsky districts (Table 3). A stable decrease in conditional infection was noted only in the Kazan district within the second group of the analyzed districts over three years. In other regions, the infection rate remained unchanged or, on the contrary, increased. In addition, they performed the analysis of newly detected animal virus carrier dynamics according to the results of serological studies in these two groups (Table 4, 5). This information reflects the proportion of newly detected virus carriers among previously healthy or RID negative animals, that is, it shows the direct infectivity of animals with leukemia.

Table 5. Dynamics of the newly detected animal carriers of cattle leukemia in the context of municipal districts (group No. 2)

District	The share of newly detected RID positive animals, %		
	2017	2018	2019
Vagaysky	1,7	0,7	1,3
Kazansky	8,8	6,8	4,6
Nizhnetavdinsky	6,6	7,0	6,9
Tobolsky	3,4	2,2	3,6
Uvatsky	1,8	6,3	8,2
Yalutorovsky	9,1	10,0	9,8
Average for group No. 2	6,7	6,2	5,9
Regional average	13,2	13,1	11,6

Figure 3: Dependence of newly detected animal virus carriers on artificial insemination coverage

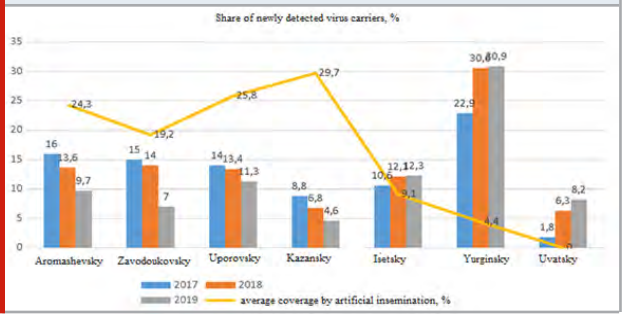
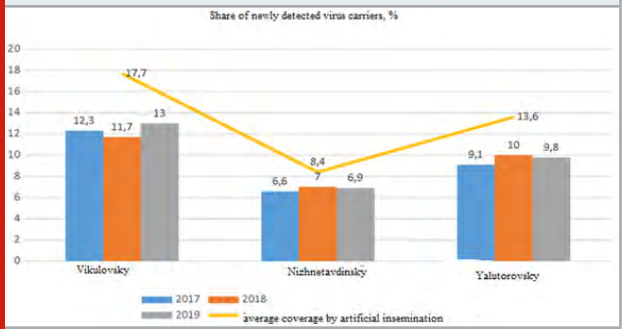


Figure 4: Dependence of newly detected animal virus carriers on artificial insemination coverage



The analysis of newly detected virus carrier share is very important, since it directly reflects the result and effectiveness of the measures taken to prevent and eliminate leukemia among cattle. The increase in re-infectivity clearly shows an unsatisfactory and insufficient complex of veterinary-prophylactic and organizational measures. A steady decline of newly detected animal virus carriers was noted for the period from 2017 to 2019 in Aromashevsky, Zavodoukovsky, Sladkovsky and Uporovsky districts. On the contrary, Vikulovsky, Isetsky and Yurginsky districts demonstrated increase of RID positive animals detected during serological studies (Pavlik et al., 2020).

In general, taking into account the analysis of data on the conditional infection of animals and the proportion of newly detected virus carriers, a positive result of infected animals stable decrease was noted in Aromashevsky, Zavodoukovsky and Uporovsky districts. An increase in the number of newly detected RID positive animals is observed in the Isetsky and Yurginsky districts, with almost unchanged level of conditional infection.There is no system dynamics in Vikulovsky and Sladkovsky districts. In some cases, there is an increase, in some cases, on the contrary, a decrease in either conditional infection or the proportion of newly detected virus carriers (Dittrich et al., 2021).

A stable decrease of newly detected animal virus carriers was noted only in the Kazan region during the period from 2017 to 2019. On the contrary, there is an annual increase of RID positive animals in the Uvatsky region, detected during serological studies. The changes are insignificant in Vagaysky, Nizhnetavdinsky, Tobolsky and Yalutorovsky districts. No systemic dynamics is observed for the rest of the regions of the 2nd group. In some cases, there is an increase, or some decrease in either conditional infection or in the proportion of newly detected virus carriers.Taking into account the analysis of the data based on the results of the study, an inversely proportional relationship is observed between the dynamics of leukemia infection among animals and the number of animals that are artificially inseminated. Some increase in the coverage of the cattle population with artificial insemination in dynamics leads to the decrease of both newly detected virus carriers and the conditional infection of animals, determined on the coverage of the cattle population by hematological studies.

This tendency was revealed both in group No. 1 with a high degree of livestock infection - in Aromashevsky, Zavodoukovsky and Uporovsky districts, and in group No. 2 with a low degree of infection - in Kazan district. Accordingly, a lower coverage of the livestock of animals by artificial insemination leads to newly detected virus carrier increase and to the conditional infection increase

among animals. This trend was also found in both groups. Group No. 1 - in the Isetsky and Yurginsky districts, group No. 2 - in the Uvatsky district, where, despite the very low infection of animals with leukemia in the absence of artificial insemination, the share of newly detected animal virus carriers increased to 8.2% per year or in 4.5 times over three years (Figure 3).

In group No. 1 with a high degree of infection of the livestock, and in group No. 2 with a low degree of infection, the areas were identified where the coverage by artificial insemination averages from 8.4% to 17.7% (Vikulovsky, Nizhnetavdinsky and Yalutorovsky districts). The dynamics of the conditional infection of animals and the proportion of newly detected animal virus carriers in these areas fluctuates either upward or downward (Figure 4). There is no systemic dynamics.

Thus, it can be concluded that the factor of sexual transmission in case of bovine leukemia is present, and in order to obtain a positive result from artificial insemination for the prevention of bovine leukemia and to minimize the factor of the disease transmission during free mating, the coverage of artificial insemination should be at least among 18% of the available livestock. During the analysis, infectious nodular dermatitis was first registered in the Tyumen region in 2019. In total, 11 cases of the disease were recorded, including the Kazansky district - 6 cases; Abatsky district - 1 case; Sladkovsky district - 4 cases. By the decisions of the Veterinary Directorate of the Tyumen Region, restrictive measures were established in all cases, a complex of veterinary preventive measures was taken to eliminate and prevent the disease spread.

To find out the causes of the disease onset, they analyzed the documents on the establishment of restrictive measures, the acts of epizootic examination, expert evaluations, accompanying documents to the selected samples, the submissions from state veterinary inspectors, and veterinary reporting data (Sąjjadi and Moosavi, 2019). The facts that give reason to assume that the infection of animals occurred sexually, are not confirmed in any of the recorded cases. There were no facts of artificial insemination and sexual intercourse during the period of 30 days before the detection of diseases. The absence of disease sexual transmission factor is also confirmed by the absence of contagious nodular dermatitis infection cases among other susceptible animals during the outbreak (Dittrichet al., 2021).

The most likely route of infection is transmissible, i.e., mechanical transfer of various species by arthropods. All cases of the disease were detected during the period from 05/23/2019 (1 case) to 10/04/2019 (the last case), when an active flight of blood-sucking insects was observed. With the cessation of flight of insects, the cases of infectious nodular dermatitis were no longer recorded among cattle (Pavlik et al., 2020). Thus, we can conclude that in cases of cattle infection with nodular dermatitis in the Tyumen region, the factor of the disease sexual transmission has not been established.

CONCLUSION

Based on the study results, they studied the features of infectious diseases among cattle, the transmission of which are possible sexually. These are infectious, including especially dangerous diseases such as anthrax, epizootic aphthae, infectious nodular dermatitis, tuberculosis, brucellosis and leukemia. The monitoring of diseases of infectious etiology in the Tyumen region allows us to state only isolated cases of infectious nodular dermatitis - 11 cases in 2019. At the same time, the cases of bovine leukemia are detected regularly and everywhere throughout the region - from more than 5 thousand cases annually at the beginning of 2000 and up to 620 cases by the end of 2019. When analyzing control and prevention measures, taking into account the constantly changing epizootic situation, constant monitoring of these diseases is necessary, and it is advisable to introduce additional and systemic preventive measures to prevent cattle leukemia.

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Biomedical
Communication

Comparative Analysis of Codon Usage of Dengue and Chikungunya Viruses with Human Host and Vector

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ABSTRACT

Chikungunya (CHIKV) and Dengue (DENV) are common arboviruses usually found in tropical countries. To understand the molecular evolution pattern and regulation of viral genome the codon usage analysis serve as an important tool. This study utilized the two important parameters Nucleotide composition (Nc) and Relative Synonymous Codon Usage (RSCU) of codon usage analysis for detailed understanding. We have processed and analyzed 382 sequence of DENV and 641 sequences of CHIKV for Nucleotide composition (Nc) and Relative Synonymous Codon Usage (RSCU). A comparative analysis of RSCU between both pathogens (DENV and CHIKV) and between vector and host i.e. *Ades aegytpti* and *Homo sapiens* was carried out. It is hypothesized because DENV and CHIKV both are sharing common host and vector therefore the findings of our study will provide a detailed insight towards an understanding of molecular evolution between the virus vector and its host. The study suggests that the codon usage patterns of these viruses are a combination of coincidence and antagonism. The observations recorded indicate that the codon usage pattern of DENV and CHIKV is dominated by mutation pressure under the influence of natural selection from its hosts. In conclusion, the overall codon usage bias is low in DENV and CHIKV i.e. mutation pressure is playing a key role in evolution. To the best of our knowledge, this is the first report describing the comparative analysis of codon usage in CHIKV and DENV genomes. The findings from this study are expected to increase our understanding of factors involved in viral evolution, and fitness towards hosts and the environment.

KEY WORDS: DENGUE VIRUS, CHIKUNGUNYA VIRUS, CODON USAGE BIAS, MUTATIONAL PRESSURE, NATURAL SELECTION.

INTRODUCTION

Dengue virus (DENV) is a major threat to worldwide human health. Over 50 million cases are reported per year, it is most prevalent in tropical and sub-tropical countries, itconsists of four serotypes, DENV1 - DENV4. DENV is a member of the *Flaviviridae* family (Chen et al.,2013; Dwivedi et al.,2017.). The transmission of the virus to humans is by a vector that is a mosquito, primary vector *Aedesaegypti* and secondary vector *Aedesal bopictus*. The viral genomes consist of a positive single-stranded RNA encoding for ten proteins; three structural proteins capsid (C), membrane protein (M), envelope protein (E), and seven non-structural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. These proteins are translated as a polyprotein, which is cleaved into individual proteins during maturation by the host proteases, (Behura and Severson, 2013, Simet al.,2015, Sim and Hibberd, 2016 Pollettet al.,2018).

Chikungunya virus (CHIKV) is a member of the *Togaviridae* family genus *alphavirusit* is a single stranded, enveloped, positive-sense RNA virus (Galánet al.,2015). The genome is 12 kb in size and contains two open reading frames encoding non-structural and structural proteinsCHIKV has caused several outbreaks in several Southeast Asian countries and emerge as a severepublic health concern (Dutta et.al.,2018). It is also anarthropod-borne virus, the mode of transmission is the mosquitoes of the Aedes spp. where *Aedesaegypti* and *Aedesal bopuctus* serve as primary and secondary vectors and humans serve as hosts

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similar to the DENV virus (Volket al.,2010, Wimalasiri et al.,2019).

All amino acids, except methionine and tryptophan, are coded by more than one codon. This phenomenon of using more than one codon for each amino acid is termed as synonymous codon usage (Jun-Jun et al.,2013). The synonymous codons do not occur equally they follow a pattern in which some codons are preferred over others and this is termed as codon usage bias (Zhouet al.,2012; Hemert and Berkhout,2016). Several of factors are known to add to codon usage bias, such as translational and transcriptional selection, mutational bias, protein structure, RNA stability, GC content, tRNA abundance, etc. The study of codon usage bias can provide insights into the molecular evolution of these viruses (Butt et al., 2014).There are individual studies on the codon usage bias of DENV and CHIKV but here, we have performed a comparative study between the codon usage bias of these two important viruses with their common vector and host. Considering the increase in the occurrence of DENV and CHIKV comparative analysis of codon usage bias will be important for understanding viral evolution, as there is no specific drug and effective vaccine for DENV and CHIKV it is very important to analyse and understand the molecular evolution of the virus (Wimalasiri et al.,2019).

The literature studies reflect that much research work has been done for the codon usage analysis of CHIKV and DENV independently, though the CHIKV and DENV are members of two different families but share common host and vector. And this important correlation between the two pathogen is almost not considered. There is a lacuna towards the understanding between the two pathogen and their vector and host. Very few studies are available which address the three components of transmission cycle of virus i.e. virus, vector and host in the study, but again not for the pathogens from different origin but sharing common host and vector.An attempt has been made to understand the relationship between the codon usage of virus vector and host. So far, no work has been done to understand and compare the codon usage pattern of viruses from two different families. Therefore, understanding the role of codon usage in viruses, host and vector interaction will explore the relationship of different virus with common vector and host. This relationship will give a detailed insight towards understanding the evolutionary pattern and relationship between different genotypes of virus. An attempt has been taken to include the three components of transmission cycle of virus that are virus, vector and host in the study.

MATERIAL AND METHODS

Sequence data

Sequences: Complete genome sequences of Dengue (DENV-1 90, DENV-2 94 and DENV-3 105, DENV-4 93) and CHIKV (641) viruses were downloaded from the National Centre for Biotechnology (NCBI) database (http://www.ncbi.nlm.nih.gov) in FASTA format. The accession numbers of the selected genomes were listed in thetable (Supplementary Table.1).

Compositional Analysis: To understand the frequencies of occurrence of each nucleotide composition analysis was conducted. The overall frequency of occurrence of the nucleotides (A %, C %, U %, and G %) was calculated along with the frequency of each nucleotide at the third site of the synonymous codons (A_3, C_3, U_3, and G_3). The termination codon UAA, UAG, and UGA do not encode any amino acids and codons AUG and UGG are the only codons for Met and Trp, respectively, therefore, these five codons are excluded from the analysis (Loboet al.,2009; Feng et al.,2013; Hemert and Berkhout 2016).

Relative synonymous codon usage (RSCU): The RSCU values of codons for DENV and CHIKV were calculated to determine the characteristics of synonymous codon usage. The synonymous codons with RSCU values > 1.0 have positive codon usage bias, while those with RSCU values < 1.0 have negative codon usage bias. When the RSCU value is 1.0, it means there is no codon usage bias for that amino acid and the codons are chosen equally. The synonymous codons with RSCU values >1.6 and <0.6 were treated as over-represented and under-represented codons, respectively. The RSCU values for codon were calculated using the formula given below:

$RSCU = \frac{g_{ij}}{\Sigma_j^{ni} g_{ij}} n_i$ Where g_{ij} is the observed number of the ith codon for the jth amino acid, which has n_i types of synonymous codons (Zhonget al.,2007;Mune et al.,2017).

RESULTS AND DISCUSSION

Nucleotide composition of 382 sequences of DENV and 641 sequences of CHIKV was analyzed to understand its effect on preference for one type of codon over another, can be influenced greatly by the overall nucleotide composition of genomes (Jenkins et.al.,2003) and the nucleotide composition study reveals that codon usage pattern is influenced greatly by the overall nucleotide composition of genomes (Jun-Jun et al.,2013; Butt,et al.,2014).So First,the nucleotide composition of four serotypes of the DENV genome followed by the CHIKV genome was analyzed. Shown in (table 1).

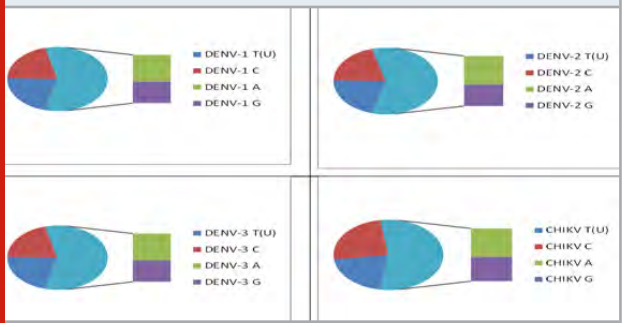
We have found that the pattern of nucleotide composition of these two viruses is quite similar. In (table1) the mean nucleotide of A% of all the 4 serotypes of DENV and CHIKV was highest followed by G%, the mean nucleotide of C% is lowest in DENV and with the U% is lowest in CHIKV. The mean composition of GC and GC3 of all 4 serotypes of DENV and CHIKV virus were also given in (Table1). The study of codon usage patterns of viruses can disclose more useful information about overall viral survival and evolution (Singh etal.,2016). In this study the pattern of nucleotide composition of these two viruses is quite analogous. This research seems to propose that there might be equal distribution of A,U,G and C nucleotides among codons of DENV and CHIKV

, with a preference towards A-ended codon followed by G/C- ended codons (figure1).

Table 1. The mean of nucleotide composition analysis of DENV 1-4 and CHIKV genomes (%)

Nucleotide composition (mean)	DENV1	DENV2	DENV3	DENV4	CHIKV
A%	32.3	33.24	32.17	31.05	29.37
G%	25.85	25.22	25.92	26.28	25.37
C%	20.58	20.64	20.55	20.79	24.68
U%	21.54	21.08	21.35	21.89	20.44
GC	46.44	45.68	46.47	47.05	50.05
GC3	45.73	46.64	43.55	48.13	48.70

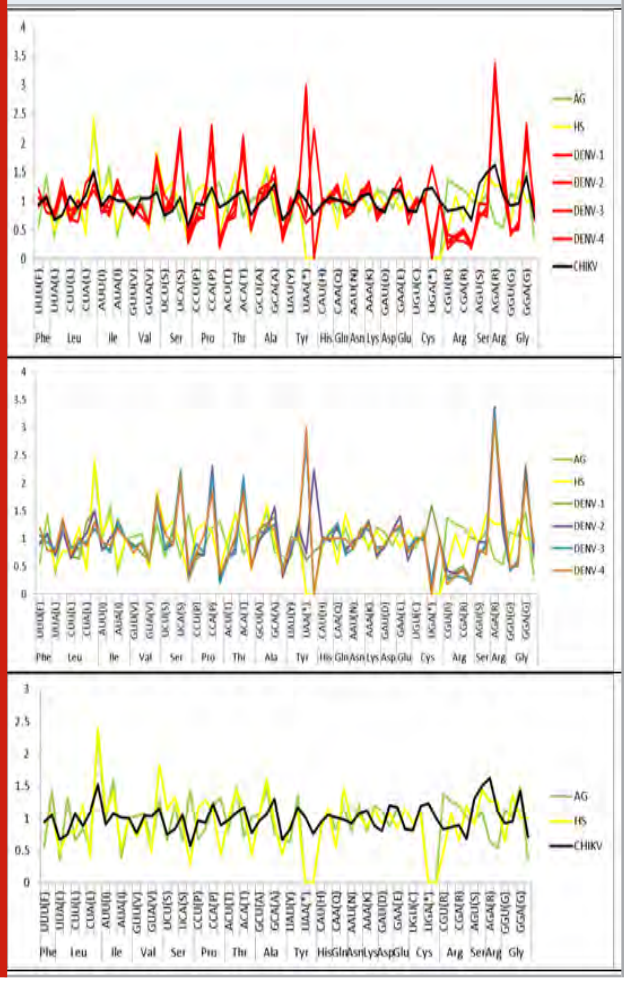
Figure 1: Comparative Analysis Nucleotide Composition of all four serotypes of DENV and CHIKV Genomes: common pattern is observed in both the viruses the mean A% was the highest, followed by G%.



To understand the patterns of codon usage and evaluate the effect of nucleotide composition on codons usage pattern, we performed RSCU analysis and calculated the RSCU values (Table 2). Among the 16 most preferred codons in DENV genomes, six (UUG, CUG, GUG, GCC, AAC, and GAC) were G or C-ended (C-ended: 3; G-ended: 3) and the remaining ten (CUA, AUA, UCA, CCA, ACA, GCA, AAA, GAA, and AGA, GGA) were A-ended codons. There is no preferred codon ending with U. We observed that DENV exhibits higher codon usage bias towards G/C- and less towards A-ended codons.Among 59 codons, six codons had RSCU value greater than or equal to 1.6are over represented codons AGA (Arg), GGA (Gly), CCA (Pro), UCA (Ser), ACA (Thr), GUG (Val). And five under-represented codons had RSCUvalue equal to or less than 0.6 GCG (Ala), CGU (Arg), CCG (Pro), UCG (Ser), ACG (Thr). We observed that the codon usage pattern of virus and human host is quite similar in comparison to the codon usage pattern of virus and vector (Figure 2).

Among the 19 most preferred codons in CHIKV genomes, ten (UUC, CUG, AUC, GUG, GCC, UAC, UGC, CAC, AAC, and GAC) were G or C-ended (C-ended: 8; G-ended: 2) and the remaining seven (ACA, GCA, UCA, AGA, AAA, GAA, GGA and CCA, CAA) were A-ended codons (Table 2). None of the preferred codons were U-ended. We observed that CHIKV exhibits higher codon usage bias towards G/C- and less towards A-ended codons. This indicates that the G/C content at the third position of the codons influences the codons usage patterns. Among 59 codons, only CUG (Leu) and AGA (Arg) had RSCU value greater than or equal to 1.6 are overrepresented codons. However, the underrepresented codons (RSCU,0.6), were identified as follows: UUA (Leu), GCG (Ala), and CGG (Arg). We observed that the codon usage pattern of virus and human host is quite similar in comparison to the codon usage pattern of virus and vector (Figure 2).

Figure 2: Comparative analysis of relative synonymous codon usage (RSCU) patterns. (1) Between DENV and CHIKV, Homo sapiens (HS) and *Aedesaegypti* (AG). (2) Between DENV, Homo sapiens (HS) and *Aedesaegypti* (AG) (3) Between CHIKV, Homo sapiens (HS) and Aedesaegypti (AG)



DENV and CHIKV both viruses belong to different families but share a common vector and host. When we analyzed the most abundantly used codons we observed a common pattern is both the viruses. The viruses exhibit a higher codon usage bias towards G/C- and less towards A-ended codons (Figure 2). This suggests that GC composition plays an important role in crafting the codon usage pattern of both viruses. Another common

feature we observed is that the codon usage pattern of virus and human host is quite similar in comparison to the codon usage pattern of virus and vector. The overall codon usage bias in DENV and CHIKV is low. The result is similar to other RNA viruses like polioviruses, H5N1 influenza virus, and SARS-Covs, respectively, we assume that the weak codon bias in RNA virus is advantageous to replicate efficiently in host cells (Hu et.al 2011; Huipeng et .al, 2019).

Table 2. The synonymous codon usage patterns of DENV and CHIKV, its hosts (Human) and vector (*Aedesaegypti*).

AA	Codon	AG	HS	DENV-1	DENV-2	DENV-3	DENV-4	CHIKV
Phe	UUU(F)	0.56	0.92	0.95	0.9	1.06	1.2	0.94
	UUC(F)	1.44	1.08	1.05	1.1	0.94	0.8	1.06
Leu	UUA(L)	0.36	0.48	0.71	0.69	0.81	0.76	0.67
	UUG(L)	1.32	0.78	1.17	1.15	1.31	1.37	0.75
	CUU(L)	0.66	0.78	0.69	0.65	0.84	0.7	1.08
	CUC(L)	0.84	1.2	0.64	0.91	0.91	1.01	0.88
	CUA(L)	0.54	0.42	1.3	1.1	0.95	0.87	1.1
	CUG(L)	2.28	2.4	1.5	1.5	1.18	1.3	1.52
Ile	AUU(I)	0.99	1.08	0.82	0.79	0.96	0.94	0.9
	AUC(I)	1.59	1.41	0.82	1.01	0.74	0.88	1.08
	AUA(I)	0.39	0.51	1.36	1.19	1.3	1.17	1.01
	AUG(M)	1	1	1	1	1	1	1
Val	GUU(V)	1.04	0.72	0.89	0.85	0.84	0.74	0.77
	GUC(V)	1.08	0.96	0.74	0.92	0.93	0.93	1.05
	GUA(V)	0.6	0.48	0.6	0.59	0.59	0.62	1.04
	GUG(V)	1.28	1.84	1.78	1.64	1.64	1.7	1.15
Ser	UCU(S)	0.66	1.14	0.84	0.82	0.8	1.1	0.75
	UCC(S)	1.2	1.32	1.09	0.89	0.98	0.89	0.83
	UCA(S)	0.66	0.9	2.23	2.04	2.17	2.07	1.06
	UCG(S)	1.44	0.3	0.26	0.35	0.44	0.42	0.57
Pro	CCU(P)	0.68	1.16	0.63	0.73	0.9	0.72	0.96
	CCC(P)	0.84	1.28	0.74	0.7	0.75	1.08	0.93
	CCA(P)	1.2	1.12	2.29	2.31	2.17	1.84	1.22
	CCG(P)	1.32	0.44	0.34	0.26	0.19	0.36	0.89
Thr	ACU(T)	0.8	1	0.72	0.63	0.66	0.66	0.97
	ACC(T)	1.48	1.44	0.96	0.84	0.73	1.04	1.09
	ACA(T)	0.72	1.12	1.82	2.03	2.13	1.82	1.17
	ACG(T)	1.01	0.44	0.51	0.5	0.48	0.48	0.77
Ala	GCU(A)	1.08	1.08	0.96	0.96	1.13	1.17	0.96
	GCC(A)	1.48	1.6	1.27	1.16	1.13	1.29	1.07
	GCA(A)	0.76	0.92	1.39	1.57	1.27	1.16	1.3
	GCG(A)	0.68	0.44	0.38	0.31	0.47	0.38	0.67
Tyr	UAU(Y)	0.64	0.88	1.04	0.77	0.89	0.97	0.83
	UAC(Y)	1.36	1.12	0.96	1.23	1.11	1.03	1.17
	UAA(*)	0	0	0.62	0.73	2.75	3	1.01
	UAG(*)	0	0	0.77	2.24	0.03	0	0.76
His	CAU(H)	0.84	0.84	0.89	1.04	0.91	1	0.94
	CAC(H)	1.16	1.16	1.11	0.96	1.09	1	1.06
Gln	CAA(Q)	0.82	0.54	1.16	1.23	1.29	1.01	1.02
	CAG(Q)	1.18	1.46	0.84	0.77	0.71	0.99	0.98
Asn	AAU(N)	0.8	0.94	0.89	0.97	0.83	0.83	0.92
	AAC(N)	1.2	1.06	1.11	1.03	1.17	1.17	1.08
Lys	AAA(K)	0.8	0.86	1.34	1.3	1.16	1.24	1.12
	AAG(K)	1.2	1.14	0.66	0.7	0.84	0.76	0.88

Continue Table 2

Asp	GAU(D)	1.12	0.92	0.89	0.85	0.87	0.89	0.8
	GAC(D)	0.88	1.08	1.11	1.15	1.13	1.11	1.2
Glu	GAA(E)	1.16	0.84	1.21	1.4	1.17	1.25	1.17
	GAG(E)	0.84	1.16	0.79	0.6	0.83	0.75	0.83
Cys	UGU(C)	0.84	0.92	1.04	0.99	1	0.94	0.81
	UGC(C)	1.16	1.08	0.96	1.01	1	1.06	1.19
	UGA(*)	0	0	1.6	0.03	0.23	0	1.23
	UGG(W)	0	0	1	1	1	1	1
Arg	CGU(R)	1.38	0.48	0.3	0.42	0.28	0.18	0.83
	CGC(R)	1.26	1.08	0.41	0.37	0.32	0.33	0.86
	CGA(R)	1.2	0.66	0.51	0.45	0.31	0.44	0.89
	CGG(R)	1.02	1.2	0.26	0.18	0.21	0.21	0.68
Ser	AGU(S)	0.96	0.9	0.74	0.95	0.7	0.8	1.3
	AGC(S)	1.11	1.44	0.83	0.94	0.91	0.72	1.5
Arg	AGA(R)	0.64	1.26	3.15	3.37	3.3	3.14	1.62
	AGG(R)	0.54	1.26	1.36	1.2	1.58	1.71	1.11
Gly	GGU(G)	1.12	0.64	0.48	0.46	0.41	0.5	0.92
	GGC(G)	1.04	1.36	0.51	0.55	0.63	0.54	0.95
	GGA(G)	1.48	1	2.34	2.26	2.12	2.03	1.42
	GGG(G)	0.36	1	0.67	0.74	0.84	0.93	0.71

In this study, the comparative analysis of RSCU of DENV and its vector and host, CHIKV and its vector and host, and DENV and CHIKV was done to understand the patterns of codon usage and evaluate the effect of nucleotide composition on the codons usage pattern. Both the viruses exhibit a higher codon usage bias towards G/C- and less towards A-ended codons. This suggests that GC composition plays an important role in crafting the codon usage pattern of both viruses. The findings propose that even though RNA viruses have a high mutation rate, DENV and CHIKV has evolved with a moderately stable genetic composition at specific levels of codon usage. The jist of, combining nucleotide composition and RSCU analysis, it is determined that the selection for preferred codons is influenced by nucleotide compositional constraints, which also suggests the presence of mutational pressure. However, it is not the claimedthat the nucleotide compositional constraints are the sole factor associated with codon usage patterns in DENV and CHIKV, because though the RSCU values could disclose the codon usage pattern genome of DENV and CHIKV, (Hu et.al 2011; Huipeng et .al, 2019).

CONCLUSION

The study shows that the comparative analysis of the codon usage pattern in DENV and CHIKV is slightly biased. The nucleotide composition and mutation pressure act as a key factor in shaping codon usage patterns in both DENV and CHIKV. Our data suggested that the codon usage pattern of DENV and CHIKV is evolving to re-adapt to different vector and hosts. Though, further detailed analysis is required to understand the relationship of codon choices between viruses and hosts. To the best of our knowledge, this is the first study comparing the codon usage of DENV and CHIKV with their common host and vector and it is anticipated to increase our understanding of the evolution of DENV and CHIKV. However, The information from this research may not only help to understand the molecular evolution of these two viruses, but it will also contribute to the further development of the virus vaccine and antiviral drug (from Genome to Hit).To achieve the goal for viruses ; the preferred codon information is going to play a key regulating role to develop a therapeutics. It is well stated that the preferred codon are conserved throughout thespecies, whereas the remaining genome content of virus is highly unstable that is susceptible to the environment.

Ethical Statement: This article does not contain any studies with human participants or that were performed on animals.

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Biomedical
Communication

Analysis on the Novel Approach of Using Colloidal Silver Against *E. coli* Persisters to Ampicillin

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ABSTRACT

Bacterial persisters are phenotypic variants that form under stress, cause recurrent infections and also possess antibiotic tolerance. The use of colloidal silver to combat persisters seems to be a promising alternative since bacterial tolerance towards metals or metal ions has not been reported. The objective of this study was to determine the effect of colloidal silver on *E. coli* K-12 NCIM 2665 persisters to Ampicillin. The study determined the effect of colloidal silver on *E. coli* persisters to Ampicillin. The combined action of Ampicillin and silver against persisters was determined by checkerboard assay. Tolerance of log phase population of *E. coli* K-12 NCIM 2665 to silver and whether persisters to silver are formed was also determined. The Fractional Inhibitory Concentration (ΣFIC) index for Ampicillin and colloidal silver was determined to be ≤ 1which indicates an additive effect. A five-log reduction in log phase population and two log reduction in antibiotic persisters was observed after one hour exposure to 16ppm silver; indicating the effectiveness of colloidal silver. Colloidal silver decreases the formation of Ampicillin persisters as well as prevents the survival of existing Ampicillin persisters. In order to combat recurrent bacterial infections, methods need to be found to reduce the formation of pathogenic bacterial persisters or to enhance the susceptibility of persisters to antibiotics. The results of the present study imply that colloidal silver can be used as an anti-persister strategy directly or in combination with an antibiotic.

KEY WORDS: COLLOIDAL SILVER, E.COLI K-12, PERSISTER CELLS.

INTRODUCTION

The host immune system has a variety of mechanisms that can combat bacterial infections. However, pathogens like *Pseudomonas aeruginosa, Mycobacterium tuberculosis, Helicobacter pylori*, *Candida albicans* not only evade the host immune system, but also persist in the host even after antibiotic treatment resulting in recurrent infections (Jayaraman 2008; Dawson et al. 2011; Fauvart et al., 2011; Hong-Geller and Micheva-Viteva 2015; Fisher et al., 2016; Moorhouse et al., 2016). One of the main factors that contribute to the recurrence of infection is persistence. Persistence was first observed as a phenomenon by Bigger when a population of *S. aureus* survived after treatment with Penicillin (Bigger 1944). Bacterial persisters are distinct physiological variants that exhibit tolerance to antimicrobial agents. Tolerance towards antimicrobials makes treatment of such recurrent infections difficult.

Persistence differs from antibiotic resistance since resistant cells grow in the presence of higher doses of antibiotics while persister cells remain dormant. Multidrug tolerance of persisters is one of the major factors for recurrent infections and the inability of antibiotics to eliminate the pathogen (Waters and Bassler, 2005; Lewis, 2008; Moker et al., 2008; Lewis, 2010). Many antibiotics have been shown to be active only against dividing bacteria. Persister cells are known to greatly slow down essential cellular functions that are generally targeted by antibiotics such as transcription, translation, cell wall synthesis and DNA replication (Kwan et al., 2013). New methods to enhance the susceptibility of persisters to antibiotics or to reduce the formation of pathogenic bacterial persisters are required to combat recurrent

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bacterial infections (Defraine et al., 2018). The methods for eliminating persisters can be categorized based on the mechanism of action-Direct killing of metabolically dormant persister cells, Sensitization of persisters to activate the target site for antibiotics, Stimulation of antibiotic influx and Use of methods that can interfere with or reduce the formation of persister cells (Defraine et al., 2018).

Metal and metal ions exert bactericidal effect through multiple mechanisms in contrast to site specific action of an antibiotic. Metals are involved in high-affinity, energetically favourable reactions with a number of target biomolecules (Harrison et al., 2005). Silver or silver ions have been known to possess a broad spectrum of strong inhibitory and microbicidal effects (Lansdown 2006). Silver ions react with proteins by combining with the thiol (–SH) group leading to their inactivation (Russell and Hugo 1994; Feng et al., 2000). Colloidal silver has been shown to be very effective as an antibacterial agent (Morrill et al. 2013; Domínguez et al., 2020). A colloid consists of solid particles (1 nm to 1000 nm) suspended in a liquid. Colloidal silver comprises of two distinctly different forms of silver; metallic silver particles and silver ions.

The total amount of silver that is reported as the silver concentration in parts per million (ppm) is the sum total of the silver contained as particles as well as ions. Colloidal silver has multiple modes of action which makes it more effective (Silverbiotics.com). The nano colloidal silver particles get attracted to the surrounding water molecules and become a part of the structure of the water. This makes the colloidal silver much more stable and bio available than other forms of silver. It has the ability to attract and accept multiple electrons compared to ionic silver which can accept only one electron. Treatment with colloidal silver also leads to an increase in production of Reactive Oxygen Species (ROS) further enhancing the killing of bacterial cells (Domínguez et al. 2020).

Overall, colloidal silver disturbs the structure of biomolecules resulting in cell death (Domínguez et al. 2020). The present study describes the effect of colloidal silver against *E. coli* persisters to Ampicillin. Though there are recent reports on methods to eliminate persisters (Defraine et al., 2018; Khan et al., 2021). must come here after persisters" this study is the first of its kind investigating the use of colloidal silver to combat persisters to antibiotics.

MATERIAL AND METHODS

Bacterial strains and growth medium: *E. coli* NCIM 2665 (*E. coli* K-12) was obtained from NCCS Pune, India. Luria Bertani (LB) broth and Luria Bertani (LB) agar were used as growth media. Log phase culture was prepared by inoculating 500µl of a saline suspension (OD_{530} - 0.4) of overnight grown culture into 100ml of sterile LB broth and incubating for 3hrs at 37°C, 220 rpm. Log phase was achieved at OD_{530} - 0.5, as determined from growth curve experiments.

Antibiotics and Colloidal silver solution: Ampicillin sodium salt was obtained from Hi Media laboratory, India. Stock solutions of Ampicillin (10mg/ml) were prepared in sterile saline and stored at -18°C. The stock solution was freshly diluted before each experiment with sterile saline or sterile LB broth. Colloidal silver solution was obtained from Viridis Biopharma Pvt. Ltd, India. It was diluted in sterile distilled water or sterile LB broth.

Minimum Inhibitory Concentration (MIC): The MIC of Ampicillin for *E. coli* NCIM 2665 was determined by the broth dilution method according to Clinical and Laboratory Standards Institute (CLSI) Standards (CLSI 1991, 2015). The concentration range used was 2-10µg/ml of Ampicillin. Presence of growth was measured as Optical density (O.D) at 530 nm after 24hours. The presence of *E. coli* NCIM 2665 persisters to Ampicillin was determined by dose dependent and time dependent kill curve.

Dose dependent kill curve: To determine the antibiotic concentrations that result in the survival of only drug-tolerant persister cells, killing curves were performed. Dose-dependent study was done using different concentrations (40x, 80x, 100x MIC) of Ampicillin against *E. coli*. Respective antibiotic concentrations were added to 10 ml of log phase culture and further incubated at 37°C, 220 rpm for one hour. The medium was separated by centrifugation at 8000 rpm for 10 mins and the cell pellet obtained was washed thrice with sterile saline and then suspended in 10ml sterile saline. Cell counts were determined in triplicates by drop plate technique on LB agar plates using 10µl of the dilutions (Miles et al.1938; Moker et al., 2010).

Time dependent kill curve: The antibiotic concentration determined by the dose dependent kill curve was added to log phase culture and incubated at 37°C, 220 rpm for time intervals of 1, 2 and 3 hours. After the designated incubation time, samples were centrifuged at 8000rpm for 10 mins. The cell pellet was washed thrice with sterile saline, and then suspended in 10ml sterile saline. Cell counts were determined in triplicates by drop plate technique on LB agar plates using 10µl of the dilutions to determine the number of surviving persister cells.

Persister cells are obtained by exposure of log phase cultures to a certain concentration of antibiotics for a given time interval. Based on dose and time dependent kill curve experiments, the log phase cultures of *E. coli*

were treated with 400µg/ml of Ampicillin for 1 hour. The high concentration of antibiotic would kill non persister population; resulting in survival of only persister cells. After the antibiotic treatment, the cells were pelleted by centrifugation at 8000rpm for 10 mins. The cell pellets were washed thrice with sterile saline and then suspended in sterile saline. Persister cell numbers were determined by drop plate technique using 10µl of the diluted cell suspension on LB agar plates. The number of colonies obtained represents the number of cells that survived the high antibiotic exposure for one hr and thus were considered as persisters.

Minimum Inhibitory Concentration (MIC) of Colloidal silver for *E. coli* NCIM 2665: MIC was determined by the broth dilution method according to CLSI Standards. The concentration range of colloidal silver used was 1- 10ppm. Presence of growth was measured as Optical density (OD) at 530 nm after 24hours.

Determination of the effect of colloidal silver on formation of persister cells to Ampicillin: Silver at MIC concentration was added to the log phase culture in three different ways: i) 4ppm colloidal silver was added to culture at log phase followed by addition of 400µg/ml Ampicillin after 1 hr incubation, ii) 400µg/ml Ampicillin was added to culture at log phase followed by addition of 4ppm colloidal silver after 1 hr incubation, iii) 400µg/ml Ampicillin and 4ppm colloidal silver were added simultaneously at log phase. Each set was incubated at 37°C, 220 rpm for further one hour and persister cell count was performed in triplicates.

Synergistic action of Ampicillin and colloidal silver against persister cells: Synergistic action was determined using the checkerboard assay in microtitre plates (Balouiri et al. 2016). Persister cells to Ampicillin were obtained as per the earlier mentioned protocol and used as inoculum for the checker board assay. Stock solutions of both Ampicillin and colloidal silver were prepared in LB medium. The reaction volume in each well in the microtiter plate was set as 150µl of the given concentration of Ampicillin in LB medium + 150µl of the given concentration of colloidal silver in LB medium + 20µl of persister cell suspension ($3x10^5$ cfu/ml). The concentrations of Ampicillin used were 0-20µg/ml while that of colloidal silver were 0-5ppm.

After incubation at 37°C for 24hr, the growth was determined by measuring optical density at 530nm and further confirmed by addition of 10µl of Triphenyl tetrazolium chloride TTC solution (1%w/v) to each well. Conversion of colourless TTC solution to red Formazan indicated growth. The Formazan intensity was measured at 490nm. According to the CLSI guideline, synergy between drugs is determined by calculating the Fractional Inhibitory Concentration (ΣFIC) index. ΣFIC index represents the sum of the FICs of each drug tested, where the FIC is determined for each drug by dividing the inhibitory concentration of each drug when used in combination by the MIC of each drug when used alone. In the given study ΣFIC = FIC Ampicillin + FIC colloidal silver. The combination is considered synergistic when the ΣFIC is = 0.5; additive, when the ΣFIC is >0.5 to < 2, and antagonistic when the ΣFIC is = 2.

Determination of persistence/tolerance of *E. coli* NCIM 2665 to colloidal silver: Log phase cultures of *E. coli* NCIM 2665 were treated with colloidal silver at concentration range of 4 – 20 ppm and incubated at 37°C, 220 rpm for one hour. After incubation, the cell pellets obtained after centrifugation were rinsed with sterile saline thrice to remove traces of colloidal silver and suspended in sterile saline. Enumeration of surviving cell number was done in triplicates by the drop plate method.

Effect of colloidal silver on *E. coli* NCIM 2665 persisters to Ampicillin: *E. coli* NCIM 2665 persisters formed to 400µg/ml Ampicillin were inoculated in LB medium containing colloidal silver at concentration range of 4 – 20 ppm. Exposure to colloidal silver was done for 1 hour. Enumeration of cell number was done in triplicates by drop plate method.

Statistical analysis: Graphs were prepared using Microsoft Excel 2016. Error bars in the graphs were expressed as standard error of the mean ± (SEM). For effect of colloidal silver on persister formation, mean values were compared within and between groups using one-way analysis of variance (ANOVA) followed by Bonferroni's test using SPSS software (file version 1.0.0.118). P value less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Minimum Inhibitory Concentration (MIC) of Ampicillin: The MIC value of Ampicillin for *E. coli* NCIM 2665 as determined by the broth dilution method was found to be 5µg/ml.

Dose dependent kill curve: Dose dependent study was done using different concentrations (40x, 80x, 100x MIC) of Ampicillin. Fig. 1 depicts the survival of *E. coli* population on exposure to the different concentrations of Ampicillin for 1 hr. Increase in the Ampicillin concentration to 400µg/ml resulted in a rapid killing of the majority of the population followed by a plateau consisting of surviving drug-tolerant persister cells with further increase in drug concentration (Fig. 2). Concentration of Ampicillin for obtaining persister population was selected within the plateau region of the survival curve. With further increase in Ampicillin concentration beyond 400µg/ml there was no significant decrease in surviving population count.

Hence, 400µg/ml of Ampicillin was used for the isolation of persister cells throughout the study.

Figure 1: Dose dependent kill curve. Log phase culture was treated with different concentrations of Ampicillin. Cell numbers are plotted as log CFU/ml. The values are average of three independent experiments. Error bars indicate ± SEM.

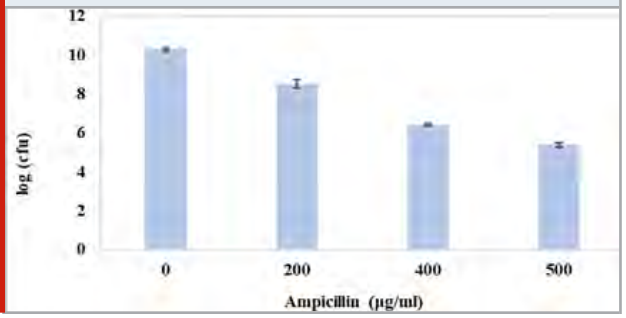
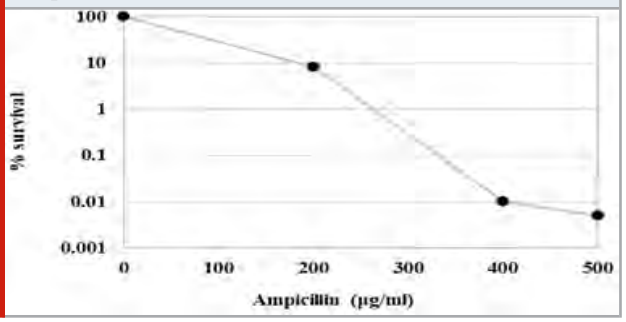


Figure 2: Percentage survival for dose dependent kill curve. Untreated control sample (0µg/ml) was taken prior to antibiotic treatment. Cell numbers are plotted as percentage survival at different concentration of Ampicillin.



Time dependent kill curve: Survival of *E. coli* population on exposure to 400µg/ml Ampicillin at different time intervals (1hr, 2hrs and 3hrs) is presented in Fig. 3. The control was log phase culture incubated without any antibiotic for the same time period. The time kill curve in Fig. 4 shows a typical biphasic curve with rapid decrease in population at the end of 1 hour; followed by constant surviving population count with further increase in time of exposure to antibiotic. As exposure time of one hour resulted in maximum persister cell number; for further experiments, persister population was obtained by exposing log phase culture to 400µg/ml of Ampicillin for 1 hr at 37°C and 220rpm.

Minimum Inhibitory Concentration (MIC) of Colloidal silver: The MIC of Colloidal silver for *E. coli* NCIM 2665 determined by the broth dilution method was found to be 4ppm.

Effect of colloidal silver on formation of persisters to Ampicillin: Colloidal silver was seen to have an effect on formation of *E. coli* NCIM 2665 persisters to Ampicillin (Fig. 5).

Figure 3: Time dependent kill curve at 400µg/ml Ampicillin. Control indicates untreated culture. Test indicates log phase culture treated with 400µg/ml of Ampicillin and incubated for different time intervals. Cell numbers are plotted as log CFU/ml. The values are average of three independent experiments. Error bars indicate ± SEM.

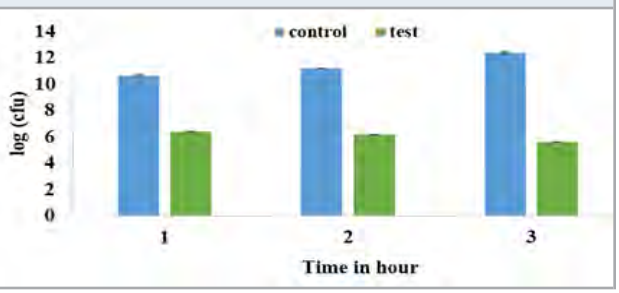


Figure 4: Percentage survival for time dependent kill curve at 400µg/ml Ampicillin. Untreated control sample (0µg/ml) was taken prior to antibiotic treatment. Cell numbers are plotted as percentage survival after 1, 2, 3 hours of exposure to 400µg/ml of Ampicillin

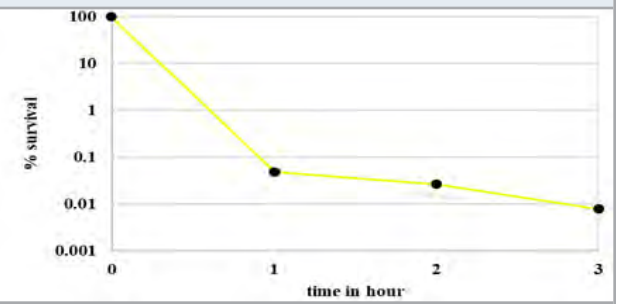
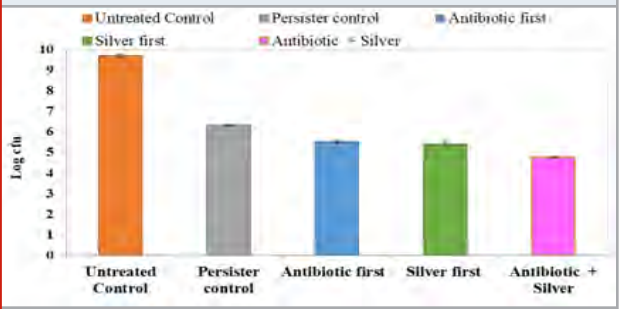


Figure 5: Effect of colloidal silver on persister formation in *E. coli* NCIM 2665. Untreated control indicates log phase culture not treated with antibiotic. Persister control indicates log phase culture treated with only 400µg/ml Ampicillin. Antibiotic first indicates addition of Ampicillin at log phase followed by silver. Silver first indicates addition of silver at log phase followed by Ampicillin. Antibiotic + silver indicates addition of both simultaneously at log phase. Cell numbers are plotted as log CFU/ml. The values are average of three independent experiments. Error bars indicates ± SEM.



The number of persisters decreased under both conditions namely when silver was added prior to the addition of Ampicillin or when it was added after the antibiotic. Also, when Ampicillin and silver were added simultaneously at log phase, persister population decreased further significantly. Fig. 6 represents the percentage survival under the three different experimental conditions. The persister control is the number of cells that survived after exposure to 400µg/ml of Ampicillin for one hour.

Figure 6: Effect of colloidal silver on persister formation in *E.coli* NCIM 2665 (Percentage survival). Persister control indicates population that survived after treating log phase culture with 400µg/ml of Ampicillin only. For treatment methods -Antibiotic first, Silver first and Antibiotic + silver, the percentage survival was calculated in comparison to persister control. The values are average of three independent experiments. Error bars indicate ± SEM. a, b, c indicates P<0.001 compared to persister control for each treatment method respectively.

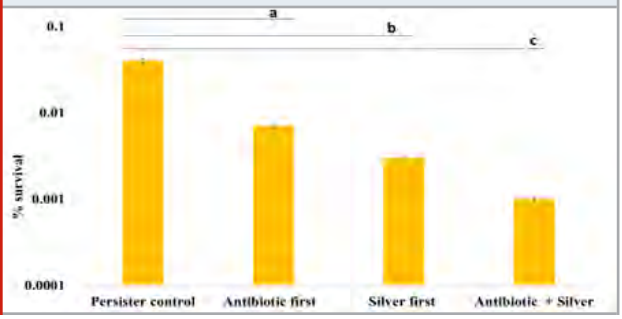
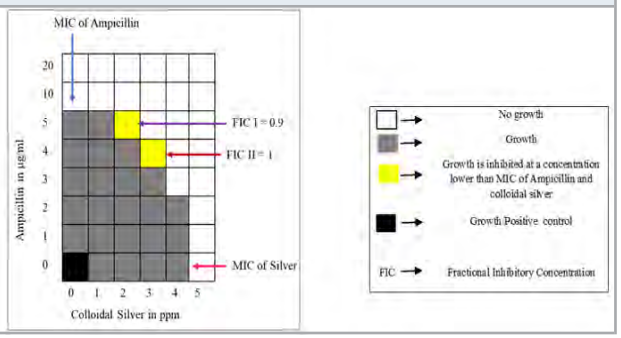


Figure 7: Checkerboard assay for Ampicillin and colloidal silver against *E. coli* persister. Ampicillin and colloidal silver was used at 1-20µg/ml and 1-5ppm concentration respectively. Growth control indicates maximum growth and viability of persister cells. FIC was calculated for two combinations I & II where growth was inhibited at a concentration lower than MIC of Ampicillin and colloidal silver.



The percentage of persister cell survival in the presence of 400µg/ml of Ampicillin was found to decrease in the presence of silver under all three experimental conditions. It can be concluded that 4ppm colloidal silver was able to significantly decrease the formation of *E. coli* persisters to Ampicillin. The results obtained by the three experimental set ups (Fig. 6) were compared with the persister control and statistically evaluated using one way ANOVA followed by Bonferroni post hoc test. For all the three sets using silver, decrease in the population was found to be statistically significant (P<0.001).

Synergistic action of Ampicillin and colloidal silver against persisters: The results of the microtitre plate checker board assay to determine the synergistic action of Ampicillin and colloidal silver on *E.coli* persisters to Ampicillin are represented in Fig. 7. Ampicillin and colloidal silver in combination inhibited the persisters at concentrations lower than their respective MIC concentrations. The ΣFIC was calculated for the two combinations showing inhibition as indicated in the figure.

$$\Sigma FIC = \frac{\text{Inhibitory concentration of Ampicillin in combination}}{\text{MIC concentration of Ampicillin}} + \frac{\text{Inhibitory concentration of Silver in combination}}{\text{MIC concentration of Silver}}$$

ΣFIC I = 5/10 + 2/5 = 0.9 ΣFIC II = 4/10 +3/5 = 1

A combined action is considered additive, if the ΣFIC lies between 0.5 to 2. Hence, colloidal silver and Ampicillin were found to exert an additive effect on *E.coli* persisters to Ampicillin. Colloidal silver significantly reduced the inhibitory concentration of Ampicillin.

Determination of persistence/tolerance of *E. coli* NCIM 2665 to colloidal silver: As the MIC concentration of 4ppm of colloidal silver was used to study the inhibitory effect of colloidal silver on the formation of *E. coli* persister to Ampicillin, it was imperative to study the tolerance of *E.coli* to silver and whether persisters to silver get formed. Log phase cultures of *E. coli* were treated for 1 hr with colloidal silver ranging from 4ppm to 20ppm at intervals of 2ppm.

E. coli NCIM 2665 was found to tolerate up to 16ppm of colloidal silver (Fig. 8); albeit a significant decrease in population. At 16ppm, a five-log reduction was observed as compared to untreated control log phase population. There was total killing of cells on exposure to 18ppm for 1 hour. For isolation of persisters to Ampicillin; *E. coli* NCIM 2665 was exposed to 80xMIC (400µg/ml) and 100xMIC (500µg/ml) for 1 hr. However, *E. coli* was able to tolerate 1 hr exposure to only 16ppm of colloidal silver which is 4x MIC and 18ppm brought about total killing in 1 hr. It seemed evident that *E.coli* NCIM 2665 does not form persisters to colloidal silver.

Effect of colloidal silver on *E. coli* NCIM 2665 persisters to Ampicillin: Ampicillin persister cells were exposed to different concentrations of colloidal silver for 1 hr. Similar to the log phase *E. coli* population, the Ampicillin persister cells were also found to tolerate up to 16ppm of colloidal silver (Fig. 9). At 16ppm, a two-log reduction

was observed as compared to untreated persister control while the log phase *E. coli* population showed a five log reduction at 16ppm (Fig. 8).

Figure 8: Tolerance of *E. coli* NCIM 2665 to colloidal silver. Log phase culture was exposed to different concentrations of colloidal silver. No growth indicates growth is inhibited by colloidal silver. Cell numbers are plotted as log CFU/ml. The values are average of three independent experiments. Error bars indicate ± SEM.

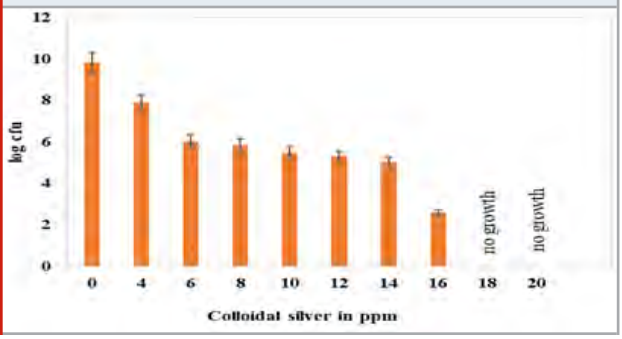
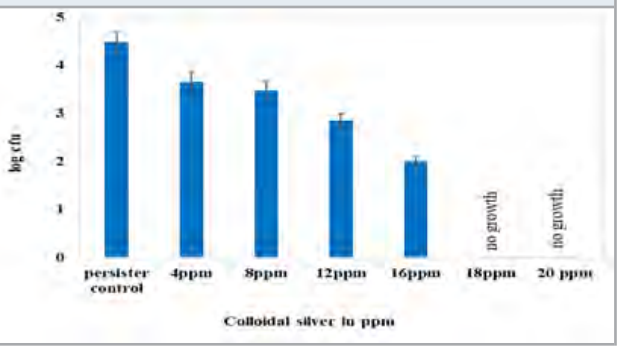


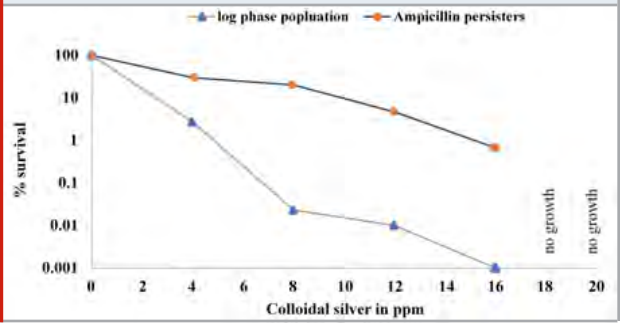
Figure 9: Effect of colloidal silver on *E.coli* Ampicillin persisters. Persister control indicates persister cells unexposed to colloidal silver. Persister cells were exposed to 4-20ppm of colloidal silver. Cell numbers are plotted as log CFU/ml. No growth indicates growth is inhibited by colloidal silver. The values are average of three independent experiments. Error bars indicate ± SEM.



Similarly, total killing of the Ampicillin persisters was seen after 1 hr exposure to 18ppm of colloidal silver as was observed with log phase *E. coli*. Fig. 10 shows percentage survival of log phase population and Ampicillin persisters of *E. coli* in the presence of various concentrations (4ppm to 16 ppm) of colloidal silver. Both, the log phase *E. coli* population as well as the Ampicillin persisters were able to tolerate 16ppm colloidal silver for 1 hr. However, the log reduction was much greater in the case of normal cells as compared to the two-log reduction for persister population.

Bacterial persisters are phenotypic variants that form under stress and possess antibiotic tolerance (Bigger 1944; Lewis 2010; Fisher et al. 2017; Van den Bergh et al. 2017). Bacterial persistence is an emerging problem in addition to drug resistance. Recurrence of an infection indicates survival of the pathogen in the host for longer duration (Levin and Rozen 2006; Michiels et al. 2016; Meylan et al. 2018; Bartell et al. 2020). Increasing the dosage or duration of the antibiotics not only increases tolerance towards the drugs but also promotes persistence.

Figure 10: Survival percentage of log phase population and Ampicillin persisters of *E. coli* in the presence of colloidal silver. The population counts taken prior to the treatment with colloidal silver were considered as 100%. No growth indicates no viable cells or zero percent survival.



Different strategies to eliminate bacterial persister cell sinclude the direct killing of metabolically dormant persister cells, sensitization of persister cells for the stimulation of antibiotic influx, and the use of molecules capable of interfering with or reducing the formation of persister cells (Gefen and Balaban 2009; Allison et al. 2011b; Feng et al., 2015; Kwan et al., 2015; Chowdhury et al. 2016; Orman and Brynildsen 2016; Liu et al.,2020; Khan et al., 2021). Also, using combination of a strong and weak metabolism-dependent antibiotic has shown to decrease persister population effectively (Zheng et al., 2020).

Polymyxin B, poly-L-lysine and phenothiazine have also known to reduce the persistent phenotype in *Escherichia coli* and *Pseudomonas aeruginosa* (Mohiuddin et al., 2020). Electrochemical methods combined with antibiotics have been suggested as an effective alternative for persister cell elimination (Sultana et al., 2016). The use of copper to control persisters has been recently shown to be effective (Moreira et al., 2020). Silver is known to enhance antibiotic activity against Gram-negative bacteria (Morones et al. 2005; Morones et al., 2013).

However, till date the use of silver against persisters is not known. Hence the use of colloidal silver in this study is a novel alternative approach to control bacterial persister cells (Moreira et al. 2020). In the present study, *E. coli* NCIM 2665 persisters were obtained at Ampicllin concentration of 400µg/ml. The cell population so obtained on exposure to 400µg/ml of Ampicillin for 1

hr were considered to be persisters since they exhibited a biphasic killing curve (Keren et al., 2004; Moker et al., 2010; Kamble and Pardesi 2020).

The MIC of colloidal silver used in the present study was found to be 4ppm for *E. coli* NCIM 2665. Effect of colloidal silver on persister formation was studied under antibiotic stress. Survival of persister population decreased with prior silver treatment but when silver and antibiotic were added simultaneously at log phase, persister population decreased significantly ($P<0.001$). Checkerboard assay indicated additive effect and therefore the colloidal silver can be administered along with antibiotic. Colloidal silver decreased the survival of both log phase culture and Ampicillin persister population. Both log phase population and antibiotic persisters were able to tolerate 1 hour exposure to colloidal silver up to 16ppm. However, antibiotic persisters showed a higher percentage of survival compared to the non persister population which confirms their rigid persistence feature.

A drastic five log reduction in log phase population and two log reduction in antibiotic persisters at 16ppm of colloidal silver, indicates its effectiveness. There was total killing of log phase culture as well as Ampicillin persisters at 18 ppm silver. Overall decrease in persisters observed after simultaneous addition of silver and Ampicillin may be attributed to the killing effect exerted by colloidal silver on log phase cells as well as on the antibiotic persisters once formed. Additive effect as seen in the checkerboard assay may also contribute to it. Colloidal silver may be modifying the physiological state of the cells or the state of the environment such that persistence is not promoted.

CONCLUSION

This study confirms that colloidal silver can be considered as a promising anti persistence strategy. It can be used either as the sole bactericidal agent or can be used to enhance the effectiveness of an antibiotic. The key advantage of colloidal silver besides its inhibitory effect is that persister cells to the colloidal silver do not seem to be formed.

Conflict of Interests: There was no conflict among the interests of the participating authors.

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Microbiological
Communication

Detection and Evolutionary Genetical Identification of Some Fungal Spoilage Fish Species from the Red Sea

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ABSTRACT

The high nutritional value of fish makes it highly perishable because it provides favorable medium for the growth of micro-organisms after catching. In addition, they can also act as carriers of several microbial and other health hazards. Many mould growth on foods stored at low temperature is common and recurring problem. Certain molds are known to be capable of producing mycotoxins at low temperature as low as (-2 to 10°) C. That encouraging us to perform this study which aimed to survey, isolation and perform different further genetic identification of fungal spoilage species of some marine fish from Saudi Arabia markets. 100 samples from 5 types of fishes; Salmon "*Salmoniformes*", Red sea Bream "*Pagrus pagrus*", *Rabbitfish "Siganus rivulatus*", *Spanish mackerel "Scomberomorous commerson*", Red mullet "*Mullus surmuletus*", collected from Jeddah fish markets, then prepared aseptically and plated on Potato Dextrose Agar (PDA) medium incubated for 3-7 days/ 28±2°C and examined daily Macroscopically, microscopically and genetically. The results recorded 4 species of fungi; *Aspergillus eucalypticola* as one of (Black *Aspergilli*), *Aspergillus oryzae* as (white *Aspergilli*), *Penicillium digitatum* as (green fungi) and *Byssochlamys spectabilis*. This is the first report of *Penicillium digitatum* and Byssochlamys spectabilis in fish. Furthermore, the research identified each fungi gene cluster. More attention in fish rearing facilities, caution should be taken by consumers in preparation and applying perfect cooking in consuming fish and more education and efforts should be developed by fish farmers to avoid fishponds contamination. We recommended to further research should be done on fungal contaminations.

KEY WORDS: ASPERGILLUS EUCALYPTICOLA, ASPERGILLUS ORYZAE, PENICILLIUM DIGITATUM, BYSSOCHLAMYS SPECTABILIS, GENETIC DIVERSITY, NCBI BLAST.

INTRODUCTION

Recently, the awareness about the nutritional and health benefits of fish consumption were developed due to its richness in; good quality protein with essential amino acids such as; lysine, with vitamin A, E, ß- complex (B12, B6) and calcium, phosphorus fluorine, iodine which are needed development of strong teeth and the prevention of goiter in man, ω-3 and ω-6 fatty acids that known to support good health while, containing low saturated polyunsaturated fatty acids which are known to reduce the risks of coronary heart diseases. All that increased the dietary and health significance of seafood consumption (Adeyeye, et. al., 2015 and FAO, 2016, Neo 2019).

The coastal countries become capture about 50% of the world harvest and a large proportion of the catch are consumed internally, more than 30% of fish for human consumption comes from aquaculture. In many Asian countries over 50% of the protein intakes comes from fish while in Africa the proportion is 17.50% (Wogu & Maduakor, 2010, Neo 2019). The high nutritional value of fish makes it highly perishable because it provides

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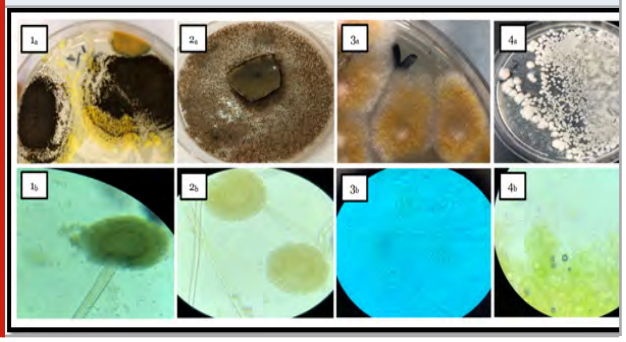
favorable medium for the growth of micro-organisms after catching. In addition, they can also act as carriers of several microbial and other health hazards. The greatest risk to human health is due to the consumption of raw or insufficiently processed fish and fish products. Contamination is a very important aspect as this is the mode that most unwanted microorganisms may be transmitted onto seafood and other food products and may occur at various stages of handling and processing (Al-Ghabshi et al., 2012).

Figure 1: Macroscopic and Microscopic features of fungal genera isolated from tested fish spp. samples as following:
1)Pictures from (1a & 1b) group declared the macroscopic and microscopic view of *Aspergillus eucalypticola* cultureplates on PDA respectively.
2)Pictures from (2a & 2b) group viewed the macroscopic and microscopic view of *Aspergillus oryzae* cultureplates on PDA respectively.
3)Pictures group 3 (3a & 3b); showed the macroscopic and microscopic view of *Penicillium digita* tumplates on PDA respectively.
4)Pictures group 4 (4a & 4b); revealed the macroscopic and microscopic view of *Byssochlamys spectabilis* that cultured on PDA respectively



Fish is more likely to be spoiled by fungi than by bacteria. The occurrence of fungi and fungus-like organisms in water reservoirs is of great importance for sanitary and epidemiological reasons. Many mould growth on foods stored at low temperature is common and recurring problem. Certain molds are known to be capable of producing mycotoxins at low temperature as low as (-2 to 10° C). Fungi and fungus-like organisms regarded as important etiological factors of mycotic infections are identified in fresh and saltwater. The most commonly encountered fungi in various ecosystems include such pathogenic species as *Aspergillus* sp, *Penicillium* sp. In the case of *Penicillium* species that can grow under refrigeration, spoilage of these products may happen more frequently during the colder months. Fluctuation of temperature in the packaging areas must be avoided since it can result in condensation.

The condensation on surfaces (walls, ceilings, overhead piping, etc.) can be conductive for mould growth. Furthermore, moisture condensation inside the package, due to packaging of the products prior to being completely cooled, may accelerate mould growth and spoilage (Srinivasan & Saranraj, 2017). *Aspergillus eucalypticola* is one of (Black *Aspergilli*) which consider one of the food spoilage fungi in addition to their mycotoxin production in different food items (Gil-Serna, et. al., 2019). While, *Aspergillus oryzae* related to the Flavi species of Aspergillus (white *Aspergilli*) which produce very toxic compounds known as (aflatoxins) and highly spread among food elements. Furthermore, those microbes highly infected to immunocompromised consumers, causing "aspergillosis" (Kjærbølling, et. al., 2020).

Table 1. Results of ncbi BLAST query for the 11 fungi sequences isolated from Examined Fish Samples

Isolate	Description	Query Coverage%	Identity %
WA1	*Aspergillus oryzae*	98	97
WA2	*Aspergillus eucalypticola*	97	97
WA3	*Aspergillus oryzae*	84	97
WA4	*Aspergillus eucalypticola*	97	97
WA5	no match was found		
WA6	*Penicillium digitatum*	98	89
WA7	*Aspergillus eucalypticola*	89	97
WA8	*Aspergillus oryzae*	81	98
WA9	*Penicillium digitatum*	89	96
WA10	*Byssochlamys spectabilis*	84	98
WA11	*Penicillium digitatum*	93	97
WA12	*Aspergillus oryzae*	88	98

On the other hand, *Penicillium digitatum* is one mesophilic fungus from the most common blue and green "*Penicillium*" molds although this mold more common on fruits, this is one of the rarely report of this fungi in fish samples. *Penicillium digitatum* can causing mycosis or allergies for consumers and causing decaying of fish meat (Palou, 2014). *Byssochlamys spectabilis* able to spoil food and producing heat resistant ascospores and widely spread within food causing spoilage (Houbraken et al., 2008). Fungal spoilage of fish imposes significant annual global revenue for the food and beverage industries. Mould spoilage can also be a food safety issue due to the production of mycotoxins or allergens by these moulds. To avoid or reduce mould spoilage, several hurdles can be used: (1) reducing the water activity of the food, (2) thermal processing, (3) addition of preservatives, (4) reduction of oxygen in; the packaging using vacuum, oxygen scavengers or modified atmosphere packaging (MAP), and (5) refrigerated storage. However, the addition of each of these hurdles will be selecting for a different group of spoilage fungi (Rico-Munoz, et al., 2019).

There were a surveillance shortage in fish spoilage fungus species in marine fish generally and marketed fish species in Saudi Arabia especially, In addition to nearly complete absence of studies on the genome sequences for this fungus specially in fish, which have

great importance on the fish quality and consumers' health. All that encouraging us to perform this study which aimed to survey, isolation and perform different further identification of fungal spoilage species of some marine fish from Saudi Arabia markets.

Figure 2: Distribution of 11 fungyisolates

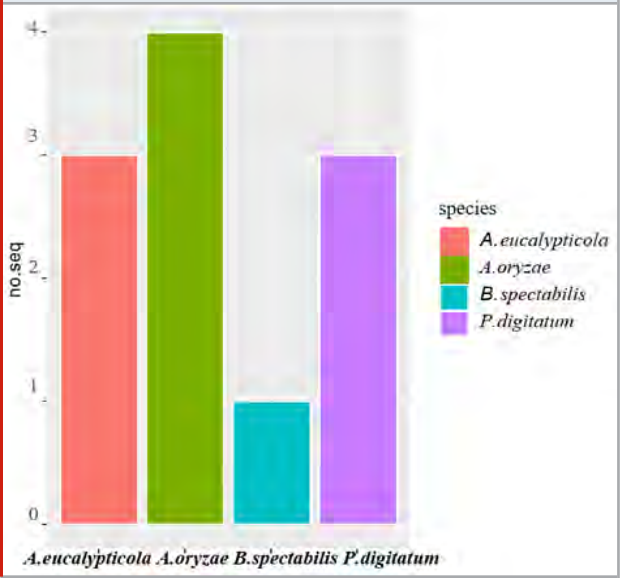


Table 2. Sequence length & GC content of 11 sequences

Isolate	Length	GC%
Aspergillus eucalypticola	1352	47
Aspergillus eucalypticola	1502	48
Aspergillus eucalypticola	1426	48
Aspergillus oryzae	1262	48
Aspergillus oryzae	1448	47
Aspergillus oryzae	1467	48
Aspergillus oryzae	1439	48
Penicillium digitatum	1361	47
Penicillium digitatum	1471	48
Penicillium digitatum	1411	48
Byssochlamys spectabilis	1306	47

MATERIAL AND METHODS

Study area, collection of samples: A total of one 100 samples from 5 types of fishes; Salmon "Salmoniformes", Red sea Bream "*Pagrus pagrus*", *Rabbitfish "Siganus rivulatus", Spanish mackerel "Scomberomorous commerson"*, Red mullet "*Mullus surmuletus*", 20 fish from each fish type. samples of different types of marine fish samples collected from Jeddah fish markets, about 150g/fish packaged in polyvinyl chloride films and transferred on ice box container as soon as possible to Faculty of science, University of Jeddah, on one of the postgraduate microbiology laboratories.

Examination of Samples: Collected samples were prepared aseptically according to the technique recommended by (USDA, 2012). 25 grams of the sample were transferred into a sterile plastic bag of the stomacher (Seward laboratory services, 400R Auckland) then 225 ml. of sterile 0.1% peptone water were added. The 2 minutes stomached sample was adequately dispersed to provide the homogenate; which represent the dilution of 1:10 (10-1) and plated in triplicate over plates containing on different culture media Potato Dextrose Agar (PDA) medium incubated for 3-7 days/ 28±2 ^{0}C and examined daily. Macroscopic identification made by observing the colony colour and texture then prepared slides with lacto phenol cotton blue to detect fungal structures covered with a cover slip, identified microscopically according to the morphology of colony and spores.

Figure 3: Boxplot of Sequence of 11 isolates

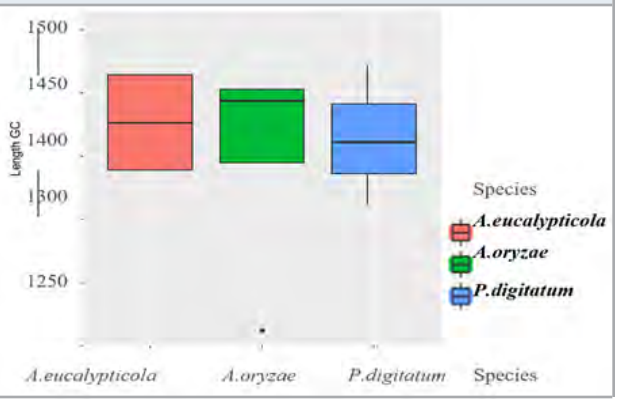


Table 3. Distribution of 11 fungi isolates in 2 clusters

Species	Cluster	
A.eucalypticola	1	0
A.eucalypticola (2)	1	0
A.eucalypticola (3)	1	0
A.oryzae	1	0
A.oryzae (2)	1	0
A.oryzae (3)	1	0
A.oryzae (4)	1	0
B.spectabilis	0	1
P.digitatum	1	0
P.digitatum(2)	1	0
P.digitatum(3)	1	0

Serological examination: All the 11 sequences were subjected to ncbi BLAST search tool http:// blast.ncbi.nlm.nih.gov to detect non-chance sequence similarity. BLAST search was restricted to Reference genomic sequence and fungi database, where models (XM/XP) as well as unclultured/environmental samples were also filtered out, such that more reliable results would be attained. Each individual sequence was solely blastd, where blast hit with the lowest expect- value (which

indicate number of non-chance alignments) was picked. In order to ensure that Blast out puts were governed by expected-value (aka e-value), Blast algorithm parameter was decreased such the expected threshold was set to more stringent value of $1e^{-6}$.

Figure 4: Base frequencies of 11 isolates

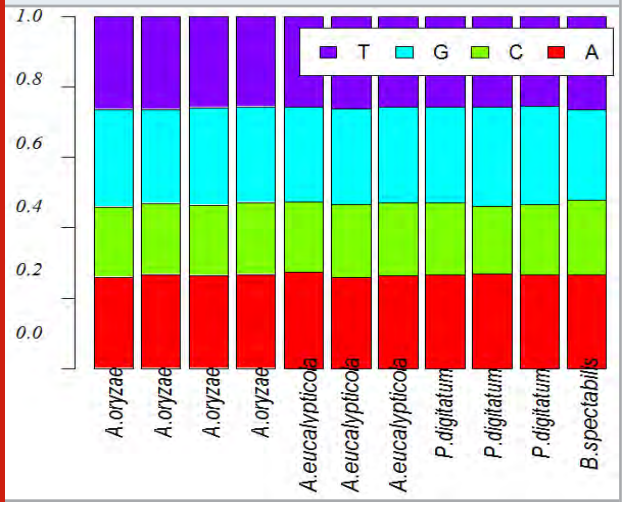


Table 4. Estimated parameters of DNA polymorphism 11 fungi isolates

No. Haplotype	Haplotype diversity±sd	Nucleotide diversity π	Average number of nucleotide differences	11	1±0.04

Alignment of the 11 sequence was carried out using version 2 of Clustalx (Larkin et al., 2007) Exploratory data and phylogenetic analyses were carried out under R Project for Statistical Computing (R Core Team, 2019). Where Exploratory data analysis was done using Seqinr (Charif & Lobry, 2007) R package. Phylogenetic analysis was carried out by ape package (Paradis et al., 2004). Re- construction of the phylogenetic tree was done using Neighbor joint method (Nei, 1987). DnaSP (Librado & Rozas, 2009) software was used to analyze the haplotype diversity (Hd), the average number of nucleotide differences, the average number of nucleotide differences, the nucleotide diversity (π). The polymorphic site (S), the singleton variable sites (SP), and the parsimony informative sites (PIP) for each gene, and the average number of nucleotide substitutions per site between species (D_{xy}) (Tajima, 1983).

Statistical Analysis: The statistical program, SPSS version 16 for window, was used for determination of means, standard error and analysis of variance (ANOVA) using the one way (mean at significance level of ($P<0.05$). Statistical significance was tested at the 5% level of significance in this study.

RESULTS AND DISCUSSION

Phenotyping of the different fungal species in examined fish samples Figure (1) shows the macroscopic and microscopic features of fungal genera isolated from tested fish spp. samples as following: (1a & 1b) pictures declared the macroscopic and microscopic view of *Aspergillus eucalypticola* culture plates on PDA respectively which belongs to black *Aspergilli* with their morphological feature as black colonies which roughen with maturity and black dark conidial heads with dark spores as almost members of Nigri family. While, Pictures group (2a & 2b) viewed *Aspergillus oryzae* on PDA as; pale grey to black colonies with coarsely wall of conidial heads and short column. *Penicillium digitatum* declared in (3a & 3b) as; woolly growth, velvety texture colonies. The white color firstly turns to yellowish green with white center or olive gray, microscopically appeared as; branched hyaline, with cup shape phialides and brush-like clusters which known as "penicilli". Meanwhile morphological feature of *Byssochlamys spectabilis* in (4_a, 4_b) as pale yellow-brown, smooth-walled irregularly branched cylindrical conidia.

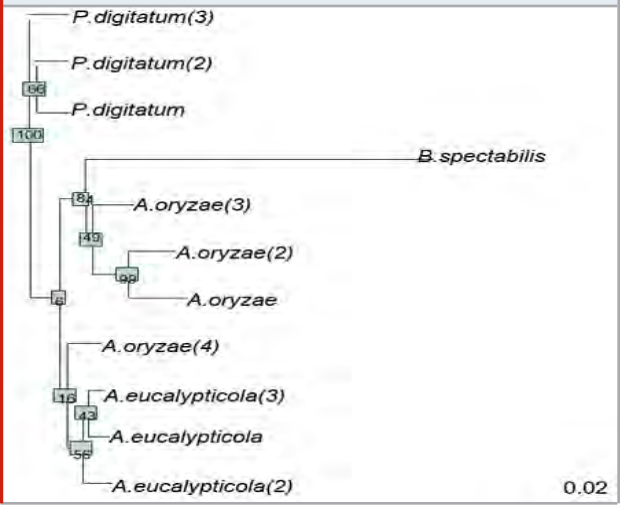
NCBI Blast Query : Table (1) shows results of ncbi BLAST query for the 36 sequenced isolates. The criteria used for query sequence aimed to narrow down the search space (database), as the smaller the database the more likely to contain sequence of interest. For all the 11 queries zero E-values were attained indicated that all alignments were non-chance alignments. The percentages of query coverage ranged from 81 to 98%, where identity % were also high which ranged from 89 to 97%. The distribution of isolates/species/distribution is presented graphically in Figure (2). For the 11 isolates, the NCBI query resulted in 4 fungi species namely 4 isolates belonged to *Aspergillus oryzae*, 3 isolates belonged to *Aspergillus eucalypticola* and 3 isolates belonged to *Penicillium digitatum* oryzae and only one isolate belonged to *Byssochlamys spectabilis*.

Exploratory Data Analysis: The base frequencies of the 11 all species showed, no noticeable differences in the frequency of all 11 isolates, neither in GC content Table (2). Sequence length varied greatly among the 11 isolates. Sequence length ranged from 1262 to 1502bp. The sequence length *Byssochlamys spectabilis* of species were also varied, for the range of sequence length 1306bp. While, *Aspergillus eucalypticola* ranged from 1352 to 1502bp, where the range of sequence length of *Aspergillus oryzae* ranged from 1262 to 1467bp. Meanwhile, *Penicillium digitatum* sequence length ranged from 1361-1471bp Figure (3) declared the boxplot sequence length of the fungi isolates.

Cluster Aphylogenetic Analysisnd: Cluster analysis was carried out as pre-processing step to glean an insight into the data distribution. Results of cluster analysis are shown graphically in Table (3) and figure (4). The resulted dendrogram of comprised 2 large clusters are shown, the first cluster merely includes only one species *Byssochlamys spectabilis*, where the rest of the 3 species were comprised in the second cluster. One of *Aspergillus oryzae* was clustered together with the other *Aspergillus eucalypticola* isolates in the same clade.

Figure (5) shows the evolutionary history of the 11 isolates was inferred based on Neighbor-Joining (NJ) method (Saitou & Nei, 1987). The optimal tree with the sum of branch length = 0.3 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. This analysis involved 11 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1558 positions in the final dataset. Evolutionary analysis was conducted by ape package (Paradis et al., 2004).

Figure 5: Neighor-joining phylogenetic tree of 11 fungi isolates

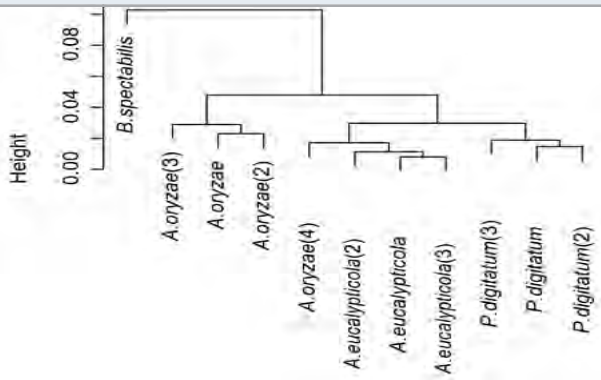


Polymorphism and Genetic Diversity among species: The 11 sequences were also analyzed to characterize the sequence diversity. The results of the analysis are presented table (4) the haplotype diversity was 1±0.04 and nucleotide diversity was only 0.04 where the average number of nucleotides diversity was 45.0nly one conserved region was found among the 11 isolates in the region between 30 to 1017. Measurements of conservation (C)=0.97, homozygosity =0.99 and P-value <0.001. Conservation (C) is calculated as the proportion of conserved sites in the alignment region, where homozygosity is measured as 1- heterozygosity.

Table 5. Estimated parameters of the polymorphic sites for 11 fungi isolates.

No. Sites	No. monomorphic Informative sites	No. Polymorphic sites	Parsimony informative sites	Singletone variable sites
1564	1081	142	63	79

Figure 6: cluster analysis of 11 fungi isolates



General information about the polymorphisms on the 11 fungi isolates is shown in table (5) the number of sites was 1564. The number of polymorphic sites was 142, number of parsimony-informative sites (i.e. sites that have a minimum of two nucleotides that are present at least twice) was 63 and the number of singletons was 79. The NJ tree declared in figure (6) where comprised from two large clusters. The *P.digitatum* species occupied one cluster on its own, where the rest of the 3 species clustered in the second cluster. This second cluster involved two large clades; the first clade contains *A.eucalypticola* species along with one of *A.oryzae* species. The rest the A.oryzae species shared another clade with *B.spectabilis*. This inferred NJ tree is very similar to the cluster analysis except for that *B.spectabilis* did not has a separate cluster, this difference might be due to differences in the used algorithms.

Fungal spoilage of fish imposes significant annual global revenue losses due to the production of mycotoxins by moulds. The main fungal groups associated with spoilage are the xerophilic, heat-resistant, preservative-resistant, anaerobic and psychrophilic fungi (Rico-Munoz et al., 2019). Results recorded the percentages of query coverage ranged from 81 to 98%, where identity % were also high which ranged from 89 to 97%. The distribution of isolates/species/distribution is presented graphically for the 11 isolates, the NCBI query resulted in 4 fungi species namely 4 isolates belonged to *Aspergillus oryzae*, 3 isolates belonged to *Aspergillus eucalypticola* and 3 isolates belonged to *Aspergillus oryzae* and only one isolate belonged to *Byssochlamys spectabilis*. Different results have been reported by Donnenberg, (2005) who assessed the fish (*Trachurus trachurus*) and reported some spoilage moulds such as *Apergillus flavus* (33.3%), *Penicillium* sp. (16.7%) and *Neurospora* sp. (16.7%).

Aspergillus eucalypticola is one of (Black *Aspergilli*) which consider one of the food spoilage fungi in addition to their mycotoxin production in different food items (Gil-Serna, et. al., 2019). While, *Aspergillus oryzae* related

to the Flavi species of Aspergillus (white *Aspergilli*) which produce very toxic compounds known as (aflatoxins) and highly spread among food elements. Furthermore, those microbes highly infected to immunocompromised consumers, causing "aspergillosis" (Kjærbølling, et. al., 2020). from the other hand, *Penicillium digitatum* is one mesophilic fungus from the most common blue and green "*Penicillium*" molds although this mold more common on fruits, this is one of the rarely report of this fungi in fish samples. *Penicillium digitatum* can causing mycosis or allergies for consumers and causing decaying of fish meat (Palou, 2014). *Byssochlamys spectabilis* able to spoil food and producing heat resistant ascospores and widely spread within food causing spoilage (Houbraken. et. al., 2008).

According to Nishihara et al., (2008) and Samaha, et. al., (2015) fungal contamination of fishes prone in the field during; harvest, transport, marking and with the consumer. The most common moulds isolated were; *Aspergillus* species consist of *Aspergillus flavus, A. niger, A. sydowii, A. wentii*, and *A. penicilloides*. Several *Penicillium* spp. were also isolated, Iqbal & Saleemi, (2013) isolated four types of fungi from the fish; orange (37.83%), black (35.13%), grey (24.32%) and white (2.70%) appeared on agar plates.

Three fungal genera; *Aspergillus* spp. (78.5%), *Blastomyces* sp. (7.5%), *Penicillium* sp. (3.5%) and unidentified fungal hyphae (10.5%) were isolated from the fish. Recently Akwuobu, et. al., (2019) have reported different types of fungal agents associated with the contamination of fish sold in open markets in Makurdi, Benue State. A total of 100 randomly selected fish samples from the 3 major fish markets in the study area were used for the study. Fungi were detected in 74 of the 100 fish samples from the 3 markets surveyed with isolation rates ranging from 67.6% to 84.8%. A total of 77 fungal isolates were recorded from the 74 positive samples the predominant isolated fungi were; *Aspergillus* spp. (28.6%), *Penicillium* spp. (18.2%). The base frequencies of the 11 all species showed, no noticeable differences in the frequency of all 11 isolates, neither in GC content Table (2). Sequence length varied greatly among the 11 isolates. Sequence length ranged from 1262 to 1502bp. The sequence length Byssochlamys spectabilis of species were also varied, for the range of sequence length 1306bp. While, Aspergillus eucalypticola ranged from 1352 to 1502bp, where the range of sequence length of *Aspergillus oryzae* ranged from 1262 to 1467bp. Meanwhile, *Penicillium* digitatum sequence length ranged from 1361-1471bp Figure (3) declared the boxplot sequence length of the fungi isolates.

Recntly, different results have been reported by Kjærbølling, et. al., (2020), who sequenced about 19 genomes for *Aspergillus* spp. which were isolated from spoiled food. However, the genome covered 99.78% and concluded that the size of *Aspergillus* spp. genome was an important aspect. They added that the average genome of *A. oryzae* was about (37.96Mbp). The branching of the tree about (100 straps in each branch) was present. According to Houbraken, et. al., (2008) *Byssochlamys spectabilis* is heat resistance mould, producing heat-resistant ascospores in raw food and can tolerate cooking temperatures and producing mycotoxins. There is a need to develop new antimicrobials to ensure food safety and extend shelf life. The use of antimicrobial agents directly added to foods or through antimicrobial packaging is one effective approach. In recent years, the use of inorganic antimicrobial agents in nonfood applications has attracted interest for the control of microbes (Wilcznski, 2000 and Tilman et al., 2011).

CONCLUSION

The presented study provides 11 new genomes, which have been added to the fungal genetic diversity. We identified the isolated fungi phenotypes macroscopically, microscopically and genetically. This study also was concerned with the isolation and proper identification of fungus in fish from Saudi Arabian markets. The results recorded 4 species of fungi; *Aspergillus eucalypticola* as one of (Black *Aspergilli*), *Aspergillus oryzae* as (white *Aspergilli*), *Penicillium digitatum* as (green fungi) and *Byssochlamys spectabilis*.

This is the first report of *Penicillium digitatum* which commonly found in citric fruit. The result also has the first report of *Byssochlamys spectabilis* as heterothallic mould species which causing food spoilage in fish. The four sequenced species compared with the already sequenced Aspergillus species and determined the genetic diversity. The phylogram constructed for whole-genome basis, showing the taxonomy tree of each isolated fungus genus. Furthermore, the research identified each fungi gene cluster. More attention in fish rearing facilities, caution should be taken by consumers in preparation and applying perfect cooking in consuming fish and more education and efforts should be developed by fish farmers to avoid fishponds contamination. We recommended to further research should be done on fungal contaminations.

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Ecological
Communication

Seasonal Fluctuations in Physicochemical Parameters in Relation to Fish Diversity in Muragacha Beel, West Bengal India

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ABSTRACT

The present study was made to find out the health status of Muragacha Beel, District North 24 Parganas, West Bengal, India. The study focused on the seasonal fluctuation of physicochemical parameters of water and diversity of fish fauna during pre-monsoon, monsoon, and post-monsoon from March 2019 to February 2020. Water samples and fish specimens were collected from three different sites of the beel every month to evaluate the correlation between physicochemical properties of water and the indices of fish diversity. A significant seasonal fluctuation in fish diversity (2.762-2.796) in relation to physicochemical parameters (temperature 20.2-32.8 ^{0}C, turbidity 23.1-30.1 cm, pH 7-8.5, carbon dioxide0-15.6 mg/l, dissolved oxygen 4.5-5.9 mg/l, biochemical oxygen demand 2.6–7 mg/l, alkalinity 148-194 mg/l, hardness 106-168 mg/l, phosphate 0.43-1.25 mg/l and nitrate 0.17-0.45 mg/l of water) was recorded during the study period. In respect of fish faunal diversity, a total of 25 species belonging to 6 orders and 16 families were identified. Maximum representatives belong to the order Cypriniformes having 6 species with 44.6% Relative Abundance, followed by Clupeiformes with 2 species with 18.6% Relative Abundance. The beel was heavily infested with aquatic vegetation creating complex environments for the growth of different. The present study indicates that the beel is moderately productive and suitable for commercial aquaculture. The effect of seasonal variations on the assemblage of fish composition is found to be important and it should be considered into account when developing initiatives that will be taken in the future to support the productivity and biodiversity of the wetland.

KEY WORDS: PCA, PEARSON CORRELATION, PHYSICOCHEMICAL PROPERTIES, SEASONAL FLUCTUATION, SHANNON-WEINER INDEX.

INTRODUCTION

Wetlands are highly diversified and productive ecosystems. Huge numbers of biodiversity are found within the lap of the wetland (Groombridge and Jenkins 1998; Ramachandra et al., 2006). The existence of aquatic life is completely dependant on the health status of wetlands. Constant surveillance is needed to conserve and protect this treasure of nature (Ramachandra et al. 2006; Ramesh et al. 2007). Freshwater wetlands in India are not only rich in fish biodiversity but also support a fashionable source of zooplankton, phytoplankton and macro invertebrate species. The primary aim to maintain the health status of any wetland is to keep its physicochemical parameters within desirable limits (Khan 2002; Garg et al., 2010).

The diversity of the aquatic community varies greatly with seasonal fluctuation. The fluctuation of water quality parameters is considered a vital factor to analyze the species' biodiversity (Mondal et al. 2010; Jagadeeshappa and Kumara 2014). The physicochemical parameters play an important role to study any aquatic environment (Jagadeeshappa and Kumara 2014). The alteration in the physicochemical status of water directly influences the composition of aquatic flora and fauna. There are more than 150 floodplain lakes in West Bengal, covering a 42,000-ha area, contributing to about 22 percent of the

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freshwater habitat of the entire state (Bhaumik et al., 2003; Singh et al., 2017).

Among them, the North 24 Parganas district possesses a huge amount (8861ha) of water resources. Subsequent deterioration of water quality could be a serious threat to the fish biodiversity and productivity of floodplain lakes in the state. The present study was administrated to measure the magnitude of the physicochemical parameters of water and its relation with the fish biodiversity indices of Muragacha Beel. The area is mainly rain-fed. The fisherman utilizes this wetland for fish capture. Sewage from the municipality, domestic disposal directly mixes up in this wetland. The reason behind the selection of this particular beel is to assess its health status and its suitability for fish culture (Mondal and Kaviraj, 2008; Mondal et al., 2010).

In India, multiple studies have been carried out to analyze the physicochemical parameters of aquatic bodies (Azmi et al., 2015). Ansari (2017) carried out the water quality analysis of Surajpur wetland, UP to check the relationship among various physicochemical parameters in both temporal and spatial scales. Multiple pieces of research are done to analyze the water samples on several wetlands in the state. But inadequate information is found regarding the physicochemical properties and fish assemblage of this particular wetland (Azmi et al., 2015; Ansari 2017). So, an investigation has been done to research the physicochemical status of water and diversity of fish fauna during pre-monsoon, monsoon and post-monsoon from March 2019 to February 2020.

MATERIAL AND METHODS

Muragacha beel (Latitude 22°41'58.7"N; Longitude 88°25'3.8"E) (Figure 1) falls under the North 24 Parganas districts of West Bengal, India, at an altitude of 6 meters above sea level. Muragacha Beel spreads over an area of 10.3 hectares.

Figure 1: Map (source: Wikipedia and Google Satellite Map) of the study area with three sites pointed as Site1, Site2 and Site3.

The study was conducted on monthly basis from March 2019 to February 2020 for the analysis of physicochemical parameters and the relationship of those parameters with fish diversity. A total of three representative sampling sites (Site1, Site2 and Site3) were selected. The most changeable and sensitive water quality parameters such as Water Temperature, pH, Transparency (Turbidity), Dissolved Oxygen (DO) and Free CO_2 were measured in-situ, while rest parameters (BOD, Total Hardness as $CaCO_3$, Total Alkalinity as $CaCO_3$, Nitrate, and Phosphate) were analyzed in the laboratory. The collection of water samples was done between 5 am to 8am. Sample preservation and evaluation of different water quality index were performed according to standard procedures (APHA, 2005; APHA, 2012; Chattopadhyay, 1998).The outcomes of the physicochemical study of the water sample were matched with the World Health Organisation (2011) standard (Panigrahi and Subhasini, 2020).

Table 1. Water quality parameters as per World Health Organization (2011)

Sl No	Physicochemical Parameters	Permissible Limits*
1	Water Temperature (0C)	<40
2	pH	6.5 – 8.5
3	Turbidity (Transparency) (cm)	<100
4	Dissolved oxygen (DO) (mg/l)	3.0 – 6.0
5	Free CO2(mg/l)	<12
6	Biochemical oxygen demand (BOD) (mg/l)	2.0 – 8.0
7	Total hardness as $CaCO_3$ (TH)(mg/l)	200
8	Total alkalinity as $CaCo_3$ (TA) (mg/l)	200
9	Phosphate (PO43-)(mg/l)	5
10	Nitrate (NO_3-)(mg/l)	<45.0

S.W. Species Diversity index $\overline{H} = -\sum_{i=1}^{s}\left(\frac{Ni}{N}\right)\log_2\left(\frac{Ni}{N}\right)$	Where S is the total number of species; N is the total number of the individual; Ni is the number of specimens in each species.
Evenness Index (J) = $\overline{H}/\log_2 S$	Where $\overline{H}$ is the S.W. Species Diversity Index; S is the total number of species.
Index of Dominance (ID) = $\Sigma\left(\frac{Ni}{N}\right)^2$	Where N is the total number of the individual; Ni is the number of specimens in each species.
Relative Abundance (%) = N^{th}/N X 100	Where, N^{th} = Total Number of individual species; N = Total Number of the species population.

The fishes were captured at three stations with local nets and their identification was confirmed by the method used by Armantrout et al., (1994). The diversity and evenness indices were calculated by the references of Shannon et al., (1950). Pearson linear correlation was carried out to see the connection between various physicochemical parameters. To evaluate the diversity indices, random fish samples were extracted from five nettings across each site were collected to produce a 1kg sample of small wild fish for every site per month. From these data Shannon-Weiner species diversity index($\bar{}$H), Evenness index (J), Index of Dominance(ID) and Relative Abundance(RA)were determined using the following equations (Shannon et al., 1950; Armantrout et al.,1994):

The limnological data of the study period was assembled for three seasons and assessment was done for seasonal variations, viz., Pre-monsoon (from March to June), Monsoon (from July to October), and Post-monsoon (from November to February). Mean, Standard Error of Mean and One-way analysis of variance (ANOVA) for various parameters for three seasons and multivariate statistical analysis PCA (Principal Component Analysis) were performed using XLSTAT 2020 and Graph Pad Prism9. Tukey HSD tests were carried out to measure the significant difference at the 5% probability (α) level (Shannon et al., 1950; Armantrout et al., 1994).

RESULTS AND DISCUSSION

Seasonal variation of the physicochemical parameters of water (water temperature, pH, turbidity, dissolved oxygen, free carbon dioxide, biochemical oxygen demand, total alkalinity, total hardness, nitrate, and phosphate) of Muragacha beel in pre-monsoon, monsoon, and post-monsoon season are showed in table 2.

In study the range of the physicochemical parameters viz., water temperature 20.2-32.8 °C, turbidity 23.1-30.1 cm, pH 7-8.5, carbon dioxide0-15.6 mg/l, dissolved oxygen 4.5-5.9 mg/l, biochemical oxygen demand 2.6–7 mg/l, alkalinity 148-194 mg/l, hardness 106-168 mg/l, phosphate 0.43-1.25 mg/l and nitrate 0.17-0.45 mg/l was found. The results of the Pearson correlation test were further verified through Principal Component Analysis (PCA). In Muragacha beel water temperature showed a positive correlation with turbidity and CO_2 whereas a negative correlation with pH, dissolved oxygen, BOD, total alkalinity, nitrate, and phosphate (Table 3). Ziauddin et.al., (2013) reported that minimum and maximum temperature in beels of West Bengal varied from 17.5 to 35.0 °C, which conforms with the present study.

Table 2. Different letters (a-b) indicates a significant difference ($p<0.05$) within the same row (One way ANOVA followed by Tukey test)

	Pre-Monsoon	Monsoon	Post-Monsoon
W T (°C)	28.63 ± 2.76a	30.08 ± 1.8a	22.28 ± 2.35b
pH	7.16 ± 0.12a	7.43 ± 0.21a	8.18 ± 0.23b
TUR (cm)	25.98 ± 2.02a	29.54 ± 0.46a	26.98 ± 2.35a
DO (mg/l)	5.32 ± 0.78a	6.67 ± 1.02b	8.29 ± 0.58b
CO_2 (mg/l)	8.46 ± 3.01a	6.09 ± 2.42a	2.6 ± 1.39b
BOD (mg/l)	4 ± 0.99a	5 ± 0.77a	6 ± 0.63a
TH (mg/l)	133 ± 7.22a	125 ± 6.64a	144 ± 24.5a
TA(mg/l)	159 ± 7.6a	176 ± 10.97a	178 ± 17.18a
NO_3 (mg/l)	0.21 ± 0.02a	0.3 ± 0.09a	0.22 ± 0.03a
PO_4^- (mg/l)	0.59 ± 0.06a	0.71 ± 0.2a	0.67 ± 0.09a

Table 3. Correlation matrix among the physicochemical parameters and Shannon-Wiener diversity Index of Muragacha beel wetland fromMarch 2019 to February 2020.

	W T	pH	TUR	DO	CO_2	BOD	TH	TA	NO_3	PO_4^-	SW I
W T (0C)	1.00										
pH	-0.91	1.00									
TUR (cm)	0.41	0.00	1.00								
DO (mg/l)	-0.58	0.87	0.50	1.000							
CO_2 (mg/l)	0.83	-0.99	-0.16	-0.94	1.00						
BOD(mg/l)	-0.99	0.85	-0.53	0.47	-0.75	1.00					
TH (mg/l)	-0.97	0.77	-0.64	0.35	-0.66	0.99	1.00				
TA(mg/l)	-0.40	0.75	0.66	0.98	-0.85	0.28	0.16	1.00			
NO_3 (mg/l)	0.62	-0.24	0.97	0.28	0.08	-0.72	-0.80	0.46	1.00		
PO_4^- (mg/l)	0.00	0.41	0.91	0.81	-0.56	-0.13	-0.26	0.91	0.78	1.00	
SW I	-0.52	0.82	0.57	1.00	-0.91	0.40	0.28	0.99	0.35	0.86	1.00

** WT=Water Temperature, TUR=turbidity, DO=Dissolve Oxygen, CO_2=Carbon Di Oxide, BOD= Biochemical Oxygen Demand, TH=Total Hardness, TA=Total Alkalinity, PO_4^- =Phosphate, NO_3= Nitrate, SWI = Shannon Wiener Index.

The higher pH was recorded in post-monsoon due to the low level of water, higher nutrient content. The range of pH showed sub-alkaline in nature (7.0 to 8.5) that was within the permissible limit as prescribed by WHO (2011). In Muragacha beel, pH showed a positive correlation with DO, BOD, total hardness and total alkalinity while showed a negative correlation (Table–3, Fig. 2) with nitrate, water temperature and CO_2. The results of the present investigation are comparable with Panigrahi and Debnath (2013). A water body's transparency usually suggests its effectiveness. Throughout the monsoon month, it was found to be a maximum that may be attributed to the entry of rainwater from the surrounding area. Throughout pre-monsoon and post-monsoon it was generally low which shows similarities to the earlier work of Kumar et al. (2019). In Muragacha beel, turbidity showed a positive correlation (Table–3, Fig. 2) with dissolved oxygen, nitrate and phosphate whereas it showed a negative correlation with CO2, BOD and total hardness (Panigrahi and Debnath 2013).

Figure 2: Heatmap of Pearson correlation matrix between different physicochemical parameters and Shannon-Wiener diversity Index of Muragacha beel from March 2019 to February 2020.

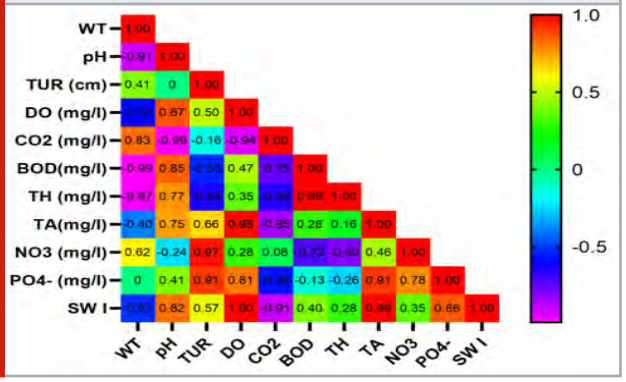
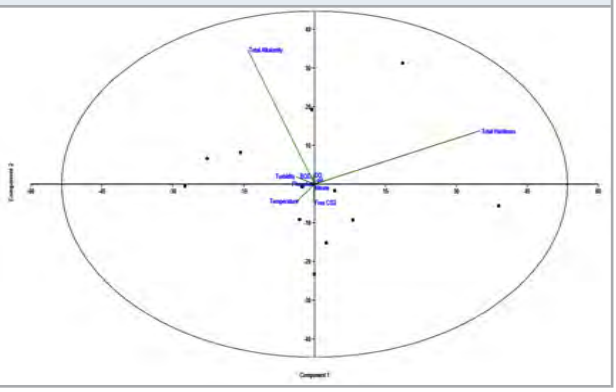


Figure 3: Quadrant distribution of different water physicochemical parameters of water of Muragacha beel throughout the year via PCA.



Dissolved oxygen attributes are inversely related to the temperature cycle, differing from post-monsoon upside to pre-monsoon lowest point. Chaurasia and Tiwari (2011), also reported similar observations in a freshwater beel of Bhagalpur in Bihar. In the present investigation, dissolved oxygen of the wetlands shows a negative correlation (Table-3, Fig.2) with temperature, CO_2 and nitrate but showed a positive correlation with pH and phosphate (Chaurasia and Tiwari 2011).

Free CO_2 was relatively high during the summer months, which can be due to the faster decomposition of organic matter and high temperature. Lacking free carbon dioxide in other seasons could be due to its utilization by phytoplankton and other aquatic plants via photosynthesis. The free carbon dioxide shows a significant positive correlation with temp and nitrate while negative with the rest of the parameters which similar to Hassan et al., (2011) and Shinde et al., (2011).

Figure 4: Scree plot showing the eigenvalues, principal components and broken stick derived from PCA regarding the different water physicochemical parameters of water of Muragacha beel

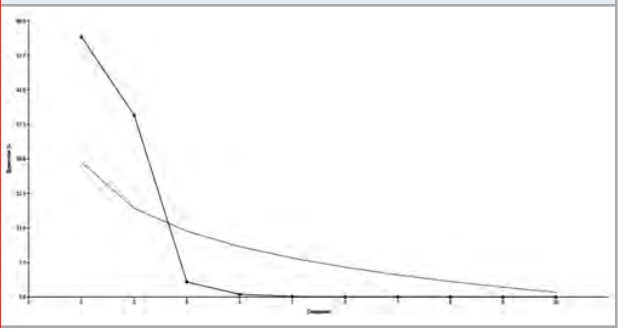
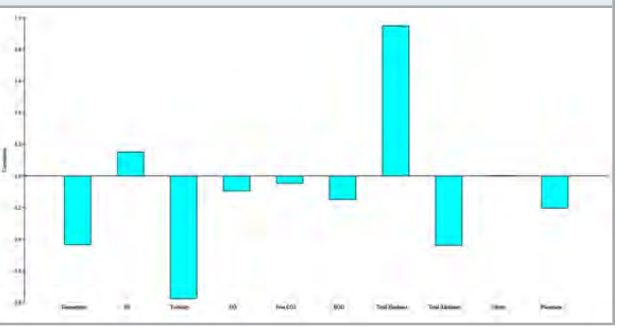


Figure 5: Factor loading plot of principal component 1 showing different water physicochemical parameters of water of Muragacha beel derived from PCA



The maximum total hardness was obtained in post-monsoon due to some construction activity. Ansari(2017), also reported similar observations in the Surajpur wetland. In Muragacha beel, total hardness showed a negative correlation with water temp, turbidity, CO_2 and phosphate but showed a positive correlation with pH, BODand DO. The results of total alkalinity obtained from the present study were in close conformity with the findings of Mishra et al., (2014) and Arya et al., (2011). In Muragacha beel, total alkalinity showed a positive correlation with pH, DO, BOD, nitrate and phosphate but showed a negative correlation with water temperature and CO_2 (Jain et al., 1996; Gupta et al., 2016).

Table 4. Species diversity indices in three different seasons

	Shannon-Wiener Index	Evenness Index	Index of Dominance
Pre-Monsoon	2.762	0.858	0.080
Monsoon	2.789	0.866	0.078
Post-Monsoon	2.796	0.868	0.077

The amounts of nitrate in all three sites of this beel were found very low. During the monsoonperiod, however, the level of nitrate was found a bit high due to the surface runoff and some microbial activity. In monsoon, the activities of these microbes go down resulting in a higher value of nitrate (Kaur et al., 1996). In Muragacha beel, nitrate showed a positive correlation with phosphate, TA and water temp but a negative correlation with pH, DO and BOD. Phosphorus in water commonly exists as phosphate. The phosphate concentration in water above 0.5 mg l-1 indicates pollution (Jain et al., 1996). Maximum phosphate was observed in Muragacha beel during the monsoon and minimum during the pre-monsoon. In Muragacha beel, phosphate showed a negative correlation (Table-3, Fig.2) with water BOD, Total hardness and CO2 while apositive correlation with pH, DO and nitrate was observed. The concentration of phosphate which was moderate throughout the year indicates that this beel is mesotrophic. A mesotrophic wetland system has intermediate levels of phosphorus and is suitable for the growth of aquatic plants and fishes (Gupta et al., 2016).

The results described above indicate that the physicochemical parameters studies were within acceptable limits and the water quality of this beel was good enough to support rich high species diversity and suitable for fish culture. This is also relevant to Bhatnagar and Devi (2013),where they found most of the physicochemical parameters were within the target range (Bhatnagar and Devi 2013). 25 species of fishes belonging to 6 orders, 16 families and 22 genera were obtained from the Muragacha beel wetland. Out of those fishes, Cypriniformes was the most dominating order having 6 species with 44.6% Relative Abundance, followed by Clupeiformes with 2 species and 18.6% Relative Abundance, Siluriformes with 5 species and 15.2% Relative Abundance, Perciformes with 9 species and 14.8% Relative Abundance, Synbranchiformes with 2 species and 5.5% Relative Abundance and Osteoglossiformes with 1 species and 1.3% Relative Abundance(Roy et al. 2013).

Shannon-Wiener diversity index (H) was obtained highest (2.796) in Post-Monsoon and lowest (2.762) in pre-monsoon. Similarly, the species evenness was found highest in post-monsoon (0.868) and lowest in pre-monsoon (0.858). Both Shannon-Wiener diversity index (H) and evenness index highest during post-monsoon, while the index of dominance was lowest during post-monsoon(0.077) and similarly it was observed that the index of dominance highest during pre-monsoon(0.080) but the evenness index and Shannon-Wiener diversity index lowest (Table 4). The Shannon-Wiener diversity index shows a negative correlation with water temperature and CO2 while positive relation with the other parameters like pH, DO, BOD, and phosphate. The dominance index increases with the harshness of the environment (Karr, 1971)while the evenness index decreases with the increase in stress in the environment (Chapinet al. 2000). Natural and anthropogenic activities directly impact the relative abundance of species until it becomes an endangered species(Roy et al. 2013). In this present study, the Shannon-Wiener diversity indices suggest that the water of Muragacha beel is healthy, which was compared with Jewel et al. (2018).

CONCLUSION

This is the first report ofMuragacha beel which showed that this beel has immense potential to sustain good biodiversity andideal for aquaculture. Most of the physicochemical parameters were within the target range of water quality guidelines of the Central Pollution Control Board, India. Results of phosphate and nitrate concentration in the present study and result of fish diversity indicate that the beel is Mesotrophic. However, there is significant seasonal fluctuation in the water quality along with fish diversity. Finally, it is concluded that the beel is productive and suitable for pisciculture.

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Ethical Clearance Statement: The Current Research Work Was Ethically Approved by the Institutional Review Board (IRB) of University of Burdwan, West Bengal, India.

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Toxicological Communication

Ameliorative Effects of Aqueous Garlic Extract and the Haematology of Arsenic-Induced *Channa punctatus*

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ABSTRACT

Blood parameters and the bioaccumulation of metals in different organs are the most important tools that can be used as effective and sensitive index to monitor physiological and pathological changes in fishes. In this study the effects of sublethal concentration of sodium arsenite were studied to evaluate heavy metal toxicity stress symptoms in fish blood. The relevant aspects of the therapeutic potential of aqueous garlic extract (AGE) against arsenic induced toxicity in the fresh water fish *Channa punctatus* have been evaluated. It was observed that haemoglobin content and red blood cells decreased significantly while the white blood cells and clotting time increased for the arsenic treated fishes. However, the haematological parameters were found to be stabilised in the revival group treated with garlic. The accumulated arsenic in liver, gill, and kidney tissues of *Channa punctatus* was estimated by determining total arsenic concentration through Atomic Absorption Spectroscopy. The highest total arsenic content in the liver tissue of the fishes exposed to arsenic was 29.25 ± 0.15 ppb while the AGE treated group was found to be 27.96 ±0.13 ppb. In the gill tissue arsenic treated group accumulated 36.31 ± 0.21 ppb and arsenic + AGE treated group accumulated 34.94 ± 0.12 ppb of total arsenic. The total arsenic concentration in the kidney tissue was 21.8 ± 0.23 ppb for the arsenic treated group and arsenic content reduced to 20.27 ± 0.02 ppb for arsenic + AGE. Accumulation of arsenic found in liver, gill and kidney in the present study confirms the fact that arsenic toxicity was responsible for the physiological changes in the fishes. The results of the present study also signify that the haematological disturbances are caused by arsenic and its consequences could be reverted to a large extent by using aqueous garlic extract.

KEY WORDS: AAS, ARSENIC, GARLIC, HAEMATOLOGICAL PARAMETER.

INTRODUCTION

Fishes are reasonably sensitive to the changes in their adjoining environment. Therefore, fish health is assumed as a reflection of the physical condition of a specific aquatic ecosystem. The freshwater fish *Channa punctatus* has been used as a bio indicator in toxicological studies since it is a very common species and it has higher tolerance for any stressed condition. Several studies conducted on mice, rats and chickens have reported that an oral garlic oil supplement affords significant protection against the toxicity of hepatotoxins including heavy metals (Senapati et al. 2001; Arora et al. 2004; Olaganathan and Patterson 2013). Haematological parameters are one of the most important tools that can be used as effective and sensitive index to monitor physiological and pathological changes in fishes.

The blood parameters of different fishes exposed to different water pollutants and toxicants, such as metals, biocides, pesticides, chemical industrial effluents, etc. had been used as sensitive indicator of stress for the toxicity evaluation studies of these pollutants (Singh et al. 2008; Podeti and Benarjee 2017). The aquatic biotope, fish species, age, sexual maturity and health status of the fishes were found to affect the haematological parameters (Radu et al., 2009; Patriche et al., 2011). Appreciable changes were observed in haematological parameters and biochemical profiles of the lithium induced *Channa punctatus* and *Oreochromis niloticus* (Thanga Malathi and Anuradha 2020).

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The relationship of blood characteristics to the habitat and adaptability of the fish to the environment can be understood by working on the haematological and biochemical parameters (Podeti and Benarjee 2017). In order to understand the fish physiology and pathology different investigators had estimated the various blood parameters in fishes (Va´zquez and Guerrero 2007; Satheesh kumar et al. 2012). Blood constituents of fishes are directly or indirectly influenced by the quality of water, temperature, food availability and physiological status of the fishes (Dheer et al., 1988; Bala et al., 1994; Iqbal et al., 1997). It was also observed that many factors including environmental factor influence the haematological parameters of the fish.

When the fish, *Channa punctatus* was treated with both copper and chromium a fall in RBC count, Hb% and PCV% along with acute anaemia was noticed (Pandey 1977; Singh 1995). The oxygen carrying capacity of blood, the iron content of the blood and number of red blood cells in fishes apparently depend on the life history, stage, habitat and environmental conditions (Singh et al., 2008). It was observed that acute sub-lethal concentrations of lead, copper and zinc can produce haemolytic anaemia which associated with the decrease in Hb%, PCV% value and the number of erythrocytes in *Colisa fasciatus* and *Oreochromis mossambicus* (Soiveo and Nikinmaa 1981; Sampath et al., 1998).

In the last few decades, the concentrations of heavy metals in fish had been extensively studied in different parts of the world (Elnabris et al. 2013). Most of these studies concentrated mainly on the heavy metals in the edible part (fish muscles). However, other studies reported the distribution of metals in different organs like the liver, kidneys, heart, gonads, bone, digestive tract and brain. It was observed that arsenic has the tendency to accumulate in bottom sediments (Smedley and Kinniburgh 2002). Assimilation of metals in fish takes place through ingestion of food, ingestion of material suspended in water; absorption on the tissue and membrane surfaces and exchange of ions dissolved in water across lipophilic membranes (e.g. gills).

Distribution of metal in different tissues depends on the mode of exposure (i.e., dietary exposure) and can be considered as an indicator of pollution (Alam et al. 2002). Therefore, bioaccumulation of metals has been considered as an index of the pollution status of the related water body and used as a tool to study the biological role of the metals present at elevated levels in aquatic organisms, particularly fish (Tariq et al., 1991; Mehra and Chadha 2020). Further, any alteration in biochemical parameters can have serious outcomes in the form of various diseases in both the animal and its consumers (Prakash and Verma 2020).

For determining the total arsenic (As) concentration in different fish tissue, several techniques such as inductively coupled plasma mass spectro-photometry (ICP-MS), electrothermal atomic absorption spectro-photometry (ETAAS) has been used (Gong et al., 2002). The generation of hydride is also an important process for the determination of As (Moretto and Cadore 2004). Microwave digestion processes have also been used in these purposes due to the advantages of this technique, which include the speed of digestion and less risk of contamination during the process. Atomic absorption spectrometry through low injection with hydride generation has been used successfully for the determination of arsenic in biological samples (Ybanez et al., 1992; Navarro et al. 1992; Soylak et al., 2004) and it is possible to use for a few microlitres of sample. In order to determine the total arsenic concentration in different fish tissues, it is essential to assure complete mineralization of the samples using non-destructive technique (Jalbaniet et al., 2007). Bioaccumulation and concentration level of other heavy metals such as chromium, cadmium, lead etc. in the riverine water and edible fish such as *Channa punctatus* were also analyzed by using Atomic Absorption Spectrophotometer (Idrees et al., 2020).

Fishes are relatively situated at the top of the aquatic food chain; therefore, they can normally accumulate heavy metals from food, water, and sediments and they are considered as a good indicator of heavy metal contamination in water (Yilmaz et al., 2007; Zhao et al., 2012; Voegborlo et al., 2012). However, to the best of our knowledge, bioaccumulation studies on the *Channa punctatus* under the influence of any therapeutic agent such as aqueous garlic extract (AGE) are not available in the literature. Hence, the present study aims to investigate the effect of arsenic in fresh water teleost *Channa punctatus* and to assess the impact of aqueous garlic extract on the revival of arsenic toxicity by evaluating the changes in the haematological parameters. Atomic absorption spectroscopy (AAS) was done to determine the total arsenic concentration accumulated in the liver, gill, and kidney tissues of *Channa punctatus* under the influence of aqueous garlic extract (AGE).

MATERIAL AND METHODS

In the present study *Channa punctatus* was selected as an experimental animal. Healthy and disease free fishes were collected from local markets in Guwahati. Their weight was approximately in between 25-45 gm and average length was 14 cm. Fishes were brought to ambient laboratory condition. Fishes were disinfected with a dip of 2% potassium permanganate (KMnO4) solution the fishes were acclimatised in aquaria (75×30×60 cm) for two weeks before initiation of experiment. The fishes were fed everyday with fish food *(Tokyu pallete*) during acclimatization period.

Following the standard procedure given by APHA the physicochemical parameter of the test water was monitored (Das and Goswami 2020). Sodium Arsenite (NaAsO2), molecular weight- 129.91 Merck, India (Ltd.) was procured for performing the experiment. A stock solution was prepared by adding 5 mg of sodium arsenite to 100 ml of distilled water. The test concentration was prepared by diluting the stock solution with appropriate

amount of distilled water. Each experimental aquaria contained 8 litres of water each. Sub-lethal doses of 2.5 ppm/litre were prepared by adding 20 ppm in 8 litres of water for each experimental setup arsenic (Das and Goswami 2020).

Physicochemical parameters of the water used in test solution were maintained according to the standard procedures given by APHA, 2005. The control group of fishes were kept in similar conditions without adding sodium arsenite. The second group of fishes were exposed to sub-lethal dose of arsenic, the third group was exposed simultaneously to arsenic and AGE. The fourth group was exposed to only garlic extract without arsenic (Das and Goswami 2020). To prepare aqueous solution of garlic extract the outer layer of garlic (*Allium sativum* L.) cloves were removed, and crushed mechanically in a mortar-pestle with 100 ml of autoclaved distilled water for 1 gm of garlic. The homogenate was shaken for 20 minutes, filtered successively through gauze and 0.22 micron membrane filter to obtain the aqueous garlic extract (AGE) (Chowdhury et al. 2008; Das and Goswami, 2020).10 ml of AGE / litre or 80 ml of AGE in 8 litres was added in each aquaria during the experimental procedure.

Fish specimens from the three groups were collected for haematological study. Blood samples were collected by piercing the caudal peduncle using plastic disposable syringe fitted with a needle which was moistened with heparin.To estimate the RBC from the fish blood Acid haematin technique has been used. Blood collected was quickly drawn into the cleaned and dried RBC pipette up to 0.5 or 1 mark. The extra blood was wiped off the outer surface of the pipette and immediately erythrocyte or red blood cell (RBC) fluid was drawn up to the 101 mark. The contents of the pipette were thoroughly mixed by rotating with thumb and fingers for 5 minutes. The blood was drawn up to 1 mark to make dilution 100-fold. Diluted blood solution was filled up between the cover slip and the counting chambers of the haemocytometer by the capillary action. The haemocytometer was allowed to stand for few minutes and transferred carefully on the plate form of a microscope. The erythrocytes were counted in the four corner squares and in the central square. The cells were enumerated by using the Neubauer haemocytometer (Singh et al., 2008).

Blood collected was quickly drawn into the cleaned and dried WBC pipette and sucked the blood in the pipette up to 0.5 mark without any air bubble. The extra blood was wiped off the outer surface of the pipette and immediately diluting fluid was drawn up to the 11 mark. The contents of the pipette were thoroughly mixed by rotating with thumb and fingers for 5 minutes. It provides 20 times dilution of blood. The first one or two drops were discarded and then filled the Neubauer counting chamber. Diluted blood solution was filled up between the cover slip and the counting chambers of the haemocytometer by the capillary action. The haemocytometer was allowed to stand for 3-5 minutes to settle the leucocytes. Haemocytometer was carefully transferred on the platform of a microscope. They were counted in four corners of 1 square mm in the central ruled area on both the sides of the counting chamber of the haemocytometer.

The haemoglobin concentration was evaluated to study the stress induced due to arsenic exposure on *C. punctatus*. Haemoglobin (gm/percent) was determined using haemometer (Humtsoeet al. 2007). 0.1 N HCl was filled up to lowest mark (20 % mark). Blood from the control and treated fish was collected and sucked into the Hb pipette up to 20 micron litre (µl). The blood was carefully sucked to avoid any air bubbles. The blood was then transferred from the blood pipette into the acid at the bottom of the graduated tube and was shacked thoroughly. The mixture was allowed to stand for 10 minutes so that the haemoglobin gets converted to the dark brown coloured acid haematin. The solution was diluted by adding few drops of distilled water carefully and mixing with reaction mixture until the colour matches with that of the standard tube (Humtsoe et al., 2007).

The level of the fluid at its lower meniscus was noted and the reading corresponding to this level on the scale was recorded in g/dl (Humtsoe et al. 2007). The heart of the control and treated fish were pierced by a needle to ensure free flow of blood and the time when the wound was made was recorded by starting a stopwatch. The first drop of blood was rejected and the second drop of free flowing blood was allowed to fill readily the capillary tube by capillary action. After every half a minute about 1 cm of the capillary tube was broken down so as to find whether fibrin string was formed or not. When the fibrin string appeared in the capillary piece then the total time required for clotting was noted down by stopping the stop-watch (Singh et al., 2008). After a period of 15 days, surviving fish from experimental and control groups were sacrificed for the estimation of arsenic in the liver, gill and kidney tissue. Total arsenic concentration in the tissues was determined on a Varian Atomic Absorption Spectrophotometer (AAS), Model No.: Spectra AA 220 FS (Netherland). Analysis of heavy metal was carried out following the standard methodology of Angerer and Schaller (1988) and Zwart and Trivedi (1995).

RESULTS AND DISCUSSION

The 96 hours LC_{50} of sodium arsenite for *Channa punctatus* was found to be 25 ppm through probit analysis and it is discussed in details elsewhere (Das and Goswami 2020). The present toxicity studies carried out on 10 % of the 96 hour LC_{50} value (i.e 2.5 ppm) which was selected as sublethal concentration of sodium arsenite for 15 days exposure to the fish. In the present study, exposure of fish to sub-lethal concentration of arsenic for 15 days caused significant alterations in haematological parameters of freshwater fish, *Channa punctatus*. The changes observed in various haematological parameters, such as RBC, WBC, Hb% and clotting time of blood in the control and experimental groups were recorded (Table 1) (Das and Goswami 2020).

Figure 1: RBC profile of *C. punctatus* exposed to (10%) sub lethal concentrations of arsenic and Aqueous Garlic Extract (AGE) for 15 days along with control group. Values are significant at P < 0.05. (*indicates that the values are significantly different at P < 0.05 level compared to the values of control group determined by one way ANOVA analysis)

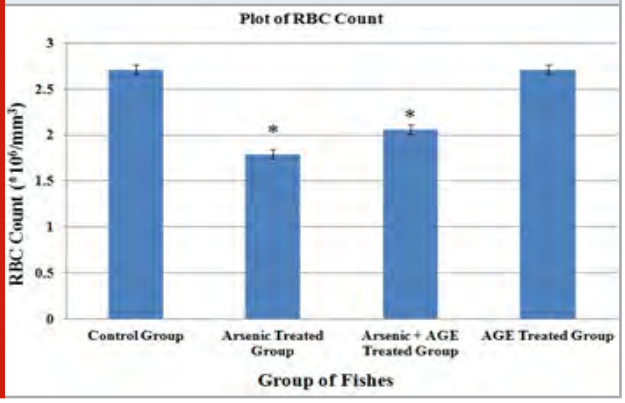
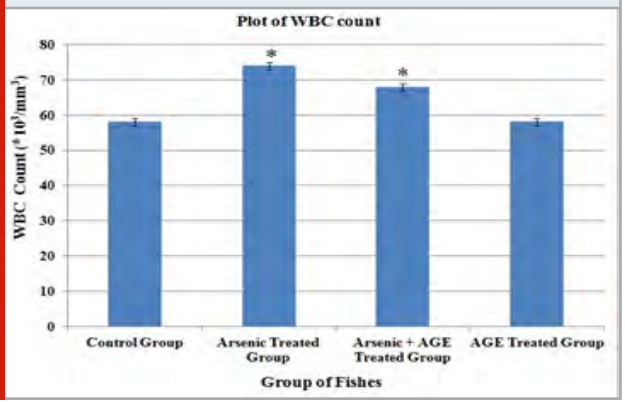


Figure 2: WBC profile of *C. punctatus* exposed to (10%) sub lethal concentrations of arsenic and Aqueous Garlic Extract (AGE) for 15 days along with control group. Values are significant at P < 0.05. (* indicates that the values are significantly different at P < 0.05 level compared to the values of control group determined by one way ANOVA)



In the control group of fishes in the present experimental set up RBC was found to be (2.71±0.08)×10^6/mm^3, WBC (58.08±0.13) ×10^3/mm^3, haemoglobin content (10.54±0.06) gm % and clotting time (27.20±0.15) second. In the group which was exposed to sub-lethal concentration (10 % of LC_{50} value) of sodium arsenite (Das and Goswami 2020). RBC counts were found to decrease to (1.79±0.02) ×10^6/mm^3, while WBC counts increased to (73.96±0.14) ×10^3/mm^3. The values were significant at p< 0.05. Haemoglobin was found to decrease to (8.25±0.07) % and clotting time was raised to (38.03±0.16) second. When the fishes were treated with arsenic and garlic extract after 15 days of exposure, RBC was found to be (2.06±0.04) ×10^6/mm^3, WBC decreased to (67.89±0.19) ×10^3/mm^3. Haemoglobin content was found to increase to (9.24±0.06) gm % and clotting time was reduced to (33.05±0.10) second. The values were significantly different from control values (p< 0.05) (Table 1). When the data were plotted for each parameter significant change in each group was observed (Figure 1-4) (Das and Goswami 2020).

Figure 3: Haemoglobin profile of *C. punctatus* exposed to (10%) sub lethal concentrations of arsenic and Aqueous Garlic Extract (AGE) for 15 days along with control group. Values are significant at P < 0.05. (* indicates that the values are significantly different at P < 0.05 level compared to the values of control group determined by one way ANOVA)

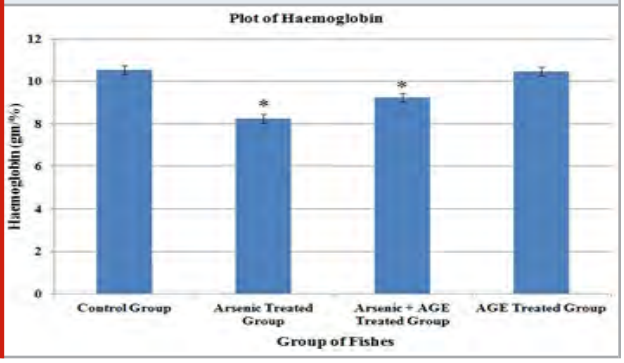
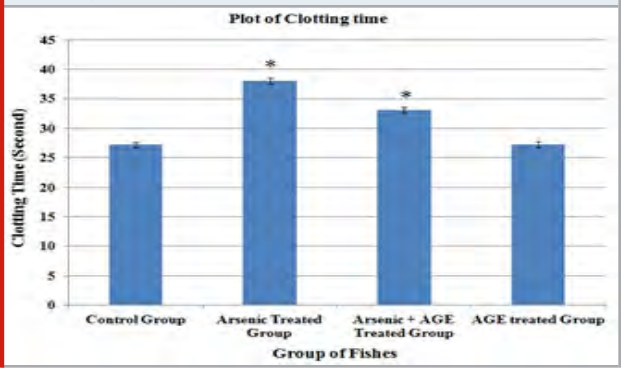


Figure 4: Clotting time profile of *C. punctatus* exposed to (10%) sublethal concentrations of arsenic and Aqueous Garlic Extract (AGE) for 15 days along with control group. Values are significant at P < 0.05. (* indicates that the values are significantly different at P < 0.05 level compared to the values of control group determined by one way ANOVA)



Considering the role of arsenic in the field of eco-toxicology, the present study had been undertaken to understand the accumulation of arsenic, when sodium arsenite was induced to fishes in laboratory conditions. The remedial measure with garlic extract was also aimed. So, liver, gill and kidney tissues from the four groups were taken for the present experimental set up (Table 2, 3 & 4). Accumulation of arsenic in treated group had given a very significant result. In control and only garlic treated group had shown that there was negligible amount of arsenic in the liver tissue, but it rose to 29.25 ± 0.15 ppb in arsenic treated group. However, the arsenic + AGE treated group had shown minimal decrease in arsenic accumulation (27.96 ± 0.13 ppb) (Figure 5).

The total arsenic content accumulated in the gill tissues was found to be 36.31 ± 0.21 ppb while there was very little decrease in arsenic concentration in the arsenic + AGE (34.94 ± 0.12) treated group. Normal control and garlic treated group had shown no accumulation of arsenic (Figure 6).

Figure 5: Total arsenic concentration in liver tissue of four groups of *C. punctatus* (Group 1-control, Group 2- Treated with sodium arsenite, Group 3- Treated with sodium arsenite + AGE, Group 4 – Treated with only AGE)

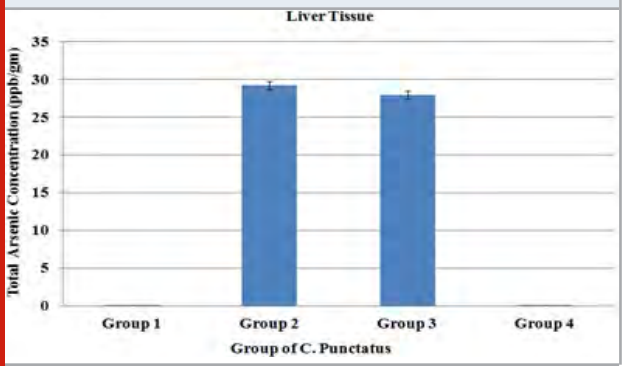
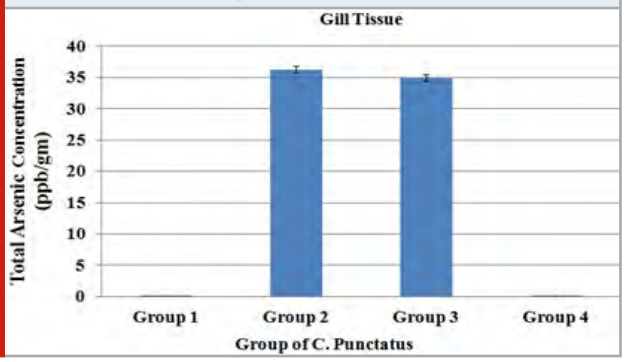


Figure 6. Total arsenic concentration in Gill tissue of four groups of *C. punctatus* (Group 1- control, Group 2- Treated with sodium arsenite, Group 3- Treated with sodium arsenite + AGE, Group 4 – Treated with only AGE)



Arsenic concentration when analysed in kidney tissue from different groups of *Channa punctatus*, it was found that total arsenic accumulated was 21.8 ± 0.23 ppb in the kidney in the sodium arsenite treated group, whereas in the sodium arsenite + AGE treated group, arsenic content decreased to 20.27 ± 0.02 ppb. Control group and garlic treated group had shown no arsenic in their tissues (Figure 7). A comparative account of arsenic content of tissues in four different Groups showed that the total arsenic accumulated was highest in the gill tissue as it had the maximum accumulation followed by liver and kidney tissues (Figure 8).

Similar trend was also observed in the sodium arsenite + AGE treated group but the amount of arsenic accumulated was less than in the sodium arsenite treated group. A negligible amount of arsenic content is observed in different tissues of control group of fishes and the group of fish treated with only AGE. This negligible value in the control and AGE treated group proved that fishes were collected from water bodies free from any arsenic contamination.

Figure 7: Total arsenic concentration in kidney tissue of four groups of *C. punctatus* (Group 1- control, Group 2- Treated with sodium arsenite, Group 3- Treated with sodium arsenite + AGE, Group 4 – Treated with only AGE)

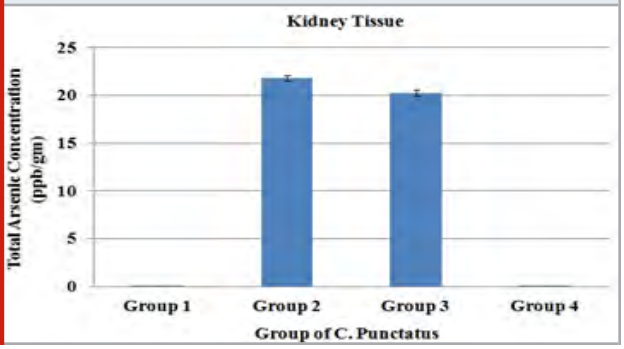
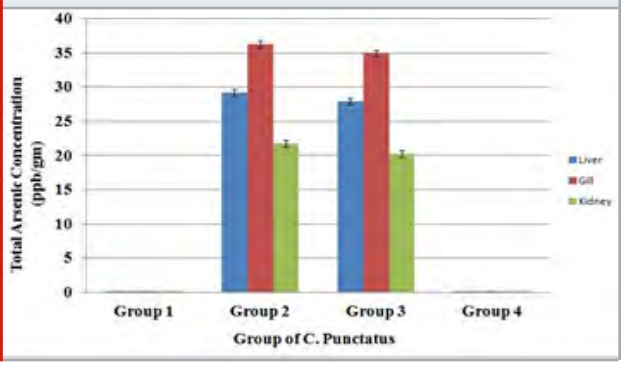


Figure 8: A comparison of total arsenic concentration in liver, gill and kidney tissues of four groups of *C. punctatus* (Group 1- control, Group 2- Treated with sodium arsenite, Group 3- Treated with sodium arsenite + AGE, Group 4 – Treated with only AGE).



It was observed here that Hb% and RBC decreased significantly while WBC and clotting time increased for arsenic treated group. Similar alternations of these parameters were also observed in the group of fishes exposed simultaneously to arsenic and AGE. Although it was found that changes of the haematological parameters in the Arsenic + AGE treated group was less significant. The exposure of *Channa punctatus* to sub-lethal concentration of arsenic significantly decreased Hb% and RBC count leading to anaemia and it can be considered as early manifestation of acute and chronic intoxication of heavy metals. Anaemia under arsenic induced stress may also be due to the injury in the blood cell and disruption in the heamoglobin synthesis (Mckim et al., 1970; Gross et al. 1975).

Many Researchers previously reported similar results with significant reduction of RBC and Hb% content in fishes exposed to different heavy metals (Goel et al. 1985; Goel and Sharma 1987; Pamila et al., 1991). Joshi et al.

(2002) stated that impaired intestinal absorption of iron exposure could decrease the RBC and Hb%. Pamila et al., (1991) suggested that the reduction in haemoglobin in fish blood exposed to heavy metal could be due to the inhibitory effect of the toxic substance on the enzyme system responsible for synthesis of haemoglobin. Christensen et al., (1972) stated that the changes in the haematological parameter could be understood in terms of the reduction of oxygen consumption in fish resulting in death due to heavy metal contamination (Christensen et al.,1972; Joshi et al., 2002).

According to Vanitha et al. (2017) the reduced RBC content in the blood under the effect of arsenic may be due to inhibition of erythropoiesis or by the destruction of blood cells. Saravanam et al. (2011) reported that disruption of haemopoietic processes and accelerated disintegration of erythrocyte cell membrane caused the reduction of haemoglobin content in toxicant exposed fishes. In this study, the observed decrease in RBC count and haemoglobin content in the arsenic treated *C.punctatus* might have resulted from destruction of RBC's due to erythroblastosis leading to anaemia (Saravanam et al., 2011; Vanitha et al., 2017). White blood cells are involved in the regulation of immunological functions and increase in their number under the exposure of any toxicant is a protective response in fish to stress conditions (Mishra and Niyogi, 2011).

It was stated that increase in the white blood cell counts might be the indication of damages of body tissues due to infection, severe physical stress and as well as leukemia. In the present work white blood cell counts were found to be increased for arsenic treated group. Similar findings were also observed by other researchers in the fishes exposed to different heavy metals. In the present study white blood cell counts were found to be increased for both arsenic treated and arsenic + AGE treated group (Nath and Banerjee, 1995; Mazon et al., 2002; Singh et al., 2008; Vanitha et al., 2017).

The increase of WBC count for the arsenic + AGE treated group is less as compared to the arsenic treated group. In the present study it was observed that *C. punctatus* under sublethal exposure of arsenic exhibits a long clotting time. When a lesion is made in any blood vessel a clot is formed as the end product of blood coagulation. Prothrombin, a blood clotting substance is present in high percentage in fish blood (Vanitha et al. 2017). A substance called thrombosthenin released by the platelet is responsible for clot retraction (Pandey and Shukla, 2005). Analogous results could also be seen in *Labeo rohita* exposed to copper sulphate, and *Catla catla* exposed to cadmium (Vincent et al., 1996; Sinha et al., 2000). Singh et al., (2008) studied the impact of copper on haematological profile of freshwater fish, *Channa punctatus*.

The exposure of fish to sub-lethal concentration of copper for 15, 30 and 45 days caused significant alterations in haematological parameters of Indian freshwater fish, *Channa punctatus*. Hb% and RBC decreased significantly after 15, 30 and 45 days of exposure periods, respectively, in comparison with control. On the contrary, WBC were found significantly increased after 15, 30 and 45 days of exposure, as compared to control. Exposure of fish to copper showed a significant decrease in the haemoglobin (Hb) content, red blood cells (RBC) at the end of 45th day as compared to control. Whereas the white blood cells (WBC) increased clotting time (CT) erythrocyte sedimentation rate and mean corpuscular volume increased significantly with increase in exposure periods. Amsath et al. (2017) studied the effect of Arsenic on haematological parameters of freshwater air breathing fish, *Channa punctatus* (Bloch) (Pragnya et al. 2020).

Exposure of fish to 10% sub-lethal concentration of arsenic for 10, 20 and 30 days caused significant alterations in hematological parameters in *C. punctatus* along with development of lesion in epidermis. The exposure of *C. punctatus* to 10% sub lethal concentrations of arsenic trioxide for 10, 20 and 30 days showed significant decrease in RBC count and Hb%. While WBC count increased significantly following arsenic exposure (Amsath et al. 2017). Jha et al. (2017) studied the toxicological effects of arsenic exposure on haematology of fresh water fish *Channa punctatus*. In their investigation the effect of subchronic exposure of arsenic induces significant alteration in hematological parameters when compared to control group. RBC count decreased corresponding increase in the exposure level, whereas, WBC count was increased at all concentration of arsenic treatment suggesting dose dependent response. Arsenic exposure may cause anaemic conditions and from their study it was found fish treated with subchronic doses of arsenic showed low Hb level resulting in anaemic behaviour. Mukherjee et al., (2015) studied arsenic toxicity on the Indian Murrel, *C. punctatus* at the haematological level that was under taken to assess the induction of stress on the fishes in controlled laboratory condition.

The exposure of *C. punctatus* to sub lethal concentration of sodium arsenite exhibited significant decrement in RBC count that might have led to anaemia. Anaemia under arsenic induced stress may be due to blood cell injury. White blood cell counts were found to increase significantly following arsenic exposure. The number of lymphocytes in arsenic exposed blood of fresh water teleost increased in compared to control (Mukherjee et al., 2015; Pragnya et al., 2020). The eosinophils have been implicated in inflammation and the percentage of this cell type exhibited a dose dependent increase in respect to control. Monocytes and neutrophils are important white blood cells to protect the body through their elevated phagocytic activity against opportunistic pathogen and parasite infection.

The percentage of both monocytes and neutrophils decreased in a dose dependent manner in respect to control. Basophil count also showed an increasing tendency under sublethal concentration of arsenic toxicity. Haematological parameters of fish can be helpful to identify the target organs of toxic effects and also the

general health condition of harmful changes in stressed organisms.It is clear from the study that the changes in all the haematological parameters (RBC, WBC, Hb% and Clotting time) for the arsenic + AGE treated group of fishes is less than the arsenic treated group of fishes from its control value.

It signifies a protective role of garlic extract against the toxicological effect of arsenic on the *C. punctatus*. Present investigation has also highlighted the use of garlic for de-toxification of arsenic which is safe and possibly useful to prevent adverse effects with arsenic exposure. In the present study the accumulated the highest total arsenic content in the liver tissue was 29.25 ± 0.15 ppb and it was found in the group treated with sodium arsenite. The total arsenic concentration in the liver tissue treated concurrently with sodium arsenite and Aqueous Garlic Extract (AGE) were found to be 27.96 ±0.13 ppb which is less than the total arsenic found in the earlier group.

In the gill tissue it was observed that arsenic treated group accumulated 36.31 ± 0.21 ppb of total arsenic and arsenic + AGE treated group accumulated 34.94 ± 0.12 ppb of total arsenic. The results of total arsenic concentration analysis in the kidney tissue showed that 21.8 ± 0.23 ppb total arsenic accumulated for the arsenic treated group and arsenic content reduced to 20.27 ± 0.02 ppb for the group treated simultaneously with arsenic and Aqueous Garlic Extract (AGE). The accumulated arsenic was found to be maximum in gill tissue and followed by liver and kidney. Other researchers also reported similar trend of arsenic content in fish and shellfishes (Pragnya et al., 2020).

Table 1. Haematological profile of *C. punctatus* exposed to (10%) sub lethal concentrations of Arsenic and Aqueous Garlic Extract (AGE) for 15 days.

Haematological Parameter	Control Group	Arsenic Treated Group (10% of LC_{50})	Arsenic + AGE Treated Group	AGE Treated Group
RBC (*106/mm3)	2.71 ± 0.08	1.79 ± 0.02 *	2.06 ± 0.04 *	2.71 ± 0.03
WBC (*103/mm3)	58.08 ± 0.13	73.96 ± 0.14 *	67.89 ± 0.19 *	58.08 ± 0.14
Hb gm (%)	10.54 ± 0.06	8.25 ± 0.07 *	9.24 ± 0.06 *	10.47 ± 0.06
Clotting Time (Second)	27.20 ± 0.15	38.03 ± 0.16 *	33.05 ± 0.10 *	27.23 ± 0.13

Table 2. Total arsenic concentration in the liver tissue of four different groups of *Channa punctatus*. Values are represented as mean ± MSE, n=5, Values are significant at P <0.05.

Accumulation of total Arsenic (micro gram/gm or ppb) in Liver			
Control Group (Group 1)	Treated Group (Sodium arsenite) (Group 2)	Treated Group (Sodium arsenite+AGE) (Group 3)	Treated Group (Only AGE) (Group 4)
0.01 ± 0.003	29.25 ± 0.15	27.96 ±0.13	0.02 ± 0.002

Table 3. Total arsenic concentration in the Gill tissue of four different groups of *Channa punctatus*. Values are represented as mean ± MSE, n=5, Values are significant at P < 0.05.

Accumulation of total Arsenic (micro gram/gm or ppb) in Gill			
Control Group (Group 1)	Treated Group (Sodium arsenite) (Group 2)	Treated Group (Sodium arsenite+AGE) (Group 3)	Treated Group (Only AGE) (Group 4)
0.02± 0.001	36.31 ± 0.21	34.94 ± 0.12	0.02± 0.004

The values of arsenic content observed in different tissues from this study were compared with available literature data (Vukadin et al., 1995; Engman and Jorhem 1998; Storelli and Marcotrigiano 2000). High arsenic concentrations in the edible muscle tissue of freshwater fish from arsenic-contaminated and non-

contaminated sites in Thailand, was reported by Jankong et al., (2007). Moretto et al., (2004) reported total arsenic in fish muscles. Al Rmalli et al., (2005) observed total arsenic content (0.097–1.32) µg/g; in muscles tissue of the fresh water fishes on the foodstuffs on sale in the United Kingdom and imported from Bangladesh (Al Rmalli et al., 2005; Pragnya et al., 2020).

Table 4. Total arsenic concentration in the kidney tissue of four different groups of *Channa punctatus*. Values are represented as mean ± MSE, n=5, Values are significant at P < 0.05.

Accumulation of total Arsenic (micro gram/gm or ppb) in kidney			
Control Group (Group 1)	Treated Group (Sodium arsenite) (Group 2)	Treated Group (Sodium arsenite+AGE) (Group 3)	Treated Group (Only AGE) (Group 4)
0.019 ± 0.001	21.8 ± 0.23	20.27 ± 0.02	0.02± 0.001

De Rosemond et al. (2008) investigated five freshwater fish species from Back Bay near Yellowknife, NT, Canada, and reported total arsenic in muscles, intestine and liver. Delgado-Andrade et al. (2003) investigated total arsenic content in muscles tissue of fishes from south-east Spain. Has-Schon et al. (2006) investigated total arsenic content in five fish species from River Neretva, Croatia and observed in muscles, gill and liver tissue. Juresa and Blanusa (2003) observed high concentration of mercury, arsenic, lead and cadmium in fish and shellfish from the Adriatic Sea. Shah et al. (2009) evaluated the total arsenic (As) in five tissues (gills, mouthpiece, intestine, liver and muscles) of 10 different fresh water fish species caught from arsenic contaminated Manchar Lake, Sindh, Pakistan during 2006–2007 (Shah et al. 2009; Pragnya et al. 2020).

Using hydride generation atomic absorption spectrometry (HG-AAS) method they have obtained arsenic concentration to be highest in liver followed by muscles, mouth pieces, intestine and gill(Shah et al. 2009; Pragnya et al. 2020). This survey of literature shows that the total arsenic content observed in the present study in different tissue of *Channa punctatus* was comparable to the available literature data. In the present study it was observed that arsenic concentration of all the tissues in the group treated with arsenic + garlic was found to be little less than the group treated with only arsenic and the arsenic content were found to be maximum in gill for all groups of fishes except the control group.

A negligible amount of arsenic content is observed in different tissues of control group of fishes. This negligible value of arsenic may be because of some instrumental error, as control group of fishes were completely free from any arsenic contamination. The arsenic content in the group treated with arsenic + AGE slightly decreased than the group treated with only arsenic, which signifies that garlic has some restorative properties to minimise the damages caused by arsenic. Analogous results of bioaccumulation of heavy metals (Zn, Pb, Cd, Co, Cu, and Fe) were also observed in fish tissues of various organs of three fish species, i.e., *Labeo rohita, Pangasius hypophthalmus* and *Katsuwonus pelamis* (Pragnyaet al. 2020).

CONCLUSION

In the present work white blood cell counts and clotting time were found to be increased whereas the RBC count and haemoglobin content were found to be decreased for the arsenic treated group. However the haematological profile of the fishes in the revival group was restored to some extent. From the results of the present investigation it could be concluded that stress due to heavy metals present in the water does create hematological disturbances, erythrocyte destruction (hemolysis), and leukocytosis in fish population which affects the immune system and makes the fish vulnerable to diseases. The results of the present study also signify that the haematological disturbances are caused by arsenic and its consequences could be reverted to a large extent by using aqueous garlic extract.

The observed value of total arsenic accumulation was below the permissible limit set by the Bureau of Indian Standards (50 ppb) but more than the international limit of 10 ppb. The accumulation of arsenic found in liver, gill and kidney in the present study confirms the fact that arsenic toxicity was responsible for the physiological changes in the fishes. It also indicates the protective role of garlic against arsenic toxicity and how its long term use can be beneficial to eliminate arsenic from blood and soft tissues. There is a scope of further investigation in to the mechanism of reduced arsenic accumulation by prolonged AGE treatment. These results may be used to guide ecological monitoring programs that measure the bioavailability of arsenic in freshwater fishes.

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Agricultural
Communication

Efficiency of the Initiation Methods of Fruits in the Young Intensive-Type Apple Orchard

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ABSTRACT

The intensification of horticulture involves denser plantings, optimal provision with heat, light, moisture, and other life factors. Growing apples in intensive plantings require control and regulation of plant nutrition, the use of rational types of tree crowns, and pruning techniques. The study's main aim is to investigate the efficiency of the initiation methods of fruits in the young intensive-type apple orchard. An intensive-type unsupported apple orchard at the Agro centre Research and Production Complex of the Saratov State Agrarian University was planted in the fall of 2014 according to a 4x2.5 m scheme with 1000 trees per hectare. Rootstock - 54-118. The research was carried out in 2019 and 2020 on apple varieties of autumn (Zhigulevskoe, Orlik, Gubernskoe, Shafran Saratovsky) and winter (Kulikovskoe, Kutuzovets, Honey crisp, Berkutovskoe) ripening. On the example of 8 apple varieties (Zhigulevskoe, Orlik, Gubernskoe, Shafran Saratovsky, Kulikovskoe, Kutuzovets, Honey crisp, and Berkutovskoe), this article attempts to prove that green operations (June pruning of shoots more than 25 cm long for 4-6 buds) in a young intensive-type apple orchard leads to a decrease in the growth activity of plants, an increase in the specific foliage and the specific provision of branches with fruit formations. Foliar dressing of apple plants with an organic microelement complex during intensive growth provides an increase in plant foliage, the initiation of a more significant number of fruits, and the higher quality yield. Simultaneously, in the studied apple varieties, the significance of differences in changes in growth activity has not been proven. Based on the results, green operations and foliar dressing promote the formation of the potential for higher productivity of the apple tree.

KEY WORDS: APPLE TREE, VARIETIES, FRUITS, GREEN OPERATIONS, NUTRITION, PRODUCTIVITY.

INTRODUCTION

The successful growth and initiation of fruits require a balanced diet for apple trees, including various mineral elements, such as nitrogen, phosphorus, potassium, calcium, iron, magnesium, manganese, zinc, copper, boron, cobalt, molybdenum, etc. Each mineral element performs its function during the growing season of plants. Their reasonable combination is considered optimal. For example, the use of only phosphorus and potassium fertilizers without nitrogen is ineffective (Kondratiev and Eskov, 2017; Ryabushkin et al., 2020). The use of organic fertilizers alone is not always effective. In this regard, organometal fertilizers, which contain both organic substances and minerals, have become widespread in agronomic practice. They have no negative effect and can completely replace mineral fertilizing. Organo mineral fertilizers (for example, Raikat Razvitie, Aminokat) nourish plants without oversaturation with elements and do not pollute the environment (Khilko, 2017; Jia et al., 2020).

Recent studies have shown the promise of using trace elements with organic biologically active compounds (amino acids and their protein derivatives). These complex compounds (produced, for example, by Bioamid JSC, Saratov) can easily penetrate the cell walls and be absorbed by the body. Simultaneously, the increased strength of the bonds of the metal with amino acids significantly reduces the possibility of undesirable side processes with the participation of the microelement. At present, organic trace element complexes (OTEC) based on L-asparaginase are successfully used both in premixes for feeding farm animals and as fertilizers for growing cultivated plants. The fertilizer contains iron (Fe), zinc (Zn), copper (Cu), manganese (Mn), cobalt (Co), molybdenum (Mo), and boron (B) (Williams et al., 2020).

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The role of iron (Fe) is significant in oxidative and energy metabolism in the formation of chlorophyll. Therefore, organic compounds, which include iron, are primarily important for the biochemical processes of plants occurring during respiration and photosynthesis (Sajjadi and Moosavi, 2019). With a lack of zinc (Zn), fruit crops have smaller, lighter leaves with scarce irregular fruits (Sheikhshoaie et al., 2018). Copper (Cu) makes possible the processes of respiration and photosynthesis, carbohydrate metabolism and synthesis of fats, and the formation of certain vitamins. Manganese (Mn) provides the normal course of photosynthesis, the accumulation of chlorophyll in the leaves, the synthesis of sugars, vitamin C. By participating in redox reactions, it contributes to the active development of plants, increases their productivity, regulates the water regime, and resistance to adverse factors. Cobalt (Co) in plants is necessary for the fixation of molecular nitrogen; it accumulates in pollen and accelerates its germination, stimulates plant growth processes (including stretching of cell membranes). This microelement is involved in cell reproduction of leaves. (Grace and Wilson, 2021).

Molybdenum (Mo) participates in metabolic, recovery, and energy processes, protein synthesis. It is part of the enzymes that regulate nitrogen metabolism, improves calcium nutrition of plants, participates in the formation of chlorophyll, in the development of the root system, as well as in exchange of phosphorus compounds and carbohydrates (Ibrakhimov et al., 2020). Boron (B) is necessary for plants throughout their life, participating in the transport of carbohydrates, in particular sugars, and the synthesis of cell walls. It affects the intensity of photosynthesis, improves hydrocarbon, nucleic and protein metabolism activates enzymes (Ryabushkin et al., 2020). This article provides data on the assessment of the effect of summer pruning of shoots and foliar feeding with a product made based on an organo mineral complex on the growth and initiation of apple tree fruits in the intensive garden of the Agrocenter Research and Production Complex of Saratov State Agrarian University.

MATERIAL AND METHODS

Soil maintenance system: the aisles - natural ramping with systematic mowing and leaving of grass as mulch, the near-trunk strip - herbicide-treated fallow. According to the fusiform type (new Russian spindle), formation of plants with the implementation of green operations in June (4-6 buds pruning of young growths more than 25 cm long). Drip irrigation: Treatment of plants with a product based on an organomineral complex was carried out twice during intensive plant growth (in June) with an interval of 10 days. The drug flow rate was 430 g/ha, the spray material flow rate was 1000 l/ha.

Experimental design:
Variant 1. – Control. Plants were not subjected to green operations or foliar dressing.

Variant 2. – Plants with fruit branches subjected to green operations (4-6 buds pruning of annual growths). Treatment with an organic microelement complex was not carried out.

Variant 3. – Plants with fruit branches subjected to green operations (4-6 buds pruning of annual growths) and treatment with an organic microelement complex.

Field experiment was taken as a methodological basis for research. The main surveys and observations were carried out in accordance with the program and methodological instructions for the various study of fruit, berry and nut crops (Grace and Wilson, 2021). The leaf surface area was determined by the method of carvings according to the method developed by Fulga, 1961. The analysis of the initiation of fruits was carried out on well-lit fruit branches located in the middle part of the crown on the eastern side.

RESULTS AND DISCUSSION

The objective of the research was to reveal the effectiveness of green operations and an organic microelement complex by Bioamid JSC (Saratov) on changes in growth activity and the initiation of fruits in intensive-type apple plantations. The studies have shown that significant changes in plant parameters occur under the influence of the studied objects. Gardeners largely form the shape and size of the crown during tree pruning, so these indicators were not considered when analyzing the growth parameters of apple trees under the influence of green operations and foliar fertilizers. More objective is the analysis of the annual growth of shoots and the diameter of the stem under the influence of the studied factors (measurements were taken at the end of the growing season in 2019 and 2020). The increase in the diameter of the stem over the year was characterized by the maximum values in the control variant, when part of the increased annual shoots was not removed and foliar feeding was not carried out (Table 1). This pattern can be traced for all studied varieties. Thus, in Zhigulevskoye trees (autumn variety), the increase in the diameter of the trunk during the growing season under the influence of the June pruning decreased by 2.2-2.6 mm (by 17.6-20.8%); in the Orlik variety - by 4.4 -4.7 mm (by 42.7-45.6%), in the Gubernskoe variety - by 6.2-7.7 mm (by 32.6-40.5%), in Safran Saratovsky varieties - by 4.2-4.8 mm (35.6 -40.7%).

This pattern is also typical for trees of winter ripening varieties: In the Kulikovskoe variety, under the influence of chasing, there is a decrease in the growth of the stem diameter by 4.0-3.9 mm (by 47.1-45.9%), in the Kutuzovets variety - by 4.4-4.1 mm (by 21.3-19.8%), in the Berkutovskoe variety - by 2.4-3.1 mm (by 24.0-31.0%), in Honey crisp - by 4.3-4.1mm (34.7-33.1%). No significant differences in the increase in the diameter of the stem with foliar feeding in the studied apple varieties have been proven (see Table 1). Observations of the formation of growths on model tree branches using June 4-6 buds pruning of shoots showed the following. 2-3 weeks after trimming the shoots, the length of which exceeded 25 cm, on the remaining parts of the shoots from the upper buds, new shoots develop, the size of which is significantly inferior to the control by the end of the growing season (Table 2). As a rule, the size of these increments corresponds to the size of fruit formations: dards (up to 25-30 cm), spurs (up to 15 cm), ringlets (up to 3 cm). There were no significant changes in the length

of shoot growth under the influence of foliar dressing. Evaluation of the development of the leaf apparatus on model branches according to the variants of the experiment indicates some advantages of plants treated with an organic microelement complex (Table 2).

Table 1. Annual increase in the diameter of the trunk in apple trees under the influence of June pruning of shoots and foliar dressing with an organic microelement complex

Variety	Increase in the trunk diameter, variant			HCP_{05}
	1	2	3	
Autumn ripening varieties				
Zhigulevskoe	12.5	10.3	9.9	1.4
Orlik	10.3	5.9	5.6	1.6
Gubernskoe	19.0	12.8	11.3	2.3
Shafran Saratovskii	11.8	7.6	7.0	1.9
Winter ripening varieties				
Kulikovskoe	8.5	4.5	4.6	1.6
Kutuzovets	20.7	16.3	16.6	1.8
Berkutovskoe	10.0	7.6	6.9	1.1
Honey crisp	12.4	8.1	8.3	1.3

Table 2. Growth and leafiness of apple shoots on model branches

Parameters	Variant	Autumn ripening varieties				Winter ripening varieties			
		Zhigu levskoe	Orlik	Gubern skoe	Shafran Saratovskii	Kuliko vskoe	Kutu zovets	Honey crisp	Berku tovskoe
Average shoot length, cm	1	30.3	51.3	52.7	42.7	57.0	65.7	40.7	47.3
	2	20.7	16.0	17.7	19.7	31.3	35.3	13.8	12.3
	3	17.0	16.9	16.7	17.3	32.0	40.3	13.0	12.2
	HCP_{05}	4.1	4.9	6.7	3.8	4.9	5.8	4.8	4.9
Number of leaves, pc.	1	21.7	29.0	19.0	19.0	25.7	31.7	25.3	20.7
	2	15.3	12.0	13.0	13.0	18.7	21.3	11.3	10.9
	3	14.7	13.2	12.7	9.7	18.3	20.0	11.3	12.5
	HCP_{05}	3.2	2.7	2.1	2.2	2.1	3.8	3.1	1.9
Leaf surface area, dm^2	1	11.6	15.5	11.4	11.2	12.9	11.2	8.7	8.9
	2	9.4	4.9	4.4	5.4	7.5	8.3	5.1	4.3
	3	8.8	5.6	5.1	5.0	8.1	8.8	4.9	4.9
	HCP_{05}	1.2	1.7	1.5	1.7	1.1	1.4	1.1	0.9
Leaf surface area per 1 cm shoot, cm^2	1	38.3	30.2	21.6	26.2	22.6	17.0	21.4	18.8
	2	45.4	30.6	24.9	27.4	24.0	23.5	37.0	35.0
	3	51.8	33.1	30.6	28.9	25.3	21.8	37.7	40.2
	HCP_{05}	4.0	3.7	3.2	2.7	2.1	2.8	3.9	3.7
Lamina area, cm^2	1	53.5	53.4	60.0	58.9	50.2	35.3	34.4	43.0
	2	61.4	40.8	33.8	41.5	40.1	39.0	45.1	39.5
	3	59.9	42.4	40.2	51.5	44.3	43.8	43.4	39.2
	HCP_{05}	7.3	4.8	6.2	5.5	4.1	4.2	5.0	3.8

If the control variant shows a significant superiority of indicators in the number of leaves per one-year growth and the total leaf area on the model branch, then the analysis of the specific foliage of 1 cm of shoot growth and the average leaf size indicates a tendency towards an increase in these indicators after foliar treatment of plants with fertilizer. In autumn cultivars Zhigulevskoe, Gubernskoe and in winter cultivars Berkutovskoe, the significance of differences in foliage per unit of shoot growth under the influence of foliar fertilization was proved mathematically. In terms of the size of the lamina, significant advantages of the option with feeding with an organic microelement complex are noted in Gubernskoe, Shafran Saratovskii, and Kutuzovets. The fruits of an apple tree, as a rule, are formed on dards, spurs, ringlets, the presence of which determines the potential productivity of trees.

Horticulture uses various agricultural practices to initiate these fruits: selection of varieties, formation and pruning of plants, their nutrition. Studies of an earlier period indicate a high varietal specificity of the formation of fruit formations, especially at a young age (Ryabushkin et al., 2020). These studies have shown that there is a steady pattern of increase in the total number of fruit formations after green operations in all apple varieties (Table 3). A significant increase in the number of formed fruit formations under the influence of foliar treatment of plants with fertilizer is observed only for the Gubernskoe, Shafran Saratovskii and Berkutovskoe varieties. For the rest of the varieties, we can only talk about the tendency for the formation of a larger number of fruit formations under the influence of the studied fertilizer. This pattern can also be traced in the analysis of the specific load of fruit formations of one running meter of the fruit branch.

Table 3. Initiation of fruits on young apple trees

Variety	Variant	Number of fruits on a fruit branch				Fruit branch length, cm	Number of fruits per 1m of fruit branch, pcs
		Spurs, pcs	Dards, pcs	Fruit dards, pcs	Total, pcs		
Zhigulevskoe	1	6.0	1.7	0.7	8.4	139.0	6.0
	2	12.7	3.3	1.0	16.0	134.3	11.9
	3	13.0	3.6	0.8	17.4	136.1	12.8
HCP_{05}		1.5	0.3	0.1	1.9	-	1.4
Orlik	1	6.0	2.0	1.3	9.3	129.7	7.2
	2	8.7	2.3	0.3	11.3	143.3	7.9
	3	8.3	1.3	1.3	10.9	130.7	8.3
HCP_{05}		0.8	0.3	0.1	1.2	-	0.6
Gubernskoe	1	7.0	0.7	0	7.7	131.1	5.9
	2	9.0	2.0	0	11.0	130.0	8.5
	3	8.7	2.7	0	11.4	123.3	9.2
HCP_{05}		0.7	0.2	-	1.0	-	0.6
Shafran Saratovskii	1	10.0	1.0	1.0	12.0	138.0	8.7
	2	9.0	0.5	1.0	10.5	120.0	8.8
	3	7.7	3.3	0.7	11.7	123.3	9.5
HCP_{05}		0.7	0.3	0.1	1.1	-	0.5
Kulikovskoe	1	5.9	2.1	1.6	9.6	133.2	7.2
	2	6.5	1.6	2.0	10.1	125.1	8.1
	3	7.0	2.3	1.4	10.7	130.1	8.2
HCP_{05}		0.6	0.3	0.2	0.9	-	0.5
Kutuzovets	1	5.8	2.0	2.0	9.8	166.3	5.9
	2	6.0	2.2	1.8	10.0	150.2	6.7
	3	7.6	1.8	1.0	10.4	153.0	6.8
HCP_{05}		0.5	0.2	0.2	1.0	-	0.7
Honey crisp	1	6.8	1.7	0.0	8.5	111.3	7.6
	2	8.2	0.8	0.3	9.3	90.2	10.3
	3	7.7	1.5	0.7	9.9	92.3	10.7
HCP_{05}		0.8	0.2	0.1	0.7	-	1.1
Berkutovskoe	1	6.1	0.8	1.0	7.9	98.6	8.0
	2	5.3	1.8	0.0	7.1	78.1	9.1
	3	6.8	1.6	1.1	9.5	85.6	11.1
HCP_{05}		0.7	0.2	0.1	1.1	-	1.2

Additional plant nutrition, as a rule, improves the quality of the grown products. Our studies showed no significant increase in fruit weight after two-time treatment of apple trees with OTEC (Table 4). Based on the results obtained, most varieties (Gubernskoe, Shafran Saratovskii, Kutuzovets, Honey crisp, Berkutovskoe) tend to form larger fruits only, which confirms the relevant studies' results (Jia et al., 2020; Grace and Wilson, 2021). However, the exception is the Kulikovskoe variety, which had fruits in the fertilization variant (variant 3) 45% larger (on average 131.7 g) than in the variant without fertilization (90.9 g). Obviously, this is due to the fact that trees of the Kulikovskoe variety, under the influence of fertilizers, formed 10.5% larger leaves (see Table 2). In addition, Zhigulevskoe and Orlik, after foliar fertilization, show a slight decrease in fruit weight, which given the relevant studies, stands to reason (Ryabushkin et al., 2020; Williams et al., 2020, Grace and Wilson, 2021).

In conclusion, given the outcomes, it can be concluded that 4-6 buds pruning of shoots in June leads to a temporary stop in the growth of shoots and the subsequent formation of growths, the size of which is 2-3 times less than in the control variant. These increments

are more consistent with the size of fruits (dards, spurs, fruit spurs) in terms of parameters. Simultaneously, the size of plants decreases due to a decrease in the size of annual increments, a decrease in the increase in the trunk diameter. And the specific provision of the tree crown with a leaf canopy increases. The total number of fruit formations on the tree, depending on the variety, increases by 2...90%.

Table 4. Influence of plant feeding with OMEK fertilizer on changes in average fruit weight

Variety	Average fruit weight, g		
	Variant		HCP_{05}
	2	3	
Autumn ripening varieties			
Zhigulevskoe	170.5	167.5	Ff<Ft
Orlik	120.7	111.6	Ff<Ft
Gubernskoe	119.6	124.3	Ff<Ft
Shafran Saratovskii	111.4	119.4	Ff<Ft
Winter ripening varieties			
Kulikovskoe	90.9	131.7	14.6
Kutuzovets	98.7	101.7	Ff<Ft
Berkutovskoe	135.0	143.7	Ff<Ft
Honey crisp	123.3	114.7	Ff<Ft

CONCLUSION

The significance of differences in the change in growth activity (increase in the diameter of the stem, increase in shoots) has not been proven when dressing the plants with OTEC in the studied apple varieties. There are tendencies to increase plant foliage, the initiation of a larger number of fruits, and the higher quality yield. In general, green operations and foliar dressing promote the formation of the potential for higher productivity of the apple tree. Similar studies need to continue to develop practical recommendations for the use of OTEC for apple trees.

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Pathological
Communication

On the Efficiency of Silver Nanoparticles Synthesized using *Streptomyces* spp. against Human Pathogens

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ABSTRACT

Silver nanoparticles have changed the scenario of applications in various fields like food, medical, textile, health care, bio-sensing systems, etc. This work is dedicated to find out the Invitro killing ability profiles of silver nanoparticles producedby actinomycetes (*Streptomyces* AK3) against human pathogens and contributes to the enhancing knowledge about the effectiveness of silver nanoparticles for the treatment of human diseases. The better relevant strain of actinomycetes has been examined and sequenced (GenBank MT626067). The soil sample was collected from Tapi river, Surat. The river is polluted with heavy metals and can bear the heavy metal resistant bacteria. *Streptomyces* spp. AK3 strain was isolated with the actinomycetes isolation media and then sub cultured in starch casein broth. The broth was filtered then cell free filtrate was added with $AgNO_3$ and kept in shaking incubator. Intensity of colour change from yellow to brown was measured with Shimadzu UV-Vis 1601 spectrophotometer. Size distribution and Zeta potential of silver nanoparticles were known with Malvern, and TEM analysiswas done to have a detailed account of prepared silver nanoparticles' characteristics. The diffusion method was used for analyses of efficiencies shown by the prepared silver nanoparticles and the antibiotics against various human pathogens. In result, the zones of inhibition were formed with strain synthesized silver nanoparticles as 22 mm, 24 mm, 25 mm and 24 mm for *Salmonella*, Co-agulase negative *Staphylococcus, Klebsiella*, and *Enterococcus* respectively. In conclusion, silver nanoparticles synthesized by *Streptomyces* spp. AK3 have been found a better alternative to antibiotics in terms of their efficiencies against human pathogens and side effects.

KEY WORDS: SILVER NANOPARTICLES, STREPTOMYCES SPP. AK3, TEM ANALYSIS, ZETA POTENTIAL ANALYSIS, ZONES OF INHIBITION.

INTRODUCTION

Use of silver dates back to 69 B.C.E.The use of silver has been happening for a long time and was reported from Before Christ to mentioned year (Hill et al.,1939; Alexander 2009). However chemical reduction has been the major technique, production of nanoparticles is possible with various life forms (Mohanpuria et al. 2008; Kholoud et al.,2010). NADH co-factor of nitrate reductase plays a role in the reduction process (Husseiny et al., 2007). AgNO3 concentration, pH, and reaction temperature are decisive in obtaining the silver nanoparticles of desired size (Gurunathan et al., 2009). Bio-synthesized nanoparticles should not only be effective against pathogens but also be nontoxic to the patient and this property is possessed by silver nanoparticles (Song et al., 2009). *Pseudomonas stutzeri* can produce AgNPs in its periplasmic space, whereas *Verticillium* has the ability to produce them on the surface of mycelia as its surface contains nitrate reductase, inturn, this enzyme can bind silver ions with its negatively charged groups and gives the output as reduced silver ions (Klaus et al., 1999; Senapati et al., 2004; Krishnamurthy et al., 2010). Silver nanoparticles can accumulate inside the bacterial cells and kill them, in turn; these killed cells can act as reservoirs of AgNPs and release silver cations slowly in the environment of pathogenic bacteria(Mohamed et al., 2020).

Microbes arthrospira commonly usedas dietary supplements have also been found to have the ability with some biochemical alterations during the process to produce nanoparticles (Cepoi et al., 2015). AgNPs are

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potential agents for treating viruses, cancers of breast, liver, skin and blood (Wei et al., 2015). High doses of antibiotics have side effects, if silver nanoparticles added to antibiotics cause the reduction of antibiotics' dose with the same or increased efficiency as compared to the previous high dose of antibiotics' alone, harmful effects of high doses can be minimized (Singh et al., 2015). Not only the enzymes like nitrate reductase but also the substances having carboxyl, amino, or hydroxyl groups have been noticed to reduce and cap the ions (Abdel-Raouf et al., 2017). Due totheir smaller size, Silver nanoparticles can cross the membranes of pathogens and cause the production of free radicals (Siddiqi et al., 2018). Since silver nanoparticles in high doses can cause the leakage of hemoglobin from erythrocytes hence exhaustive examination of the In Vivo effects must be followed (Hamouda et al., 2019). This work presents a comparative account of lethal effects of the strain synthesized AgNPs and antibiotics against human pathogens.

MATERIAL AND METHODS

The soil sample was collected at the bank of the heavily polluted Tapi River, Surat (Gujarat). The sample was sprinkled on actinomycetes isolation agar media, four strains naming AK1-AK4 were isolated, transferred to starch casein broth in Erlenmeyer flasks and grown at 120 rpm, and 30 ^{0}C for 72 hours. Preliminary identification of these strains was done with citrate utilization, methyl red, indole, urea hydrolysis, and nitrate reduction tests. Broths of flasks; AK1-AK4 in two sets, were filtered through bacteriological filter paper, and two types of filtrates; cell filtrate and cell-free filtrate, were obtained. One set was left as control.10ml of each filtrate; AK1-AK4, from one set was made up to 48.5 ml with phosphate buffer of 0.1M and then 1ml of 1mM $AgNO_3$ each and 0.5 ml of 1mM of methionine and cysteine each were added to these filtrates. The same was followed with a set of controlsexcept the addition of $AgNO_3$. Flasks of these filtrates were kept in a shaking incubator at 120 rpm and 30 ^{0}C for 72 hours and any colour change to brown; an indication of silver nanoparticles' synthesis, was noticed (Sukanyaet et al., 2013).

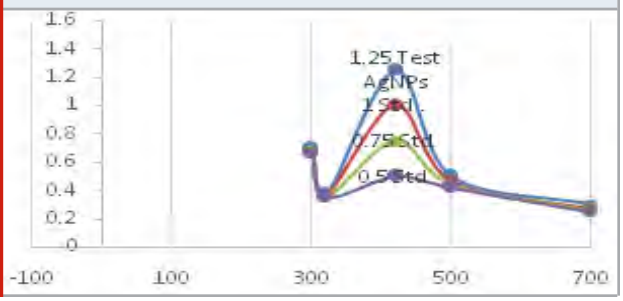
This 50 ml was evaporated to have 5 ml as a semi-final volume and a further 10 times dilution of this semi-final volume for readings with spectrophotometer was done leaving one set as undiluted semi-final volume for the highly resistant bacteria. Separation of antibiotics was done with a dialysis membrane and phosphate buffer of 6.5pH as silver nanoparticles are conjugated with bio-polymers and cannot pass through the pores of the membrane. Spectrophotometric analysis was done at 420 nm. On finding themaximum absorbance given by the product of strain AK3, the candidate was got sequenced for 16S rRNA with ABI 3130 genetic analyzer at Biokart Ltd. Bangaluru and zeta potential and size distribution of silver nanoparticles synthesized by *Streptomyces* spp. AK3(Gen Bank MT626067) were measured and checked for the degree of agglomeration at PERD, Ahmedabad. Strain AK3 was analyzed for shape and size with TEM at Sprint testing, Powai, Mumbai after some improvement in procedure with the addition of amines to prevent the agglomeration. *Enterococcus foecails*, Coagulase negative *Staphylococci* spp., *Klebsiella pneumoniae, Staphylococcus aureus* and *Salmonella* spp. were selected for testing the silver nanoparticles' activity. These microbes were grown on Petri plates with different concentrations of synthesized silver nanoparticles; given in table-1, and antibiotics in the wells.

RESULTS AND DISCUSSION

Morphological and physiological analyses: Colonies were wrinkled, raised, opaque and, white andstrainswere visualized as long positive rods. Strains could grow in the range between 26 ^{0}C to 40 ^{0}C and the best growth was observed at 32 ^{0}C. Production of AgNPs was checked at pH ranging from 4.5 to 8.5. The optimum pH for production was found as 6.5. Slightly acidic pH may impart a stronger binding ability to capping agents, in turn; smaller sized nanoparticles are formed (Gan et al., 2012). 2% NaCl was the optimum concentration at which strain can grow the best as opposed to halophytic actinomycetes which grow at 15%-25% salt concentration (Jiang et al., 2016).

Biochemical tests: The strain showed positive results for citrate utilization, methyl red, urea hydrolysis and nitrate reduction, and negative for indole.

Figure 1: Absorbance on Y axis at wavelengths in nm



UV-Visible spectroscopic analysis of synthesized nanoparticles:

Figure of absorbance versus wavelength was developed with Shimadzu UV-1601 spectrophotometer. An inference can be made for the synthesis of silver nanoparticles with the colour change to light brown. The test sample was diluted ten times and readings were noted with standards of 40 µg/ml, 30 µg/ml and 20 µg/ml as 1.25, 1, 0.75, and 0.5 absorbances respectively from the test sample to the least concentrated standard. The test sample was diluted later also as per the matches with the most effective antibiotics available in terms of efficiencies against human pathogens, whose data are described in this document. Surface plasma resonance of AgNPs synthesized by *Streptomyces* spp. ranges from 440-450 nm (Eid et al., 2020).

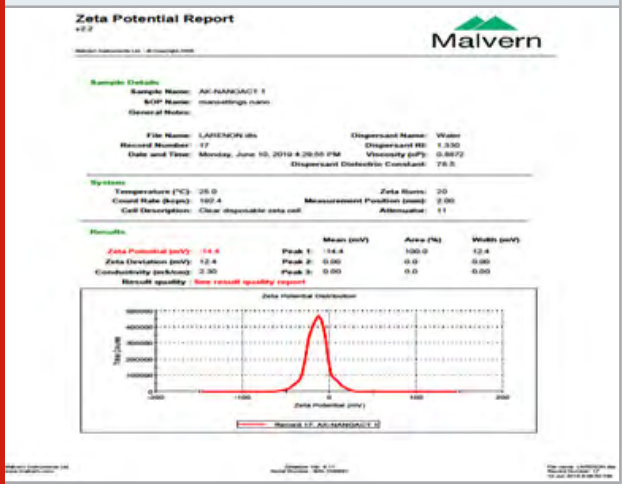
16S rRNA sequencing of strain AK3: Strain AK3 (GenBank MT626067) was found as *Streptomyces* spp. closer to *Streptomyces atacamensis*. A few species of *Streptomyces* are known to have the ability to convert silver ions to silver nanoparticles. Primers such as 27F and 1492R can be used for the amplification of *Streptomyces* spp.(Khadyat et al., 2020).

Zeta potential silver nanoparticles: Zeta potential was-14.4 which shows agglomeration-free silver nanoparticles to the most extent.AgNPs can be sterically stabilized by natural surfactants (Chartarrayawadee et al., 2020).

Features of produced silver nanoparticles known with TEM: TEM produced the images with 20 nm-sized silver nanoparticles having various morphological characteristics such as spherical, prism-shaped etc. A role against destabilization of silver nanoparticles is played by sulphur-containing amino acids, thiols amines, alcohols to some extent as different results are seen for control and stabilizers added silver nanoparticles synthesizing cell-free filtrate (Iravani et al. 2014). Properties can be manipulated by changing the concentration of capping agents, however the efficiency of silver nanoparticles against pathogens is affected with increased concentration adversely. Antimicrobial activity of silver nanoparticles depends on the shapes also. Dispersity of AgNPs can be modulated by the flow rate in a continuous flow tubular microreactor (Dawadi et al., 2021).

Comparative antibacterial activities of synthesized silver nanoparticles and antibiotics: Well diffusion method was performed and data of affected microbes by different antibiotics and SNPs with zones of inhibition have been represented here as Petri plates and the table is shown as well below them.

Figure 2: Synthesized silver nanoparticles' Zeta potential measured by Zeta sizer



24 (a)-*Enterococcus foecalis* with SNPs, LE5, and HIG-120
24 (b)-*Enterococcus Foecalis* with LZ30, AMP10, TEI30, IPM10, VA30, and CIP5
346-Co-agulase negative *Staphylococci* spp. with SNPs, LZ30, TEI30, VA30, DO30, and TE30
1019-*Klebsiella pneumoniae* with SNPs, CPT30, CFM5, C30, TOB10, TFC15
13-*Staphylococcus aureus* with SNPs, TE30, LE5, CIP5, TEI30, DO30, and MI30
341-*Salmonella* spp. with SNPs, PB300, CL10, CFS30, TE10, CFM5, AZM15, and PF5

Figure 3: Images of silver nanoparticles with TEM

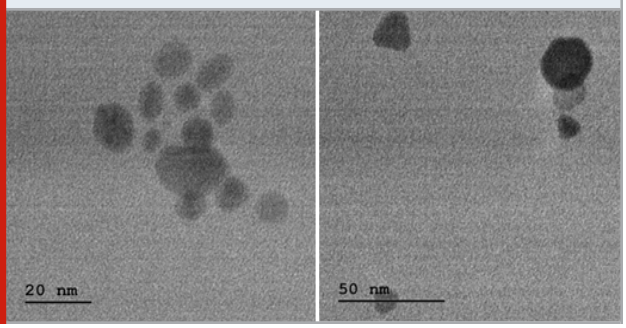
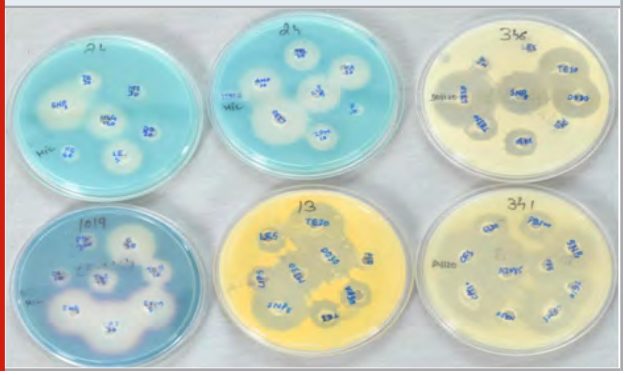


Figure 4: Inhibition zones of SNPs andantibiotics



1. *Staphylococcus aureus*, *Salmonella typhi, Pseudomonas aeruginosa, E.coli*, Co-agulase negative *Staphylococci, Enterococcus foecalis*, and *klebsiella pneumoniae* were selected as test pathogens. 2. *Staphylococcus aureus, Salmonella typhi, Pseudomonas aeruginosa, E.coli,* and Co-agulase negative *Staphylococci* were tested on Mueller Hinton agarwhereas *Enterococcus foecalis* and *klebsiella pneumoniae* were tested on Hi-chrome media. Plates were placed in the incubator for 20 hours at 370 C. 3. *Staphylococcus aureus* was highly sensitive to tetracycline 30, almost resistant to penicillin-G 10, and sensitive to silver nanoparticles (10 µg/ml) with a 25 mm zone of inhibition. Addition of silver nanoparticles to the tetracycline, produced in the current work, could have enhanced lethal effect on *S. aureus* (Hussein et al. 2019). Effect of silver nanoparticles on the closer species *Staphylococcus epidermidis* has been reported (Swolana et al., 2020). 4. Test organism *Salmonella* was the most sensitive to cefapazone sulbactam 30, the least sensitive to polymyxin B300 and sensitive to silver nanoparticles with a 22 mm inhibition zone.

Table 1. Effect of *Streptomyces*'s strain AK3 synthesized Silver Nanoparticles (SNPs) on test organisms

Test Organism	Antibiotics-µg/ SNPs	Zone of inhibition (mm)	Plate No.
Staphylococcus aureus	Tetracycline 30 (TE30)	28	13
	SNPs	25	
	Levofloxacin 5 (LE5)	12	
	Ciprofloxacin 5 (CIP5)	9	
	Penicillin-G 10 (P10)	7	
	Vancomycin 30 (VA30)	15	
	Teicoplanin 30 (TEI 30)	14	
	Doxicycline 30 (DO 30)	13	
	Minocycline 30 (MI 30)	23	
	Clindamycin 2 (CD2)	18	
Salmonella spp.	SNPs	22	341
	Polymyxin B 300 (PB300)	15	
	Colistin 10 (CL10)	17	
	Cefopazone sulbactum30 (CFS30)	24	
	Tetracline 10 (TE10)	17	
	Cefixime 5 (CFM5)	25	
	Azithromycin 15 (AZM15)	16	
	Pefloxacin 5 (PF5)	18	
Coagulase negative *Staphylococci*	Linezolid 30 (LZ30)	26	346
	SNPs	24	
	Teicoplanin 30 (TEI30)	15	
	Vancomycin 30 (VA30)	16	
	Doxycycline 30 (DO30)	20	
	Tetracycline 30 (TE30)	23	
Enterococcus faecalis	Linezolid 30 (LZ30)	25	24 (a)
	Ampicillin10 (AMP10)	19	
	Teicoplanin 30 (TEI30)	17	
	Imipenam 10 (IPM10)	15	
	Vancomycin 30 (VA30)	19	
	Ciprofloxacin 5 (CIP)	20	
Enterococcus faecalis	SNPs	24	24 (b)
	Levofloxacin 5 (LE5)	18	
	High level gentamycin	20	
	120 (HIG120)		
Klebsiella pneumoniae	SNPs	25	1019
	Ceftaroline 30 (CPT30)	27	
	Cefixime 5 (CFM5)	22	
	Chloramphenicol 30 (C30)	22	
	Tobramycin 10 (TOB10)	15	
	Tigecycline 15 (TGC15)	13	

The sensitivity of *Salmonella* to silver nanoparticles (10 µg/ml)has been documented as the "sensitivity depends on the strain, and dose" (Losasso et al. 2014; Petrus et al. 2011).When *Salmonella braenderup* was exposed to AgNPs, membranes of the microbes were ruptured (Diego et al., 2020).

5. Co-agulase negative *Staphylococci* can cause infections when they reach the blood stream. An iinhibition zone of 25 mm was produced by prepared AgNPs. Linezolid was the only antibiotics with a bigger zone of 26 mm than that of AgNPs.

6. Treatment of *Enterococcus foecalis* is difficult when it reaches the urinary tract (Kau et al., 2005). Linezolid 30 is effective against this species. In the current study, Linezolid 30 produced a 25 mm of zone of inhibition. Prepared AgNPs (30µg/ml) had almost the same effect

as that of Linezolid with a 24 mm zone of inhibition. Traditional approaches are less effective in treatment of *Enterococcus foecalis* how ever; silver nanoparticles could be a potent solution to treat them (Sadony et al., 2019).

7. *Klebsiella pneumoniae* produces extended spectrum β-lactamase, in turn, this compound can breakdown many medicines. Carpabenam has been found effective against ESBL-producing *Klebsiella pneumonia* (Patersonet al. 2004). However, *Klebsiella pneumoniae* shows greater resistance against considerably safe AgNPs but at higher doses as 100µg/ml, AgNPs formed a 25 mm zone of inhibition. *Klebsiella pneumoniae* has been resistant to ampicillin and AgNPs were tested against this species to get effective results (Hamida et al., 2020).

CONCLUSION

Streptomyces Spp. AK3 has been found to synthesize expected quality silver nanoparticles. Synthesized silver nanoparticles with this strain are much closer to the most effective antibiotics against human pathogens in terms of the formation of zones of inhibition as represented in table-1. The strain synthesized silver nanoparticles should be preferred to antibiotics for the sake of having lesser side effects, being cost-effective, and working as a tool against multi-drug resistant bacteria.

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Biotechnological Communication

Molecular Characterization of Phosphate Solubilizing Endophytic Fungi and its Effect on Growth of the Maize, *Zea mays*

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ABSTRACT

In most of the agricultural soils phosphorus is present in considerable amounts but its availability to plants is limited because it is fixed in soil as insoluble phosphate. The over application of chemical phosphorus fertilizers does not increase the bioavailability of phosphorus but the leads to adverse effects on soil fertility. Phosphate solubilizing microorganisms (PSMs) can hydrolyse phosphorus compound into soluble form and make them available to plants. There is need of identifying such microbes which can be used as bioinoculants in agricultural soils to increase plant growth and productivity. In this study 64 endophytic fungi were isolated from maize plants by inoculating surface sterilized parts on potato dextrose agar medium. The phosphate solubilization index of fungal cultures was determined by measuring the hole zone formed around cultures on Pikovskaya's agar medium and the morphological identification of cultures was based on study of colony morphology and microscopic characters. It was seen that about 41% of the cultures had phosphate solubilization potential. The morphological characterization of cultures revealed that 23 cultures were of *Aspergillus niger*, 2 cultures were of *Penicillium oxalicum* and 1 culture was of *Curvularia* sp. aff. *C. Verruculosa* Tandon & Bilgrami ex M. B. Ellis. *Penicillium oxalicum* (E 137) and two culture of *Aspergillus niger* (E 204 and E 215) having high PSI were identified using molecular techniques and the effect of addition of spore suspension of these cultures on seed germination, seedling growth and plant growth was determined. The results of the study indicate that the addition of fungal spores significantly increases the seedling growth and plant growth in pot culture experiments suggesting that they may serve as potential biofertilizer/ bioinoculants.

KEY WORDS: ENDOPHYTIC FUNGI, PHOSPHATE SOLUBILIZING MICROORGANISMS, PLANT GROWTH, SEED GERMINATION, ZEA MAYS.

INTRODUCTION

Agriculture is the utmost important area of Indian economy and it accounts for 18% of GDP (Gross domestic product). About 50% of Indian population depends upon agriculture for their livelihood and increasing agricultural production is crucial for feeding current and future population of humans (Madhusudhan 2015). Phosphate is one of the major limiting factors for proper plant growth due to its low availability in agricultural fields. Phosphorus forms an essential constituent of many cellular molecules including nucleic acids, phospholipids, ATP's and enzymes. It is needed in almost every aspect of plant growth like growth of roots, shoots, formation of flowers, seeds, energy production, nitrogen fixation, etc. and it accounts for 0.2-0.8% of dry weights of plants (El-Hamshary et al., 2019; Kalayu 2019).

Most of the soils contain considerable amount of phosphate but its availability becomes limited because a large portion of phosphorus is fixed as insoluble phosphate of iron, aluminium and calcium. Organic

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matter present in soil is a major source of immobilized phosphate and 20-80% of phosphate present in soil is in this form. The soluble phosphate is highly reactive with other elements andin acidic soils phosphate is complexed with iron and aluminium compounds while in calcareous soils calcium phosphate is predominately formed (Rashmi et al., 2018).The acidic soils found in tropical and subtropical regions are often phosphorus deficient because they have high phosphate fixation capacity (Rashmi et al., 2018). Most of the former use chemical overcome phosphorus deficiency (Chouyia et al., 2020; Mazrou et al., 2020).

The addition of chemical fertilizers to overcome the phosphate availability in soil results not only in increase in agricultural cost but also causes adverse effect on soil health. The addition of phosphorus fertilizers in soils does not increase its bioavailability as 75-90% of phosphate fertilizers are precipitated by formation of metal ion complexes and result in fixation in soil and only 5% of added phosphorus is available to plants (Pande et al. 2017). The repeated non judicial application of phosphate containing chemical fertilizers result in loss of soil fertility by decreasing the microbial diversity. It has been suggested that accumulated phosphate in agricultural soils can sustain crop yields over 100 years (Gizaw et al. 2017). Phosphate solubilizing microorganisms are a group of microorganisms which help in solubilizing the phosphate present in soil and make it available for plant growth (Rashmi et al. 2018). The addition of PSMs to soil is an eco-friendly alternative which can make the phosphorus available to plant and can decrease the agricultural cost and toxic effect of indiscriminate use of chemical fertilizers (Qarni et al., 2021; Raymond et al., 2021).

The knowledge about phosphate solubilizing microorganisms has gradually increased over the last few years and a large number of bacteria and fungi have been identified which have high phosphate solubilizing activity. Fungi can travel long distances and are more important and they form about 0.1-0.5% of the total fungal soil population in nature (Mahadeva murthy et al., 2016; Zhu et al., 2017; Mazrou et al. 2020).Bioprospection of phosphate solubilizing endophytic microbes has become extremely relevant as they can colonize plants without inducing any apparent disease symptoms and benefit the host plant by providing protection against stress and pathogens (Zheng et al. 2016; Mazrou et al. 2020). They produce various secondary metabolites and help in different process of plant including nitrogen fixation, phosphate solubilization etc. (Santoyo et al. 2016). Phosphate solubilizing endophytic fungi are very competitive and aggressive colonizers. The common phosphate solubilizing endophytic fungal genera are *Aspergillus, Penicillium, Curvularia* and *Piriformospora*, where as the common phosphate solubilizing endophytic bacterial genera are *Bacillus, Pseudomonas* and *Rhizobium* (Matos et al. 2017; Singh et al., 2020; Abawari et al. 2021; Fouda et al., 2021).

An important constraint in use of biological organisms in agricultural fields is their inability to grow and adapt to different habitats. Hence, it is necessary that indigenous microorganisms may be isolated and identified which canbe used for inoculation in agricultural fields. The indigenous microorganisms are native to the particular environment as they areadapted for growth at particular climatic condition. The application of indigenous microorganisms as biofertilizers/ bioinoculants/ biocontrol agent is crucial for sustainable agriculture (Kumar and Gopal 2015; Jan et al. 2020; Fouda et al. 2021).

In view of above it was of interest to isolate and identify indigenous fungal genera having high phosphate solubilization potential, so they may be used in agricultural fields as bioinoculants for promoting sustainable agriculture in local fields. In the present study we have isolated and identified 26 end ophytic fungi having phosphate solubilizing activity from maize (*Zea mays*) plants growing agricultural fields of Ratadiya Village near local fields. Three cultures showing high phosphate solubilizing index, namely E 204 (*Aspergillus niger*), E 215 (*Aspergillus niger*) and E137 (*Penicillium oxalicum*) were characterized at molecular level and their effects as bioinoculants on seedling growth and plant growth were studied.

MATERIAL AND METHODS

Plant material was collected in July, 2017 from the agricultural fields of Ratadiya village near Ujjain (M.P.). Randomly growing sixteen completely healthy and mature maize plants along with roots were uprooted, packed in sterilized polythene bags and brought to the laboratory. The plant samples were stored at room temperature and processed within 24 hours. Different parts of plant i.e., roots and leaves were used for isolation of endophytic fungi by following the modified method of Han et al., (2013).

The plant material was thoroughly washed with tap water and cut into 1-1.5 cm pieces and surface sterilization was done using 70% ethanol and 0.1% $HgCl_2$ for 2 minutes and then washed twice with sterilized distilled water. The plant pieces were placed on potato dextrose agar (PDA) medium containing chloramphenicol (40µg/ml) and incubated at 28 ± 2°C for 5 to 7 days. The hyphal tips which emerged from the plant material were aseptically cut and transferred to fresh PDA medium slants and incubated at 28 ± 2°C to obtained fungal cultures. Sub culturing of cultures was done to obtain pure cultures of endophytic fungi.

Morphological identification of fungal cultures was done based on study of colony characteristics and arrangement of spores using fungal keys. A small amount of culture was used for preparing wet mount on sterile glass slide. The culture was stained with lactophenol cotton blue (LCB) and the slide was examined under light microscope at different magnifications (10X, 45X and 100X). The identification was confirmed by National Fungal Culture Collection of India (NFCCI), Pune, India. The fungal

cultures were preserved in PDA slants and stored at -20°C (Visagie et al. 2014; Nyongsa et al. 2015). Molecular characterization of three cultures was done. Genomic DNA was extracted by using 5 to 7 days old fungal cultures grown on PDA medium. The DNA was amplified using primers ITS4(R-5'TCCTCCGCTTATTGATATGC3') and ITS 5(F-5'GGAAGTAAAAGTCGTAACAAGG3') and the PCR product were purified and sequenced. The resulting sequencing wasmatched in BLAST analysis software at NCBI (https://blast.ncbi.nlm.nih.gov) and phylogenetic tree were constructed using MEGA X software.

Fungal cultures were deposited at NFCCI (National Fungal Culture Collection of India), Pune, India, for obtaining accession numbers (Singh et al., 2020). The phosphate solubilizing potential of endophytic fungal cultures was determined by using modified method of Mazrou et al., (2020). The cultures were grown in Petri dishes containing Pikovskaya's agar medium at28 ± 2°C for 5 daysand the halozone formed around the fungal colony was measured. The phosphate solubilization index (PSI) was calculated by using the following formula:

$$PSI = \frac{\text{Colony diameter + Halo zone diameter}}{\text{Colony diameter}}$$

The spore suspension of fungal cultures was prepared in sterile distilled water. The fungal cultures of *Aspergillus niger* (E 204 and E 215) and *Penicillium oxalicum* (E 137)were grown in 250 ml conical flask containing 50 ml PDA medium for 10 days at 28 ± 2°C and the spores were harvested using 50 ml sterile distilled water. Concentration of spores per ml was determined using Haemocytometer and a final spore suspension 10^5-10^6 spores per ml was made. The spore suspensionwas used for bioinoculation on same day on which it was prepared (Mahadevamurthy et al., 2016). The seeds of *Zea mays* variety MRM 3777 were purchased from local market and stored in air tight containers at room temperature.

The maize seeds were surface sterilized using 70% ethanol and 0.1% $HgCl_2$ and washed twice with sterile distilled water. The seeds were soaked in spore suspension for 24 hours at room temperature and washed once with sterile distilled water. Three seeds were placed in sterilized Petri dishes containing sterilized cotton layer. The Petri dishes were incubated at 28 ± 2°C for 5 days. The experiments were done in triplicate and seeds treated with distilled water were used as control. After 5 days of growth the germination percentage and the length of root and shoot was measured. The plant parts were kept at 80°C for 2 hours after which their dry weights were determined. The vigor index was calculated using the mathematical expression described below (Mahadevamurthy et al. 2016).

Vigor index = Seed germination (%) x [Mean Root Length + Mean Shoot Length]

Soil from agricultural field of Ratadiya village was used for performing pot culture experiments. The air dried and sieved soil samples were used for determining pH, available potash, available phosphorus, available nitrogen and organic carbon (Wagh et al., 2013; Das et al., 2017). The soil used in experiments was sterilized three times with an interval of 24 hours between each sterilization cycle. This was done to remove microbes present in soil so that only the effect of inoculated fungal cultures could be observed. The experiments were performed in 1L poly propylene autoclavable beakers having capacity of containing 1kg soil. In each pot (beaker) 6 seeds were placed 1 cm below the top layer and 5 ml spore suspension (10^5-10^6 spores per ml) was added on the top of each seed then the seeds were covered with soil layer.

The pots were kept at room temperature and exposed to natural conditions throughout the day. The soil in pots was kept moist by adding 50-100 ml water every day according to weather conditions. After 7 days the plants were removed from the soil and the plant height, root length and area of largest leaf were determined. The plants parts were wrapped in aluminum foil and kept at 80°C for 24 hours and dry weights were determined to measure the biomass formed. The pot culture experiments were performed in the month of October and November (Singh et al., 2018). All the experiments were performed in triplicates and statistical analysis was done using one-way analysis of variance (ANOVA) followed by Tukey's HSD test. The mean values of samples and standard deviation were calculated. The values at $P \leq 0.01$ and $P \leq 0.05$ were considered as significant.

RESULTS AND DISCUSSION

Isolation and identification of phosphate solubilizing endophytic fungi: In the present study 64 endophytic fungal cultures were isolated from different parts of maize. Out of these cultures 20 (31.25%) were isolated from roots and 44 (68.75%) were isolated from leaves (Table 1). The phosphate solubilizing potential of fungal cultures was determined by measuring the halo zone formed around the cultures. It was seen that 26 (40.62%) fungal cultures had phosphate solubilizing activity (Table 1). The morphological identification of these cultures showed that 23 (35.93%) cultures belonged to *Aspergillus niger*, two (3.12%) cultures belonged to *Penicillium oxalicum* and one (1.56%) belonged to *Curvularia* sp. aff. *C. Verruculosa* Tandon & Bilgrami ex M. B. Ellis (Table 1). E137 (*Penicillium oxalicum*) showed highest phosphate solubilization index followed by E 215 (*Aspergillus niger*) and E 204 (*Aspergillus niger*) (Figure1 and Table 1). Molecular characterization two cultures of *Aspergillus niger* (E 204, E 215) and one culture of *Penicillium oxalicum* (E 137) was done using 18S rDNA sequencing and phylogenetic trees constructed using MEGA X software are shown in Figure 2, Figure 3 and Figure 4.

The accession numbers of cultures obtained from NFCCI, Pune are NFCCI 4851 (E 137: *Penicilliumoxalicum)*, NFCCI 4852 (E 204: *Aspergillus niger*) and NFCCI 4853 (E

215: *Aspergillus niger*). Earlier study of rhizospheric soil samples from different plants in Jimma town and Manna district farmlands in Ethiopia revealed that *Aspergillus* spp., *Penicillium* spp. and *Fusarium* spp. had phosphate solubilization activities (Elias et al., 2016). Cultures of *Aspergillus niger* having high phosphate solubilization potential were also isolated from rhizospheric soil of *Cucumis, ocimum and* Eruca *plants* in Jeddah, Saudi Arabia (El-Hamshary et al., 2019).

Phosphate solubilizing endophytic fungi belonging to *Penicillium oxalicum*, *P. citrinums* and *Aspergillus* sp. Were also reported from sea weeds collected from Rameswarem coastal regions in India (Noorjahan et al., 2019). Culturable endophytes belonging to species of *Aspergillus niger* and *Penicillium oxalicum* having phosphate solubilizing potential have been reported from *T. wallichiana* (Adhikari and Pandey 2018).

Figure 1: Morphological characterization and phosphate solubilization potential of fungal cultures. A. Halo zone around colony of *Aspergillus niger* (E 204); B. Conidiospores of *Aspergillus niger* (E 204); C. Halo zone around colony of *Aspergillus niger* (E 215); D. Conidiospores of *Aspergillus niger* (E 215); E. Halo zone around colony of *Penicillium E. Halo zone around colony of Penicillium oxalicum (E 137); F. Conidiospores of Penicillium oxalicum (E 137)*

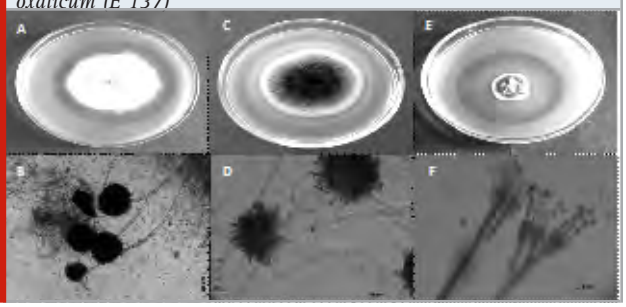


Table 1. Phosphate solubilization index of endophytic fungi isolated from different parts of maize

S. No.	Culture ID	Plant part used for isolation	Name of fungi	Phosphate Solubilization Index (PSI) on 5th Day
1	E 198	Root	*Aspergillus niger*	1.32±0.02
2	E 204	Root	*Aspergillus niger*	1.42±0.06
3	E 205	Root	*Aspergillus niger*	1.35±0.01
4	E 209	Root	*Aspergillus niger*	1.14±0.02
5	E 210	Root	*Aspergillus niger*	1.13±0.02
6	E 215	Root	*Aspergillus niger*	1.67±0.08
7	E 216	Root	*Aspergillus niger*	1.08±0.03
8	E 218	Root	*Penicillium oxalicum*	1.06±0.03
9	E 224	Root	*Aspergillus niger*	1.08±0.03
10	E 102	Leaf	*Aspergillus niger*	1.17±0.02
11	E 105	Leaf	*Aspergillus niger*	1.07±0.03
12	E 114	Leaf	*Aspergillus niger*	1.10±0.02
13	E 115	Leaf	*Curvulariasp.* aff. *C. verruculosa* Tandon & Bilgrami ex M. B. Ellis	1.02±0.02
14	E 118	Leaf	*Aspergillus niger*	1.04±0.03
15	E 122	Leaf	*Aspergillus niger*	1.27±0.03
16	E 123	Leaf	*Aspergillus niger*	1.23±0.04
17	E 124	Leaf	*Aspergillus niger*	1.10±0.02
18	E 125	Leaf	*Aspergillus niger*	1.37±0.04
19	E 128	Leaf	*Aspergillus niger*	1.12±0.04
20	E 135	Leaf	*Aspergillus niger*	1.10±0.02
21	E 137	Leaf	*Penicillium oxalicum*	2.93±0.05
22	E 139	Leaf	*Aspergillus niger*	1.04±0.03
23	E 142	Leaf	*Aspergillus niger*	1.35±0.01
24	E 147	Leaf	*Aspergillus niger*	1.08±0.03
25	E 158	Leaf	*Aspergillus niger*	1.08±0.03
26	E 164	Leaf	*Aspergillus niger*	1.16±0.04

Figure 2: Phylogenetic tree of the fungal culture E 204 (NFCCI 4852) using ITS region of rDNA. The tree was constructed using Maximum Likelihood method and Tamura-Nei model and the highest log likelihood (-4618.01) is shown in the figure. In this analysis 24 nucleotide sequences were used and evolutionary analysis was conducted in MEGA X

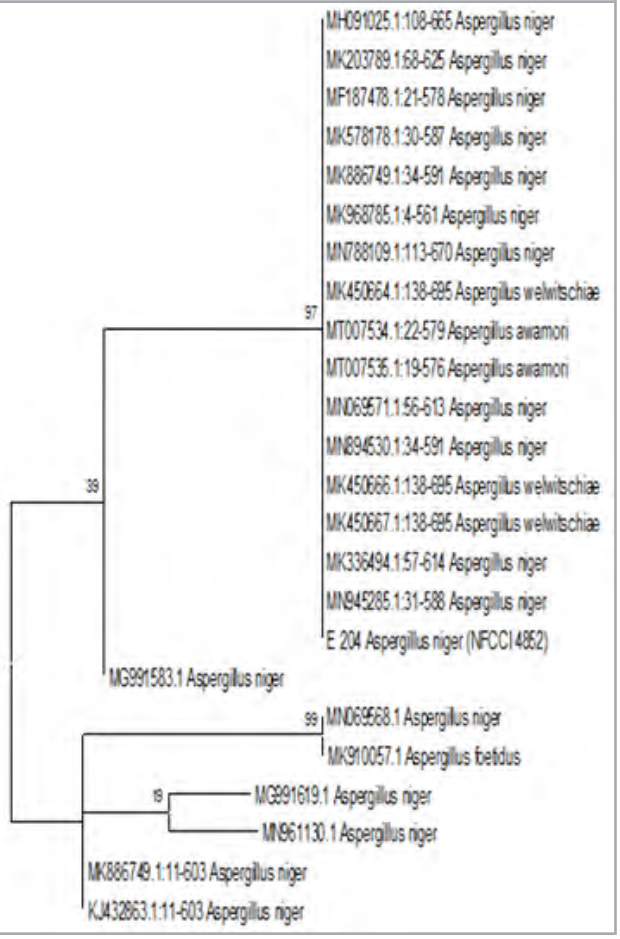
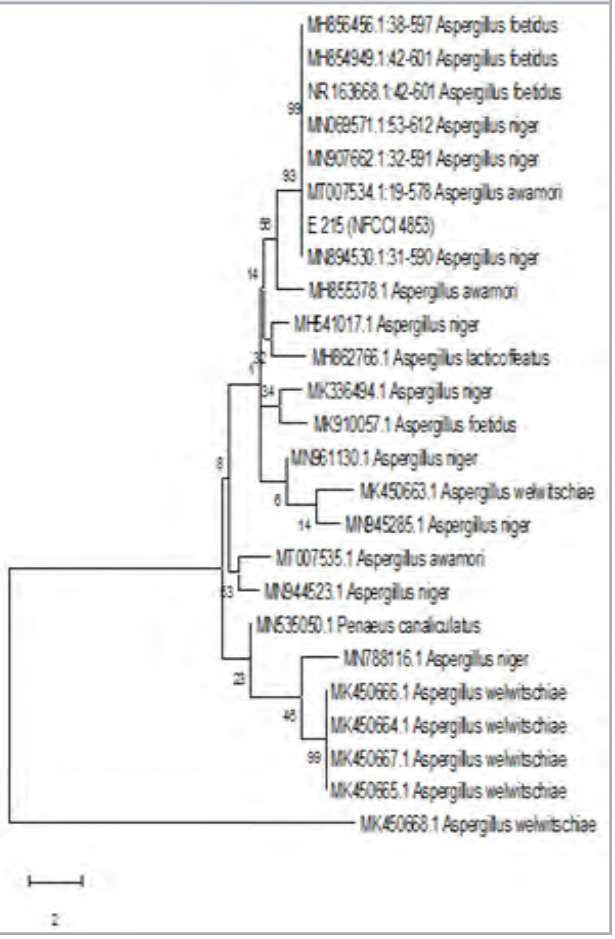


Figure 3: Phylogenetic tree of the fungal culture E 215 (NFCCI 4853) using ITS region of rDNA. The tree was constructed using Maximum Likelihood method and Tamura-Nei model and the highest log likelihood (-13958.58) is shown in this figure. In this analysis 24 nucleotide sequences were used and evolutionary analysis was conducted in MEGA X



In the present study also phosphate solubilizing potential was seen in endophytic fungal cultures belonging to *Aspergillus niger*, *Penicillium oxalicum* and *Curvularia* sp. aff. *C. verruculosa* Tandon & Bilgrami ex M. B. Ellis. Thus, the results of present study corroborate with the findings of earlier worker and strengthen the fact that cultures of *Aspergillus* spp. and *Penicillium* spp. have high phosphate solubilizing potential. It is suggested that the application of these fungi as bioinoculants in agricultural fields can act as alternatives of chemical fertilizers and help in promoting sustainable agriculture practices (Baron et al., 2018; Noorjahan et al., 2019). The potential of phosphate solubilizing microorganisms as biofertilizers / bioinoculants was also been suggested by other workers (Mazrou et al., 2020; Abawari et al., 2021).

Effect of fungal treatment on seed germination, seedling growth and plant growth: The effect of E 204 (*Aspergillus niger*), E 215 (*Aspergillus niger*) and E 137 (*Penicillium oxalicum*), as bioinoculants on seed germination and seedling growth was studied in Petri dishes (Figure 5). It was seen that when spore suspension containing 10^5-10^6 spores per ml was used then percentage of germination in control (seeds treated with sterile distilled water) and seeds treated with fungal spores remained 100% indicating that these fungi do not have negative effect on germination of seeds when added in this concentration.

However, the application of spore suspension above this concentration resulted in damage to seeds. The effect of fungal treatment on root length and shoot length is shown in Figure 6. The analysis of results reveal that these fungal cultures significantly enhance the root length and shoot length. The mean dry weights of roots in control seeds were0.087±0.003 mg and the mean dry weights of roots of seeds treated with E 204 (*Aspergillus niger*), E 215 (*Aspergillus niger*) and E 137 (*Penicillium oxalicum*) was 0.147±0.004 mg, 0.183±0.003 mg and 0.135±0.003 mg respectively. Similarly, the mean dry weights of shoots in control seeds were 0.673±0.004

mg and the mean dry weights of shoots of seeds treated with E 204 (*Aspergillus niger*), E 215 (*Aspergillus niger*) and E 137 (*Penicillium oxalicum*) was 0.762±0.003mg, 0.883±0.004 mg and 0.889±0.004 mg respectively.

The seedling vigor also increased as a result of fungal treatment. The vigor index of control seeds was 1886 and the vigor index increased on treatment with fungal spores. The highest vigor index was seen by treatment with E 204 (3603) followed by E 137 (2856) and E 215 (2759). These results indicate that treatment of maize seeds with fungal spores significantly improves seedling growth and vigor of seedlings.

Figure 4: Phylogenetic tree of the fungal culture E 137 (NFCCI 4851) using ITS region of rDNA. The tree was constructed using Neighbor-Joining method. The sum of branch length = 0.27637271 is shown in tree. In this analysis 38 nucleotide sequences were used and evolutionary analysis was conducted in MEGA X

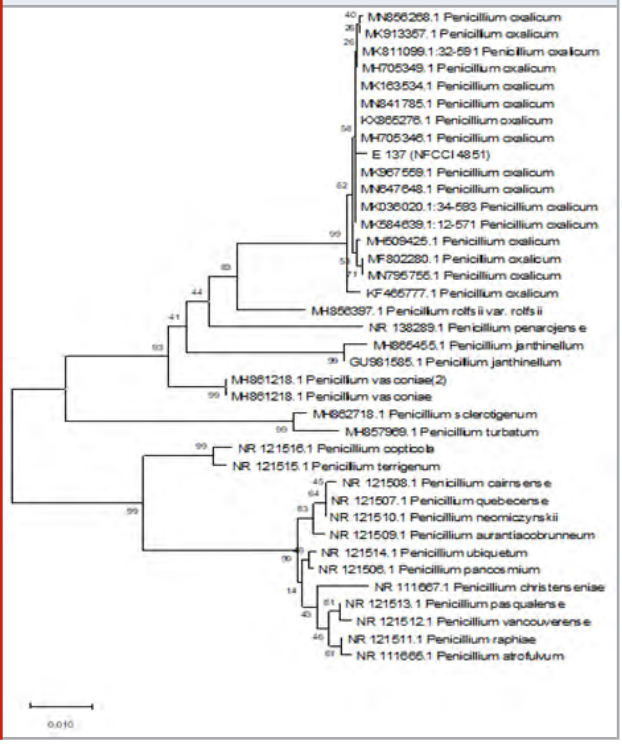
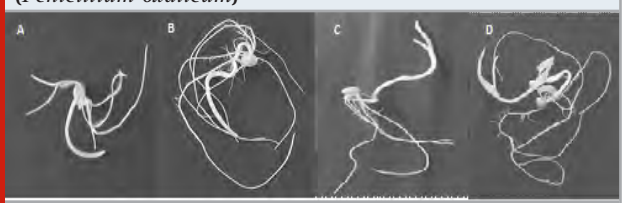


Figure 5: Effect of fungal spore suspension on seedling growth of maize. A. Control (Distilled water); B. E 204 (*Aspergillus niger*); C. E 215 (*Aspergillus niger*); D. E 137 (*Penicillium oxalicum*)



The effect of fungal treatment on plant growth was studied in pot culture experiments (Figure 7). The maize plants were grown in sterilized soil having pH 7.61, organic carbon 0.86%, available nitrogen 291 kg/ha, available phosphorus 36.33kg/ha and available potassium 723.33 kg/ha. This indicates that the agricultural field soil contains high level of phosphorus and other nutrients which could be due to continuous addition of chemical fertilizers in the agricultural fields. The plant height, root length, area of largest leaf and dry weights of roots and shoots were compared in control plants and in plant grown in soil supplemented with fungal spores of E 204, E 215 and E 137 (Yin et al., 2015).

Figure 6: Effect of fungal spore treatment on root length and shoot length of maize seeds

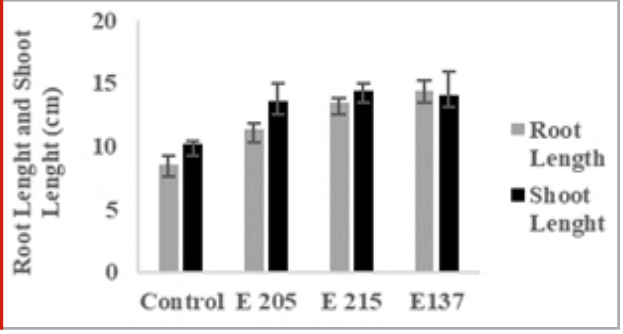
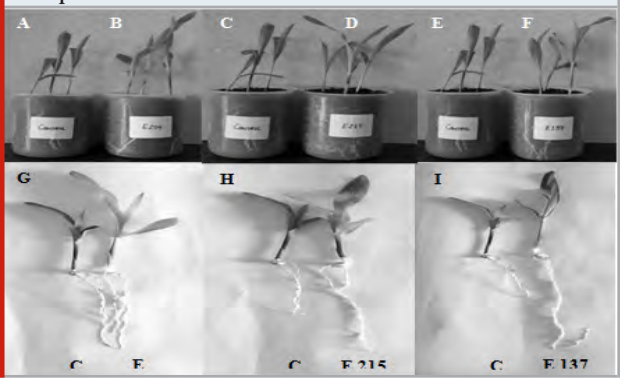


Figure 7: Effect of fungal treatment on growth of maize plants. A, C and E: Control; B, D and F: Treated with E 204 (*Aspergillus niger*), E 215 (*Aspergillus niger*) and E 137 (*Penicillium oxalicum*) respectively. G, H and I: Comparison

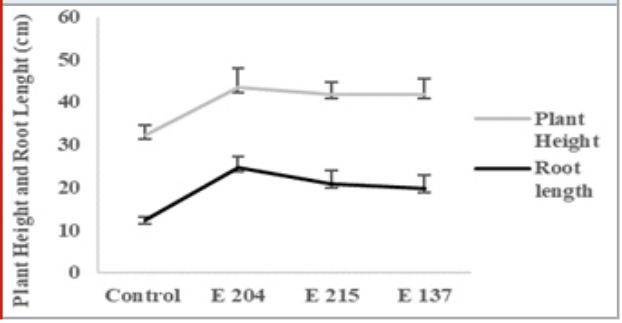


It was seen that root length and shoot length significantly increased inall the fungal treatmentsin comparison to control (Figure 7 and Figure 8). E 204 (*Aspergillus niger*) culture showed best results and there was 1.34 folds increase in plant height and 1.58 folds increase in root length when treated with E 204 (*Aspergillus niger*). There was 1.29 folds increase in plant height when treated with E 215 (*Aspergillus niger*) and 1.29 folds increase in plant height when treated with E137(*Penicillium oxalicum*). The root length increased 1.66 folds when treated with E 215 (*Aspergillus niger*) and 1.97 folds whentreated with E137(*Penicillium oxalicum*). Further, the mean dry weight of control plants was 0.117± 0.004 mg and the mean dry weight plants grown in soil containing E 204 (*Aspergillus niger*), E 215 (*Aspergillus niger*) and E137(*Penicillium oxalicum*) was found to 0.123± 0.005

mg, 0.132± 0.004 mg and 0.123± 0.005 mg respectively (Jan et al. 2020).

Mean dry weight of roots of control plants was 0.122± 0.005 mg while the mean dry weight roots grown in soil containing E 204 (*Aspergillus niger*), E 215 (*Aspergillus niger*) and E137(*Penicillium oxalicum*) was seen to be 0.130± 0.004 mg, 0.140± 0.004 mg and 0.172± 0.004 mg respectively. The leaf area of the longest leaf of control plant was 81.9 cm^2 and it increased significantly when treated with fungal cultures.The largest leaf area was found by treatment with E 137 (170.0 cm^2) followed by E 215 (116.9 cm^2) and E 204 (109.2 cm^2). These results indicate that addition of fungal spores in soil positively influence the growth of maize plants which could be due to phosphate solubilizing ability of these fungal cultures or due to other plant growth promoting activities of these endophytic fungi (Yin et al. 2015).

Figure 8: Graph showing difference in plant height and root length of maize plants



Over application of chemical fertilizers for increasing the agricultural productivity adversely affects agricultural sustainability and causes harmful effects on environment. Hence, steps are needed to develop alternative strategies for improving plant nutrition and increasing agricultural production (Yin et al. 2015). There is a global trend for bioprospecting indigenous microorganisms which may be used for promoting sustainable agriculture practices and help in decreasing the use of chemical fertilizers in agricultural fields. It has been suggested that bioformulations of indigenous microbes having phosphate solubilizing activities may be used for promoting plant growth in view of food security scenario. The application of indigenous microbes is an ecofriendly, environmentally safe and healthy practice having potential to create better crops. It has been observed that endophytic microbes have several plant growths promoting activities including phosphate solubilization, siderophore production, IAA production, etc (Kumar and Gopal 2015; Ahkami et al., 2017; Jan et al., 2020).

The relationship of host plant with endophytic microorganisms is very special and significantly influence the formation of different metabolites in plant which provide various benefits to plants (Jia et al., 2016). In this study it has been demonstrated that bioinoculation of endophytic fungi belonging to *Aspergillus niger* and *Penicillium oxalicum* significantly improves growth of maize plants. Earlier studies have also demonstrated that inoculation of endophytic bacteria and fungi have positive influence on plant growth (Matos et al., 2017; Adhikari and Pandey 2018). It has been reported that endophytic fungi *Cladosporium cladosporiodes* positively influenced the shoot growth while *Aspergillus amstelodami* positively influenced the root length in the rice seedlings (Lalngaihawmi et al., 2018).

Study by Yin et al. (2015) reported that *Penicillium oxalicum* iscapable of promoting maize growth in calcareous soils and studies in Egypt have demonstrated that inoculation of *Penicillium crustosum* and *Penicillium chrysogenum* significantly increase root length in maize (Hassan et al., 2017). The plant growth promotion activities of various bacteria and fungi have also been reported by other (Banu et al., 2019; Aliyat et al., 2020, Chouyia et al., 2020; Turbat et al., 2020). Hence, our results are in accordance to earlier studies. Moreover, the fungal cultures isolated in this study are indigenous to this region andit appears that they may be used as bioinoculants for improving soil health and increasing plant growth. However, further studies are needed to examine the effects of these cultures in unsterilized soil and in field conditions (Turbat et al. 2020).

CONCLUSION

In this study 26 phosphate solubilizing endophytic fungi have been isolated and identified. Three fungal cultures having high PSI have been identified as *Aspergillus niger* (E 204 and E 215) and *Penicillium oxalicum* (E 137) using 18S rDNA sequencing. These three fungi positively influence the seedling growth and growth of maize plants in pot culture experiments when used as bioinoculants. The findings suggest that these fungi may be used as bioinoculants, biofertilizers for promoting sustainable agriculture. However, further studies are needed to strengthen these findings and examine the effect of these cultures in field conditions in unsterilized soils before theymay be used as bioinoculants in field conditions.

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Conflict of Interests: The authors declare no conflict among their interests while completing this research.

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Biotechnological Communication

Quality Rating and Shelf-Life Prediction of Curd Products with Biocorrective Action

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ABSTRACT

The effective functioning of the body's physiological systems is determined by the presence of essential substances, including macro- and trace elements, whose deficiency leads to various homeostasis disorders. In this regard, the development of new daily consumed foods with targeted corrective action on the human body's nutritional status is an essential and urgent task. Fortification of curd products with biologically active substances of plant origin makes it possible to obtain economically widely available bio corrective food systems. The study objects were curd products with a 5% fat mass fraction without adding and with adding 20% plant composition based on flours made of walnut, sesame, and pumpkin seeds. The storage duration was 90 days at minus 20±2 ^{0}C. Qualities and quantities of individual groups of high volatile compounds (HVC) in the equilibrium gas phase (EGP) over the samples of curd products were assessed using an odor analyzer "MAG-8" with the electronic nose (e-nose) methodology. It was found that samples No. 1 and 2 are identical by more than 85% in quality of the EGP. The data obtained demonstrate the prevalence of common to curd products organoleptic characteristics, which are in line with traditional ones expected by the consumer. The composition and content of HVC in air samples over the test samples during the entire period of storage, determining the degree of rancidity of the fat fraction and being indicators of shelf life, were assessed. Based on the test data obtained, an information system for modeling and predicting the shelf life of curd food systems was developed. It includes input parameters – type of packaging, storage conditions (temperature and relative humidity of ambient air), and the plant component content. The output parameter is the area of the "visual imprint" of the maximum sensor signals in the EGP of the study sample assessing the total intensity of odor proportional to the concentration of HVC.

KEY WORDS: VEGETABLE BIOCORRECTORS, CURD PRODUCTS, FOOD STATUS, ODOR ANALYZER, SHELF LIFE.

INTRODUCTION

Adjusting the diet through the introduction of effective sources of vital biocorrective nutrients (mineral elements, vitamins, and other biologically active substances) into the list of ingredients of daily consumption is a pressing technological and nutritional challenge (Rodionova et al., 2016; Antipova et al., 2018; Eamonn, 2019; Bouillon, 2020). The enrichment of curd products with biologically active substances opens up new possibilities of a directed positive biocorrective effect on various functions of the body to prevent and treat a wide range of diseases of nutritional nature (Dmitrieva, 2018). One of the most promising sources of biologically active substances and minerals of natural origin is partially defatted flours made of walnut, sesame, and pumpkin seeds (Yu et al., 2020). Walnut flour contains sphingolipids, phytosterols, phospholipids, carotenoids, and a high lysine level, thus accelerating the protein digestion process.

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It has an antineoplastic action (Rodionova, 2019; Donskaya and Drozhzhin, 2020).

Sesame seed flour is a valuable source of calcium and phosphorus and is recommended for treating osteoporosis, strengthening bones and tooth enamel, normalizing lipid metabolism, preventing atherosclerosis, hypertension, and arthritis (Donskaya and Drozhzhin, 2020). Pumpkin seed flour is a source of native proteins and contains a significant amount of dietary fiber, vitamins A, E, D, K, and B-group vitamins, essential amino acids, essential polyunsaturated fatty acids ω-3 and ω-6, sugars, phytosterols, organic acids, salts of phosphoric and silicic acids, macro- and trace elements, among which potassium, phosphorus, magnesium, sodium, and zinc should be highlighted (Antipova et al., 2018; Aydar et al., 2021). The work aims to assess the storage stability and model the storage process of new curd and vegetable products using an e-nose system based on piezo sensors. The study objects were curd products with a 5% fat mass fraction without adding (sample No. 1, control) and with adding 20% functional plant composition based on partially defatted flours made of walnut, sesame, and pumpkin seeds (sample No. 2). The plant composition formulation was balanced by the content of polyunsaturated fatty acids ω-6 and ω-3 (the ratio was 5.8:1) as a result of mathematical processing, using a developed software product implementing the object-oriented programming (software languages Ruby 2.2, Ruby on Rails 4.2) (Rodionova & Alekseeva, 2015; Liu et al., 2021). The study objects were stored for 90 days at minus 20±2°C.

MATERIAL AND METHODS

Scent research of test samples was carried out on a laboratory odor analyzer "MAG-8" with the e-nose methodology (Yu et al., 2020; Aydar et al., 2021). Eight sensors based on VAW-type piezoelectric quartz resonators with a reference oscillation frequency of 10.0 MHz with various film sorbents on the electrodes were used as a measuring array. Coatings were chosen according to the test task (possible emission from samples of different organic compound classes): sensor 1 – polyvinylpyrrolidone (PVP); sensor 2–bee glue (BG); sensor 3– dicyclohexano-18-crown-6 (DCH18C6); sensor 4–bromocresol green (BCG); sensor 5– polyethylene glycol succinate (PEGS); sensor 6 – polyethylene glycol PEG-2000 (PEG-2000); sensor 7–Tween-40 (Tween); sensor 8 – trioctylphosphinoxide (TOPO).

During experimental studies, test samples weighing 5.00 g were placed in glass samplers, sealed tightly, and incubated at room temperature (20±1°C) for at least 30 min to saturate the EGP over samples (Liu et al., 2021). 3 cm3 of EGP was taken through the membrane with individual syringes and injected into the detection cell. The sensor array background was from 15 to 30 Hz.s. The measurement duration was 60 s, the recording mode of sensor responses was uniform at 1 s intervals, and the measurement error was 5-10%. The initial primary analytical information of the e-nose system is a chrono-periodogram. It is the piezo sensor output curve for the time of measuring the dependence of changes in each sensor oscillation frequency on time (figures from the device software). Further, the selective information of the chrono-periodograms is used for processing and decision making (Donskaya and Drozhzhin, 2020; Yu et al., 2020).

The sum analytical signal was formed using an integral signal processing algorithm of 8 sensors represented by a "visual imprint". In order to determine the total odor composition of samples, full "visual imprints" of the maximums (the largest responses of 8 sensors) were used. "Visual imprints" of the maximums are constructed according to the maximum responses of the sensors in the EGP of samples during the measurement time (60 s). They make it possible to establish the similarity and difference of the high volatile odor fraction composition over analyzed samples (Tan & Xu, 2020). The areas of the figures are calculated automatically in the device software. The "visual imprint" shape with characteristic distributions along the response axes, determined by the set of compounds in the RGF, was chosen as the criterion for qualitative assessment of the difference in the odor of the analyzed samples.

Identification parameters Aij, calculated from the sensor signals in analyzed samples, were used to recognize individual classes of compounds in the mixture. The quantitative characteristics were: 1) SΣ, Hz.s – the full "visual imprint" total area, evaluating the total intensity of the odor, proportional to the concentration of HVC, including water; it is built according to all signals of all sensors for the full measurement time; 2) the maximum signals of sensors with the most active or specific sorbent films ΔFmax, Hz to assess the content of individual classes of organic compounds in the EGP by rationing method (Yu et al., 2020; Liu et al., 2021). The sensor responses were recorded, processed, and compared in the analyzer "MAG Soft" software.

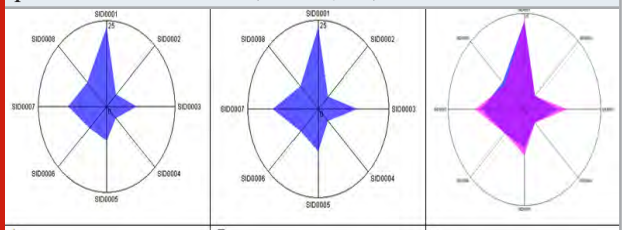
RESULTS AND DISCUSSION

With the aim to solve the set task, the changes in the e-nose responses in time for test samples are monitored. In order to establish the differences in the composition and content of HVC in the EGP over samples of curd products, the primary e-nose information – the response values of the selected sensors in the array and the value of the quantitative integrated e-nose signal – the "visual imprint" area of the maximum responses is compared (Table 1). Odor intensity difference of samples without and with the introduction of a functional plant composition is 6.6%, and it is not significant (Sidnyaev, 2018). In order to establish subtle differences in quality and quantity of the high volatile odor fraction of samples studied, the change in the total content of HVC in the EGP over the samples is monitored (Figure 1). According to the shape of the "visual imprint" figure of the sensors' responses in the array, no critical differences in all points in the EGP chemical composition over the samples are found out.

Table 1. Average sensor responses (±1 Hz) and "visual imprint" area of sensor signals in the EGP over samples (Sσ ± 30, Hz.s)

Sample No.	S1 – PVP	S2 – BG	S3 – DCH18C6	S4 – BCG	S5 – PEGS	S6 – PEG-2000	S7 – Tween	S8 – TOPO	Sσ, Hz.s
1	23	5	11	5	10	9	14	10	304
2	23	5	14	5	12	9	16	9	324

Figure 1: "Visual imprints" of maximum sensor signals in the EGP over samples No. 1 (a), No. 2 (b); comparison of samples No. 1 and 2 (c)
(On the circular axis – numbers of sensors in the array, on the vertical axis – maximum sensor responses at a certain point of measurement (ΔFmax, Hz).



It is found that the "visual imprint" area of the maximum sensor signals in the EGP over sample No. 1 was 304.41 Hz.s, No. 2 – 324.56 Hz.s, and the absolute difference between the areas – 20.15 Hz.s. The air quantitative composition changes (assessed by the normalization method) over the studied samples are traced then by the content ratio of the major HVC classes, for which the sensor array is tuned (Table 2).

Table 2 shows that samples No. 2 and No. 1 differ in the content of the major classes of organic compounds. It confirms the change in both quality and quantity of the high volatile fraction of the odor samples. Thus, slightly high content of nitrogen-containing compounds, acids, alcohols, and ketones, and low content of water and specific aromatic compounds are fixed in the EGP over sample No. 2. However, such changes in the content indicate only a redistribution of the HVC of test samples instead of new groups of compounds that may appear due to the introduction of a functional plant composition (Yu et al., 2020; Aydar et al., 2021). The parameter $A_{i/j}$, which shows the ratio constancy of concentrations of HVC separate classes in the EGP (Table 3), helps to monitor changes in the EGP quality over samples and the appearance or disappearance of HVC fraction.

Table 2. The content ratio of components in samples, ω (±0.5) % wt.

Medium, sample No.	S1 – PVP	S2 – BG	S3 – DCH18C6	S4 – BCG	S5 – PEGS	S6 – PEG-2000	S7 – Tween	S8 – TOPO
	Water	Alcohols, ketones,	Alcohols, ketones, acids	Nitrogenous bases	Amines, ketones	Alcohols, acids	Acids	Aromatic, sulfur-containing
1	26.4	5.7	12.6	5.7	11.5	10.3	16.1	11.5
2	24.7	5.4	15.1	5.4	12.9	9.7	17.2	9.7

If the parameters $A_{i/j}$, criteria of odor stability, are close or coincide for compared samples, then the content ratio of these groups of compounds in the samples is the same. If the ratio of the signals differs from such for the samples, the concentration ratio of these groups is different against the corresponding standard (Table 3). If more than 40% of parameters (3-4 parameters out of 7 selected) significantly change, then in the odor samples there are significant quality changes – groups of HVC appear or disappear, which with high probability are recorded in the organoleptic assessment of consumers and tasters (narrative descriptors and a score for the intensity of standard descriptors change) (Yu et al., 2020; Aydar et al., 2021).

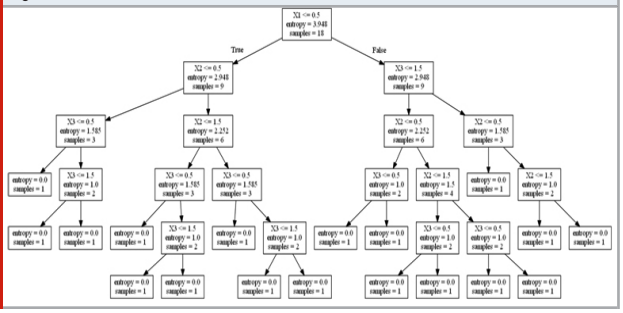
It was found out that samples No. 1 and 2 are identical by more than 85% in quality of the EGP. Higher content of alcohols, acids, and a smaller number of sulfur-containing compounds is fixed in sample No. 2 that is consistent with the quantitative composition. It can be stated for sample No. 2 a decrease in the odor of the compounds reinforcing assessments of negative descriptors (e.g., "bitter," "rancid," "another"). The next step in the experimental studies was to assess the composition and content of HVC in air samples over test samples of curd products during the entire period of storage, which determines the rancidity degree of the fat fraction and are indicators of shelf life.

Based on the experimental data obtained, an information system for modeling and predicting the shelf life of curd food systems was developed. It includes input parameters – a type of packaging (presence/absence of vacuum packaging, X1); storage conditions (temperature and relative humidity of ambient air, X2); content of plant component, X3) and output parameter – a value of quantitative integrated signal of an array of eight mass-sensitive piezo sensors (Y) (Python 3.6 software language) (Rodionova 2019; Donskaya and Drozhzhin, 2020). For modeling the storage process and visualizing the experimental dependencies, a machine learning method, the solver tree, was used (Figure 2).

Table 3. Ratio of signals of several sensors in the matrix for the test samples

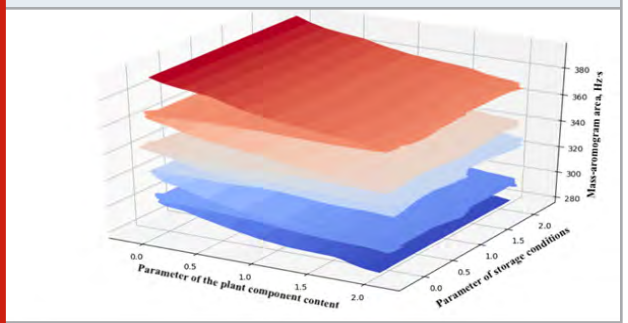
Sample No.	Odor stability-indicating parameter $A_{i/j}$					
	Tween/ PVP	BG/ PEG-2000	BCG/ PEGS	DCH18C6/ Tween	TOPO/ PVP	TOPO/ DCH18C6
1	0.61	0.56	0.50	0.79	0.43	0.91
2	0.70	0.56	0.42	0.88	0.39	0.64

Figure 2: Basic diagram of information system organization for modeling and predicting the shelf life of curd food systems

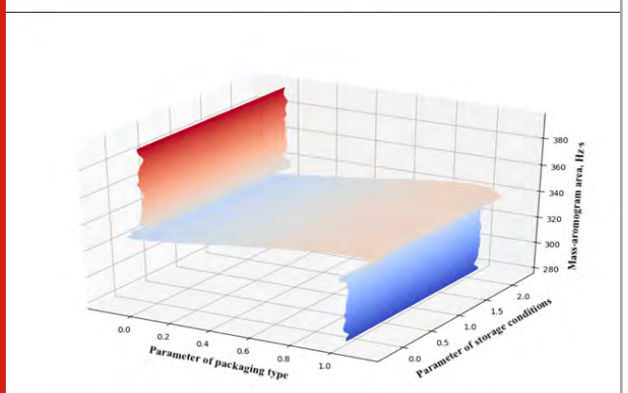


The software result is a graphical display of three-dimensional dependencies of the mass-aromogram area on the storage conditions, plant component content, and type of packaging. Figure 3 shows the software result on the example of the dependencies *Y* (X_2, X_3) and Y (X_1, X_2). The developed mathematical model makes it possible to obtain, with high precision, the values of the "visual imprint" area of the maximum sensor signals assessing the total odor intensity proportional to the concentration of HVC in the EGP of the study sample depending on specific conditions – a type of packaging, temperature and relative humidity of the ambient air, the content of the plant component, and also to plan storage regimes in the controlled environments.

Figure 3: The software results represented by three-dimensional dependences of the mass- aromogram areas on storage conditions and the plant component content (a); packaging type and storage conditions (b)



A



B

CONCLUSION

As a result of the scientific and technological research, the ingredient composition and expediency of using vegetable biocorrectors that generate deficient vitamins, macro- and trace elements, dietary fiber, and expand the range of fermented-milk food systems of functional action has been substantiated. Analysis of components and quantities of HVC in the EGP over test samples, carried out using a complex of sensitive piezo sensors of the odor analyzer, has shown that the introduction of functional plant composition does not have a significant effect on organoleptic parameters of curd products. Application of piezo sensory analysis made it possible to predict the shelf life of curd semi-finished products based on the assessment of composition and content of HVC in air samples over test samples during storage, determining the rancidity degree of the fat fraction.

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Dental
Communication

Comparison Between first and Second Premolar Extraction: Effects on Soft Tissue Profile Changes After Orthodontic Treatment in Patients with Bimaxillary Protrusion

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ABSTRACT

Orthodontic corrections involving tooth size arch length discrepancies (TSALD) often require the extraction of premolar teeth, to achieve the desired profile changes. General guidelines, suggest extraction of first premolars when the TSALD source area is primarily in the anterior portion of the arch. However, the basic indication for second premolar extraction is when there is moderate anterior crowding with no protrusion and the patient has good facial balance. Cephalometric radiographs of 60 adult BMP patients who underwent orthodontic retraction of anterior teeth following extraction of all first premolars or all second premolars reporting to a private dental center between January 2013 and January 2019 and fulfilling the inclusion criteria were included for the study. Analysis of the digital cephalometric radiographs was done using Dolphin Imaging® Software, Version 10.0 (Dolphin Imaging and Management Solutions, Chatsworth, California, USA). Paired comparison between pre-treatment and post-treatment cephalometric values, following 1st premolar and 2nd premolar extraction, were done using IBM SPSS Statistics Version 20. The statistical analysis of extracted data revealed that 1st premolar extraction treatment resulted in greatest change in upper and lower incisor inclination per unit change in upper or lower incisor retraction, as predicted through regression analysis. In the present study, the lower incisor retraction and proclination has proved to be a predictor of the need for second premolar extraction ($p < 0.05$). Hence the, decision to extract first or second premolar can be predicted on the pre-treatment position of the lower incisors and the desired amount of lower incisor tooth retraction.

KEY WORDS: ORTHODONTIC CORRECTIONS, CEPHALOMETRIC, PAIRED COMPARISON.

INTRODUCTION

The predominant reason to seek orthodontic treatment in patients with bimaxillary protrusion (BMP) is dental crowding and malalignment (Abdullah 2015). These malocclusions are commonly due to the discrepancies in tooth crown dimensions and the available space of the supporting alveolar arches (Abdullah 2015). In standard orthodontic protocols, extraction of premolars are advised to create space and enable correction of bimaxillary protrusion (Al-Anezi 2011). During orthodontic treatment involving the extraction of teeth, arch dimensional continue to change following active treatment (Ong and Woods 2001). Literature fails to provide a predicable guide to predicting the use of maxillary or mandibular extraction spaces (Aldosari et al., 2020).

Williams and Hosila (1976) showed varying amounts of molar and incisor movement during extraction space closure after first-premolar extraction cases (Keim et al., 2002). As against the conventional practice, extraction of second premolar is sometimes preferred to salvage first premolar when the second premolar has deep caries requiring root canal treatment or with poor prognosis. Once the extraction decision has been made there are several factors that influence how the teeth are aligned in the arches (Albarrak et al., 2019).

Majority of the practicing clinical practitioners advocate that BMP cases require premolar extractions for treatment (Aldosari et al., 2020). Further, it is estimated that one-

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third of all orthodontic patients have such a severe malocclusion that some pattern of premolar extraction is deemed necessary to resolve the problems and align the teeth (Proffit et al., 2013). Tooth size-arch length discrepancy (TSALD) is the most important factor necessitating the decision whether first- or second-premolars should be extracted in the maxilla and/or in the mandible (Correia et al., 2014). In addition, the extent of correction needed in the upper or lower anterior proclination to achieve the desirable profile changes is often considered a major determinant.

General guidelines suggest extraction of the first premolars when the TSALD source area is primarily in the anterior portion of the arch (Alqahtani et al., 2019). Removing the first premolars is the prescribed way to correct anterior crowding, excessive overjet and protrusion (Anthopoulou et al., 2014). This correction works by making space for the alignment of teeth or the retraction of canines and incisors. Extracting premolars close to the area of crowding is beneficial because of the minimal post extraction space that remains to be closed (Alqahtani et al., 2020). The use of premolar extractions for orthodontic treatment is still considered controversial (Akyalcin et al., 2011). Nevertheless, previous works have demonstrated that premolars are the most common teeth removed for orthodontic treatment (Sharma et al., 2014, Ong and Woods 2001). Conveniently located between the anterior and posterior segments, premolars would appear to be the obvious choice for correcting crowding and anterior-posterior discrepancies (Shirazi et al., 2016).

However, the basic indication for second premolar extraction is when there is moderate anterior crowding with no protrusion and the patient has good facial balance (Aljhani and Aldrees 2011). Some subjectivity of these guidelines is shown because de Castro (1974) describes this instance of "moderate" crowding as being when there is a TSALD of 5 mm or more, while Schoppe (1964) describes it as being a TSALD of 7.5 mm or less (Meyer et al. 2014). Other considerations for removing second premolars instead of first premolars include posterior crowding, anterior open bite, Class III correction, and facilitation of intentional anchorage slippage (Sandler et al., 2014). When second or third molars are crowded, ectopic, or impacted, they can be helped by increasing space in the posterior segments.

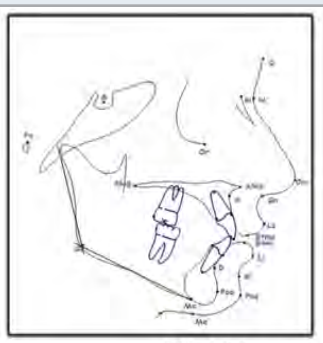
This space is created by extracting second premolars so that the first molar can be migrated mesially (Alqahtani et al. 2019). The dilemma is when the indication for extraction is for the 1st premolars but the 2nd premolars have poor prognosis due to carious lesion or large restoration, or the indication is for extraction of 2nd premolars but the 1st premolars have poor prognosis. Here comes the question of how much difference will it make on the facial profile or on incisors inclinations if extraction of 1st premolars or 2nd premolars will take place. In the present study, we compared the post orthodontic profile changes among patients who underwent first or second premolar extraction and establish the possible predictor variables for premolar extraction.

MATERIAL AND METHODS

Following ethical approval from the Institutional Review Board (CDRC Approval No. FR-0439 / IRB. No. E-18-3029), cephalometric radiographs of adult BMP patients who underwent orthodontic retraction of anterior teeth following extraction of all first premolars or all second premolars were included. The sampling frame included all patients reporting to a dental center between January 2013 and January 2019, and fulfilling the following inclusion criteria:

- Harmonious facial profile with an ANB (A point–Nasion–B point) angle of 3°± 2.3 and an SN-MP (Sella-Nasion to Mandibular plane) angle of 32°± 5.
- Class I molar relationship with an interincisal angle of 110.4° ± 6, overjet of 3 ± 1 mm, and overbite of 1.4 ± 1 mm (Aldrees and Shamlan 2010).
- Treated using fixed orthodontic appliance and availability of pre- and post-treatment cephalometric radiographs of adequate diagnostic quality.
- Absence of functional appliance therapy or orthognathic surgical procedures as a part of treatment.
- Absence of congenitally missing teeth (excluding third molars).
- No medical history of pharyngeal pathology and/or nasal obstruction, snoring, obstructive sleep apnea, adenoidectomy, or tonsillectomy.

Figure 1: Lateral cephalometric tracing dental and soft tissue profile measurements.



Hard Tissue Landmarks

S: *sella*, the center of the sella turcica.
N: *nasion*, the most anterior point of the frontonasal suture.
Pn: *porion* (anatomic porion), the midpoint of the upper contour of the external auditory canal.
Or: *orbitale*, the lowest point on the inferior orbital margin.
Pog: *pogonion*, the most prominent point on the hard tissue contour of the chin.
ANS: *anterior nasal spine*, the tip of the anterior nasal spine of the palatal bone in the hard palate.
PNS: *posterior nasal spine*, the tip of the posterior nasal spine of the palatal bone in the hard palate.
Go: *gonion*, point on the curvature of the angle of the mandible, located by bisecting the angle formed by lines tangent to the posterior ramus and the inferior border of the mandible.
A: *point A*, the greatest concavity of the alveolar bone overlying the maxillary incisors.
B: *point B*, the point of greatest concavity of the alveolar bone overlying the mandibular incisors.

Soft Tissue Landmarks

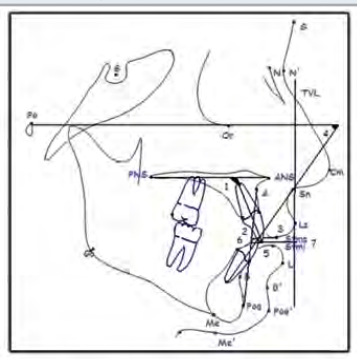
N': *Soft tissue nasion*, the deepest point of the concavity between the forehead and soft tissue contour of the nose.
Pog': *soft tissue pogonion*, the most prominent point on the soft tissue contour of chin.
G': *glabella*, the most prominent point of the soft tissue drape of the forehead, in midsagittal plane.
Cm: *columella*, the most anterior soft tissue point on the columella of the nose.
Sn: *subnasale*, the point where the upper lip meets the inferior border of the nose.
Me': *soft tissue menton*, the lowest point of the soft tissue chin contour.
Stms: *stomion superius*, the lowermost point on the vermillion of the upper lip.
Stmi: *stomion inferius*, the uppermost point on the vermillion border of the lower lip.
Ls: *labrale superius*, the most prominent point in the upper lip.
Li: *labrale inferius*, the most prominent point in the lower lip.
B': *soft tissue point B*, the point of greatest concavity on the contour of the lower between Li and Pg'.

Based on evidence from previously published data (Trisnawaty et al. 2013, Solem et al. 2013, Yasutomi et al. 2006), and assuming a statistical power of 80% with 95% confidence level, the sample size was estimated

to be 30 patients for group I (First premolar extracted) and 30 patients for group II (Second premolar extracted) Patient records identified using the inclusion criteria were assigned unique reference numbers by a blinded operator and were randomly included to the study using an online random number generator (RANDOM.ORG, Dublin, Ireland).

Pre- and post-treatment digital cephalometric radiographs of the study patients were collected for analysis. All the radiographs were obtained using a Planmeca Proline XC CEPH Digital X-Ray Unit (Planmeca OY, Helsinki, Finland) set at 80 kV with total filtration 2.5 mm Al and 1500 VA 50 Hz. Bias arising as a result of differing treatment methodologies was avoided by selecting records of patients treated by a single orthodontist with fixed edgewise (0.022" slot) mechanotherapy using maximum anchorage (Nance appliance) in the upper arch. Analysis of the digital cephalometric radiographs was done using Dolphin Imaging® Software, Version 10.0 (Dolphin Imaging and Management Solutions, Chatsworth, California, USA). The magnification probability was eliminated through calibration of the actual length of the ruler on the head positioner with simultaneous identification of the two ends of the rulers and the anatomical landmarks (Fig. 1 and Fig. 2).

Figure 2: Lateral cephalometric tracing showing the dentoalveolar and angular measurements



Dentoalveolar Measurements

1. **Upper incisor retroclination* (U1-PP)(°):** the angular difference in the pre- and post-treatment inclination of the upper incisors.
2. **Upper incisor retraction (U1-APog) (mm):** the linear difference in the pre- and post-treatment position of upper incisors in relation to A-Pog (point A to pogonion) line.
3. **Upper incisor retraction (U1-TVL) (mm):** the linear difference in the pre- and post-treatment position of the upper incisors in relation to TVL (true vertical line).
4. **Lower incisor retroclination* (LI-FMIA)(°):** the angular difference in the pre- and post-treatment inclination of the lower incisors.
5. **Lower incisor retraction (LI-APog) (mm):** the linear difference in the pre- and post-treatment position of the upper incisors in relation to the A-Pog line.
6. **Lower incisor retraction (LI-TVL) (mm):** the linear difference in the pre- and post-treatment position of the upper incisors in relation to TVL.

** Angular measurements which became more obtuse during following treatment will have a positive value.*

To assure examiner reliability, samples of 10 randomly selected cephalometric radiographs were traced and measured by the same investigator, who would eventually trace all the radiographs. Identification of the cephalometric landmarks and measurement of the variables were carried out in two different sessions separated by a period of two weeks. In order to ascertain the test-retest reliability, the mean values of the variables obtained during the two sessions were compared using paired t-tests. Furthermore, Pearson's correlation was done to evaluate the relationship between the first and second readings and negligible error was assumed for a minimum correlation coefficient (Pearson's r) of 0.85.

Pre- and post-treatment cephalometric data were analyzed using the SPSS PC+ version 21.0 for Windows, (IBM SPSS Statistics, Armonk, NY, USA). Descriptive statistics (mean and standard deviation) were calculated for all the quantitative outcome variables (dental and airway). The pre- and post-test mean values of the quantitative variables were compared using a student's paired t-test, with the resulting difference being the variable of interest. To quantify the correlation between the difference in the pre- and post-treatment values of the variables, Pearson's correlation was used. Linear regression analysis was used to identify the independent predictor variables (changes in dental measurements) for the dependent outcome variables (changes in pharyngeal airway measurements) of interest. The statistical significance of the results was fixed at a p-value < 5% ($\alpha = 0.05$) and at 95% confidence interval.

Statistical analysis: All cephalometric data were exported to a spreadsheet software (MS Excel 2016, Microsoft, Redmond, WA, USA). Data were grouped into dental and soft-tissue related cephalometric variables and duplicates were identified and removed. Descriptive analysis and statistical comparisons assuming a 95% level of significance ($p < 0.05$) were performed using statistical package software (IBM SPSS Statistics Version 20, IBM, Armonk, NY, USA). Shapiro-Wilk test for normality of sample distribution was done for all variables and a p-value less than 0.05 was indicative of non-parametric distribution.

Paired comparison between pre-treatment and post-treatment cephalometric values, following 1st premolar and 2nd premolar extraction, were done using paired samples t-test (normal distribution) and Wilcoxon signed rank test (non-parametric distribution). Magnitude of change following treatment (difference between pre and post-treatment values) by 1st premolar or 2nd premolar extraction was compared using independent samples t-test (normal distribution) and Mann-Whitney-U test (non-parametric distribution). Pearson's correlation based on post-orthodontic treatment change in cephalometric measurements was also performed.

RESULTS AND DISCUSSION

Since time immemorial, practitioners have often compared the efficacy of orthodontic treatment between extraction or non-extraction cases (Keim et al. 2002). However, current day literature analyzes the preferential extraction of first or the second premolar. In our retrospective study we aimed to assess the possible difference in the dental and soft tissue profile changes between the two treatment groups. Premolar extraction is preferred on the basis of its favorable position between the anterior and posterior teeth especially when the focus is on the correction of crowding and anteroposterior discrepancies (Proffit et al., 2006).

Table 1. Normality of sample distribution tested using Shapiro-Wilk test, showing p-values for each variable. A p-value less than 0.05 indicates non-normal distribution.

	1st Premolar Extraction (df = 25)		2nd Premolar Extraction (df = 29)		Pre - Post Treatment Change	
Cephalometric Variables	Pre-Treatment	Post-Treatment	Pre-Treatment	Post-Treatment	1st Premolar Extraction	2nd Premolar Extraction
Dental Variables						
Interincisal Angle (UI-LI) (Deg)	0.759	0.910	0.763	0.242	0.709	0.171
Overbite (mm)	0.239	0.877	0.383	0.995	0.860	0.906
Overjet (mm)	0.974	0.124	0.203	0.869	0.154	0.344
Upper Incisor Protrusion (UI-APog) (mm)	0.152	0.131	0.349	0.519	0.830	0.053
Upper Incisor Inclination (UI-APog) (Deg)	0.215	0.903	0.530	0.385	0.229	0.112
Lower Incisor Protrusion (LI-APog) (mm)	0.433	0.028	0.951	0.012	0.368	0.952
Lower Incisor Inclination (LI-APog) (Deg)	0.743	0.882	0.228	0.643	0.225	0.806
IMPA (LI-MP) (Deg)	0.553	0.067	0.705	0.008	0.398	0.135
FMIA (LI-FH) (Deg)	0.338	0.769	0.291	0.769	0.327	0.934
UI - FH (Deg)	0.028	0.543	0.464	0.235	0.808	0.166
FMA (MP-FH) (Deg)	0.113	0.481	0.501	0.916	0.121	0.208
UI - Occlusal Plane (Deg)	0.289	0.416	0.177	0.283	0.014	0.818
UI - Nasion Perpendicular (Deg)	0.028	0.543	0.464	0.235	0.808	0.166
LI - Occlusal Plane (Deg)	0.141	0.139	0.470	0.655	0.373	0.270
LI - SN (Deg)	0.950	0.011	0.243	0.460	0.394	0.970
LI - GoGn (Deg)	0.278	0.165	0.727	0.005	0.185	0.049
UI - NA (mm)	0.060	0.897	0.587	0.557	0.845	0.162
UI - NA (Deg)	0.084	0.652	0.227	0.114	0.511	0.061
LI - NB (mm)	0.286	0.156	0.173	0.204	0.053	0.636
LI - NB (Deg)	0.923	0.636	0.598	0.506	0.795	0.550
Soft-tissue Variables						
Upper Lip Length (Sn – St sup.) (mm)	0.098	0.139	0.668	0.943	0.954	0.494
Lower Lip Length (St inf. - Me) (mm)	0.932	0.141	0.836	0.630	0.116	0.953
Interlabial Gap (St sup. – St inf.) (mm)	0.045	0.000	0.084	0.229	0.056	0.165
Upper Lip Thickness @ A Point (mm)	0.372	0.082	0.249	0.000	0.943	0.055
Upper Lip Thickness @ Ver. Border (mm)	0.959	0.293	0.645	0.429	0.618	0.066
Upper Lip to E-Plane (mm)	0.498	0.178	0.874	0.217	0.893	0.095
Lower Lip to E-Plane (mm)	0.529	0.302	0.326	0.076	0.775	0.007
Superior Sulcus Depth (mm)	0.385	0.484	0.837	0.052	0.934	0.140
Subnasale to H-Line (mm)	0.543	0.879	0.909	0.054	0.752	0.683
Lower Lip to H-Line (mm)	0.332	0.525	0.613	0.992	0.593	0.047
Inferior Sulcus to H-Line (mm)	0.619	0.995	0.551	0.692	0.550	0.954
Soft-tissue Facial Angle (FH-N'Pog') (Deg)	0.143	0.065	0.420	0.990	0.234	0.059
Convexity (A-NPog) (mm)	0.170	0.391	0.777	0.975	0.071	0.897
Convexity (NA-APog) (Deg)	0.305	0.502	0.467	0.681	0.072	0.667

UI – Upper incisor; LI – Lower incisor; APog – Point A-Pogonion line; IMPA – Incisor mandibular plane angle; MP – Mandibular plane; FMIA – Frankfort mandibular incisal angle;
FH – Frankfort horizontal; FMA – Frankfort mandibular angle; SN – Sella-Nasion line; GoGn – Gonion-Gnathion line; NA – Nasion-Point A line; NB – Nasion-Point B line; Sn – Subnasale; St sup. – Stomion superior; St inf. – Stomion inferior; Ver. Border – Vertical border; NPog – Nasion-Pogonion line; N'Pog' – Soft-tissue Nasion-Soft-tissue Pogonion line

In general, extraction of first premolars is advised when the conditions like anterior crowding, excessive overjet and severe protrusion of teeth prevail. In the other hand, clinical scenarios with mild anterior crowding, posterior crowding or an anticipated loss of molar anchorage is needed then extraction of 2nd premolars is needed (Alqahtani et al., 2020). Out of the 60 patients enrolled in the study, a total of 25 patients completed orthodontic treatment with 1st premolar extraction and 29 patients completed treatment with 2nd premolar extraction. Pre- and post-treatment cephalometric data of all the patients who completed treatment were available in the form of digitized patient records. The pre-treatment, post-treatment and magnitude of change data were predominantly normally distributed for most of the variables (Table 1).

Table 2. Paired samples comparison (paired t-test) of pre-treatment and post-treatment values for dental and soft-tissue cephalometric variables, in patients with 1st premolar extraction orthodontic treatment. (n = 25)

Cephalometric Variables	Pre - Treatment		Post-Treatment		Mean Difference	Significance (p-value)
	Mean	S.D.	Mean	S.D.		
Dental Variables						
Interincisal Angle (UI-LI) (Deg)	108.360	6.753	120.250	6.205	-11.888	0.000
Overbite (mm)	1.120	1.793	0.896	1.184	0.224	0.424
Overjet (mm)	4.316	1.419	3.424	0.877	0.892	0.014
Upper Incisor Protrusion (UI-APog) (mm)	10.300	1.946	6.460	2.086	3.840	0.000
Upper Incisor Inclination (UI-APog) (Deg)	39.132	5.110	31.316	4.057	7.816	0.000
Lower Incisor Protrusion (LI-APog) (mm)*	5.964	2.127	3.080	2.060	2.884	0.000
Lower Incisor Inclination (LI-APog) (Deg)	32.520	4.700	28.420	4.335	4.100	0.001
IMPA (LI-MP) (Deg)	101.220	6.406	96.504	6.151	4.716	0.000
FMIA (LI-FH) (Deg)	45.892	6.377	49.712	5.341	-3.820	0.001
UI - FH (Deg)*	117.520	6.172	109.460	5.732	8.060	0.000
FMA (MP-FH) (Deg)	32.892	7.252	33.788	5.531	-0.896	0.252
UI - Occlusal Plane (Deg)	128.960	4.434	124.130	4.966	4.824	0.001
UI - Nasion Perpendicular (Deg)*	27.520	6.172	19.460	5.732	8.060	0.000
LI - Occlusal Plane (Deg)	57.328	5.283	64.388	4.192	-7.060	0.000
LI - SN (Deg)*	39.332	5.735	44.272	5.755	-4.940	0.000
LI - GoGn (Deg)	103.980	6.368	99.456	5.825	4.520	0.000
UI - NA (mm)	6.576	3.301	3.052	2.466	3.524	0.000
UI - NA (Deg)	28.920	7.209	22.192	6.326	6.728	0.000
LI - NB (mm)	8.500	2.016	6.008	2.091	2.492	0.000
LI - NB (Deg)	38.040	5.037	32.940	4.966	5.100	0.000
Soft-tissue Variables						
Upper Lip Length (Sn – St sup.) (mm)	21.384	2.586	20.592	2.504	0.792	0.021
Lower Lip Length (St inf. - Me) (mm)	37.948	3.523	38.956	3.593	-1.008	0.103
Interlabial Gap (St sup. – St inf.) (mm)*	7.060	3.391	3.304	1.315	3.756	0.000
Upper Lip Thickness @ A Point (mm)	14.228	2.402	13.636	2.046	0.592	0.258
Upper Lip Thickness @ Ver. Border (mm)	10.500	1.672	10.764	1.631	-0.264	0.449
Upper Lip to E-Plane (mm)	-0.536	2.200	-2.464	2.300	1.928	0.000
Lower Lip to E-Plane (mm)	2.684	3.086	-0.280	2.679	2.964	0.000
Superior Sulcus Depth (mm)	2.876	1.436	1.252	1.352	1.624	0.000
Subnasale to H-Line (mm)	7.148	2.045	5.188	2.307	1.960	0.000
Lower Lip to H-Line (mm)	3.032	2.140	1.344	1.467	1.688	0.000
Inferior Sulcus to H-Line (mm)	3.524	1.566	3.444	1.522	0.080	0.752
Soft-tissue Facial Angle (FH-N'Pog') (Deg)	86.712	4.340	85.700	3.366	1.012	0.138
Convexity (A-NPog) (mm)	4.584	3.038	4.244	2.530	0.340	0.430
Convexity (NA-APog) (Deg)	10.200	6.722	9.136	5.329	1.064	0.284

*Non-parametric paired comparison using "Wilcoxon signed rank test".
UI – Upper incisor; LI – Lower incisor; APog – Point A-Pogonion line; IMPA – Incisor mandibular plane angle; MP – Mandibular plane; FMIA – Frankfort mandibular incisal angle; FH – Frankfort horizontal; FMA – Frankfort mandibular angle; SN – Sella-Nasion line; GoGn – Gonion-Gnathion line; NA – Nasion-Point A line; NB – Nasion-Point B line; Sn – Subnasale; St sup. – Stomion superior; St inf. – Stomion inferior; Ver. Border – Vertical border; NPog – Nasion-Pogonion line; N'Pog' – Soft-tissue Nasion-Soft-tissue Pogonion line

Table 3. Paired samples comparison (paired t-test) of pre-treatment and post-treatment values for dental and soft-tissue cephalometric variables, in patients with 2nd premolar extraction orthodontic treatment. (n = 29)

Cephalometric Variables	Pre - Treatment		Post-Treatment		Mean Difference	Significance (p-value)
	Mean	S.D.	Mean	S.D.		
Dental Variables						
Interincisal Angle (UI-LI) (Deg)	109.340	5.872	122.100	7.527	-12.759	0.000
Overbite (mm)	0.997	1.651	0.841	0.939	0.155	0.553
Overjet (mm)	3.986	1.439	3.259	0.921	0.728	0.030
Upper Incisor Protrusion (UI-APog) (mm)	10.224	1.873	6.335	1.639	3.890	0.000
Upper Incisor Inclination (UI-APog) (Deg)	38.638	4.443	30.783	4.868	7.855	0.000
Lower Incisor Protrusion (LI-APog) (mm)*	6.228	2.016	3.124	1.928	3.104	0.000
Lower Incisor Inclination (LI-APog) (Deg)	32.031	4.660	27.110	4.523	4.921	0.000
IMPA (LI-MP) (Deg)*	100.540	6.996	94.928	6.359	5.612	0.000
FMIA (LI-FH) (Deg)	47.028	5.463	51.279	5.407	-4.252	0.000
UI - FH (Deg)	117.680	5.146	109.190	6.443	8.493	0.000
FMA (MP-FH) (Deg)	32.445	5.477	33.793	5.351	-1.348	0.009
UI - Occlusal Plane (Deg)	129.120	3.801	123.770	4.985	5.348	0.000
UI - Nasion Perpendicular (Deg)	27.679	5.146	19.186	6.443	8.493	0.000
LI - Occlusal Plane (Deg)	58.448	4.968	65.872	4.666	-7.424	0.000
LI - SN (Deg)	39.517	5.118	45.314	5.670	-5.797	0.000
LI - GoGn (Deg)*	103.590	6.895	98.059	6.250	5.531	0.000
UI - NA (mm)	6.545	2.828	2.990	2.465	3.555	0.000
UI - NA (Deg)	28.593	6.713	21.797	6.517	6.797	0.000
LI - NB (mm)	8.731	1.951	5.779	1.706	2.952	0.000
LI - NB (Deg)	37.514	4.495	31.735	5.239	5.779	0.000
Soft-tissue Variables						
Upper Lip Length (Sn – St sup.) (mm)	21.972	2.697	20.928	2.693	1.045	0.004
Lower Lip Length (St inf. - Me) (mm)	37.683	4.197	38.872	4.291	-1.190	0.019
InterLabial Gap (St sup. – St inf.) (mm)	7.603	3.584	3.555	0.767	4.048	0.000
Upper Lip Thickness @ A Point (mm)*	13.886	1.722	13.517	1.928	0.369	0.179
Upper Lip Thickness @ Ver. Border (mm)	10.552	1.438	10.628	1.473	-0.076	0.810
Upper Lip to E-Plane (mm)	-0.483	2.247	-2.717	2.411	2.234	0.000
Lower Lip to E-Plane (mm)	3.017	2.946	-0.107	2.467	3.124	0.000
Superior Sulcus Depth (mm)	3.028	1.565	0.935	1.478	2.093	0.000
Subnasale to H-Line (mm)	7.597	2.120	5.331	2.384	2.266	0.000
Lower Lip to H-Line (mm)	3.366	1.993	1.735	1.329	1.631	0.000
Inferior Sulcus to H-Line (mm)	3.083	1.454	3.179	1.401	-0.097	0.655
Soft-tissue Facial Angle (FH-N'Pog') (Deg)	87.100	3.357	85.638	3.361	1.462	0.000
Convexity (A-NPog) (mm)	4.541	2.787	4.176	2.706	0.366	0.245
Convexity (NA-APog) (Deg)	10.035	6.140	8.990	5.709	1.045	0.147

*Non-parametric paired comparison using "Wilcoxon signed rank test".
UI – Upper incisor; LI – Lower incisor; APog – Point A-Pogonion line; IMPA – Incisor mandibular plane angle; MP – Mandibular plane; FMIA – Frankfort mandibular incisal angle; FH – Frankfort horizontal; FMA – Frankfort mandibular angle; SN – Sella-Nasion line; GoGn – Gonion-Gnathion line; NA – Nasion-Point A line; NB – Nasion-Point B line; Sn – Subnasale; St sup. – Stomion superior; St inf. – Stomion inferior; Ver. Border – Vertical border; NPog – Nasion-Pogonion line; N'Pog' – Soft-tissue Nasion-Soft-tissue Pogonion line.

Literature reveals that clinical scenarios where the TSALDs and anterior protrusion are the treatment objective, first premolar extraction is considered ideal (Qamaruddin et al., 2018). In addition, dental and skeletal anteroposterior discrepancies is commonly treated with extraction of premolars (Sheerah et al., 2019). However, there is lack of advocacy and standard protocol regarding the preference between first and second premolar extraction (Akyalcin et al., 2011). Varying patterns of extraction are adopted with the primary aim of creating space for desired tooth movement (Aljhani and Zawawi 2010). The current study evaluated the profile changes through pre and post treatment occlusal records of two treatment groups with bimaxillary protrusion treated with first and second premolar extraction to show how much changes in the incisors inclination between the

groups and how much effect on the facial profile. In future perspective this assessment can provide a reference guide for clinicians towards expected treatment outcomes based on the determined teeth will be extracted.

Table 4. Independent samples comparison (independent t-test) of magnitude of post-treatment change for dental and soft-tissue cephalometric variables, following 1st premolar and 2nd premolar extraction orthodontic treatment.

Cephalometric Variables	Post Treatment Change				Mean Difference	Significance (p-value)
	Pre - Treatment		Post-Treatment			
	Mean	S.D.	Mean	S.D.		
Dental Variables						
Interincisal Angle (UI-LI) (Deg)	-11.888	8.366	-12.759	9.189	0.871	0.719
Overbite (mm)	0.224	1.376	0.155	1.390	0.069	0.856
Overjet (mm)	0.892	1.677	0.728	1.714	0.164	0.724
Upper Incisor Protrusion (UI-APog) (mm)	3.840	1.876	3.890	1.464	-0.050	0.913
Upper Incisor Inclination (UI-APog) (Deg)	7.816	4.882	7.855	4.948	-0.039	0.977
Lower Incisor Protrusion (LI-APog) (mm)	2.884	2.078	3.103	1.844	-0.219	0.683
Lower Incisor Inclination (LI-APog) (Deg)	4.100	5.343	4.921	5.494	-0.821	0.582
IMPA (LI-MP) (Deg)	4.716	4.292	5.614	4.756	-0.898	0.473
FMIA (LI-FH) (Deg)	-3.820	5.010	-4.252	5.209	0.432	0.759
UI - FH (Deg)	8.060	6.683	8.493	5.828	-0.433	0.800
FMA (MP-FH) (Deg)	-0.896	3.815	-1.348	2.598	0.452	0.609
UI - Occlusal Plane (Deg)*	4.824	6.327	5.348	5.799	-0.524	0.822
UI - Nasion Perpendicular (Deg)	8.060	6.683	8.493	5.828	-0.433	0.800
LI - Occlusal Plane (Deg)	-7.060	5.060	-7.424	5.423	0.364	0.801
LI - SN (Deg)	-4.940	4.440	-5.797	4.861	0.857	0.505
LI - GoGn (Deg)*	4.520	4.372	5.528	4.556	-1.008	0.828
UI - NA (mm)	3.524	3.201	3.555	2.472	-0.031	0.968
UI - NA (Deg)	6.728	7.407	6.797	6.205	-0.069	0.971
LI - NB (mm)	2.492	1.795	2.952	1.776	-0.460	0.350
LI - NB (Deg)	5.100	4.139	5.779	4.832	-0.679	0.585
Soft-tissue Variables						
Upper Lip Length (Sn – St sup.) (mm)	0.792	1.605	1.045	1.773	-0.253	0.588
Lower Lip Length (St inf. - Me) (mm)	-1.008	2.970	-1.190	2.566	0.182	0.810
InterLabial Gap (St sup. – St inf.) (mm)	3.756	3.219	4.048	3.463	-0.292	0.751
Upper Lip Thickness @ A Point (mm)	0.592	2.555	0.369	2.019	0.223	0.722
Upper Lip Thickness @ Ver. Border (mm)	-0.264	1.713	-0.076	1.680	-0.188	0.686
Upper Lip to E-Plane (mm)	1.928	1.237	2.235	1.132	-0.306	0.346
Lower Lip to E-Plane (mm)	2.964	1.752	3.124	1.634	-0.160	0.730
Superior Sulcus Depth (mm)	1.624	1.399	2.093	1.329	-0.469	0.213
Subnasale to H-Line (mm)	1.960	1.592	2.266	1.339	-0.306	0.447
Lower Lip to H-Line (mm)	1.688	1.336	1.631	1.285	0.057	0.874
Inferior Sulcus to H-Line (mm)	0.080	1.251	-0.097	1.152	0.177	0.592
Soft-tissue Facial Angle (FH-N'Pog') (Deg)	1.012	3.294	1.462	1.965	-0.450	0.538
Convexity (A-NPog) (mm)	0.340	2.117	0.366	1.656	-0.026	0.961
Convexity (NA-APog) (Deg)	1.064	4.859	1.045	3.774	0.019	0.987

*Non-parametric comparison using "Mann-Whitney U test".

UI – Upper incisor; LI – Lower incisor; APog – Point A-Pogonion line; IMPA – Incisor mandibular plane angle; MP – Mandibular plane; FMIA – Frankfort mandibular incisal angle; FH – Frankfort horizontal; FMA – Frankfort mandibular angle; SN – Sella-Nasion line; GoGn – Gonion-Gnathion line; NA – Nasion-Point A line; NB – Nasion-Point B line; Sn – Subnasale; St sup. – Stomion superior; St inf. – Stomion inferior; Ver. Border – Vertical border; NPog – Nasion-Pogonion line; N'Pog' – Soft-tissue Nasion-Soft-tissue Pogonion line

Paired samples comparison of cephalometric variables before and after 1st premolar extraction treatment, showed significant change for almost all the dental and soft-tissue variables, except overbite, FMA, lower lip length, upper lip thickness (both at A-Point and Vertical border), inferior sulcus to H-line distance, soft tissue facial angle and convexity (both linear and angular) (Table 2). Earlier researches revealed that

there were significant changes in arch width and depth with extraction orthodontics (Bindayel 2019). Among the predictive variables the most commonly assessed in previous literature like Upper Incisor Protrusion (UI-APog), Upper Incisor Inclination (UI-APog), Lower Incisor Protrusion (LI-APog), Lower Incisor Inclination (LI-APog), IMPA (LI-MP) and FMIA (LI-FH) was tabulated for evaluation (Aljhani and Aldrees 2011, Anthopoulou et al., 2014). Further, when comparing the first and second premolar extractions, incisor retraction in first premolar extraction group was more significant (Aldrees et al., 2015, Aljhani and Zawawi 2010).

Table 5. Correlation based on post-orthodontic treatment change in 1st premolar Extraction group

Predictor Variable	Dependent Variables	r	P- value	r^2	Predicted change in dependent variable per unit change in predictor variable
Upper Incisor Retraction (UI - Nasion Perp.) (Deg)	Upper Incisor Protrusion (UI-APog) (mm)	0.567	0.003	29.2%	2.715 mm
	Upper Incisor Inclination (UI-APog) (Deg)	0.811	<0.001	64.3%	3.633 Deg
	Lower Incisor Protrusion (LI-APog) (mm)	0.375	0.065*	-	-
	Lower Incisor Inclination (LI-APog) (Deg)	0.523	0.007	24.2%	1.150 Deg
Lower Incisor Retraction (LI – GoGn) (Deg)	Upper Incisor Protrusion (UI-APog) (mm)	0.395	0.051*	-	-
	Upper Incisor Inclination (UI-APog) (Deg)	0.224	0.281*	-	-
	Lower Incisor Protrusion (LI-APog) (mm)	0.578	0.002	30.6%	1.916 mm
	Lower Incisor Inclination (LI-APog) (Deg)	0.743	<0.001	53.2%	0.905 Deg

*No significant correlation between predictor and dependent variable

Table 6. Correlation based on post-orthodontic treatment change 2nd Premolar Extraction Group

Predictor Variable	Dependent Variables	r	P- value	r^2	Predicted change in dependent variable per unit change in predictor variable
Upper Incisor Retraction (UI - Nasion Perp.) (Deg)	Upper Incisor Protrusion (UI-APog) (mm)	0.584	0.001	31.7%	2.790 mm
	Upper Incisor Inclination (UI-APog) (Deg)	0.850	<0.001	71.1%	1.450 Deg
	Lower Incisor Protrusion (LI-APog) (mm)	0.485	0.008	20.7%	1.954 mm
	Lower Incisor Inclination (LI-APog) (Deg)	0.655	<0.001	40.8%	0.296 Deg
Lower Incisor Retraction (LI – GoGn) (Deg)	Upper Incisor Protrusion (UI-APog) (mm)	0.398	0.032	12.7%	3.311 mm
	Upper Incisor Inclination (UI-APog) (Deg)	0.606	<0.001	34.3%	4.877 Deg
	Lower Incisor Protrusion (LI-APog) (mm)	0.618	<0.001	35.9%	1.971 mm
	Lower Incisor Inclination (LI-APog) (Deg)	0.871	<0.001	75.1%	0.165 Deg

In agreement, in the present study also 0.4 mm more incisor retraction was evident in first premolar extraction group as against the second premolar treatment group. Further, 2nd premolar extraction treatment resulted in significant post-treatment change for most cephalometric variables except, overbite, upper lip thickness (both at A-Point and Vertical border), inferior sulcus to H-line distance, and convexity (both linear and angular) (Table 3). Based on paired comparison of cephalometric variables, the outcomes of 1st and 2nd premolar extraction orthodontic treatment showed statistically significant post-treatment change for the dental variables like Upper Incisor Protrusion (UI-APog), Upper Incisor Inclination (UI-APog), Lower Incisor Protrusion (LI-APog) and Lower Incisor Inclination (LI-APog). In both the study groups soft tissue variables like Interlabial gap, upper lip and lower lip length to E-plane were also shown to change significantly. However, FMA angle and soft tissue angle were significantly altered only in the 2nd premolar group.

On the contrary, upper lip thickness at the level of vertical border and at point A, were more pronounced in the 1st premolar group. Comparing the change in convexity, minimal change was evidenced in both groups which was statistically insignificant. This was further confirmed by the results of the independent samples comparison test. This was in agreement with earlier study by Tae-Kyung Kim et al., who found insignificant change in facial vertical dimension in both groups. He proposed

that decision on extraction of 1st and 2nd premolar could be rather based on the need for incisor retraction, area of crowding, dimension of teeth and conditions of the premolar teeth aimed for extraction (Kim et al., 2005).

Statistically comparing the magnitude of change following 1st and 2nd premolar treatment showed no significant difference in the amount of post-treatment change between the two groups for any dental or soft-tissue cephalometric variables (Table 4). In orthodontic corrections of class II discrepancies, greater incisor retraction is generally expected in the upper arch due to the enhanced retraction of the anterior segment to reduce the overjet and less retraction of lower anterior teeth but more protraction in lower posterior teeth to achieve Class I molar relationship (Aljhani and Zawawi 2010). The present study illustrated (Table 4) compared all the dental and soft tissue variables of profile changes between the first and second premolar groups. Although post treatment data comparisons should differences between 1st premolar and 2nd premolars groups, these differences are statistically insignificant. In both the 1st and 2nd premolar groups upper incisors were retracted by an average of 3.8 mm, however insignificant statistical (p=0.913) comparison was observed.

Nevertheless, lower incisors retraction for the 1st premolar group was 0.2 mm less than the 2nd premolar group. This difference however proved statistically insignificant (p=0.683). This is in concurrence with the research outcomes of Ong and Woods (Ong and Woods 2001). In another study comparing the first and second premolar extraction, incisor crowding was found to be definitively predictive of incisor retraction (Elnour et al., 2016). This is attributed to the closure of both inter-dental spacing and premolar extraction space by the retraction of the protrusive incisor.

Pearson's correlation based on post-orthodontic treatment change in cephalometric measurements, revealed significant positive correlation between several cephalometric variables, in both the 1st premolar and 2nd premolar extraction groups. Highly significant positive correlation was observed between upper and lower incisor retraction and upper and lower incisor protrusion and inclination, respectively, in both 1st premolar and 2nd premolar extraction groups (Table 5 and Table 6). This was further confirmed through linear regression, which indicated statistically significant predictability (Table 5 and Table 6).

In both, the 1st premolar and 2nd premolar extraction groups, upper incisor retraction showed greater predictability of changes in upper incisor inclination (1st premolar "r^2" – 64.3%; 2nd premolar "r^2"– 71.1%), than changes in upper incisor protrusion (1st premolar "r^2" – 29.2%; 2nd premolar "r^2" – 31.7%). Similarly, greater predictability of changes in lower incisor inclination (1st premolar "r^2" – 53.2%; 2nd premolar "r^2"– 75.1%) than lower incisor protrusion (1st premolar "r^2" – 30.6%; 2nd premolar "r^2" – 35.9%), were observed with lower incisor retraction. Interestingly, changes in upper and lower incisor protrusion in response to upper and lower incisor retraction, respectively, were almost similar after 1st and 2nd premolar extraction orthodontic treatment (Figure 3 and Figure 4).

Figure 3: Change in upper incisor protrusion in response to change in upper incisor retraction

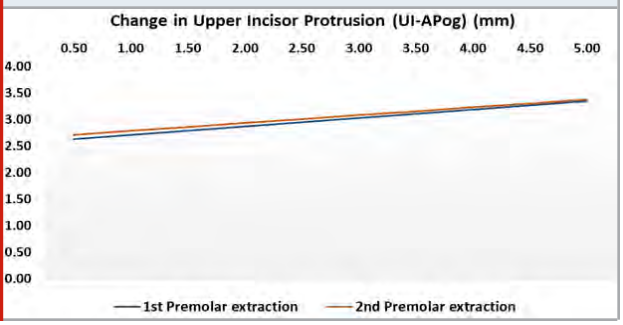


Figure 4: Change in upper incisor inclination in response to change in upper incisor retraction

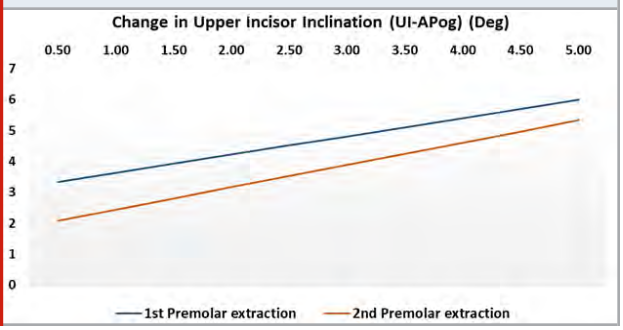
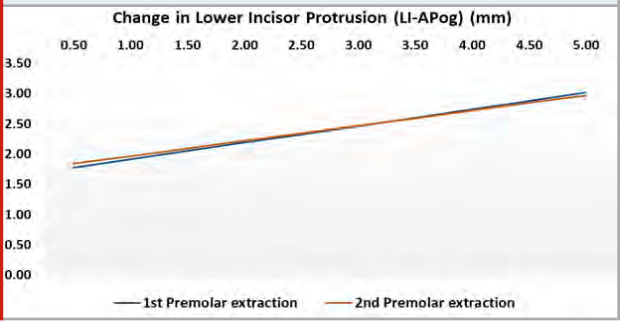


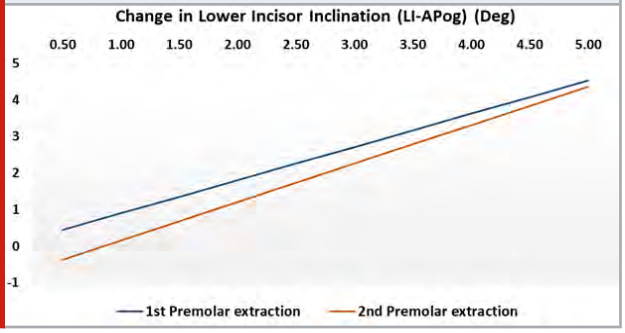
Figure 5: Change in lower incisor protrusion in response to change in lower incisor retraction



On the contrary, 1st premolar extraction treatment resulted in greatest change in upper and lower incisor inclination per unit change in upper or lower incisor retraction, as predicted through regression analysis (Figure 5 and Figure 6). Based on the present study data, unit change in the upper incisor retraction measured angularly by the "UI – Nasion perpendicular angle", predicted a 3.63 degree change in upper incisor inclination (UI – APog angle) following 1st premolar extraction orthodontic treatment (r^2– 64.3%). While similar unit change in "UI – Nasion perpendicular angle" after 2nd premolar extraction treatment, predicted only 1.45 degree change in upper incisor inclination (UI – APog angle) (r^2 – 71.1%). Similarly, unit change in

lower incisor retraction measured angularly based on "LI – Gonion/Gnathion angle" predicted a greater lower incisor inclination following 1st premolar extraction (0.91 degree; r^2 – 53.2%), than after 2nd premolar extraction (0.17 degree; r2 – 75.1%) (Tables 5 and 6).

Figure 6: Change in lower incisor inclination in response to change in lower incisor retraction



These findings imply that the decision to extract first or second premolar should be based on the pre-treatment position of the upper and lower incisors and the desired amount of upper and lower incisor inclination required post-treatment. Therefore, in a clinical scenario demanding greater amount of incisor retroclination, extraction of the 1st premolars could be considered. Our findings are in accordance with the study of Ziad et al, 2018 wherein lower incisor retraction was advocated as a predictor of premolar extraction(Omar et al. 2018). In comparison, Nance et al proposed soft tissue profile change of the lips to be a more appropriate predictor of need for first premolar extraction (Ahmad et al. 2018).

CONCLUSION

Dental malocclusions can involve arch-size tooth-size discrepancies and are often managed by premolar extraction as a preferred line of treatment. The present study compared the amounts of dental and soft tissue changes after orthodontic treatment in two common extraction patterns of 1st and 2nd premolar. There was no statistical significant difference between the amount of upper and lower incisors retraction and retroclination between the 1st premolars and 2nd premolars groups. Also, there was no statistical significant difference at the soft tissue changes between the 1st premolars and 2nd premolars groups. However, there was a positive, linear relationship observed between the amount of change in the position (retraction) of the maxillary incisor teeth and the amount of change (retrusion) in profile First premolar extraction is advised when upper incisor retraction in BMP is recommended. The results of the present study need to be considered based on the geographical limitations of the study populations. However, future studies in differing populations should be considered prior to clinical extrapolation of the findings.

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Biotechnological Communication

Analysis on the Antimicrobial and Repellent Activities of Cymbopogon martinii Essential Oil

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ABSTRACT

Cymbopogon martinii (Palmarosa) is one of the lemongrass varieties native to India and Indochina. Due to the vast economical usage of its essential oil, this lemongrass species is cultivated all over the world. The Palmarosa oil has been reported to reveal remarkably good antiviral, antibacterial, antihelmintic, antifungal, antioxidant and cytotoxic properties. In the present study, the essential oil was extracted from the *Cymbopogon martinii* collected from Arunachal Pradesh, India by hydro-distillation process in *Clevenger apparatus*. The extracted essential oil was then evaluated for its antimicrobial activity against five test bacteria viz. *Bacillus cereus, Bacillus subtilis, Listeria monocytogenes, Staphylococcus aureus* and *Escherichia coli* by measuring their zone of inhibition. Three different concentrations of essential oil in acetone viz. 25 ppm, 50 ppm and 100 ppm were also evaluated for its repellent activity against Aedes aegypti mosquito by K & D module. Antimicrobial assay showed a high sensitivity against *Bacillus cereus* and *Bacillus subtilis*; a medium sensitivity against *Listeria monocytogenes* and *Staphylococcus aureus* with a diameter of inhibition17.67±1.38, 16.95±1.23, 14.92±0.68 and 12.10±0.52 respectively. However, no obvious effect was shown against the gram-negative bacteria *Escherichia coli*. The results of repellency activity revealed significant repellent activity of the oil in a dose-dependent manner. The highest concentration i.e. the 100 ppm concentration showed the highest repellent activity against the mosquitoes which decreased as the time increased. The results validated the antimicrobial and repellent properties of the *C. martinii* essential oil and showed its potency to be used both as a natural repellent and an antimicrobial agent in the future.

KEY WORDS: AEDES AEGYPTI, ANTIMICROBIAL, CYMBOPOGON MARTINII, ESSENTIAL OIL, REPELLENCY.

INTRODUCTION

The genus *Cymbopogon* (Poaceae syn. Graminaceae) consists of around 180 plant species native to the tropics and sub-tropics of Asia, America and Africa (Akhila et al. 2009; Avoseh et al. 2015; Baruah et al. 2016). India includes 45 species of the genus which makes it one of the hubs of *Cymbopogon* diversity (Bhatnagar 2018). Generally popular for the high content of essential oil, the plants included in this genus are perennial, monocotyledonous and aromatic in nature. *Cymbopogon martinii* (Roxb.) Will. Watson, commonly known as Palmarosa, is one of the lemongrass species available in north-east India. The plant is traditionally utilized for the treatment of multiple ailments such as arthritis, rheumatism, alopecia, enterosis, lumbago, dermatitis, spasms, impotence, biliousness, wound, cancer of stomach, snakebite, sore, bleeding, and pains by different communities worldwide (Boulos 1983; Dubey and Luthra 2001; Jummes et al. 2020).

Anti-inflammatory, anti-diabetic and diuretic properties are also claimed to be possessed by the plant (Duke and DuCellier 2008). Besides, scientific studies on the plant revealed its bronchodilator, vasodilator and antispasmodic properties (Janbaz et al. 2014). The Palmarosa essential oil is rich in geraniol, geranyl acetate, linalool, β-myrcene, limonene, cymene, sabinene and various other chemical compounds, with antimicrobial properties (Raina et al. 2003; Jummes et al. 2020). This essential oil of Palmarosa is commercially important due to its use in food and flavor industries, high-grade perfumes, soaps, cosmetics, toiletry and tobacco products for its rose-like aroma (Raina et al. 2003; Omar et al. 2016; Jummes et al. 2020).

Scientific studies also suggested its applicability in perfumery, cosmetics and pharmaceutical industries

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(Jummes et al. 2020). Therefore, it is cultivated on large scale in many regions in India including North-East India. Modern pharmacological studies showed the anthelmintic, antiseptic, antifungal and insect repellent activities of the Palmarosa essential oil (Nirmal et al. 2007; Prasad et al. 2010; Caballero-Gallardo et al. 2012). Geraniol is an efficient plant based antimicrobial agent and insect repellent (Bard et al. 1988; Barnard and Xue 2004). The mosquitoes are the carrier of several vector-borne diseases including malaria, yellow fever, dengue fever and many other (AI-Shehri et al. 2020).

Palmarosa oil has reported to possess repellent activities against different mosquito species (Tyagi et al. 1998). In addition, several studies demonstrated and documented the antibacterial and antifungal activity of Palmarosa oil against a variety of bacterial and fungal strains (Prashar et al. 2003; Ahmad and Viljoen 2015; Castro et al. 2020; Mutlu-Ingok et al. 2020; Scotti et al. 2021). Still there are more important bacterial strains and mosquito species against which the effects of Palmarosa essential oil are not evaluated. Therefore, the present study was designed to determine the antimicrobial activity of Palmarosa oil against four gram positive bacterial strain viz. *Bacillus cereus* ATCC 11778, *Bacillus subtilis* ATCC 6051, *Listeria monocytogenes* ATCC BA-751, *Staphylococcus aureus* ATCC 25923 and one gram negative bacterial strain, *Escherichia coli* ATCC 25922 and repellant activities of the oil against the mosquito *Aedes aegypti*.

MATERIAL AND METHODS

From the forest division of Pasighat, Arunachal Pradesh in north-east India (179 m, 28˚03''58''N, 95˚19'36''E), fresh leaves of *Cymbopogon martinii* (Palmarosa) were collected during March-April, 2019. The plant material was identified by Dr. Pankaj Chetia, Assistant Professor, Department of Life Science, Dibrugarh University, Assam, India.

The fresh leaves (800 grams) were subjected to hydro-distillation by using Clevenger Apparatus for about 3 h to obtain the essential oil. The collected essential oil sample was then stored in sealed glass vials in refrigerator prior to experimentation. Strains of four gram-positive bacteria viz. Bacillus cereus ATCC 11778, *Bacillus subtilis* ATCC 6051, *Listeria monocytogenes* ATCC BA-751, *Staphylococcus aureus* ATCC 25923 and one gram-negative bacterium, *Escherichia coli* ATCC 25922 were collected from Centre for Biotechnology and Bioinformatics, Dibrugarh University. Three well isolated colonies of the same morphological type were selected from an agar plate culture and the strains were transferred into a tube containing 4-5 ml of Luria broth (LB) after attaining the logarithmic growth phase, and then incubated at 37˚C.

To obtain turbidity optically comparable to that of the 0.5 McFarland standards to obtain approximately 106 CFU/ml of TB, the turbidity of the actively growing broth culture was adjusted with sterile distilled water. Media plates were inoculated within 15 minutes of standardizing the inoculums to avoid changes in microbial inoculum density (Abalaka et al. 2012). The antimicrobial susceptibility test was carried out by agar well diffusion method (Das et al. 2013). Fresh bacterial culture of 100 µl (106 CFU/ml) was added to the molten Muller Hilton agar medium in the Petri plates homogenously and allowed to solidify. After that with the help of a sterile gel puncture, four wells are aseptically punched on each Petri plates with a diameter of 6-8 mm. Then a 10 µl of the extracted essential oil was introduced into the well by a micropipette under aseptic conditions. 20 µg/ml of chloramphenicol was set as positive control and double distilled water (DDW) was set as negative control (Das et al. 2013).

Depending upon the test microorganisms, the plates were incubated at 37˚C for 18-36 hours and then the diameter of growth of inhibition was measured in mm. The antimicrobial activity was evaluated following the rules of extremely sensitive (>20 mm), high sensitivity (15~20 mm), medium sensitivity (10~15 mm), low sensitivity (7~10 mm) and not sensitive (<7 mm) (Zhou et al., 2020). Triplicate analysis was performed for this experiment. Duration of protection time against mosquito (*Aedes aegypti*) bite was determined by K & D module (Islam et al., 2017). Three different concentrations of *C. martinii* essential oils were prepared with acetone viz. 25 ppm, 50 ppm and 100 ppm. A volume of 25 µL of each concentration were applied randomly to the marked area of volunteer's thigh and allowed to air dry for 5 minutes. The module was then placed over the thigh and the mosquitoes were allowed to access the treated area by opening the sliding door (Zhou et al. 2020).

Ten females (5-7 days old, blood meal unfed) were randomly selected from a pool of 200 adults placed into three adjacent cells in the K & D module. Observation was done three times on each oil concentration and the number of mosquitoes landing and biting in each cell within a 5-minute exposure was recorded. A different set of mosquito population was used for the replications. Observations on the number of bites were recorded at 30 min, 1 hr, 2 hrs, 3 hrs, 4 hrs of post treatment.

RESULTS AND DISCUSSION

Assessment of Antimicrobial Activity: The antimicrobial activity of the essential oil of *C. martinii* against five test organisms was determined by measuring zones of inhibition (Table 1). The negative control showed no antimicrobial activity. In comparison to the positive control (20 µg/ml chloramphenicol) the essential oil showed a high sensitivity against Bacillus cereus and *Bacillus subtilis*; a medium sensitivity against *Listeria monocytogenes* and *Staphylococcus aureus* with a diameter of inhibition17.67±1.38, 16.95±1.23, 14.92±0.68 and 12.10±0.52 respectively. However, no obvious effect was shown against the gram-negative bacteria *Escherichia coli*. Several studies reported that essential oil showed less antimicrobial activity against gram-negative bacteria as the lipopolysaccharides in the outer membrane of these bacteria prevent the

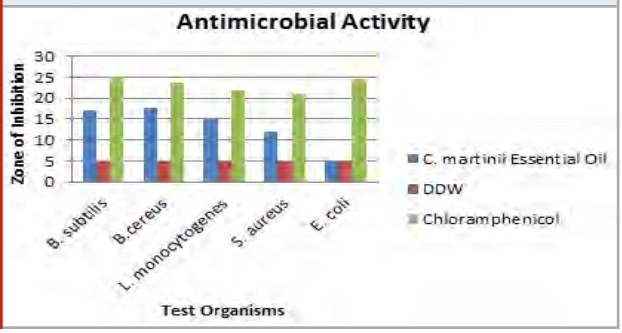
hydrophobic antimicrobial agents (Longbottom et al. 2004; Kavoosi and Rowshan 2013; Rashid et al. 2013). However, Scotti et al. (2020) found greater inhibitory effect of *C. martinii* essential oil against *E. coli* O157:H7 which also contradicts the results of Kim et al. (2016). This may be due to the higher concentrations of essential oil Scotti et al. (2020) used (Kim et al. 2016; Scotti et al. 2020).

Table 1. Zones of Inhibition of *C. martinii* essential oil, DDW and Chloramphenicol against five test organisms.

Test organisms	Zone of Inhibition (mm±SD)		
	C. martinii Essential Oil	DDW	Chloramphenicol
Bacillus subtilis (G⁺)ATCC 6051	16.95±1.23	5.00±0.00	25.19±2.31
Bacillus cereus (G⁺)ATCC 11778	17.67±1.38	5.00±0.00	23.59±0.91
Listeria monocytogenes (G⁺)ATCC BA-751	14.92±0.68	5.00±0.00	21.86±0.87
Staphylococcus aureus (G⁺)ATCC 25923	12.10±0.52	5.00±0.00	21.00±0.00
Escherichia coli (G-)ATCC 25922	5.00±0.00	5.00±0.00	24.53±1.45

(G⁺: Gram Positive; G-: Gram Negative)

Figure 1: Zones of Inhibition of *C. martinii* Essential Oil, DDW and Chloramphenicol against five test organisms



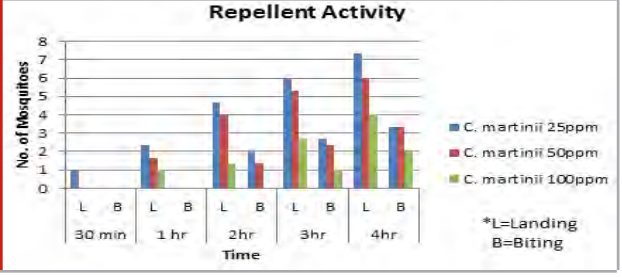
Assessment of Repellent Activity: The essential oil of *C. martinii* exhibited good repellent activity against *Aedes aegypti* mosquitoes (Table 2). The mosquitoes start landing in the cells in 30 minutes and start biting in 2 h at 25 ppm and 50 ppm concentrations. 100 ppm concentration of essential oil showed 100% repellency up to 2 h. The repellent activity of the essential oil was directly related to the concentrations of the oil as well as the time of exposure. The highest concentration i.e. the 100 ppm concentration showed the highest repellent activity against the mosquitoes which decreased as the time increased.

Table 2. Repellent activity of different concentrations of *C. martinii* essential oil against *Aedes aegypti* mosquitoes at different time intervals.

Oil Extract	Concentration	30 min		1 hr		2hr		3hr		4hr	
		L	B	L	B	L	B	L	B	L	B
Cymbopogon martinii	25ppm	1	0	2.33	0	4.67	2	6	2.67	7.33	3.33
	50ppm	0	0	1.67	0	04	1.33	5.3	2.33	6	3.33
	100ppm	0	0	1	0	1.33	0	2.67	1	4	2

(L= Landing; B= Biting)

Figure 2: Repellent activity of different concentrations of *C. martinii* essential oil against *Aedes aegypti* mosquitoes at different time intervals.



The oil exhibited great repellent activity in a dose-dependent manner. Other authors also reported the dose-response relationship of repellency of essential oils (Caballero-Gallardo et al. 2011; Al-Shehri et al. 2020). Luz et al. (2020) documented 337 essential oils from 225 plants that have been tested against *A. aegypti* and found more than 60% of these were active. Significant repellence profile of the essential oil of other *Cymbopogon* species such as *C. nardus* have been shown against *Aedes aegypti* mosquito (Muller et al. 2008; Songkro et al. 2012; Huang et al. 2015; Sajo et al., 2015; Harismah et al. 2017).

CONCLUSION

In the present study, essential oil extracted from field collected leaves of Cymbopogon *martinii* from Arunachal Pradesh are evaluated for its antimicrobial and repellent activities. The findings revealed that the oil was extremely effective against gram-positive bacteria and less effective against gram-negative bacteria. The essential oil showed great repellent activity against *Aedes aegypti* mosquitoes in a dose-dependent manner. The chemical constituents of the *C. martinii* essential oil are responsible for these activities. However, further research is required to elucidate the molecular mechanisms underlying the noteworthy antimicrobial activity. The complex composition of the Palmarosa oil depending on geographical area of origin, extraction method etc. is the main limitation in its use. This entails the necessity for precise control of the individual batches.

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Conflict of Interest: On behalf of all the authors, the corresponding author states no conflict of interests among themselves.

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Pathological Communication

Intensity of Bovine Demodicosis invasion in Northern Trans-Urals

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ABSTRACT

Among the parasitic diseases in cattle, demodectic invasion occupies a special place, and is widespread in various climatic zones of the Northern Trans-Urals, the Russian Federation, and other countries. Bovine demodicosis is an enzootic disease caused by the microscopic Demodex bovis mange, parasitizing in the hair follicles of the sebaceous and sweat glands, and localized as separate colonies on the surface of the animal's body. The disease manifests itself as focal inflammation of the skin in the form of tubercles ranging in size from 0.2 to 1 cm in diameter. In the center of the colonies, a skin scab forms; skin in these areas thickens and loses its elasticity. These areas of the skin have vortex hair formations or their absence. As a result of aggravating pathological processes, sick animals are inert, depressed, have poor appetite and fatness, and decreased milk productivity. In this regard, the objective was to study the intensity of the course of demodectic invasion in cattle in the Northern Trans-Urals. For this purpose, the cattle were clinically examined in 2002-2019 with the obligatory confirmation of the diagnosis of bovine demodicosis by microscopy of skin scrapings and the detection of demodectic mites at various stages of their development. Our studies have established that demodectic invasion in cattle develops in stages in the form of demodectic tubercle colonies: immature, mature, old, and completely developed. Most often, the disease is manifested by a clinically mild – 68.49%, moderate – 26.22% and severe demodectic invasion – 5.29%.

KEY WORDS: CATTLE, DIAGNOSTICS, DEMODICOSIS, DEMODEX BOVIS, EXTENSIVENESS OF INVASION.

INTRODUCTION

The agro-industrial complex of the Tyumen region is the most important sector of the national economy and the main source of food resources that ensure national security. The production of livestock products largely determines the economic and financial condition of the entire agro-industrial complex (Glazunov and Stolbova, 2014). At each stage of development of animal husbandry, the tasks of improving the industry are becoming more complex and broader. Their successful solution requires animals with high productivity, reproductive capacity, and strong immunity; no less important are their maintenance condition, which should be based on the biological laws of the development of the organism and fully satisfy the physiological needs of animals (Larionov and Vasilevich, 2001; Sivkov et al., 2010(b); Sivkov et al., 2010(a); Stolbova et al., 2014; ; Stolbova & Skosyrskikh, 2014; Stolbova & Skosyrskikh, 2015; Stolbova et al., 2016; Stolbova & Skosyrskikh, 2016; Olga and Stolbova, 2020; Miedzybrodzki et al., 2021).

The main reason for the emergence and development of skin pathologies of parasitic etiology in cattle today are violations of zoohygienic requirements for feeding and keeping of and caring for animals, as well as non-compliance with microclimate parameters (Sivkov et al., 2010(a); Sivkov et al., 2010(b); Skosyrskikh & Stolbova, 2011; Stolbova, 2019; Olga and Stolbova, 2020). Demodicosis is currently one of the leading invasive diseases. Bovine demodicosis is a skin disease caused by microscopic mites of the genus Demodex, localized in

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hair bulbs and follicles, and sebaceous glands (Stolbova, 2018; Stolbova et al., 2016; Olga and Stolbova, 2020; Bowman et al., 2021).

To date, issues on the etiology and pathogenesis of bovine demodicosis, as well as the confirmation criteria for the diagnosis of demodectic invasion, depending on the clinical manifestation of the disease and the intensity of the invasion still remain debatable. Thus, the objective of the research was to study the breed-specific predisposition and clinical manifestation of bovine demodicosis in the Northern Trans-Urals. The Objective of the work was to study the intensity of demodectic invasion in cattle in the Northern Trans-Urals.

MATERIAL AND METHODS

The research was carried out in 2002-2019 on the basis of the Federal State Budgetary Educational Institution of Higher Education "State Agricultural University of the Northern Trans-Urals", the departments of non-communicable diseases of farm animals and infectious and invasive diseases, the laboratory of acarology, the All-Russian Research Institute of Veterinary Entomology and Arachnology – a branch of the Tyumen Scientific Center, SB RAS, as well as in farms of different corporate forms of the Tyumen Region. Bovine demodicosis is an enzootic disease caused by the microscopic Demodex bovis mange, parasitizing as single colonies. The disease manifests itself as focal inflammation of the skin in the form of tubercles ranging in size from 0.2 to 1 cm in diameter.

Figure 1: Mild demodectic invasion in cattle

In the center of the colonies, a skin scab forms; skin in these areas thickens and loses its elasticity. In these areas of the skin, vortex hair formations or their absence are found. At the beginning, the disease is clinically unnoticeable. The intensity of invasion was assessed according to the classification proposed by Larionov by identifying a mild, moderate, and severe (generalized) degree of invasion (Larionov & Vasilevich, 2001; Sivkov et al., 2010a). In neglected cases, sick animals are inert, depressed, have poor appetite, a sharp decrease in milk and meat productivity. Differential diagnosis of demodicosis was made between psoroptosis (Sivkov et al., 2010a), hypodermatosis (Sivkov et al., 2010b), sifunculatosis (Stolbova et al., 2014), boviculosis, and ixoidosis (Sivkov et al., 2010b). The resulting digital material was subjected to statistical processing following the biometric techniques, with the calculation of arithmetic mean and root mean square errors (M±m). Values of the reliability criterion were assessed according to the Student-Fisher table of probabilities using Microsoft Excel and Biostat.

RESULTS AND DISCUSSION

Our studies have found a varying intensity of the course of bovine demodectic invasion. We have revealed three – mild, moderate, and severe (generalized) – degrees of skin damage in cattle with demodicosis (Larionov & Vasilevich, 2001; Sivkov et al., 2010(a); Bowman et al., 2021). With mild degree, up to 10 colonies were recorded on the animal's body (Figure 1); with moderate degree, up to 100 colonies were counted (Figure 2); and with severe degree, the number reached over 200 of demodectic colonies on the entire surface of the animal (Figure 3).

Figure 2: Moderate demodectic invasion in cattle

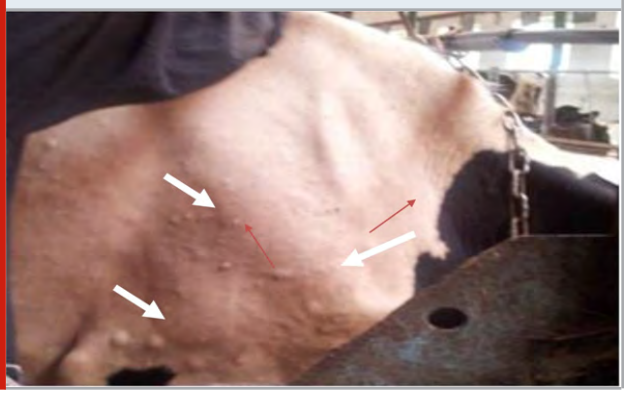


Figure 3: Severe demodectic invasion in cattle

ur studies have recorded the prevailing number of cases of mild demodectic invasion (single colonies up to 10) in dairy and meat-and-dairy cows: Black-and-White – 45.57%, Holstein – 19.83%, Simmental – 2, 94%, Ayrshire – 0.44%, and Yaroslavl – 0.27% (Figure 4). Moderate invasion (up to 100 demodectic colonies) was noted in

Black-and-White – 19.91%, Holstein – 4.47%, Simmental – 1.19%, Ayrshire – 0.22%, and Yaroslavl cows – 0.17%. Severe (generalized) demodectic invasion was noted in Black-and-White – 2.84%, Holstein – 1.46%, Simmental – 0.47%, Ayrshire – 0.15%, and Yaroslavl cows – 0.07%. Among the meet cows, Aubrac animals have up to 10 demodectic colonies in 5.71% of cases, up to 100 colonies in 3.67% of cases, and over 100 colonies in 0.82% of cases. Among the Hereford animals, 31.84% had mild invasion, 15.10% had moderate invasion, and 4.90% had severe invasion. Among the Limousine animals, 19.59% had mild invasion, 7.35% had moderate invasion, and 2.86% had severe invasion. Among the Charolais cows, 2.04% of cases had up to 10 demodectic colonies, 4.49% had up to 100 colonies, and 1.63% had over 100 papules on their body (Figure 5).

Figure 4: Severity of demodectic invasion in dairy and meat-and-dairy cattle

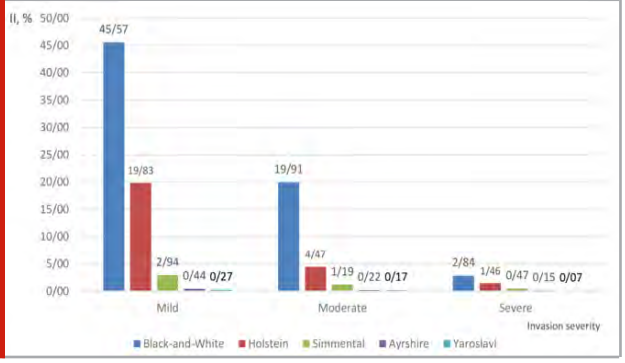
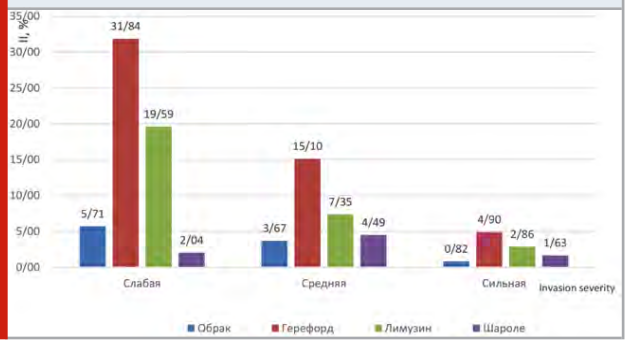


Figure 5: Severity of demodectic invasion in meat cattle



CONCLUSION

Bovine demodicosis proceeds with the formation of demodectic colonies of mild (up to 10 colonies), moderate (up to 100 colonies) and severe (more than 200 colonies) degree of damage. Demodectic invasion in animals develops in stages in the form of demodectic tubercle colonies: immature, mature, old, and completely developed. In this case, the disease is manifested by a clinically mild demodectic invasion in 68.49% of cases, moderate in 26.22%, and severe in 5.29%.

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Conflict of Interest: Author declares no conflicts of interests to disclose.

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Biomedical
Communication

The Role of Bacillibactin in the Regulation of Metabolic Processes in the Body of Animals Under Stress

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ABSTRACT

The primary aim of this survey is to investigate the role of Bacillilectin in the regulation of metabolic processes in the body of animals under stress. Evidently, under stress, the processes of lipid peroxidation (LPO) are activated in the body, which affects the structure and function of cell membranes. Of particular interest is the study of changes in the content of primary and secondary products in blood erythrocytes as the main indicators of the intensity of LPO processes and as markers of the degree of endogenous intoxication. To fulfil the aim of the study, the authors have utilized LII lectin isolated from the surface of nitrogen-fixing soil bacteria *Paenibacillus polymyxa* 1460. The studies were carried out on healthy male white outbred rats with an average body weight of 210 g. The animals were kept under standard vivarium conditions: 12-hour illumination period, temperature 20 °C, food, and water ad libitum. As a result, the study demonstrates that the lectin *Paenibacillus polymyxa*1460 (LII) plays a significant role in the regulation of metabolic processes in the body of animals against the background of swimming stress, contributing to the normalization of lipid peroxidation processes, and also participates in the normalization of the microflora of the large intestine in conditions of dysbiosis in the body of animals against the background stress, acting as a prebiotic, which in turn is widely used for the correction and restoration of the number and qualitative composition of the intestinal microflora. Furthermore, considering the results, it can be inferred that *P. polymyxa* 1460 *lectin* LII exhibits antioxidant properties and can be successfully used as a natural antioxidant, being an effective tonic, as well as bio stimulating agent.

KEY WORDS: ANIMALS' BODY, BACILLI LECTIN, LECTINOLOGY, METABOLIC PROCESSES, PAENIBACILLUS POLYMYXA.

INTRODUCTION

Lectins are carbohydrate-binding proteins of a non-immunoglobulin nature, capable of specific recognition of carbohydrates and reversible binding to them. In recent years, there has been a transition in lectinology from the study of lectins of plant and animal origin to lectins of microorganisms. Low-toxic lectins of non-pathogenic bacteria and their effect on the metabolism of an animal organism are of particular interest, (Pees et al 2021).

Therefore, studies of the effect of lectins of bacterial origin on the metabolic processes of the body of animals, both in normal conditions and in some disorders, are an interesting and urgent task and find application in biology and medicine. Lectins, being biologically active substances, have long attracted the attention of various researchers. The role of bacterial lectins in animals is not well understood to date. The ability of bacilli lectin to regulate the activity of certain enzymes, as well as the correction of important indicators of various metabolic processes in pathological conditions of the body against the background of various types of stress, opens up prospects for their possible use (Vorobiev et al., 1997; Gorelnikova, 2006; Sheikhshoaie et al., 2018; Pees et al., 2021).

As compounds of a protein nature, bacterial lectins have antimicrobial, cytokine activity, are able to change

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the adhesive function of phagocytic cells,which causes scientific and practical interest, which is constantly growing with the understanding of the role of lectins in the regulation of physiological processes and the prospect of their use as biologically active substances and effective pharmacological preparations (Wang et al., 2020; Dasgupta et al., 2020).

The normal intestinal microflora is an important link in the system of the body defense against stress and the preservation of its internal environment (Shenderov, 1987). Normally, all representatives of microflora form a biocenosis, in which each of them has a positive effect on others, ensuring the growth of microorganisms, their metabolism and resistance to damaging factors. Disturbances in the ratio between these biotic components in the intestine reflect microecological changes, which in turn contribute to the development of metabolic, regulatory, metabolic, and immunological disorders in the body and lead to quantitative and qualitative changes in normal microflora, in which not only the total number of intestinal microflora but also its individual representatives (Sajjadi and Moosavi 2018; Liu et al., 2021).

Since recently considerable attention has been paid to lectins of bacterial origin and the study of their functional role in the human and animal body to form a complete picture of the practical significance of *Paenibacillus polymyxa* 1460 lectin (LII) and its role in the regulation of metabolic processes in the body of animals, it is of great interest to study its influence on the processes of lipid peroxidation and on the natural intestinal microflora under swimming stress, which was the purpose of this work. Hence, given the significance and necessity of the issue, the authors have chosen to investigate this matter closely.

MATERIAL AND METHODS

We used LII lectin isolated from the surface of soil nitrogen-fixing bacteria *Paenibacillus polymyxa* 1460 (Karpunin et al., 1993). The studies were carried out on healthy male white outbred rats with an average body weight of 210 g. The animals were kept under standard vivarium conditions: 12-hour illumination period, temperature 20 °C, food and water ad libitum. The lectin preparation was administered to rats intraperitoneally at a dose of 2 μg per animal in physiological solution in a volume of 0.2 ml for three days daily. Swimming stress was carried out by subjecting the animals to forced swimming with weights (a load of 7% of body weight was tied to the tail) in water at a temperature of 25 °C, recording the swimming time of the animals (Dasgupta et al., 2020; Liu et al., 2021).

Determination of the activity of peroxidase in the blood of animals was carried out according to the method (Arkhipova, 1988). The decision of microflora in animals was carried out by inoculating the contents of their large intestine on Petri dishes on selective media for lactic acid bacteria, *Escherichia coli*, staphylococci by the method of serial dilutions (Kostenko et al., 2001).According to the nature of the impact, the experimental animals were divided into 4 groups: group 1 - control animals; Group 2 - animals that received an injection of a solution of lectin LII; Group 3 - animals that were stressed by swimming; Group 4 - animals that previously received an injection of a solution of lectin LII, and they were subjected to swimming stress. Statistical processing of the data obtained was carried out using the Student's t-test (Wang et al., 2020).

RESULTS AND DISCUSSION

As a result of the studies carried out in the study of the effect of bacillus lectin LII on some biochemical parameters of the blood of male rats under swimming stress, it was found that the swimming time of animals with preliminarily introduced bacillus lectin at a dose of 2 μg / animal was 1.9 times longer than the swimming time in the control group. Bacterial lectin LII increases the physical endurance of male rats. So, if the control animals swam for an average of (99.76 ± 4.26) minutes, then the rats, which were preliminarily injected with lectin of bacteria LII P. polymyxa 1460, swam (190.93 ± 1.73) minutes (Table 1), which in percentage terms was an increase in physical activity by 91.4%.

Table 1. The effect of lectin (LII) on the swimming time of male rats

The nature of the impact	Swimming time, min	Swimming time,%
Swimming	99,76±4,26	100
Lectin LII + swimming	190,93±1,73*	191,4

Note - the differences are significant at P <0.05 relative to the values: * - swimming.

The data obtained indicate the activation of metabolic processes in the body of experimental animals by lectin (LII). Thus, in the course of the experiment, it was found that intense physical activity leads to the activation of LPO processes and the accumulation of its products. Possibly, the lectin *P. polymyxa* 1460 is a natural antioxidant and mobilizes the body, normalizing the pituitary - adrenocortical system, intestinal microflora, brings the lipid peroxidation system and antioxidant defense into equilibrium (Wang et al., 2020; Liu et al., 2021). Thus, *P. polymyxa* 1460 lectin suppresses oxygen consumption and accumulation of lipid peroxidation products. The activity of blood peroxidase in the administration of *P. polymyxa* lectin to 1460 male white rats increased 2.3 times, which is a favorable moment for the activation of the antioxidant defense reaction of the body (Fig. 1).It was noted that during exercise, the activity of peroxidase decreases 1.4 times from (0.13 ± 0.015) to (0.09 ± 0.004) mmol / L * s (Fig. 1).

P. polymyxa 1460 lectin led to an increase in catalase activity by 26% relative to the group of animals that received injections of saline (Fig. 2). This can be considered a positive point, since the body's defense reaction is activated, the antioxidant system is activated.

Figure 1: Influence of bacterial lectin LII on the activity of blood peroxidase in male rats during swimming

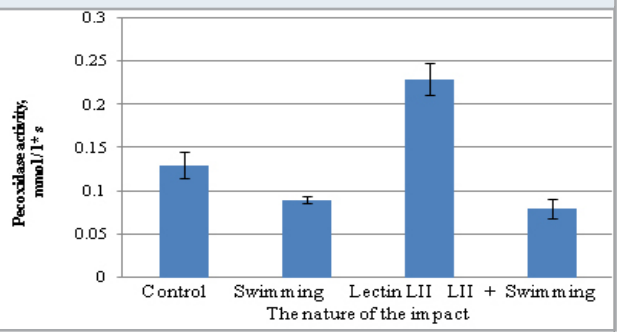


Figure 2: Effect of bacterial lectin LII *Paenibacillus polymyxa* 1460on the activity of catalase in blood plasma of male rats during swimming

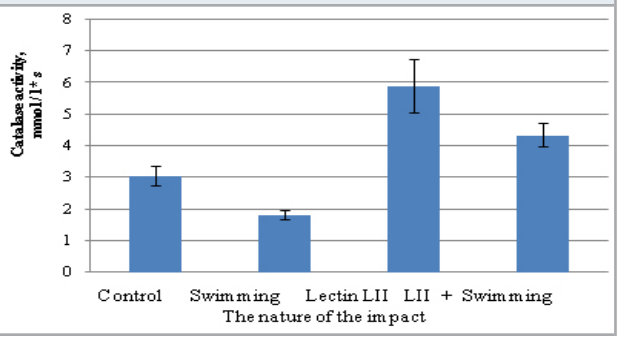
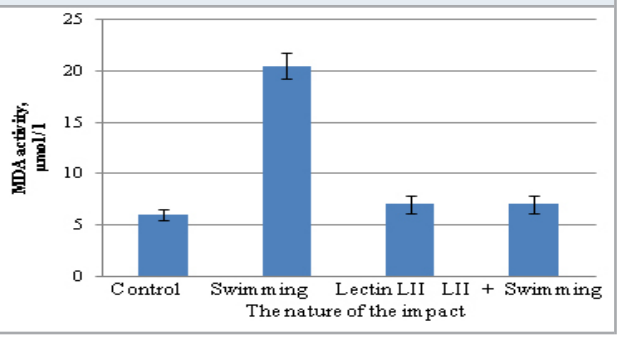
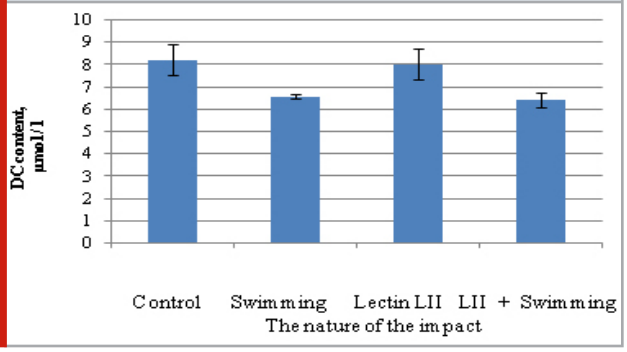


Figure 3: Influence of lectin of bacteria *Paenibacillus polymyxa* 1460on the content of malondialdehyde in the blood of rats during swimming



It was noted that during physical exertion, there is a decrease in catalase activity by 1.7 times from (3.07 ± 0.305) to (1.83 ± 0.141) μat/ L. Preliminary administration of LII lectin to male white rats before swimming leads to an increase in catalase activity from (1.83 ± 0.141) to (4.34 ± 0.370) μat / L. *P. polymyxa* 1460 lectin LII had a positive effect on the activity of antioxidant enzymes (catalase and peroxidase). Catalase activity increased from (3.07 ± 0.305) to (5.90 ± 0.850) μat/L. Thus, the activity of catalase in the blood serum of rats during swimming against the background of prolonged administration of lectin from bacteria LII *P. polymyxa* 1460 corresponded to those of animals in the control group (4.34 ± 0.370) μat/L. It was shown that the administration of *P. polymyxa lectin* LII to 1460 male white rats did not change the MDA content in blood erythrocytes. However, it was noted that during exercise, the content of malondialdehyde increases more than three times, or rather 3.4 times from (6.03 ± 0.490) to (20.46 ± 1.250) μmol / L.

Figure 4: Influence of lectin LII *Paenibacillus polymyxa* 1460for the content of diene conjugates in the blood of rats during swimming



Preliminary administration of *P. polymyxa* LII bacterial lectin to 1460 male white rats before swimming leads to a decrease in the MDA content from (20.46 ± 1.250) to (7.03 ± 0.840) μmol/L.Under combined exposure (introduction of bacterial lectin *P. polymyxa* 1460 followed by swimming) a significant change in the content of lipid peroxidation products and antioxidant activity was observed: the MDA content corresponded to the control values (7.03 ± 0.840) μmol/L.When *P. polymyxa* lectin LII was administered to 1460 male white rats, no changes were observed in the DC content in rat erythrocytes, but during swimming, the content of diene conjugates in the blood erythrocytes of male white outbred rats decreased by 1.2 times relative to the control group with (8.23 ± 0.690) to (6.59 ± 0.110) μmol/L (Fig. 4).Since diene conjugates are the primary product of lipid peroxidation, it can be assumed that their decrease is due to the fact that a sufficient amount of time has passed since the start of exposure (Pees et al., 2021; Liu et al., 2021).

Preliminary administration of *P.polymyxa* lectin LII to 1460 male white rats before swimming almost does not change the content of diene conjugates. Administration of *P. polymyxa* 1460 lectin LII does not affect the content of lipid peroxidation products in rat erythrocytes. The content of diene conjugates in the erythrocytes of the blood of male rats was (8.23 ± 0.69) μmol / L in the control group and (8.03 ± 0.70) μmol / L in the group of animals receiving lectin.

Under combined exposure (introduction of LII lectin *Paenibacillus polymyxa* 1460 followed by swimming), a significant change in the content of lipid peroxidation products and the activity of antioxidants was observed: the content of diene conjugates did not reach the control values (8.23 ± 0.690) μmol / L and remained at the level of animal values, exposed to swimming (6.43 ± 0.340) μmol/L.Taking into account the fact that LPO processes are the limiting factor in the development of fatigue, it can be assumed that the use of bacterial lectin is justified in order to increase efficiency at maximum physical exertion (Wang et al., 2020).

Table 2. Effect of *Paenibacillus polymyxa* 1460 lectin (LII) and the effect of stress on the natural microflora in the large intestine of rats

Microorganisms	Amount KOE x 10^6 /r		
	Control	Swimming	LII + Swimming
Bifidobacteria	3,80±0,20	2,30±0,10*	3,95±0,22*
Lactobacillus	3,25±0,22	2,05±0,12*	3,80±0,10*
Colibacillus	1,20±0,20	2,75±0,15	1,15±0,20
Staphylococcus	1,35±0,16	2,60±0,16	1,60±0,12

Note - * p <0.05 relative to the control group.

In the course of the studies, it was found that the introduction of bacterial lectin LII to rats led, as can be seen from Table 2, to an increase in the number of lactic acid bacteria in the large intestine (bifidobacteria by 46% and *lactobacilli* by 57%) and a decrease in the number of *E. coli* by 75% and *staphylococci* by 85%, relative to the values of the control group. In the group of animals that were subjected to swimming, a decrease in the number of bifidobacteria and lactobacilli by 39% and 37%, respectively, and an increase in the amount of facultative microflora were observed.

Perhaps, in this case, stress leads to a slowdown in the general metabolic processes in the body and to a slowdown in the growth of lactic acid bacteria. The results obtained are in good agreement with the literature data, according to which many stress factors lead to a decrease in the amount of lactic acid microflora in the intestines of animals and an increase in the pathogenic one (Pees et al., 2021). The preliminary introduction of lectin before swimming stress contributed to the normalization of the microflora of the large intestine (Table 2). Thus, as evidenced by the conducted studies, *Paenibacillusp olymyxa* 1460 lectin LII is able to normalize the microflora of the large intestine under stress by swimming (Dasgupta et al., 2020; Liu et al., 2021).

CONCLUSION

Lectin LII *P. polymyxa* 1460 suppresses oxygen consumption and the accumulation of lipid peroxidation products, which contributes to an increase in performance, endurance, the resistance of the body, increasing the swimming time. Thus, it can be assumed that *P. polymyxa* 1460 lectin LII is a natural antioxidant and thereby suppresses the accumulation of DCs in the blood erythrocytes of white male rats, which leads to a decrease in LPO products. Based on the results obtained, we can say that *P. polymyxa* 1460 lectin LII exhibits antioxidant properties and can be successfully used as a natural antioxidant, being an effective tonic, biostimulating agent, providing a complex healing effect on the body, playing an important role in the regulation of metabolic processes of the animal body against a background of stress.The data obtained indicate that *Paenibacillus polymyxa* 1460 lectin LII increases the resistance of animals in extreme conditions caused by stress.

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Biomedical
Communication

In-silico Identification of Triclosan Analogs as Novel Inhibitors of Enoyl-ACP Reductase from *Plasmodium falciparum*

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ABSTRACT

Malaria parasite resistance to currently available drugs has emerged as a major global health problem. As a result, new approaches to developing more target-specific antimalarial drugs are urgently needed. In apicoplast, the fatty acid biosynthesis enzyme enoyl-acyl carrier protein reductase (FabI) plays a vital role in the growth of malaria parasite *Plasmodium falciparum* in the liver-stage; hence it is an excellent target for the development of novel antimalarials drugs. In order to identify novel inhibitors of the FabI enzyme, a computational investigation of the fatty acid production pathway in *Plasmodium falciparum* is presented. Latest research findings has shown that antimicrobial agent triclosan inhibits the growth of *Plasmodium falciparum* by inhibiting the enzyme Fab I. Virtual screening focused on ligand was used to identify triclosan analogs against drug libraries, bioactive, commercial and virtual compounds for inhibiting PfFabI with Swiss similarity tool. Using SwissADME tool, screened compounds were subjected to physicochemical, pharmacokinetic, and toxicological analysis. Compound fulfilling ADME/Tox parameters is docked using MTiAutoDock server with binding pocket of FabI enzyme. The docked complexes were validated and enumerated based on the AutoDock scoring function to pick the best conformation. Triclosan analogs Pubchem CID 448623 and 71579715 having lowest binding energy with FabI enzyme even lower than known antimicrobial agent triclosan were predicted as novel inhibitors of PfFabI. To maximize accuracy of result, best predicted compounds again docked with FabI enzyme with different algorithm using PatchDock server. Our research has led to the discovery of two novel FabI inhibitors, which may be confirmed by laboratory experiments.

KEY WORDS: ENOYL-ACP REDUCTASE, FABI, MALARIA, PLASMODIUM FALCIPARUM, TRICLOSAN.

INTRODUCTION

Malaria is a major public health issue that has become increasingly critical and continues to promote study, impacting the world's main tropical and subtropical areas. 228 million malaria cases occurred worldwide in 2018 compared to 231 million in 2017 (WHO 2019). India and 19 countries in sub-Saharan Africa accounted for approximately 85% of the global malaria burden. An estimated 405 000 deaths from malaria were reported globally in 2018, compared with an estimated 416 000 deaths in 2017. *Plasmodium falciparum* has to be the most dominant malaria parasite in the South East Asia region of the WHO, accounting for 50% of confirmed cases of malaria in 2018 (WHO 2019).

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Anti-malarial drug formulation; vaccination and vector control are the main methods to control this parasitic disease. Parasitic drugs are between these the first line of defence. The P. falciparum 'chloroquine resistance transporter' (PfCRT) was first identified as a main determinant of chloroquine (CQ) resistance, but mutations in this protein are now known to affect the parasite's susceptibility to a variety of current and potential antimalarials (Martin, 2020). Thus, developing a new generation of anti-malarial drugs will represent a significant advance to consider. Apicoplast in malaria parasites has important consequences in combating their anti-malarial drug resistance (Gleeson, 2000; Liting and Geoffrey 2010).

Apicoplast metabolic pathways of isoprenoid precursor synthesis, fatty acid synthesis, heme synthesis and biogenesis of the iron sulfur cluster acting role in genome replication, transcription, translation, post-translational

modification and protein turnover (Ralph et al. 2004; Liting and Geoffrey 2010). The apicoplast is a remnant of an ancient cyanobacterium that incorporates enzymes and biosynthetic processes that are more bacterial than eukaryotic in nature, allowing antimalarial agents to be designed that are selective against apicoplast targets (Ralph et al. 2004; Liting and Geoffrey 2010).

Apicoplast pathways fatty acid and isoprenoid precursor synthesis are clearly bacterial in origin and are potential targets for antibiotic drugs because they differ from the eukaryotic host processes.The *Plasmodium* FAS-II pathway was of particular therapeutic significance as it is distinct from the mammalian type I (FAS-I) pathway. The fatty acid biosynthesis enzyme enoyl-ACP reductase (FabI) plays a vital role in the growth of parasites in the liver-stage (Yu et al. 2008). Among the many known PfFabI inhibitors studied so far, triclosan remains the most active with an IC50 = 14 ng / ml (50 nM), but due to human health and environmental concerns, the compound is not appropriate for medicinal application (Kapoor et al. 2004). In this study, we have incorporated ligand based virtual screening method to search out the potential triclosan analogs for the treatment against maleria targeting the *Plasmodium falciparum* enoyl-acyl-carrier-protein reductase, which were not reported previously.

Table 1. The pharmacokinetics and drug likeliness properties of triclosan analogs.

Sl. No.	Pubchem CID	BBB	GI	CYP1A2 inhibitor	CYP2D6 inhibitor	LogKp (Cm/s)	Bioavailab-ility Score	Lipinski	Ghose	Veber	Egan	Muegge
1	5564 (Triclosan)	High	Yes	Yes	No	-4.69	0.56	Yes	Yes	Yes	Yes	Yes
2	7638	High	Yes	Yes	Yes	-5.09	0.55	Yes	Yes	Yes	Yes	Yes
3	18807	High	Yes	Yes	No	-4.63	0.56	Yes	Yes	Yes	Yes	Yes
4	44129612	High	Yes	Yes	No	-5.58	0.55	Yes	Yes	Yes	Yes	Yes
5	448978	High	Yes	Yes	No	-5.04	0.56	Yes	Yes	Yes	Yes	Yes
6	447966	High	Yes	Yes	Yes	-6.02	0.55	Yes	Yes	Yes	Yes	Yes
7	2255489	High	No	Yes	No	-6.74	0.55	Yes	Yes	Yes	Yes	Yes
8	448623	High	Yes	Yes	Yes	-5.47	0.55	Yes	Yes	Yes	Yes	Yes
9	69545180	High	Yes	Yes	Yes	-6.35	0.55	Yes	Yes	Yes	Yes	Yes
10	15624	High	Yes	Yes	No	-5.17	0.56	Yes	No	Yes	Yes	No
11	16122582	High	No	No	No	-6.95	0.55	Yes	Yes	Yes	Yes	Yes
12	16220130	High	Yes	Yes	Yes	-4.69	0.55	Yes	Yes	Yes	Yes	No
13	3758	High	No	No	No	-6.62	0.55	Yes	Yes	Yes	Yes	Yes
14	5564	High	Yes	Yes	No	-4.69	0.55	Yes	Yes	Yes	Yes	Yes
15	18807	High	Yes	Yes	No	-4.63	0.55	Yes	Yes	Yes	Yes	Yes
16	5271320	High	Yes	Yes	No	-4.84	0.55	Yes	Yes	Yes	Yes	Yes
17	23656591	High	Yes	Yes	Yes	-4.94	0.55	Yes	Yes	Yes	Yes	Yes
18	852148	High	Yes	Yes	No	-5.50	0.55	Yes	Yes	Yes	Yes	Yes
19	45490026	High	Yes	No	No	-5.50	0.55	Yes	Yes	Yes	Yes	Yes
20	22947105	High	Yes	Yes	No	-4.75	0.55	Yes	Yes	Yes	Yes	Yes
21	71718966	High	Yes	Yes	Yes	-4.42	0.55	Yes	Yes	Yes	Yes	Yes
22	44410251	High	Yes	Yes	No	-5.74	0.55	Yes	Yes	Yes	Yes	Yes
23	25023958	High	Yes	Yes	Yes	-4.53	0.55	Yes	Yes	Yes	Yes	Yes
24	44405311	High	Yes	Yes	No	-5.40	0.55	Yes	Yes	Yes	Yes	Yes
25	21272512	High	Yes	Yes	No	-5.27	0.55	Yes	Yes	Yes	Yes	Yes
26	11659169	High	Yes	Yes	No	-5.28	0.55	Yes	Yes	Yes	Yes	Yes
27	6852148	High	Yes	Yes	No	-5.50	0.55	Yes	Yes	Yes	Yes	Yes
28	25023955	High	Yes	Yes	No	-5.28	0.55	Yes	Yes	Yes	Yes	Yes
29	71579715	High	Yes	Yes	Yes	-5.10	0.55	Yes	Yes	Yes	Yes	Yes
30	25023959	High	Yes	Yes	Yes	-4.23	0.56	Yes	Yes	Yes	Yes	No
31	5564	High	Yes	Yes	No	-4.69	0.55	Yes	Yes	Yes	Yes	Yes
32	94509	High	Yes	Yes	No	-4.63	0.55	Yes	Yes	Yes	Yes	Yes
33	12774295	High	Yes	Yes	No	-4.70	0.55	Yes	Yes	Yes	Yes	Yes
34	10846712	High	Yes	Yes	No	-4.97	0.55	Yes	Yes	Yes	Yes	Yes

35	10711808	High	Yes	Yes	No	-5.45	0.55	Yes	Yes	Yes	Yes	Yes
36	82267072	High	Yes	Yes	No	-5.10	0.55	Yes	Yes	Yes	Yes	Yes
37	21272514	High	Yes	Yes	No	-4.53	0.55	Yes	Yes	Yes	Yes	Yes
38	12346120	High	Yes	Yes	No	-4.61	0.55	Yes	Yes	Yes	Yes	Yes
39	82267104	High	Yes	Yes	Yes	-5.03	0.55	Yes	Yes	Yes	Yes	Yes
40	82053313	High	Yes	Yes	No	-5.39 cm/s	0.55	Yes	Yes	Yes	Yes	Yes
41	14094464	High	Yes	No	No	-5.66	0.55	Yes	Yes	Yes	Yes	Yes
42	45093134	High	Yes	No	No	-5.59	0.55	Yes	Yes	Yes	Yes	Yes
43	59319916	High	Yes	Yes	Yes	-5.04	0.55	Yes	Yes	Yes	Yes	Yes
44	156913	High	No	No	No	-4.22	0.56	Yes	No	Yes	No	No
45	627458	High	Yes	No	No	-4.52	0.55	Yes	Yes	Yes	Yes	Yes

GI-Gastrointestinal absorption, BBB-Blood Brain Barrier penetration,CYP: Cytochrome P450, Log Kp-Skin Permeation Coefficient.

MATERIAL AND METHODS

Ligand based virtual screening of triclosan against databases using Swisssimilarity web tool. Swisssimilarity tool's screenable libraries include drugs, bioactive and commercial compounds and millions of virtual compounds readily synthesizable from commercially available synthetic reagents (Zoete et al. 2016).A drug with good oral absorption must meet the following parameters: molecular weight of less than 500 Da, logP (lipophilicity) of less than five (5); maximum of five (5) groups of hydrogen donors and maximum of ten (10) groups of accepters binding intestinal permeability and consisting of the first steps towards good oral bioavailability. *In-silico* physicochemical, pharmacokinetic and toxicological properties of triclosan analogs were perfomed by SwissADME web tool. The crystal structure of plasmodium falciparum enoyl-acyl-carrier-protein reductase with triclosan (PDB ID: 1NHG) was retrieved from the Protein Data Bank (https://www.rcsb.org/). All the Nicotinamide-adenine-dinucleotide (NAD) and triclosan compound were removed and polar hydrogen added to make the structure of enoyl-acyl-carrier-protein reductase prepared for molecular docking processes (Daina et al. 2017).

inding site residues of FabI enzyme was predicted by CASTp3.0 server (Tian et al. 2018). Computed Atlas of Surface Topography of proteins (CASTp) is a web server that provides online resources to find delineate and quantify the geometric and topological properties of protein structures. The molecular docking method can be used to model the atomic level interaction between a small molecule and a protein, which enables us to describe the actions of small molecules in the target protein binding site as well as to elucidate basic biochemical processes. Screened triclosan analogs were docked with enoyl-ACP reductase using MTiAutoDock (https://mobyle.rpbs.univ-paris-diderot.fr/cgi-bin/portal.py#forms::MTiAutoDock). The most negative binding affinity score of compounds were chosen as candidate compounds (Daina et al. 2017).

RESULTS AND DISCUSSION

In Silico ADME/Tox Screening of Triclosan Analogs: About half of the drug candidates struggled during development due to ADME/Tox deficiencies. In order to prevent this development failure a series of *In-silico* ADME/Tox screens was introduced with the intention of discarding compounds during the discovery process. The ADME predictions of triclosan analogs for passive human gastrointestinal absorption (GI) and permeation of the blood-brain barrier (BBB) are both based on the BOILED-Egg model were shown in table 1. The properties of human intestinal absorption are determinant of the drug production and are required to be administered orally. The blood-brain barrier (BBB) plays a significant role in drug pharmacology (Ma et al. 2005). Carcinogenicity is a cancer-causing risk in the body. Triclosan analogs were accessed by five different rule-based filters such as Lipinski filter implemented rule-of-five, Ghose, Veber, Egan and Muegge methods, respectively and shown in table 1. The result presented in Table 1 shows that all of the investigated compounds present high gastrointestinal absorption, good skin permeation and inhibit xenobiotic metabolism involving cytochrome CYP1A and CYP2D6. At the end of the entire ADMET evaluation screening, four compounds, CID 15624, 16220130, 25023959 and 156913, failed the drug likeliness test and were deleted for subsequent analysis (Ghose et al. 1999; Egan et al. 2000; Muegge et al. 2001; Veber et al. 2002; Lipinski 2004; Ma et al. 2005).

Binding site analysis of FabI: CASTp(http://sts.bioe.uic.edu/castp/calculation.html) provides a comprehensive and detailed quantitative characterization of the inner voids and surface pockets on FabI's three-dimensional structure. Identified binding site residues of Fab I on chain A of Plasmodium FalciparumareGLY104, ILE105, GLY106, ASP107, GLY110, TYR111, GLY112, TRP113, GLY129, TRP131, VAL134, PHE167, ASP168, ALA169, SER170, HIS214, SER215, LEU216, ALA217, ASN218, ALA219, VAL222, LYS240, SER241, LEU265, THR266, TYR267, TYR277, MET281, LYS285, LEU288, ALA312, GLY313, PRO314, LEU315, SER317, ARG318, ALA319, ALA320, ALA322, ILE323.

Table 2. Triclosan analogsphysicochemical properties and binding energy with FabI

Sl. No.	Pubchem CID	Molecular weight (g/mol)	No of rotatable bonds	No of H-bond acceptors	No of H-bond donors	TPS (Å²)	Binding energy (kcal/mol)
1	5564(Triclosan)	289.5	2	2	1	29.5	-4.36
2	7638	200.23	3	2	1	29.5	-4.76
3	18807	255.09	2	2	1	29.5	-4.08
4	44129612	384.7	2	3	1	65.8	-4.42
5	448978	327.2	5	4	1	55.8	-4.42
6	447966	272.3	2	3	1	54.5	-4.71
7	2255489	239.3	2	4	2	99.3	-4.86
8	448623	338.17	3	3	2	49.3	-5.10
9	69545180	240.26	1	2	3	65.1	-3.60
10	16122582	257.31	6	5	1	94.8	-3.28
11	3758	222.24	2	3	1	69.3	-3.48
12	5564	289.5	2	2	1	29.5	-4.71
13	18807	255.09	2	2	1	29.5	-4.07
14	5271320	220.65	2	2	1	29.5	-4.14
15	23656591	303.6	3	2	1	29.5	-4.30
16	6852148	270.11	2	3	2	55.5	-4.67
17	45490026	270.11	2	3	2	55.5	-3.34
18	22947105	269.12	2	2	1	29.5	-4.45
19	71718966	270.71	2	2	1	29.5	-4.94
20	44410251	235.66	2	3	2	55.5	-4.45
21	25023958	283.1	3	2	1	29.5	-4.24
22	44405311	294.13	3	3	1	53.2	-4.52
23	21272512	271.09	2	3	2	49.7	-4.49
24	11659169	280.1	2	3	1	53.2	-4.72
25	6852148	270.11	2	3	2	55.5	-4.66
26	25023955	280.1	2	3	1	53.2	-4.66
27	71579715	271.7	2	3	1	42.2	-5.05
28	5564	289.5	2	2	1	29.5	-4.41
29	94509	255.09	2	2	1	29.5	-3.78
30	12774295	220.65	2	2	1	29.5	-4.12
31	10846712	222.67	1	2	1	29.5	-3.34
32	10711808	334.54	1	2	1	29.5	-3.60
33	82267072	221.08	2	2	1	29.5	-3.39
34	21272514	255.09	2	2	1	29.5	-3.61
35	12346120	220.65	2	2	1	29.5	-3.39
36	82267104	221.08	2	2	1	29.5	-4.27
37	82053313	186.63	1	2	1	29.5	-3.68
38	14094464	253.08	3	4	1	47.9	-3.01
39	45093134	209.023	3	3	2	49.7	-3.18
40	59319916	200.66	3	2	1	29.5	-3.39
41	627458	303.6	3	2	0	18.5	-4.20

Analysis of Molecular Docking: Forty oneADME/Tox parameters qualify triclosan were docked in binding site of FabI enzyme using MTiAutoDock server. Binding energy of triclosan analogs were shown in table 2. On analysis of binding energy of analogs, it was found that two analogs, CID 448623 and 71579715 had low binding affinity -5.10 kcal / mol, -5.05 kcal / mol with FabI, respectively, even lower than -4.36 kcal / mol with triclosan, highlighted with bold in table 1.To maximize accuracy of result, best predicted analogs again docked with FabI enzyme from different algorithm using PatchDock server using Patch Dockserver (Duhovny et al. 2002; Schneidman-Duhovny et al. 2005). The clustering RMSD was a default value of 4.0. The top 10 PatchDock solutions produced were refined using Firedock based on their binding energyand the best models had global energy of -39.04 and -43.33kcal/mol for analogs CID 448623 and 71579715 that suggests a good interaction

between ligand and protein (Andrusier et al. 2007; Mashiach et al. 2008).

Predicted Docked complexes were analyzed through Python Molecular Viewer for their interaction study shown in Figure 1 and 2. Triclosan analog was represented in sticks and balls model. Triclosan analog CID 448623 interacted with residues GLY104, LEU216, ASN218, ILE130, GLY129, TRP131, LYS240, SER170, ALA169, ASP168 and PHE167. All the residues interacting with CID 448623 belongs to predicted binding site residues of FabI except ILE130. Triclosan analog CID 71579715 interacted with residues GLY106, ARG316, ILE105, GLY106, VAL134, TRP131, ALA169, and PHE167. All the residues interacting with CID 71579715 also belongs to predicted binding site residues except ARG316. Interacting residues were represented in lines as Figure 1 and 2 (Sanner 1999; Pamudi et al. 2017).

Figure 1: Docking pose of triclosan analog CID448623in binding site of FabI enzyme.

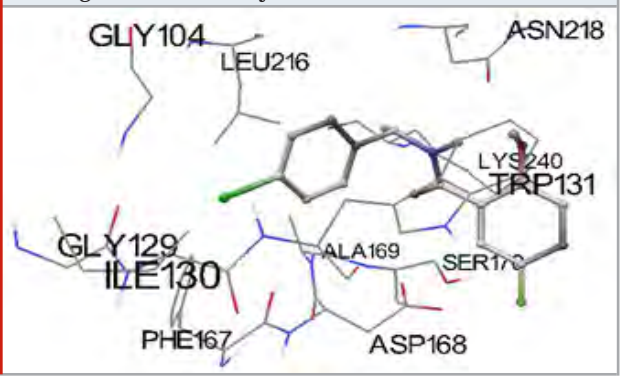
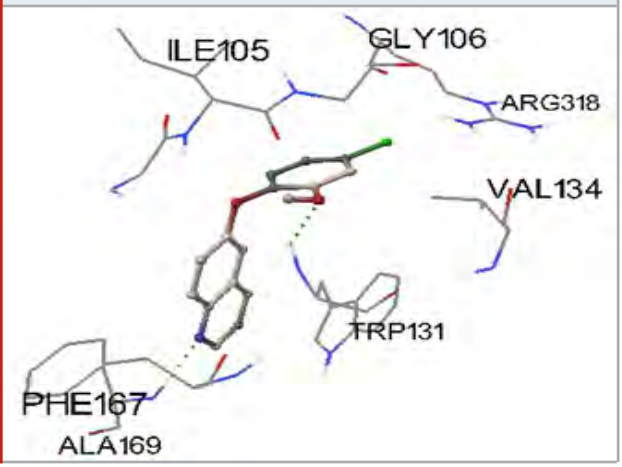


Figure 2: Docking pose of triclosan analog CID 71579715in binding site of FabIenzyme.Two H-bonds were formed between amino acid TRP131 and ALA169 of protein with compound, respectively. Hydrogen bonds are represented with spherical line.



The lack of an effective vaccine and the emergence of *Plasmodium variants* resistant to antimalarial agents emphasize the need for novel chemotherapeutic drugs. In-silico screening, also known as virtual screening, is currently being researched as a strategy for finding antimalarial medicines. Pamudi et al. (2017) explore at the usage of virtual screening to identify Enoyl Acyl Carrier Protein Reductase inhibitors in *Plasmodium falciparum*. The medicinal plants in Indonesia database were used in conjunction with a molecular docking method using GOLD software. They discovered two prospective inhibitor chemicals in tea that have the potential to be developed as antimalarial drugs: kaempferol 3-rhamnosyl-(1-3)-rhamnosyl-(1-6)-glucoside and epigallocatechin 3,5, -di-O-gallate (Pamudi et al. 2017; Dayse et al. 2020).

By using hierarchical virtual screening, Dayse et al. (2020) discovered flavonoids to be inhibitors of *Plasmodium falciparum* enoyl-acyl carrier protein (ACP) reductase (Dayse et al. 2020). A flavonoid library from the ChEMBL database was screened for physico-chemical similarity using the Euclidean distance as a criterion, and then molecular docking was performed using the GridScore scoring tool in the DOCK 6.5 software (Dayse et al. 2020). Using ligands from the Indonesian Medicinal Plants Database, in-silico screening was carried out using the AUTODOCK VINA software to identify potential *Plasmodium falciparum* enoyl-acyl carrier protein (ACP) reductase inhibitor candidates by Malau et al. (2020).

CONCLUSION

The current research utilises computer-based virtual screening to identify Plasmodium falciparum Enoyl-ACP reductase inhibitors that are required for malaria treatment. From several million chemical structures and a sequence of steps of rational refinement, including similarity search, ADME/Tox properties and molecular docking, we identified two triclosan analogs, CID 448623 and 71579715, as good inhibitors for further experimental testing.

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Biomedical
Communication

Amelioration of Streptozotocin-Induced Diabetes by *Bougainvillea spectabilis* Leaf Extract Through Modulation of Carbohydrate Metabolic Enzymes and Oxidative Stress in Rats

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ABSTRACT

Diabetes is a leading cause of the death in the world's growing population and the occurrence of diabetes in adults worldwide is estimated to rise up to 592 million in the year 2035. A number of modern drugs are available in controlling diabetes, but their long term utilization may lead to side effects, therefore interest has been focused to the consumption of medicinal plants as an alternative or complimentary treatment for diabetic medication to avoid the side effects caused by the synthetic drugs. Hence, this study was designed to evaluate the effect of methanolic extract of *Bougainvillea spectabilis* leaves (MEBS) on carbohydrate metabolism and oxidative stress in streptozotocin (STZ)-induced diabetic rats. Administration of STZ to rats demonstrated that hyperglycemia, increased lipid profile, altered carbohydrate metabolism and decreased antioxidant status in liver and kidney. On the other hand, treatment with MEBS (200 and 400mg/kg BW) to diabetic rats for 28 days suppressed the blood glucose and increased the insulin levels and thereby attenuated hyperlipidemia and lipid peroxidation. At the same time, MEBS increased the activities of glucokinase, pyruvatekinase, glucose-6-phosphate dehydrogenase and glycogen synthase and concomitantly increased the glycogen content in the liver and reduced the activities of glucose-6-phosphatase, fructose-1,6-bisphosphatase and glycogen phosphorylase. MEBS also attenuated the oxidative stress by maintaining the enzymatic and non-enzymatic antioxidants homeostasis as well as protected from the damage of pancreas, liver and kidney due to glucose toxicity which was established by the histopathological analysis. Hence, the current study recommended that MEBS contribute to preventing hepatic and renal complications associated with diabetes mellitus.

KEY WORDS: BOUGAINVILLEA SPECTABILIS, CARBOHYDRATE METABOLISM, DIABETES MELLITUS, MEDICINAL PLANTS, OXIDATIVE STRESS.

INTRODUCTION

Diabetes mellitus (DM) is a persistent multifactorial syndrome of disordered metabolism related with the metabolism of carbohydrates, proteins and fats due to insulin resistance (IR) or insulin deficiency (Kleinaki et al., 2020; Uddandrao et al., 2020a; Gokçay and Sahin 2021). The global occurrence of DM in 2019 is accounted to be 9.3% (463 million people), mounting to 10.2% (578 million) by 2030 and 10.9% (700 million) by 2045 (Saeedi et al., 2019). Uncontrolled hyperglycemia can leads to macro and micro-vascular complications such as nephropathy, retinopathy and cardiomyopathy (Parim et al., 2019). The metabolic dysregulations of glucose homeostasis are the main cause for the progress of DM and the principal reason of diabetic morbidity and death (Jiang et al., 2020).

During glucose homeostasis, the liver serves an imperative role in carbohydrate production, storage and redistribution (Jiang et al., 2020). The liver executes conflicting functions during hyperglycemic and hypoglycemic states; therefore, the physiological regulation of hepatic glucose production is a multifaceted procedure (Jiang et al., 2020). Patients with type 2 DM (T2DM) and type 1 DM (T1DM) exhibit increased hepatic glucose synthesis,

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of which numerous extra hepatic mechanisms add to the physiological regulation of hepatic glucose production (Kalidhindi et al., 2020). Conversely, evidences have been distinguished that sustain the role of oxidative stress in the pathogenesis of both T1DM and T2DM. Free radical formation in DM by glucose oxidation, increased lipid peroxidation and non-enzymatic glycation of proteins leads to damage of enzymes, cellular mechanism and also increased IR due to oxidative stress (Sathibabu et al., 2019). A number of experimental studies have recommended that the increased production of reactive oxygen species causes damage to cells and tissues like liver, pancreas and kidney throw in to diabetic complications (Uddandrao et al., 2020a).

Diet, exercise and numerous pharmacological agents are treatment strategies for DM. Taking into account the results associated with the treatment by synthetic drugs and insulin which are accessible at present, screening for safer and effective antidiabetic plant drugs are going on all over the planet. Herbal medicines play an imperative role in this part to put off adverse effects (Sathibabu et al., 2018; Parim et al., 2019). The systematic investigation of plants documented for their therapeutic value is an efficient and alternate approach for the invention of new remedial agents. Several contemporary medicines are available in controlling DM, but prolonged utilization may lead to adverse effects, therefore interest has been shifted towards medicinal plants as an alternative therapy for diabetic medication to avoid the side effects caused by the synthetic drugs (Win 2020). *Bougainvillea spectabilis* (Family: Nyctaginaceae), is one of the conventional herbal plants (Ghogar and Jiraungkoorskul 2017).

B. spectabilis is documented to have therapeutic effects which include anti-hepatotoxic, anti-inflammatory, antioxidant, antimicrobial, antihyperlipidemic, anticancer and antiulcer properties (Anisa et al. 2016). Our previous study reported that preliminary antidiabetic activity of methanolic extract of *B. spectabilis* leaves (MEBS) against streptozotocin (STZ)-induced DM in rats (Chitra Devi and Ramesh 2018). Conversely, to the best of our knowledge, the effect of MEBS as prospective antidiabetic ingredient and its effect on carbohydrate metabolic enzymes and oxidative stress are yet to be studied (Chitra Devi and Ramesh 2018). Therefore, the current study was intended to assess the impacts of MEBS on gluconeogenic and glycogenesis enzymes activities along with oxidative stress in liver and kidney of STZ-induced diabetic rats.

MATERIAL AND METHODS

For the sample collection, the leaves of *B. spectabilis* were collected from surrounding area of Kangayam, Tirupur district, Tamilnadu, India. The plant was identified by expert of Dr. K. Madhava Chetty, Plant Taxonomist, Department of Botany, Sri Venkateswara University, Tirupati. For the preparation of MEBS, the leaves of *B. spectabilis* were shade dried at room temperature. The dried plant leaves were subjected to size reduction to a coarse powder (1000 grams) by using dry grinder and passed through sieve. The powder was packed into soxhlet apparatus and extracted consecutively with petroleum ether (60-80°C) and 90% methanol. The extraction was carried out until the extract becomes colorless. The solvent was removed by distillation under reduced pressure and stored in desiccators for further experiment. For the GC-MS (Gas Chromatography-Mass Spectrophotometry) analysis, the MEBS was subjected to identify bioactive compounds by GC-MS as described by Uddandrao et al. (2020b). Interpretation of mass spectrum GC-MS was conducted by using the database of NIST (National Institute Standard and Technology) and the compounds were identified (Uddandrao et al. 2020b).

For the High-performance thin-layer chromatography (HPTLC) analysis for flavonoid profile, the HPTLC analysis was done with *B. spectabilis* leaves sample (20mg) using mobile phase for flavonoids, i.e., 90:10 ratios of chloroform and methanol solvents. The HPTLC analysis was carried out as per the protocol previously described by the Pallavi and Hemalatha (2018). For the experimental animals, male Wister albino rats (weight: 170-230gm; age: 8-12 weeks) were procured from Sri Venkateswara College of Pharmacy, Chittoor, Andhra Pradesh, India.

The animals were maintained in a well-ventilated room with at 12:12 h light, dark cycle in polypropylene cages and maintained at 22±1°C with humidity at 55±5% (Pallavi and Hemalatha 2018). They were fed balanced rodent pellet diet and tap water ad *libitum* throughout the experimental period. The protocol of this study was approved by the Institutional Animal Ethics Committee of Sri Venkateswara College of Pharmacy (Reference No: SVCOP/IAEC/002/2016-17). Induction of DM was persuaded in rats by a single intraperitoneal injection of STZ (40mg/kg body weight) in 0.1M citrate buffer (pH 4.5). DM was confirmed by the lofty glucose level in plasma which was identified after 48 hrs of STZ administration. The rats with successful elevated plasma glucose levels (above 250 mg/dL) were chosen for further treatment (Pallavi and Hemalatha 2018). For the experimental design, the rats were divided in to five groups and each comprising with 6 animals. The doses of MEBS (200 and 400mg/kg BW) were fixed based on the toxicological evaluation done (Chitra Devi and Ramesh 2018) as per Organization for Economic Cooperation and Development (OECD) guideline 423, fixed dose procedure. All the respective drugs were administered orally by using intragastric tube in a vehicle solution (normal saline) for a period of 28 days. Rats in group 1 and 2 received normal saline (2 mL/kg BW) as placebo treatment.

Group 1: Normal control rats Group 2: Diabetic control rats Group 3: Diabetic + MEBS (200mg/kg BW) Group 4: Diabetic + MEBS (400mg/kg BW) Group 5: Diabetic + Glibenclamide (600µg/kg BW) After completion of treatment duration, the animals were anaesthetised using low doses of phenobarbitone and sacrificed by cervical decapitation. Blood samples were collected by retro orbital sinus puncture method. On the other hand, organs (pancreas, liver and kidney) were straight away

dissected out, washed in ice cold saline, dried, weighed, and used for biochemical estimation. For the estimation of body weight, blood glucose, insulin and glycated hemoglobin, at the end of the experimental period, body weights of the animals were recorded. On the other hand, plasma glucose (Sigma-aldrich Kits, India), insulin (Bio-Merieux, France), and glycated haemoglobin (Hitachi 912, Germany) was evaluated by using the respective kits. To begin with oral glucose tolerance test (OGTT), overnight fasted rats were received a dose of 2.0g/kg BW glucose orogastrically and blood samples were collected from supra orbital sinuses at various intervals such as 0, 30, 60, 90, and 120 min, and the glucose levels (Sigma-aldrich Kits, India) were estimated for all time intervals.

For the biochemical analysis, liver biochemical markers such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were estimated as described by Gella et al. (1985). Lactate dehydrogenase (LDH) was measured as per commercially available kit (MAK066-1KT, Sigma-Aldrich, USA). For the estimation of serum lipid profile, the serum lipid profile markers such as total cholesterol (TC), high density lipoprotein (HDL-C), very low-density lipoprotein (VLDL-C), low density lipoprotein (LDL-C) and triacylglycerides (TG) were assessed by enzymatic colorimetric methods using kits (Nicholas Piramal India Ltd., Mumbai, India). For the determination of liver carbohydrate metabolizing enzymes activity, the carbohydrate metabolic marker enzymes namely glucokinase, pyruvatekinase, glucose-6-phosphatase, glucose-6-phosphate dehydrogenase, fructose-1,6-bisphosphatase were estimated with respective protocols (Koida and Oda 1959; Walker and Rao 1964; Gancedo and Gancedo 1971; Rudack et al. 1971; Ingeborg and Erich 1974). For the assessment of liver glycogen metabolizing enzymes activity, the liver glycogen content, glycogen synthase and glycogen phosphorylase were assessed with respective protocols (Ong and Khoo 2000; Nielsen et al. 2001; Poucher et al. 2007).

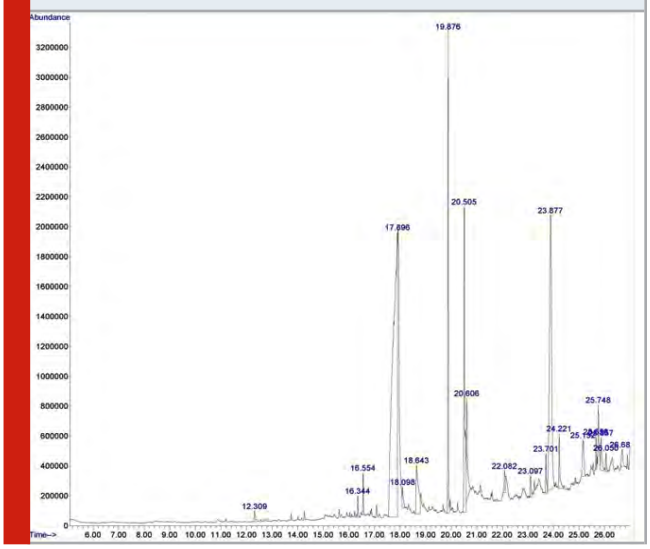
For the determination of lipid peroxidation, the level of Thiobarbituric acid reactive substances (TBARS) in serum, liver and kidney was estimated by the method of Fraga et al. (1988). For the estimation of oxidative stress markers, the oxidative stress markers in serum, liver and kidney namely reduced glutathione (GSH), vitamin A, vitamin C, vitamin E, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) were determined as described by Saravanan and Ponmurugan (2013).

For the histopathological analysis of pancreas, liver and kidney, the pancreas, liver and kidney tissues were obtained from the sacrificed rats, immediately washed by using saline solution and fixed in 10% formalin. Then, the tissues were embedded in paraffin and 5µm thick sections were prepared from the paraffinized samples and stained using hematoxylin and eosin stain. The histopathological alterations in the prepared sections were determined under light microscope. For the statistical analysis, all the results were expressed as the mean ± SEM (n=6). All the grouped data was statistically assessed with SPSS\10.0 software. Hypothesis testing methods included one way analysis of variance (ANOVA) followed by the least significant difference (LSD) test, significance level at and $P<0.05$, 0.01, were considered to point out statistical significance.

RESULTS AND DISCUSSION

Natural products of plant origin are extensively documented in the pharmaceutical manufacturing for their broad structural multiplicity as well as their wide range of pharmacological actions (Thomford et al. 2018). In the current study, GC-MS chromatogram of MEBS showed 20 peaks represented the presence of twenty compounds (Fig 1). GC-MS investigation uncovered the presence of twenty unique phytochemical compounds as described in table 1 and it was revealed that the MEBS is mainly composed of oxygenated hydrocarbons and predominantly phenolic hydrocarbons. At the same time, figure 2 and table 2 represents the HPTLC analysis of flavonoids present in the *B. spectabilis*.

Figure 1: GC-MS chromatogram of the MEBS.



These phytochemicals may responsible for various pharmacological actions like antimicrobial, antibacterial, antidiabetic and other biological activities. GC-MS analysis of phytoconstituents in plants gives a clear depiction of the pharmaceutical value of that plant. Therefore, this type of GC-MS analysis is the initial move towards indulgent the nature of medicinal properties in this therapeutic plant and this type of explore will be supportive for further comprehensive study (Uddandrao et al. 2020b). HPTLC analysis revealed that the yellowish blue colored fluorescent zone at UV 366nm mode were present in the tracks, and it was observed from the chromatogram after derivatization, which confirmed the presence of flavonoid in the given standard and as well as in the *B. spectabilis* sample (Jakimiuk et al. 2021). HPTLC is realistic for expansion of chromatographic fingerprints to find out major active constituents of

therapeutic plants. The separation and resolution are greatly superior, and the results are much more consistent and reproducible than thin layer chromatography.

Flavonoids, the most prevalent group of innate compounds, principally consist of a fused aromatic ring (A-ring) and a heterocyclic ring (C-ring) associated through a carbon-carbon bridge to an aromatic B-ring (Jakimiuk et al., 2021). Flavonoids are fitting the subject of medical research as they have been documented to own many medicinal properties, like anti-inflammatory, enzyme inhibition, antimicrobial, anticancer, antiallergy, and antioxidant properties (Jamuna and Paulsamy 2016). Other therapeutic effects include enhanced blood flow, the reticence of cholesterol absorption and guard from damage by ultraviolet B radiation. On the other hand, we also found significant amount of flavonoids by HPTLC in the *B. Spectabilis*, which indicates that this medicinal plant may have therapeutic properties as like reported earlier (Jakimiuk et al., 2021).

Table 1. Bio active compounds identified in the methanolic extract of *B. spectabilis* with GC-MS

Peak	RT	Area %	Compound name	Molecular formula
1	12.309	0.45	4-Nitrophenylglyoxylic acid	$C_8H_5NO_5$
2	16.344	0.48	Megastigmatrienone	$C_{13}H_{18}O$
3	16.554	0.80	Phytol acetate	$C_{22}H_{42}O_2$
4	17.896	42.02	Cyclobutanecarboxylic acid	$C_5H_8O_2$
5	18.098	0.72	Hexadecanoic acid, methyl ester	$C_{16}H_{32}O_2$
6	18.643	3.78	Pentadecanoic acid	$C_{15}H_{30}O_2$
7	19.876	8.82	Butyric acid, 3-tetradecyl ester	$C_{18}H_{36}O_2$
8	20.505	9.33	Oleic Acid	$C_{18}H_{34}O_2$
9	20.606	3.56	Z-1,9-Hexadecadiene	$C_{16}H_{30}$
10	22.082	0.75	Triacontyl acetate	$C_{32}H_{64}O_2$
11	23.097	0.63	Pyrazole, 5-methyl-3-(5-nitro-2-furyl)-	$C_8H_7N_3O_3$
12	23.701	0.99	7-Pentadecyne	$C_{15}H_{30}$
13	23.877	19.29	Cholestan-6-one, (5.alpha.)-	$C_{27}H_{46}O$
14	24.221	1.69	Glycerol 1-palmitate	$C_{19}H_{38}O_4$
15	25.152	1.83	Octacosanol	$C_{28}H_{58}O$
16	25.656	0.59	Squalene	$C_{30}H_{50}$
17	25.748	1.91	Oleoyl chloride	$C_{18}H_{33}Cl_0$
18	25.857	1.12	1,3,12-Nonadecatriene	$C_{19}H_{34}$
19	26.050	0.44	Cyclohexanone, 2-(2-propenyl)-	$C_9H_{14}O$
20	26.688	0.78	Fumaric acid, 3-fluorophenyl undecyl ester	$C_{16}H_{10}F_2O_4$

Table 2. Peak table with Rf values, height and area of flavonoids

Track	Peak	Rf	Height	Area	Assigned substance
STD	1	0.62	483.9	22949.6	Flavonoid standard
Sample III	1	0.25	8.7	199.6	Flavonoid 1

The development of safer medicine for DM is still a question for researchers working in this region. The research data on herbal medicine can suggest new functional leads to decrease toxicity, money and time are the three main challenges in drug detection. Hence, in the current study we made an attempt to evaluate the effect of MEBS against STZ-induced DM in rats. STZ is used as a mediator to produce DM by particular cytotoxicity effect on pancreatic b-cells. Accordingly, it affects endogenous insulin ejection and as a result increases blood glucose level (Kotb et al., 2020). Similarly, figure 3 revealed that there was a significant ($P<0.01$) increase in the blood glucose (Fig. 3A), glycated hemoglobin content (Fig. 3B) and concomitant decrease in the body weight (Fig. 3C) and insulin levels (Fig. 3D) in the STZ-induced diabetic rats. On the other hand, supplementation with MEBS to diabetic rats notably tended to bring the above parameters towards near normal levels that might be due to the occurrence of various phytochemicals in the MEBS (Chitra Devi and Ramesh 2018; Uddandrao et al., 2020b).

Figure 2: HPTLC chromatogram of MEBS showing presence of flvonoids.

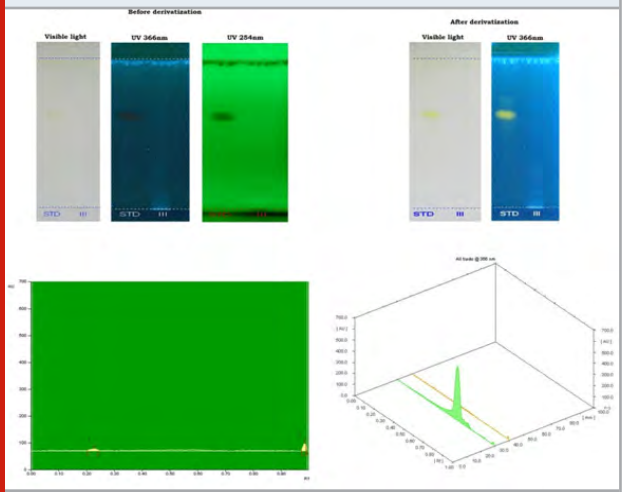
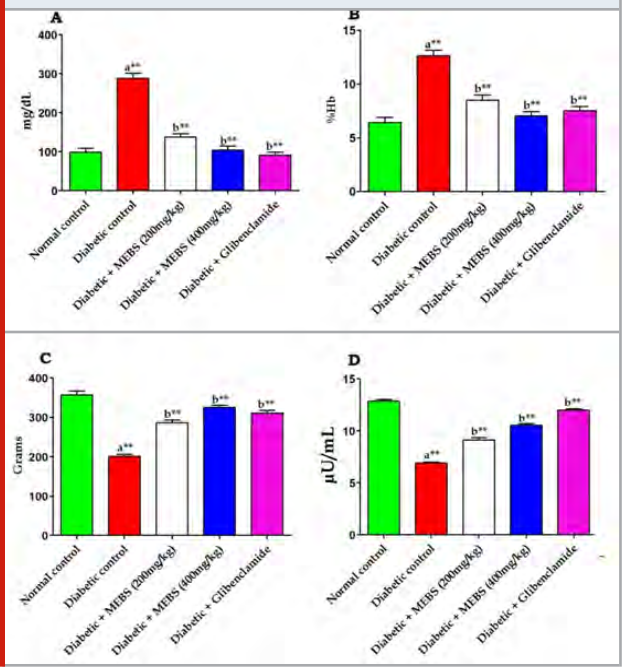


Figure 3: Effect of MEB Son (A) blood glucose, (B) glycated hemoglobin, (C) body weight and (D) insulin in control and experimental animals. All values expressed in mean ± SEM, n=6, [a]Significantly different from the normal control, [b]Significantly different from the diabetic control, **P<0.01.



Several studies have reported that phytochemicals extracted from medicinal plants display an exciting prospect for the development of new types of medicines for DM. Most extensive among phytochemical groups are the alkaloids, glycosides and phenolics such as terpenoids, flavonoids and steroids and our results are in line with this statement (Uddandrao et al., 2020b). On the other hand, OGTT in control and experimental rats established that the blood glucose levels in the control rats increased to a maximum value after 30 min of glucose load and decreased to nearly basal levels after 120 min, whereas in rats with STZ-induced DM, peak increases in blood glucose levels were noticed even after 60 min and remained high over the next 60 min. Oral supplementation of MEBS (200 and 400mg/kg) in rats with DM caused a significant decrease in the blood glucose levels after 60 min compared with those of the untreated rats with DM (Fig. 4). These results are in line with the previous studies reported by Sathibabu et al., (2019) and Uddandrao et al., (2020c).

Figure 4: Effect of MEBS on OGTT in control and experimental animals. All values expressed in mean ± SEM, n=6, [a]Significantly different from the normal control, [b]Significantly different from the diabetic control, **P<0.01.

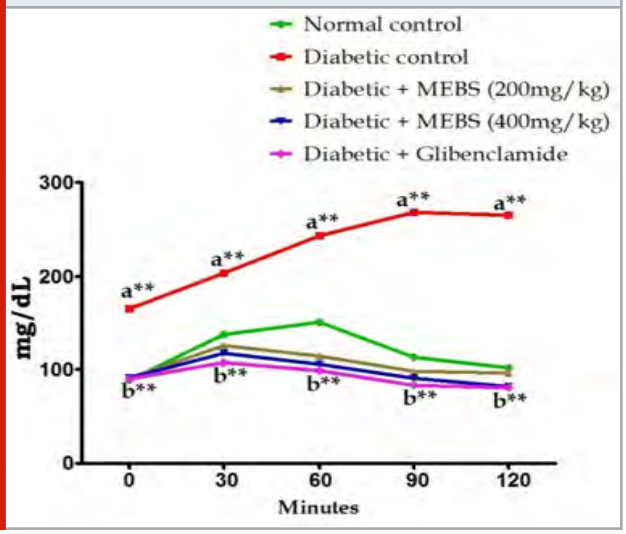
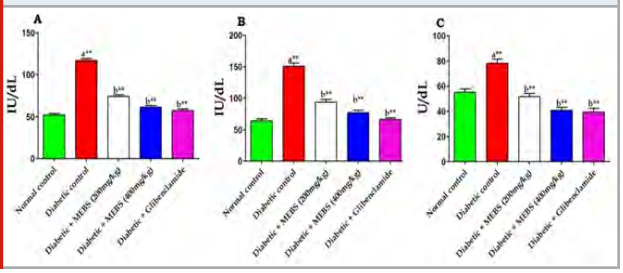


Figure 5: Effect of MEBS on liver marker enzymes (A) AST, (B) ALT and (C) LDH in control and experimental animals. All values expressed in mean ± SEM, n=6, [a]Significantly different from the normal control, [b]Significantly different from the diabetic control, **P<0.01.



In DM, several studies have demonstrated that increases in AST and ALT activities as well as changes in lipid levels (Saravanan et al. 2009; Sathibabu Uddandrao et al. 2019). An increase in these enzyme activities disclose active liver damage and rise in the activities of plasma ALT, AST, and LDH indicates liver malfunction and moreover liver was necrotized due to STZ (Saravanan et al., 2009). Similarly, figure 5 depicts that there was noteworthy (P<0.01) increase in the liver marker enzymes such as AST (Fig. 5A), ALT (Fig. 5B) and LDH (Fig. 5C) in STZ-induced diabetic rats when compared

to normal control. At the same time remarkable (P<0.01) restoration of these liver marker enzymes activities to near normal noticed when diabetic rats treated with MEBS contrasted to untreated diabetic rats. Bayramoglu et al., (2014) reported that increase in the activities of these enzymes in plasma might be principally due to the release of these enzymes from the hepatic cytosol into the blood circulation because of membrane permeability representing severe hepatocellular damage caused by DM. Our results are in line with this statement. Interestingly, the treatment of MEBS is able to defend against increase in the activity of these enzymes in diabetic rats, signifying that protective effect against liver damage and it may be used as a remedy to bring about hepatoprotective effect (Jiang et al., 2020).

Table 3. Effect of MEBS on serum lipid profile in control and experimental animals

Groups	Total Cholesterol (mg/dL)	Triglycerides (mg/dL)	LDL- C (mg/dL)	VLDL- C (mg/dL)	HDL- C (mg/dL)
Normal control	110.67±1.022	116.33±1.174	34.23±1.442	23.27±0.2348	53.17±0.4773
Diabetic control	237±1.438^{a**}	182.33±1.054^{a**}	169.87±1.520^{a**}	36.47±0.2108^{a**}	30.67±0.8819^{a**}
Diabetic + MEBS (200mg/kg)	141.50±0.9220^{b**}	152.00±1.713^{b**}	71.77±1.290^{b**}	30.40±0.3425^{b**}	39.33±0.4216^{b**}
Diabetic + MEBS (400 mg/kg)	126.33±0.4944^{b**}	130.33±0.760^{b**}	58.10±0.5905^{b**}	26.07±0.1520^{b**}	42.17±0.6540^{b**}
Diabetic + Glibenclamide	121.50±1.057^{b**}	122.33±0.8819^{b**}	46.87±1.97^{b**}	24.47±0.1764^{b**}	48.50±0.8466^{b**}

All values expressed in mean ± SEM, n=6, ^{a}Significantly different from the normal control, ^{b}Significantly different from the diabetic control, **P<0.01.

Table 4. Effect of MEBS on liver carbohydrate metabolizing enzymes activities in control and experimental diabetic rats

Groups	Glucokinase (mU/mg protein)	Pyruvatekinase (mU/mg protein)	Glucose-6-phosphatase (U/mg protein) (U/min)	Glucose-6-phosphate dehydrogenase	Fructose-1,6-biphosphatase (U/mg protein)
Normal control	27.33±0.6146	0.207±0.0016	0.412±0.0007	5.28±0.0600	0.322±0.0015
Diabetic control	8.25±0.0885^{a**}	0.076±0.0014^{a**}	0.751±0.0010^{a**}	1.98±0.0508^{a**}	0.549±0.0052^{a**}
Diabetic + MEBS(200mg/kg)	12.46±0.1358^{b**}	0.119±0.0008^{b**}	0.540±0.0018^{b**}	3.40±0.0221^{b**}	0.424±0.0032^{b**}
Diabetic + MEBS(400mg/kg)	17.07±0.1282^{b**}	0.151±0.0007^{b**}	0.339±0.00016^{b**}	4.32±0.0274^{b**}	0.381±0.0028^{b**}
Diabetic + Glibenclamide	21.32±0.3535^{b**}	0.184±0.0008^{b**}	0.378±0.0011^{b**}	4.79±0.0554^{b**}	0.361±0.0022^{b**}

All values expressed in mean ± SEM, n=6, ^{a}Significantly different from the normal control, ^{b}Significantly different from the diabetic control, **P<0.01.

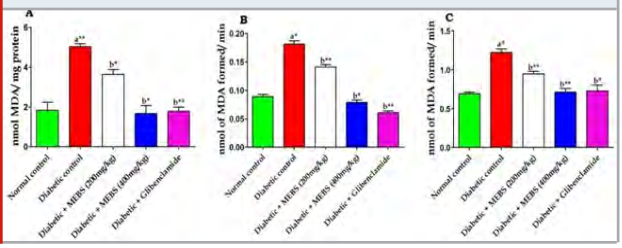
DM is associated with severe alterations in the serum lipid and lipoprotein profiles and with an increased risk of heart diseases (Hedayatnia et al. 2020). DM is frequently associated with a variety of changes in metabolic and regulatory mechanisms, due to insulin shortage or due to IR is liable for the noticed increase of lipids (Naidu et al. 2016; Swapna et al. 2019). Table 3 displays that the serum lipid profile in control and experimental animals. The data exposed that the significant (P<0.01) increase in the TC, TG, LDL-C, VLDL-C and concomitant reduction in the HDL-C in STZ-induced diabetic rats. However, treatment with MEBS (200 and 400mg/kg) predominantly (P<0.01) increased the levels of HDL-C and simultaneously reduced the levels of TC, TG, LDL-C and VLDL-C when compared to untreated diabetic rats. Swapna et al. (2019) reported that STZ-induced DM also can produce hyperlipidemia and similarly we noticed that hyperlipidemia in rats and this might be due to an enhance in the activity of hormone-sensitive lipase which catalyzes the mobilization of fatty acids from TG stored in adipocytes (Uddandrao et al., 2020c). This finding was evidenced by the results of the histopathological analysis. The organs of diabetic rats showed STZ-induced cellular damage on pancreatic islets, liver histology, and renal glomeruli and tubules (Swapna et al., 2019).

Table 5. Effect of MEBS on glycogen and glycogen metabolizing enzymes in control and experimental diabetic rats

Groups	Glycogen	Glycogen synthase	Glycogen phosphorylase
Normal control	65.82±3.46	624.93±43.66	398.79±92.64
Diabetic control	25.19±0.68^{a*}	404.72±93.48^{a*}	702.97±63.82^{a*}
Diabetic + MEBS (200mg/kg)	58.25±0.86^{b*}	523.16±44.6^{b*}	565.63±24.83^{b*}
Diabetic + MEBS (400 mg/kg)	64.16±0.58^{b**}	683.65±24.12^{b**}	399.63±25.40^{b*}
Diabetic + Glibenclamide	63.61±0.48^{b**}	622.39±34.22^{b*}	402.97±29.46^{b*}

All values expressed in mean ± SEM, n=6, ^{a}Significantly different from the normal control, ^{b}Significantly different from the diabetic control, *P<0.05, **P<0.01. Glycogen: mg of glucose/gm of liver tissue, Glycogen synthase: u moles of UDP formed/hr/mg protein, Glycogen phosphorylase: u moles of pi liberated /hr/mg protein.

Figure 6: Effect of MEBS on lipid peroxidation in (A) serum, (B) liver and (C) kidney in control and experimental animals. All values expressed in mean ± SEM, n=6, ^{a}Significantly different from the normal control, ^{b}Significantly different from the diabetic control, *P<0.05, **P<0.01.



DM has been shown to slow down the activities of pentose phosphate and glycolytic pathway enzymes while promoting lipolytic and gluconeogenic activities (Jiang et al., 2020). According to previous reports, DM was to be had with alterations in glucose homeostasis that put in to persistent hyperglycemia and liver plays a major role in the regulation of glucose metabolism (Kalidhindi et al., 2020). Table 4 represents the effect of MEBS on liver carbohydrate metabolizing enzymes activities in control and experimental diabetic rats. There was a significant (P<0.01) reduction in the activities of glucokinase, pyruvatekinase and glucose-6-phosphate dehydrogenase and simultaneous increase in the activities of glucose-6-phosphatase and fructose-1,6-bisphosphatase in STZ-induced diabetic rats when compared to normal control rats.

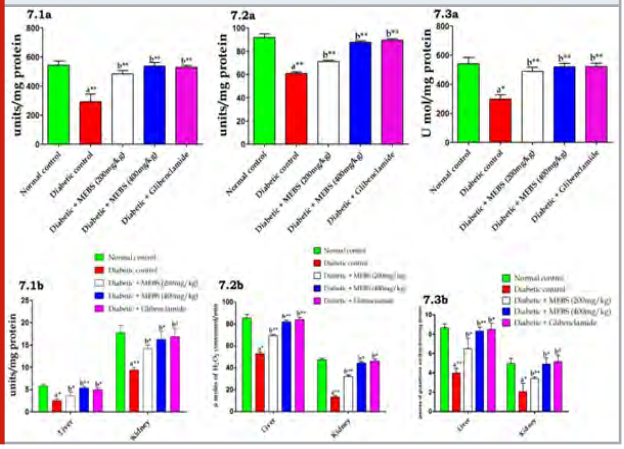
Table 6. Effect of MEBS on Nonenzymic antioxidants in liver and plasma of control and experimental diabetic rats, All values expressed in mean ± SEM, n=6, ^{a}Significantly different from the normal control, ^{b}Significantly different from the diabetic control, *P<0.05, **P<0.01. Vitamin A, C, E Units: Plasma: mg/dL, Liver: µg/mg protein. GSH Units: Serum: mg of glutathione/dL, Liver: units/mg/protein.

Groups	Vitamin A		Vitamin C		Vitamin E		GSH	
	Plasma	Liver	Plasma	Liver	Plasma	Liver	Serum	Liver
Normal control	1.95±0.03	1.87±0.08	1.48±0.02	1.34±0.07	1.92±0.21	0.92±0.05	36.95±1.42	55.12±1.98
Diabetic control	0.43±0.06^{a*}	0.65±0.06^{a*}	0.49±0.08^{a*}	0.71±0.05^{a*}	0.76±0.05^{a*}	0.51±0.08^{a*}	17.62±2.08^{a*}	15.65±2.13^{a*}
Diabetic + MEBS (200mg/kg)	0.98±0.08^{b*}	1.02±0.08^{b*}	1.18±0.065^{b**}	1.23±0.056^{b**}	1.3±0.08^{b*}	0.70±0.07^{b**}	29.85±1.96^{b*}	36.23±1.85^{b*}
Diabetic + MEBS (400 mg/kg)	1.75±0.07^{b**}	1.58±0.06^{b**}	1.59±0.07^{b*}	1.63±0.08^{b*}	1.97±0.49^{b**}	0.99±0.08^{b**}	37.83±1.86^{b**}	56.41±1.83^{b**}
Diabetic + Glibenclamide	1.89±0.08^{b*}	1.65±0.08^{b*}	1.38±0.08^{b*}	1.49±0.06^{b**}	1.89±0.12^{b**}	0.87±0.05^{b**}	38.98±1.73^{b**}	57.14±2.15^{b*}

On the other hand, treatment with high dose of MEBS (400mg/kg) predominantly (P<0.01) restored the alterations happen in these enzyme activities and brought back to near normal level. At the same time, low dose of MEBS (200mg/kg) demonstrated moderate activity in the restoration of these altered enzymes activities when compared to high dose. These results are in line with previous study reported by Kalidhindi et al. (2020). The activity of enzymes like glucokinase, pyruvate kinase, glucose-6-phosphatase, and fructose-1,6-bisphosphatase was noticeably distorted, consequential in hyperglycemia, which leads to the pathogenesis of diabetic complications (Sharabi et al. 2015; Kalidhindi et al. 2020). The altered the activity of glucokinase and pyruvate kinase, diminishing the metabolism of glucose and ATP production in diabetic conditions. The diminution in the activities of these enzymes in the liver of DM rats is a sign of increased gluconeogenesis and decreased glycolysis suggesting that these two pathways are altered in DM (Saravanan et al., 2014). The activities of regulatory enzymes in gluconeogenesis, like glucose-6-phosphatase and fructose-1,6-bisphosphatase,

are prominent in DM and improved activities of these enzymes in diabetic rats may be due to lack of insulin (Lekshmi et al., 2015).

Figure 7: Effect of MEBS on enzymatic antioxidants (7.1) SOD, (7.2) CAT and (7.3) GPX in (a) serum, (b) liver and kidney of control and experimental animals. All values expressed in mean ± SEM, n=6, [a]Significantly different from the normal control, [b]Significantly different from the diabetic control, *P<0.05, **P<0.01.



Glucose-6-phosphatase and fructose-1,6-bisphosphatase are dephosphorylating enzymes which make worse hepatic glucose consumption. Our results demonstrated that the activities of glucose-6-phosphatase and fructose-1,6-bisphosphatase were considerably diminished by the administration of MEBS. On the other hand, glycogen is a branched polymer and most significant intracellular storable form of glucose residues synthesized by the enzyme glycogen synthase. Its amount in different tissues is a straight manifestation of insulin activity, as insulin supports intracellular glycogen deposition by activating glycogen synthase and restraining glycogen phosphorylase (Nawaz et al. 2020). In the current study, STZ-induced diabetic animals are predisposed to show diminished glycogen content in liver and reduced activity of glycogen synthase and concurrent raise in the glycogen phosphorylase activity. On the other hand, supplementation of MEBS to diabetic rats increased the activity of glycogen synthase there by increased (P<0.05, 0.01) the glycogen content in the liver via reserve of glycogen phosphorylase activity (Table 5).

This might be due to the antidiabetic potential of MEBS and rise in the levels of insulin with MEBS supplementation (Devi and Ramesh 2018). On the other hand, the figure 6 showed the levels of lipidperoxidation in serum (Fig. 6A), liver (Fig. 6B) and kidney (Fig. 6C) in control and experimental diabetic rats. The STZ-induced diabetic rats had a significant (P<0.05, 0.01) elevation in the levels of lipidperoxidation when compared with the normal control rats (Devi and Ramesh 2018). Stable hyperglycemia in DM prompts intensified formation of oxygen free radicals from glycosylation of protein and autoxidation of glucose which lead to oxidative stress which is associated with diabetic complications. Phytocompounds are the bioactive molecules that have been extensively concerned in treating several clinical disorders in which their pathogenesis is directly or indirectly associated with oxidative stress (Parim et al., 2019; Ghasemi et al., 2020).

The rats treated with Glibenclamide and the high dose of MEBS (400mg/kg) showed a significant (P<0.05, 0.01) decrease and the low dose of MEBS demonstrated a moderate decrease in the levels of LPO in the serum and tissues (liver and kidney) of the rats. Brahmanaidu et al., (2017) found that lofty levels of blood glucose in DM are linked with elevated lipid peroxidation, which may put in to long-term damage of organs and interestingly, in our study revealed that STZ-administration widely increased lipid peroxidation in serum, liver and kidney and this was attenuated by the MEBS. On the other hand, this study also revealed that STZ administration developed severe oxidative stress in rats which was confirmed by the reduction of non-enzymatic antioxidants (GSH, vitamin A, C and E) and enzymatic antioxidants (SOD, CAT and GPx).

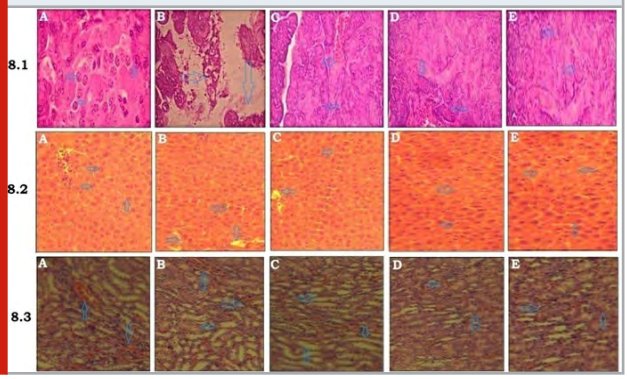
Table 6 demonstrated about the effect of MEBS on non-enzymatic antioxidant markers in control and experimental diabetic rats. There was a significant (P<0.05, 0.01) reduction in the levels of GSH, vitamin A, C and E in STZ-induced diabetic rats when compared with normal control. On the other hand, MEBS (400mg/kg) treatment to diabetic rats notably (P<0.05, 0.01) elevated the levels of non-enzymatic antioxidant markers (GSH, vitamin A, C and E) and at the same time low dose of MEBS (200mg/kg) shown reasonable elevation of these markers (Brahmanaidu et al. 2017; Devi and Ramesh 2018). In addition, the increased lipid peroxidation under DM state could be due to enhanced oxidative stress in the cell as a result of the diminution of antioxidant protection mechanisms (Sathibabu et al. 2019). Similarly we also found likewise which was demonstrated by figure 7 which exposed that the effect of MEBS on serum, liver and kidney enzymatic antioxidants in control and experimental diabetic rats. The data exposed that a noteworthy (P<0.05) reduction in the activities of SOD (Fig. 7.1a&tb), CAT (Fig. 7.2a&tb) and GPX (Fig. 7.3a&tb) in STZ-induced diabetic rats.

At the same time, diabetic rats treated with MEBS remarkably (P<0.05, 0.01) restored above enzymatic antioxidant levels to near normal. Antioxidant enzymes form the primary line of protection against reactive oxygen species in the individual include the enzymes SOD, CAT and GPx, which play a vital role in hunting the toxic intermediate of incomplete oxidation (Jansy et al. 2021). On the other hand, in our study, the treatment with MEBS restored enzymatic and non-enzymatic antioxidants in liver and kidney of diabetic rats' close proximity to normal levels, which in turn reveals the antioxidant potential of MEBS. Concomitantly with the decline in the activity of the antioxidant's enzymes, cleaned out antioxidant capability of non-enzymatic antioxidants namely GSH, vitamin A, C, and E have been documented in DM (Cammisotto et al., 2021). We also

studied histopathology of vital organs such as pancreas, liver and kidney. STZ administration caused severe pathological conditions in these organs.

Figure 8.1 summarizes the histoarchestucture of pancreas in control and experimental diabetic rats. The results revealed that the pancreatic islet cells are normal in normal control group with typically arranged islet cells, uniform sinusoid spaces with no vacuolization (Fig.8.1A). Diabetic control rats demonstrated that vascular degranulated islets with severely reduces the volume and number of islets (Fig.8.1B). Diabetic rats treated with MEBS (200mg/kg) shown mild granulation islets with moderately reduced volume and number (Fig.8.1C) (Cammisotto et al., 2021). On the other hand, diabetic rats treated with MEBS (400mg/kg) and Glibenclamide revealed well granulated and regenerated islets with normal cellular characteristics (Fig.8.1D&E). Figure 8.2 depicts the histopathological changes were noted in the liver of control and experimental diabetic rats. The liver of the normal control rats showed the normal architecture of hepatocytes with central lobules (Fig.8.2A). Liver of the diabetic control displayed abnormalities like congestion and cellular necrosis in the liver (Fig.8.2B).

Figure 8: Histopathological analysis of (8.1) pancreas, (8.2) liver and (8.3) kidney in control and experimental animals, H&E ×40, (A) Normal control, (B) Diabetic control, (C) Diabetic + MEBS (200mg/kg), (D) Diabetic + MEBS (400mg/kg) and (E) Diabetic + Glibenclamide.



Liver of the MEBS (200mg/kg) treated rats showed the hepatocytes with few lobules (Fig.8.1C). At the same time rats treated with MEBS (400mg/kg) and Glibenclamide demonstrated that heptocytes with almost a normal structure (Fig.8.2D&E). Figure 8.3 explains the histoarchestucture of kidney in control and experimental diabetic rats. The kidney of normal control rats showed the normal structure of kidney with convoluted tubules, glomerulus and tubules (Fig.8.3A). The kidney of STZ-induced diabetic rats displayed congested tubules and glomerulus (Fig.8.3B) (Cammisotto et al. 2021). The kidney of MEBS (200mg/kg) treated rats illustrated a distinguishable glomerules and tubules (Fig.8.3C). Simultaneously, MEBS (400mg/kg) and Glibenclamide treatment for 28 days to diabetic rats displayed that a similar architecture to that of the normal control rats (Fig.8.3D&E).

CONCLUSION

In conclusion, MEBS remarkably reestablished the activities of carbohydrate and glycogen metabolic enzymes and in this way kept up glucose homeostasis in diabetic rats. Moreover, MEBS had the capability to enhance enzymatic and non-enzymatic antioxidant defense system and to attenuate the lipid peroxidation and oxidative stress in liver and kidney and furthermore secured the pancreas, liver and kidney from the glucose toxicity. Taken together, these outcomes may add to a predominant comprehension of the hepatoprotective and renoprotective potential of MEBS, emphasizing the impact of this therapeutic plant and presumably preventing complications associated with DM.

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Physiological
Communication

Physiological Effects of Regular Football Training in Adolescents Using Visual Analyzer Pathology

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ABSTRACT

Recently, various pathologies of the visual analyzer are increasingly common among Russian youth. The presence of violations of the organ of vision inevitably leads to a significant decrease in physical activity, weakening of the development of the musculoskeletal system and all internal organs. This creates in this category of adolescents the risk of developing a large number of pathologies. At the same time, a systematic increase in motor activity in adolescents with reduced vision, due to regular classes in various types of adaptive sports, can help in their general health improvement. It was found that football training provides a pronounced increase in physical development and a significant increase in functional capabilities in visually impaired adolescents. In addition, regular football training helped to improve the development of the motor characteristics of the visually impaired adolescents. Regular physical activity within the framework of traditional physical education for the visually impaired could not significantly increase the strength, endurance and reserve capabilities of the body in this contingent of trainees. The obtained results of assessing the impact of dosed football trainings indicate that it is very promising to use regular physical activity in adolescents with visual impairment in this sport. They increase the general physical fitness and stimulate the functionality of the internal organs in this category of adolescents.

KEY WORDS: ADOLESCENTS, VISION, PATHOLOGY, PHYSICAL ACTIVITY, ADAPTIVE FOOTBALL, DISABLED PEOPLE.

INTRODUCTION

Currently, there is a tendency towards a deterioration in the health status of Russian youth. In recent years, a significant number of adolescents with various health disorders have been studying in the general education system (Kotova et al., 2017). Often, the pathology of the visual analyzer is encountered in Russian youth. The presence of violations of the organ of vision inevitably leads to a significant decrease in the physical activity of adolescents, weakening the development of the musculoskeletal system and all internal organs. This circumstance forms in such adolescents the risk of developing overt pathology in all parts of the body.

The situation is aggravated by the low degree of consideration in the school curriculum of the peculiarities of adolescents with physical disabilities, including those with a pathology of the organ of vision. In addition, many schools do not have enough specialized sports equipment, and the available premises do not fully meet the standards for the implementation of physical education classes with the visually impaired (Kulkova, 2013). It is known that an increase in physical activity due to physical activity, including in the framework of physical culture or sports, can very strongly stimulate all body systems in healthy adolescents and adolescents with various dysfunctions and pathologies (Vorobyeva et al., 2018 Karpov et al 2020).

At the same time, there is little information regarding the increase in the muscular activity of the visually impaired and the blind, despite the urgent need of society for their health improvement and socialization (Karpov et al., 2018). It becomes clear that in the course of systematic physical activation of blind and visually impaired adolescents, muscular sense should be used to coordinate their movements, teaching to use it as the basis for orientation in space. In this regard, such a contingent of athletes must perfectly coordinate their

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own movements in conditions of a minimum or complete absence of vision, relying on bodily sensations.

This task, in the absence of the possibility of visual imitation, is very difficult, but solvable. In this regard, the physical development of such a contingent of adolescents should be based on a gradual build-up of motor experience. To form such baggage, one should begin teaching a teenager with a pathology of the organ of vision to the basic movements: walking in a straight line, jumping in length, running in a straight line, measured movements in both directions along the stairs. These muscular actions should be brought to a level of high precision, consistency, and freedom. Only in this case, these adolescents will be able to master more complex sports movements and be able to subsequently reproduce them independently during training (Ivleva, 2019).

Increasing motor activity in adolescents with reduced vision through regular adaptive sports seems to be very promising in terms of their general improvement (Kulkova, 2013; Ivleva, 2019). At the same time, the real possibilities of this approach have not been clarified. For this purpose, the authors evaluated the results of regular adaptive football training with this contingent. Purpose of the present study was to assess the effectiveness of regular football training in relation to the development of general physical characteristics and dynamics of the main functional characteristics of the body of adolescents with visual impairment.

MATERIAL AND METHODS

The study was carried out on high school students, a total of 25 boys of 15-16 years old with visual impairment in the degree of low vision. The study took into account data from medical records and anamnesis life of the examined, including prenatal and postnatal periods. Particular attention was paid to the process of development of pathology arising during life. In most cases, adolescents with visual analyzer pathology taken into the study were exposed to a number of health hazards throughout their lives. In more than 80% of cases, prolonged or rapid labor took place, traumatic obstetric methods of obstetric assistance were used, including the application of forceps and manual stimulation of labor.

Table 1. Results of the initial testing of the subjects

Physical condition assessment option	Control group, n=13, M±m	Experimental group, n=12, M±m
Running at a distance of 30 m, s	9.82±0.87	9.98±0.77
Distance of standing jump in length, m	125.6±3.81	129.7±4.12
Throwing range of a ball weighing 3 kg, m	1.26±0.62	1.33±0.49
Leading hand wrist dynamometry level, kg	10.2±0.45	10.6±0.53
Romberg sample index, s	2.6±0.40	2.5±0.35
Flexibility index, cm	-14.7±0.86	-14.9±1.10

Note: There was no significant difference between the groups.

RESULTS AND DISCUSSION

The examined adolescents with visual pathology initially had a very reduced working capacity, they felt tired early and were quickly distracted, which lowered the quality of physical exercises. The initial physical state of the examined adolescents was comparable in all the parameters taken into account. Their physical capabilities were low, indicating their poor initial fitness (table 1). Initially, the subjects could run a distance of 30m in a little less than 10 seconds. This indicated that they had a very average running ability. Opportunities for long jump from a spot in the subjects were also low, amounting to 125.6±3.81 m and 129.7±4.12 m in both groups. The throwing range of a ball weighing 3 kg among adolescents when included in the observation group was modest, amounting to 1.26±0.62 m in the control group, and 1.33±0.49 m in the experimental group.

The muscle strength of the leading hand, which was judged by the results of dynamometry, in the outcome was low and amounted to 10.2±0.45 kg in the control and 10.6 ± 0.53 kg in the experimental group. The results of the Romberg test were low and comparable in both groups at the first examination. They pointed to the low stability of the body of adolescents with visual impairment in space. In addition, in the outcome, all subjects showed a comparably low flexibility. Regular physical activity contributed to the stimulation of their physical development in all observed. The results obtained were more pronounced in the experimental group. Table 2 shows the results of the physical testing of adolescents in the control and experimental groups at the end of the study.

Evaluating the data given in table 2, it was possible to note the statistical significance of the differences between the control and experimental groups according

to the results of all the tests used. Regular physical activity provided a more pronounced development of physical capabilities in the experimental group. So in the adolescents who made it up, more pronounced speed abilities were achieved. This was judged at the end of the observation by the acceleration of their run at a distance of 30 m for a time significantly shorter than in the control group. By the end of the observation, the jumping distance in adolescents from the experimental group was 33.3% more than in the control group. The distance the teenagers trained according to the author's scheme were able to throw a ball weighing 3 kg exceeded that in the control by 2.9 times. The power capabilities of the leading hand, assessed by dynamometry by the end of the observation, were 93.5% lower in the control group than in the control group.

The time in Romberg's test among adolescents in the experimental group increased 2.6 times during the training period, prevailing by the end of the observation over the level in the control group by 2.3 times. At the same time, the indicator of flexibility in the surveyed during their physical training has changed positively. More pronounced dynamics of this indicator was noted in the experimental group (4.2 times), which ensured its prevalence over the same indicator in the control group by 3.3 times.

The existing scientific and methodological base for the process of adaptive physical education of adolescents with visual pathology is still very modest. This speaks of gaps in the theoretical foundations of their training process, especially in the field of football. The scarce scientific literature available today, devoted to sports games for the blind and visually impaired, needs to be supplemented and seriously clarified in order to help coaches in the practical implementation of training with this contingent of athletes. In conditions of low physical activity, which is especially often the case with visually impaired, there is a high risk of many diseases and especially the cardiovascular system (Skoryatina and Zavalishina, 2017). This is facilitated by hypotrophy of the muscular system, weakening of the heart muscle and impaired vascular tone, which contributes to the development of pre-pathological conditions. In this regard, the appearance of asthenia, vegetative vascular dystonia and arterial hypertension in persons with low physical activity is very likely (Kulkova, 2013).

Table 2. Results of the final testing of the surveyed

Physical condition assessment option	Control group, n=13, M±m	Experimental group, n=12, M±m
Running at a distance of 30 m, s p<0.01	9.69±0.74	7.06±0.46
Distance of standing jump in length, m	130.4±4.62	173.8±3.82 p<0.01
Throwing range of a ball weighing 3 kg, m	1.38±0.42	3.97±0.51 p<0.01
Leading hand wrist dynamometry level, kg	10.8±0.52	20.9±0.47 p<0.01
Romberg sample index, s	2.9±0.33	6.6±0.58 p<0.01
Flexibility index, cm	-11.6±0.92	-3.5±0.50 p<0.01

Note: p is the reliability of the differences in the test results of adolescents in both groups at the end of the observation.

The assessment of the initial physical development of adolescents suffering from the pathology of the visual analyzer confirmed that it is low in all cases. As a result, they, as a rule, have weakened general functional capabilities and have a low level of motor characteristics in comparison with age norms. There is evidence that in people with pathology, including the visually impaired, at any age, the vessels often have a tendency to spasm. Because of this, there are several risk factors at once, contributing to the development and progression of various pathologies (Bespalov et al., 2018). Low muscular activity present with visual impairment leads to a progressive decrease in the available functional reserves of all internal organs, creating conditions for the appearance and strengthening of dysfunctions, and then the development of pathology in vital organs. Functionally unfavorable changes in the activity of the heart and blood vessels developing in the visually impaired are very significant in this process (Zavalishina, 2020).

In this regard, for the visually impaired and the blind, early manifestation of atherosclerosis is very characteristic, which significantly increases the risk of early angina pectoris and early myocardial infarction. Systematic physical training in adolescents, especially those with pathology, always very effectively increase the level of adaptive capabilities of initially weakened internal organs. To ensure the process of proper adaptation of the whole organism in response to physical activity, first of all, the functional reserve of all its life support systems increases. At the same time, the functional abilities of the musculoskeletal system increase rapidly, the volume of blood flow increases biologically, vascular tone is optimized, and the rheological properties of blood are adapted. All this significantly increases the volume of

oxygen delivery to all cells of the body of physically exercising adolescents (Karpov et al., 2020).

To eliminate low physical fitness in the visually impaired, it is strongly recommended to use feasible regular physical activity. At the same time, the optimal approaches to increasing the level of physical fitness and optimizing the functional capabilities of the muscles of the trunk and limbs, lungs and cardiovascular system in people with visual impairment have not yet been fully determined. This suggests the need to continue studying the issues of the influence of regular active muscular activity on the general condition of an organism with a pathology of the organ of vision (Gridneva and Nalobina, 2016). The presence of this gap in the base of accumulated physiological knowledge stimulated this study.

When performing this study, the effectiveness of two options for physical recovery of visually impaired adolescents with a risk of various somatic pathologies was followed. We compared functional changes in adolescents' bodies as a result of active football training and under the influence of standard loads for the visually impaired. Football training according to the author's scheme provided a marked increase in the level of physical development and a significant increase in the functional capabilities of adolescents in the experimental group. In addition, the complex of applied football lessons contributed to a pronounced positive development of motor abilities in adolescents from the experimental group.

The result achieved as a result of football training in visually impaired adolescents has shown a high efficiency of the football training program in terms of overall health improvement of their body (Stepanova et al., 2018). Under the conditions of long-term regular and feasible football training in adolescents, there was an increase in the fitness of the heart muscle and, apparently, a physiologically beneficial weakening of the activity of many elements of hemostasis occurred, which significantly thinned the blood and could provide optimal conditions for the work of the whole organism (Zavalishina, 2018).

Obviously, a serious positive effect in relation to the whole organism of adolescents with visual pathology of football training was realized due to a greater activation of metabolic processes than under the influence of standard physical training loads. At the same time, as a result of football training, there was a more pronounced increase in their level of fitness, which optimized overall vitality, the work of the heart and blood vessels, and significantly increased the available reserves of internal organs, endocrine glands with the achievement of a strict balance between the components of the autonomic nervous system (Kotova et al., 2017).

In the study, it was noticed that during football training, the elasticity of muscles and their strength capabilities increase rather quickly, and the entire skeletal system is significantly strengthened. Regular football loads undoubtedly led to an increase in the level of basic biologically active substances in the blood, creating conditions for the activation of anabolic processes in all tissues (Vorobyeva et al., 2018). Against the background of regular physical activity in the framework of traditional physical education for the visually impaired, representatives the control group could not significantly increase their strength and endurance and kept the reserve capacity of the body at a low level.

There is reason to believe that football training more than regular physical education stimulates the body of the visually impaired. In this regard, regular football training should be widely recommended as an activation of the muscular system in people with visual impairment. The assessment of the results of the approach to muscle stimulation used in the work gives grounds to consider it highly effective in terms of improving the health of people with visual impairments. For this reason, regular football training in adolescents with visual impairments may be in great demand to stimulate the hidden reserves of the body in this contingent.

CONCLUSION

The physical development of adolescents with visual analyzer pathology is usually low. This is due to the pronounced weakness of their musculoskeletal system. Football training was able to provide this contingent of trainees with an increase in speed characteristics, strength and flexibility. The results obtained allow us to consider the applied version of health improvement as very effective in terms of somatic strengthening of this category of adolescents. The results of the study provide a basis for a broad recommendation of football training as an option for improving the physical performance and overall health of visually impaired adolescents.

Conflict of Interest: Authors declares no conflicts of interests to disclose.

Ethical Clearance Statement: The Current Research Work Was Ethically Approved by the Institutional Review Board (IRB) of Russian State Social University, 129226, Moscow, Russia.

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Biotechnological Communication

Analysing Antibacterial Efficacy of Biosynthesized Palladium Nanoparticles Using Aqueous Leaf Extract of *Cocculus hirsutus* as the Reducing Agent

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ABSTRACT

The hunt for novel antimicrobials has become unavoidable due to the emergence of new infectious illnesses and the rise in treatment resistance among dangerous microorganisms. Nanoparticles are one of the most promising and new therapeutic agents currently available. The unique physiochemical features of nanoparticles, paired with their ability to impede microbe development, has sparked a boom in interest in nanoparticles and their potential as antimicrobials. Metals in the form of nanoparticles have recently come to the fore as possible antibacterial agents thanks to the convergence of nanotechnology and biology. The current invention and deployment of new technologies has ushered in a new age, the nano-revolution, which reveals the function of plants in bio and green nanoparticle synthesis, which appears to have piqued everyone's interest in terms of synthesizing stable nanoparticles. Although nanoparticles may be made using a variety of traditional ways, biological approaches outperform physical and chemical approaches. Plants, rather than microorganisms, are being used to synthesize nanoparticles, and the presence of a wide variety of bio-molecules in plants can function as capping and reducing agents, increasing the rate of reduction and stability of nanoparticles. Biosynthesized Palladium Nanoparticles (PdNPs) are environmentally sustainable, cost-effective, biocompatible, and expanding research areas. Because of their possible uses in the medical domain. The current research examines the possible antibacterial efficacy of biologically synthesized Palladium nanoparticles using aqueous leaf extract of *Cocculus hirsutus* as a reducing and capping agent. In a reduction reaction, silver (Pd^{+2}) ions interact in leaf extract and are reduced in solution (Pd^{+1}), resulting in the formation of stable spherical Palladium Nanoparticles. Antibacterial activity of biosynthesized palladium nanoparticles was found to be efficient and rapid against both bacterial strains ($gram^{+ve}$ and $gram^{-ve}$). Palladium Nanoparticles were found to be highly toxic to *Bacillus subtilis* and *Escherichia coli* pathogenic bacterial strains, indicating that they could be used in biomedical research.

KEY WORDS: ANTIMICROBIAL ACTIVITY, CHARACTERIZATION, COCCULUS HIRSUTUS LEAF EXTRACT, PALLADIUM NANOPARTICLES, RESULT.

INTRODUCTION

Medicinal plant mediated synthesis is a currently exploring field which gained more attention, because it can serve as an alternative option to chemical and physical methods Various types of conventional methods are available for the synthesis of PdNPs like ion exchange, Polyolmetho, chemical and electrochemical reduction and vapor deposition, thermal decomposition (Sartre et al. 1993; Son et al. 2004; Kim et al. 2003; Xiong et al. 2005; Xiong et al. 2005; Tristany et al. 2006; Iravani et al. 2011; Govindarajan et al. 2017). All of these methods are expensive and associated with toxic reducing agents (like sodium borohydride, dimethyl formamide, hydrazine etc.) stabilizers (like CTAB, dendrimers, organic ligands, etc.), high pressure, capital consumption, etc which is harmful to the environment (Govindarajan et al. 2017). So, alternative biological methods have been established which are eco-friendly and of low cost, convenient, versatile and very simple for the formation of metal nanoparticles and additionally, they can be applied for

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large scale production. Biological method involves mild reaction conditions, nontoxic solvents (eg. plant extract and water) and very simple one pot reaction setup and provides wide efficiency for biomedical applications (Abdelhalim et al. 2011; Fang et al. 2018).

Plant extracts have been found to be more reliable compared to microbes due to their easy scaling up procedures. Plant extracts exhibit reducing and stabilizing properties as they accumulate and deposit (capping) on the metal ion surface to form nanoparticles. There are so many natural, medicinal plant extracts containing primary and secondary metabolites, enzymes, proteins etc. biomolecules which are responsible for the reduction of metal ions to metal Nanoparticles (Mittal et al. 2013). The antimicrobial effect of PdNPs strongly is dependent on their size and shape. The ultra-small PdNPs with a difference of 1 nm in size showed that the smaller PdNPs were more toxic to *E. coli* than the large ones, and the ultra-small PdNPs showed a very high antimicrobial effect at even very low concentrations (10^{-9} M) (Adams et al. 2014). The Pd nanocrystals with two different shapes (i.e., Pd cubes and Pd octahedron) showed distinct antibacterial activity on Gram-positive and Gram-negative bacteria. The facet-dependent oxidase and peroxidase-like activities of Pd nanocrystal help them have excellent antibacterial properties by the generation of reactive oxygen species (ROS) (Fang et al. 2018).

To Gram-positive bacteria, the faceted Pd cubes have a more effective killing ability than faceted Pd octahedrons; meanwhile, the octahedrons can penetrate into Gram-negative bacterial membranes in a higher number than Pd nanocubes, thus resulting in higher antibacterial activity (Fang et al. 2018). PdNPs, which were synthesized by biogenic methods, also showed a good antibacterial property. For example, PdNPs, that were synthesized using biomass waste from petals of *Moringaoleifera* as a natural reducing and capping agent, showed excellent antibacterial activity against *Enterococcus faecalis*. PdNPs, which were synthesized by a green method using white tea extract (named Pd@W.tea NPs), also exhibited antibacterial activity (Anand et al. 2016; Azizi et al. 2017; Fang et al. 2018). Basically, nobel nanomaterials like palladium (Pd), iron (Fe), zinc (Zn), titanium (Ti), platinum (Pt), copper (Cu), silver (Ag) and gold (Au) have received formidable attention because of their inevitable unique physic-chemical characteristic compared to their macro scale counterparts (Parhi et al. 2012). These Nanomaterials have also exhibited outstanding pharmaceutical applications like imaging, drug delivery, bactericidal and cancer theranostics, cell labeling, anti-inflammatory, antioxidant and Surface Enhanced Raman Scattering (SERS) (Kharissova et al. 2013). Specifically, metal-oxide and metal Nanomaterials are considered to have strong potential in cancer therapy.

PdNPs are known as catalysts in various industrial applications. On the other hand, very few reports have shown the distinct characteristics of PdNPs for their utilization as a photochemical agent, drug delivery system and anti-microbial / anticancer therapy (Dumas et al. 2015). Tahir et al. (2016) demonstrated the use of biosynthesized PdNPs as antibacterial compounds against *Pseudomonas aeruginosa*. Smaller and spherical nanoparticles were observed to have more antibacterial effects compared to larger and irregular shaped Nanoparticles (Tahir et al. 2016; Hiral et al. 2020). PdNPs synthesized from *Terminalia bellirica* have also been tested for their antifungal properties against *Aspergillus niger*. At present, few reports are available which describe the biological synthesis of Novel Metal Nanoparticles using various plant extract eg. *Curcuma longer tuber, Hippophaerhammoides Linn, Diopyros kaki leaf, Banana peel, Cinnamomzeylanicum bark, Oak gum, Pistaciaatlanticakurdica gum, C.comphora leaf, Rosa caninafruit, Stachyslavandulifolia, Pectin, Crateva Religiosa, Bauhinia Variegata, Moringa Pterygosperma, Cleistanthuscollinus, Morindacitrifolia, Alternantherasessilis, Ceropegiathwaitesii, Iris germanica, Aegle marmelos* etc.

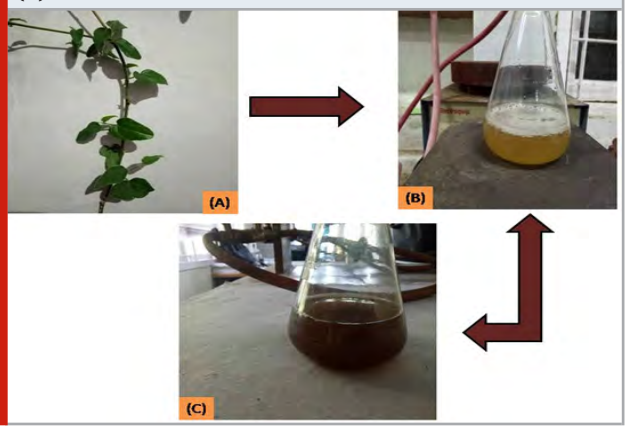
Metal Nanoparticles are made from a variety of medicinal plants (Sathishkumar et al. 2009; Sathishkumar et al. 2009; Yang et al. 2010; Banker et al. 2010; Song et al. 2010; Jeyaraj et al. 2013; Niraimathi et al. 2013; Khazaei et al. 2013; Kanipandian et al. 2014; Veisi et al. 2015; Nasrollahzadeh et al. 2015; Veisi et al. 2015; Bhakya et al. 2016; Veisi et al. 2016; Viswadevarayalu et al. 2016; Jayesh et al. 2016; Veisi et al. 2016; Hiral et al. 2017; Rahul et al. 2017; Hiral et al. 2018; Rahul et al. 2019; Rahul et al. 2020; Hiral et al. 2020). In this study, we report the plant-mediated synthesis of *Palladium* nanoparticles using the aqueous leaf extracts of *Cocculus hirsutusan* evergreen shrub which is found in many parts of India. We prepared metallic Palladium nanoparticles via green biogenic synthesis. The reduction of aqueous Pd+2 to Pd+1 ions and binded with Plant and its characterization and its anti-microbial activity tested against microorganisms namely *Escherichia coli* and *Bacillus subtilis*. The *Palladium* nanoparticles (PdNPs) synthesized was characterized by UV-Vis spectroscopy, SEM, TEM, XRD, EDAX, and FTIR. The Antibacterial/ Antimicrobial Activity effects of PdNPs.

MATERIAL AND METHODS

Plant material (*Cocculus Hirsutus*) was obtained the Department of Botany from botanical garden at Hemchandracharya North Gujarat University, Patan (Gujarat). Analytical-grade *Palladium Chloride* ($PdCl_2$) reagent was used as received from the S.d. fine chemicals (India). As a solvent, double distilled water was used in the experiment. For the *Cocculus hirsutus* Leaf Extract Preparation, the newly harvested *Cocculus hirsutus* plant material was carefully washed with tape water, supplemented by D.D. water, and dried for one month at room temperature in the shade. A grinder was used to process dried plant material. 10 gm dried plant powder was boiled at 50 ^{0}C for 30 minutes in 100 ml double distilled water. After cooling, the mixture was

filtered into Whatman filter paper no.1 and the resulting aqueous filtrate was deposited at 4°C for possible PdNPs synthesis.

Figure 1: (A) *Cocculus hirsutus* Leaf (B) Leaf extract and (C) Reaction mixture after 8 hr.



For the synthesis of PdNPs, in a conical flask, 30 ml of 1 mM $PdCl_2$ solution was slowly applied to 20 ml of prepared plant extract for biogenic synthesis of PdNPs. After applying the salt solution, the reaction mixture was placed on a magnetic stirrer with a hot plate set to 50°C for 60 minutes, and the colour of the reaction mixture changed from light orange to dark brown (Fig.1), confirming the tentative proof of PdNPs formation by reducing the ions Pd^{+2} to Pd^{+1} condition. UV-visible spectroscopy with an SPR peak at 422 nm was used to validate the results. The reaction mixture was reduced in less than eight hours. After centrifuging the final reaction mixture for 15 minutes at 6,000 rpm at 4°C, the bottom deposited nanoparticles were extracted and dried in an oven for 1-2 hours. For further characterization and biological screening, the obtained crystalline powdered PdNPs is placed in an airtight bottle.

To analyse the characteristics of PdNPs, the UV-vis spectrometer (UV-1800, Shimadzu, Japan) was used to classify biosynthesized Palladium nanoparticles in the range of 200-800 nm. A Rigaku D/MAX 40 kV diffractometer with graphite chromatography was used for the X-ray diffraction analysis. Debye Scherer's formula was used to calculate the average particle size.

$$D = \frac{0.9\lambda}{\beta \cos\theta}$$

Where D is the mean crystalline dimension, FWMH (full width at half maximum), X-ray wavelength, and diffraction angle are all constants. Field electron gun scanning electron microscopy with energy dispersive spectroscopy (FEG-SEM with EDS, JEOL JSM-7600F) and high resolution transmission spectroscopy is used to investigate the structural morphology and elemental composition (HR-TEM, Tecnai G2-F30).The functional groups present in the plant extract as biomolecules were established using Fourier transform infrared spectroscopy (FTIR) (Shimadzu, range of 400-4000 cm^{-1}) to contribute to the reduction of metal ions. Biogenic PdNPs were tested against *Bacillus subtilis* MTCC 121 (gram positive) and *Escherichia coli* MTCC 119 (gram negative) Pathogenic bacterial strains in an antibacterial assay.

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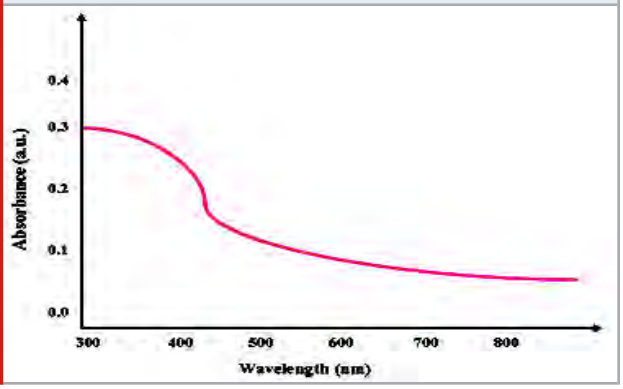
RESULTS AND DISCUSSION

UV-Vis Spectra Analysis: UV-vis spectroscopy measurements (Shimadzu UV 1800) were carried out at room temperature in the region 800–200 nm as a function of time of the reaction. The UV–Visible absorption spectrum was used for the analysis of optical properties of biogenic synthesized Palladium nanoparticles. The mono dispersed Palladium nanoparticles are shown in synthesis figure-3. The room temperature spectra exhibited strong absorption peaks at 422 nm for samples respectively, which is in good agreement with previous work (Osonga et al. 2020).

Figure 2: UV-visible spectra of Biogenic Synthesized Palladium nanoparticles



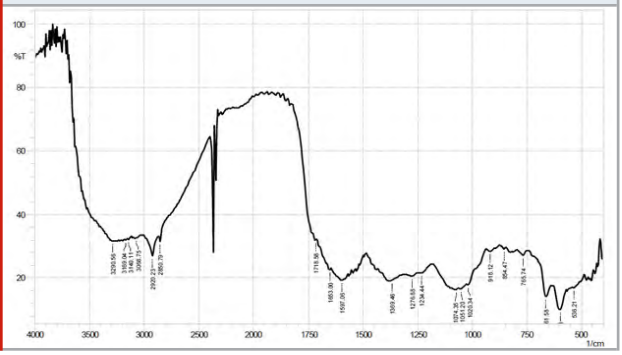
FTIR Analysis of Palladium Nanoparticles: FTIR spectroscopy analysis was carried out to identify the biomolecules responsible for the reduction of Pd^{+2} ions. FTIR spectroscopy analysis was carried out to find the biomolecules that were bound specifically on the Palladium nanoparticles surface. Fig. 4 shows the FTIR spectra of bio synthesized palladium nanoparticles. The spectrum showed sharp bands at 854 cm-1and 918 cm^{-1} corresponding to palladium nanoparticles. Strong

bands were observed at 1234 cm^{-1}, 1276 $cm^{-1}$and 1369 cm^{-1} and have been referred to as C–N stretching and N=O stretching vibrations of aliphatic amines and nitro compound, respectively. The bands obtained at 1718 cm^{-1}, 2850 cm^{-1}, 2920 cm^{-1} and 3290 cm^{-1} have been representing to stretching vibrations of C=O stretching (Aldehyde), primary alkanes and water molecules.

Table 1. FTIR spectral data for Palladium nanoparticles.

Functional Group	Wave number (cm^{-1})
Pd NPs C-N stretch	854, 918
(Aliphatic amines)	1234, 1276
N=O stretch C=O stretch	1369
(Aldehyde) C-H stretch	1718
(alkanes)	2850, 2920
O-H stretch	3290

Figure 3: FTIR spectrum of Palladium nanoparticles



XRD analysis of Palladium Nanoparticles: X-ray diffraction (XRD) measurement of the green synthesis of palladium nanoparticles carried out on a Rigaku D/max 40 kV diffractometer equipped with the graphite mono chromator and cu target. Fig. 5 shows the XRD analysis of biogenic synthesized palladium nanoparticles. This is used for further confirmation of palladium phase of nanoparticles. The observed intense peaks are 28.220, 33.610, 60.160 and 72.100 respectively representing the (100), (200), (220) and (311) reflections indicating the face centered cubic (fcc) structure of palladium nanoparticles XRD pattern reveals the face centered cubic structure indicating the crystalline nature of palladium NPs and the particle size calculated using Debye-Scherrer equation,

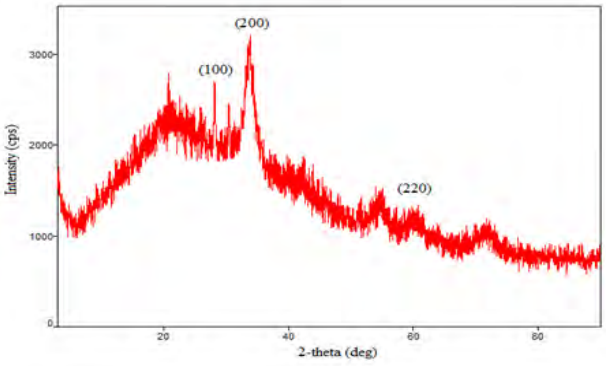
$$D = \frac{0.9\lambda}{\beta \cos\theta}$$

Where, D is average Particles size, λ is wavelength (1.5418 Å), θ is the Bragg's angle and β is full width half maximum (FWHM) of corresponding peek. The Scherer's formula was used to estimate the particles sizes and was found to around 12 nm. Field emission gun scanning electron microscope (FEG-SEM) and EDS images were recorded on a JSM-7600F series instrument. FEG-SEM spectra of biogenic synthesis of Palladium nanoparticles are shown in Fig.6. Palladium nanoparticles by green method show nearly mono dispersed distribution of particle sizes. The average particle size of the Pd nanoparticles is around 12 nm.

Table 2. XRD spectral data of palladium nanoparticles

2θ	Particle size (nm)	(h k l)
28.22	35.66	(100)
33.6	4.68	(200)
60	4.36	(220)
72	2.28	(311)
Average Particle size D = 12 nm		

Figure 4: XRD spectra of biogenic synthesized Palladium nanoparticles



The composition of Palladium nanoparticles was further probed by energy-dispersive X-ray (EDS) analysis. Fig. 7 shows the EDS pattern of Palladium NPs prepared using green synthesis, which indicates the presence of Pd and small amount of oxygen. EDS spectrum of Palladium nanoparticles shows the peaks for Palladium and respective elements indicating the formation of Palladium nanoparticles. Peak indexing of the elements is oxygen 0.5 keV and Palladium 2.8 & 3.5 keV. The compositions in the mass percentage of the elements are oxygen 41.16% and Palladium 46.21 %. The experimental composition matches with the theoretically calculated composition Rahul et al. 2019; Rahul et al. 2020; Hiral et al. 2020).

HR-TEM analysis of Palladium Nanoparticles: High-resolution Transmission electron microscope (HR-TEM) images were recorded on a Tecnai G2-F30 electron microscope. Fig. 8 shows the HR-TEM images of PdNPs prepared using *Cocculus hirsutus* plant. The sample preparation was carried out via the coating on carbon coated grid Cu Mesh 300 prior to the measurement. High-resolution Transmission electron microscopy (HR-TEM)

has been employed to characterize the size, shape and morphology of synthesized Palladium nanoparticles.

Figure 5: FEG-SEM spectra of green synthesis of Palladium nanoparticles

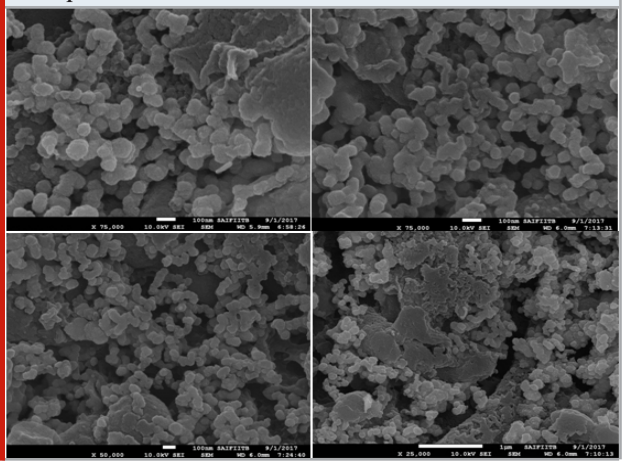


Figure 6: EDS spectra of green synthesis of Palladium nanoparticle

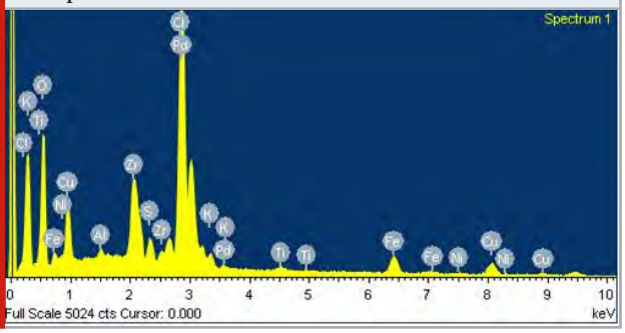


Table 3. Antimicrobial activity data for synthesized palladium nanoparticles.

Bacteria	Zone of Inhibition			Average zone of Inhibition
	1	2	3	
B. subtilis	7 mm	7.5 mm	7.5 mm	7.5 mm
E. Coli	14 mm	13 mm	13 mm	13 mm

Antimicrobial Test: Using the agar well diffusion process, biosynthesized palladium nanoparticles made from *Cocculus hirsutus* leaf extract were tested against gram^{+ve} (*Escherichia coli* MTCC 119) and gram^{-ve} bacteria (*Bacillus subtilis* MTCC 121) bacteria at different concentrations. The same procedure was used to test plant extracts.

Fresh overnight cultures of each strain were swabbed uniformly by cotton on plates containing sterile Luria Bertani agar, and 4 cup borer wells (diameter size – 6 mm) were prepared. Each well was filled with 50 microlitres of sample nanoparticles, with a commercial gentamicin disc serving as a positive monitor. It was incubated for 24 hours at 37°C, during which the diameter of the inhibition

Figure 7: HR-SEM spectra of Palladium nanoparticles

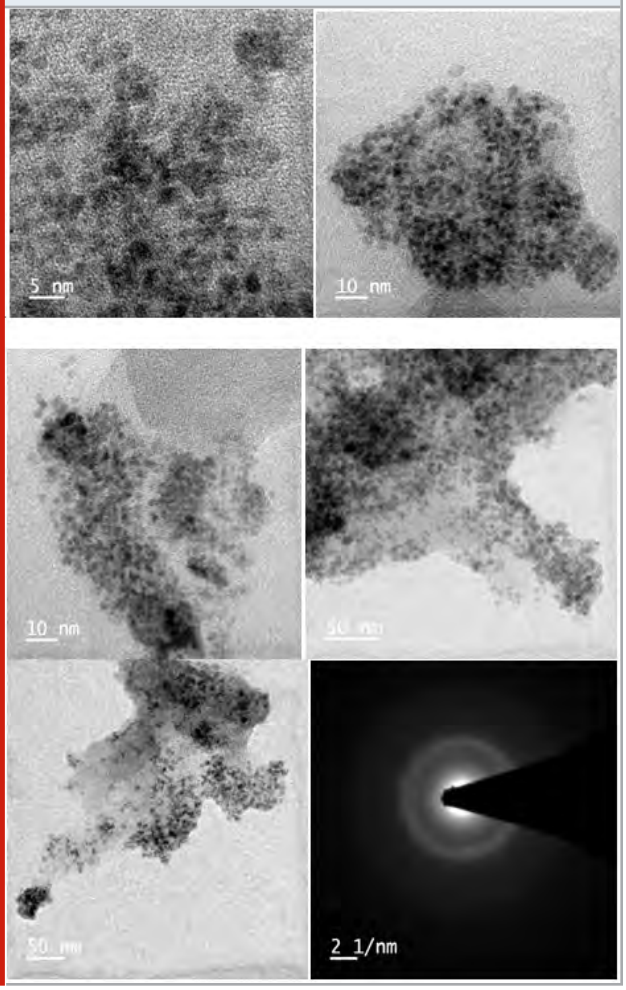
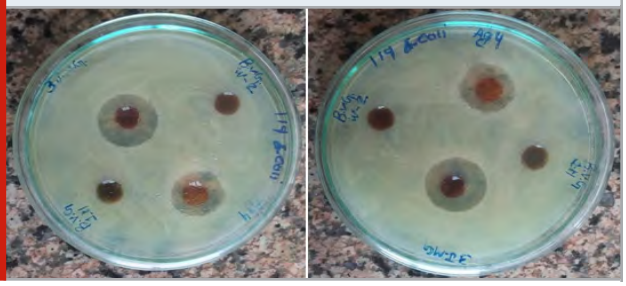


Figure 9: Palladium nanoparticle antibacterial experiments (a) Escherichia coli (b) *Bacillus subtilis*



region was measured in millimetres across the well (Fig. 9). (Table 3).The inhibitory compounds in the tested sample trigger the bacterial growth inhibition region. The antibacterial efficacy of the agar well diffusion process was found to be satisfactory. In comparison to *Escherichia coli, Bacillus subtilis* showed poor results in terms of zone of inhibition. Plant extract, on the other hand, yielded no results. The experiment was repeated three times in order to produce improved data Rahul et al. 2019; Rahul et al. 2020; Hiral et al. 2020).

CONCLUSION

The Biogenic synthesis of Palladium nanoparticles performed *Cocculus hirsutus* leaf Extracts without involving any toxic chemicals. In this reduction reaction metal ions were reduced (Pd^{+2} to Pd^{+1}) very rapidly and reaction was finally completed within 8 hours to produce Palladium nanoparticles. Different plants will take different time to complete the reaction due to have their different properties. The characterization of synthesized Palladium nanoparticles characterized by various microscopic and spectroscopic techniques which includes FEG-SEM with EDS, FTIR, HR-TEM, XRD and UV-visible confirms the formation of Palladium nanoparticles. Synthesized Palladium nanoparticles showed high stability even after few months at ordinary room temperature. XRD patterns conforms that the average particle size found to 12 nm. UV spectra shows absorption peak near 422 nm to confirm that formation of palladium nanoparticles.

ACKNOWLEDGEMENTS

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Conflict of Interests: The participating authors had no conflicts in their interests while preparing for this research.

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Psychological
Communication

Body-Shaming and its Trepidation on the Postpartum Condition of Women: A Psychological Study

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ABSTRACT

Maternal wellbeing is a pivotal subject because it involves both mother and child's health. But in most cases after giving birth to their young ones, these mothers carry the psychological brunt of being body-shamed leading to body dissatisfaction, depression, and low self-esteem. Nowadays women are given too much pressure to adhere to the standards laid by society which itself proclaims the significance of this study. They are endorsed with the additional responsibility of getting back to their pre-baby body at the earliest. Methodologically to identify the common psychological impacts of body shaming on new moms a case study of 100 new mothers from Ernakulam, Kerala were conducted after endorsement by the Institutional Human Ethics Committee, using convenience sampling. In the wake of acquiring educated assent, a semi-organized poll was utilized to gather information on self-perception discontentment. Factors responsible for pressurizing them to regain their pre-baby body were analyzed using different measurement scales.Studies based on these samples show that 81.35%of new mothers are eitherserious victims of body-shaming or are stuffed with a fearful mindset of being body-shamed during their stressful post-partum period. Development of self-recognition disillusionment was common among all. It was found that segments, for instance, higher BMI, socio-cultural strains, and sadness were all basically associated with self-observation frustration. Normally used weight control measures were eating little meals and some even practice skipping dinners83% of women eat less during the lactating period to reduce their prenatal weight gain. Enhancing the appearance and body shape where the essential clarifications behind weight control rehearses. Women undergo physical as well as mental pressures from different walks of life to work for physical perfection, no matter what stage of life they are in now; meeting these societal expectations became the top priority for modern women which acts harmful for a healthy life.

KEY WORDS: BODY DISSATISFACTION, BODY SHAMING, DEPRESSION,MATERNAL HEALTH, POST-PARTUM.

INTRODUCTION

There is a developing contention in our general public, and it's pointed unequivocally at the waistlines of our moms. No sooner than their nine months of pregnancy are finished, rather than being met with the once standard question about how much their infant's gauge, moms are being called out about what number of pounds they've picked up during their pregnancies and all the more critically, what's taking them such a long time to lose them. The strain to shed baby blues pounds has brought forth what many propose to be unreasonable assumptions regarding self-perception, blending warmed discussions about how much weight ladies should take on during pregnancy and the worthy measure of time it should take them to get it off. After conceiving an offspring, attaining quick weight reduction has become a mainstream societal fixation, with a-rundown on-screen characters, unscripted television celebrities, and performers continually gracing market tabloids covers, wearing their thinned down post-pregnancy bodies. So, what is the truth about how pregnancy weight gain and loss work? Indeed, a woman's body is not naturally wired to spring once more into pre-pregnancy shape directly after conveyance. The truth is that it took nine months to get to where you are in terms of weight gain during the pregnancy. It is probably going to take the better part of that to get you back to where you were (Matta 2019).

Many authors have discussed literature based on fat-shaming and its impact on all age groups. Fat shaming and its impact on new moms were not widely discussed,

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but recently, few articles focusing on these aspects struck my attention. Among them, the most prominent one is the fat studies journal by Taylor and Francis. In comparison to preceding studies, this study base itself on the scientific analysis based on samples of 100 new moms from Ernakulam district of Kerala who contributed their personal experiences making this study unique. Today our culture places too much importance on body weight/size and promotes specific weight gain and weight loss objectives. New moms struggle to get back to their pre-baby body within a short span of time while they are leading a stressful life adjusting themselves with their new ones. The fear of getting body shamed intensifies the psychological stress encountered by them during their post-partum. They even forget that they are lactating mothers; therefore, their body requires sufficient nutrients (Zielinski 2019).

As indicated by Body Mass Index (BMI) rules given by The American College of Obstetrics and Gynecologists and refreshed by the Institute of Medicine in 2009, underweight ladies, with a BMI underneath 18.5, should pick up between 28-40 pounds through the span of their pregnancy; the individuals who have the typical weight (BMI between 18.5-24.9) are supposed to pick-up 25-35 pounds during their pregnancy; overweight ladies with the (BMI between 25-29.9) are only supposed to gain 15-25 pounds during their pregnancy, and large ladies with a BMI of 30 and above are encouraged only to increase 11-20 pounds during pregnancy (Lee 2019). Each individual body is unique and they metabolize differently. Some people gain or lose bodyweight due to certain underlying medical conditions. For instance, patients with thyroid malady or other clinical issues may have various issues with weight (Lee 2019).

There are many works based on fat-shaming and postpartum condition of women but there was a lacuna in studying the impacts of fat-shaming on new moms. Thus, this work concentrated on the scientific study of the psychological impact of body-shaming on the post-partum condition of new moms. During the pregnancy tenure, only very few women happily embrace their weight gain. Most women live in a cocoon of fear of being body-shamed or being compared to those women who have attained their normal body size after few months of their delivery. To put it plainly, persistence rules about how soon ladies can hope to see changes in their post-child bodies. For nursing moms, a twofold portion of persistence is fundamental. At the point when the bosom is taking care of, you're going to require some additional bodyweight, muscle to fat ratio to take into consideration ideal nursing. The accentuation (for moms) ought to be on keeping up a solid eating routine and a sound way of life, both during pregnancy and a while later, realizing that proper weight gain or loss will follow (Saarikko et al. 2021).

MATERIAL AND METHODS

A case study of 100 new mothers from the Ernakulam district of Kerala was conducted. There was an estimated commonness of body image dissatisfaction and fear of being body-shamed among 81.35% of the study participants. After endorsement from Institutional Human Ethics Committee and acquiring ethical clearance, 100 new moms were taken for investigation by the convenience sampling. In the wake of acquiring educated assent, a semi-organized poll was utilized to gather information on self-perception discontentment among new moms.

They even live in fear of what others will think of them. All these concerns add to the post-partum condition encountered by most women all over the world. It was found that new moms are under the grip of certain unavoidable factors that encircle them like socio-cultural pressure, financial and media impact, statistic factors, blind belief in maintaining normal body size acquired from various spheres of life, and how does it directly affect one's confidence, out of the box body sizes may even lead to compulsiveness and depression in certain situations. Self-perception discontentment among new mothers was estimated using the Stunkard scale or the Figure Rating Scale (FRS). Females who were under the study were given the adaptations of the FRS and were approached to distinguish the figure they as of now saw themselves with, and the figure they would wish to have. Inconsistency scores were determined dependent on the contrast between the genuine and perfect figures picked. Positive scores demonstrate a longing to be littler, while negative scores recommend a craving to be bigger (Lee 2019).

The disparities between the present and wanted figures were translated as a sign of self-perception discontentment. Thus, members who had disparities in the figures picked were distinguished as having self-perception discontentment. For example, the variables related to self-perception discontentment, confidence, discouragement, socio-cultural pressure, media impact, and perfectionism were estimated utilizing recently approved surveys and utilizing Likert scale evaluations, and a score was given (Lee 2019). Rosenberg Self-Esteem Scale was utilized to quantify Self-regard with the Likert scales running from firmly concur (score 0) to unequivocally dissent (score 3) (Gray1997). Based on the responses from the questionnaire (carrying ten questions), individual scores were calculated with the total score ranging from (0-30). Those who secured (25-30) individual scores were considered to possess a higher level of confidence and higher self-esteem; only 5% secured this score (Lee 2019).

Among them, 80% secured (15-25), indicating that their confidence level was not affected. But 15% of them secured (0-15), which indicated low self-esteem and lack of confidence, which is quite 3) (was estimated utilizing the Center of Epidemiological investigations (CES)with the help of 20 questions. Depression survey was conducted with Likert scale which evaluated from once in a while to generally with the scoring of 0-3, separately (Radloff 1977). Based on the analysis, 40% of the samples secured a score of 45-60, which indicated

a higher degree of depressive symptoms, 30% got scoring30-45, indicating mild symptoms of depression. 20% secured 15-30, which is a normal range. Only 10% got 0-15 indicating no or very fewer depressive symptoms. The socio-cultural measure was estimated using SATAQ 4 with scores ranging 1to 5(Thompson 2004). The questionnaire consisted of 3 sections. The first section carries 10 questions with scores (10-50) that calculated individuals' body image perception (Zielinski 2019).

Accordingly,62.225% preferred thin/low body fat; 19.125% preferred muscular/athletic body; only18.65%was satisfied with their present body image. The following four questions measured the influence of an individual's family on forming his body image perception.75% of them got15-20, which indicated high level of influence. The next four questions are targeted to measure the peer influence. 81% agree that they are under the strong grip of their peer influence (Zielinski 2019). Media impact was estimated utilizing SATAQ 3surveys with a Likert scale evaluated with a scoring of 1-5. Four questions were targeted to extract the influence of media, and scores of 92.5% cases show that they are under the strong influence of media. Compulsiveness was estimated utilizing the EDI (Eating Disorder Inventory poll) with Likert scale evaluated on the basis of a questionnaire carrying 25 questions with a score ranging from (1 to 6) (Garner 1983). Studies show that 83% of the cases are victims of some sort of an eating disorder.

Among them 61% eat less than the required food to reduce their present body weight; 21% even skip dinner for the same reason. Extraordinary estimates like taking purgatives (17%) and vomiting after dinner (1%) were also observed. The tallness and weight of the members were estimated, and the weight list (BMI) was determined. According to determined. According, BMI<18.5 kg/m2 was named underweight, a BMI of 18.5–24.9 kg/m2 was viewed as typical, overweight is 25–29.9 kg/m2, and stoutness is ≥30 kg/m2 (Saarikko et al., 2021).

Table 1. Body image dissatisfaction and its distribution trend among new mothers based on calculated body mass index

BMI Status (kg) of new mothers	Total Percentage (Based on Body Weight)	Frequency of Body Dissatisfaction		
		Increase weight	Reduce weight	Satisfied percentage with the current bodyweight
Underweight	15%	74.5%	8.5%	17%
Normal	30%	2%	41%	57%
Overweight	45%	0%	99.5%	0.5%
Obese	10%	0%	99.9%	0.1%

Table 2. Distribution of psychological impacts pertaining to weight obsession on new mothers with different BMI scales.

BMI Status (kg)	Total Number	Psychological health issues (percentage)	Satisfied Psychological Condition
Underweight	15	70.5%	29.5%
Normal	30	65%	35%
Overweight	45	99%	1%
Obese	10	99.7%	0.3%

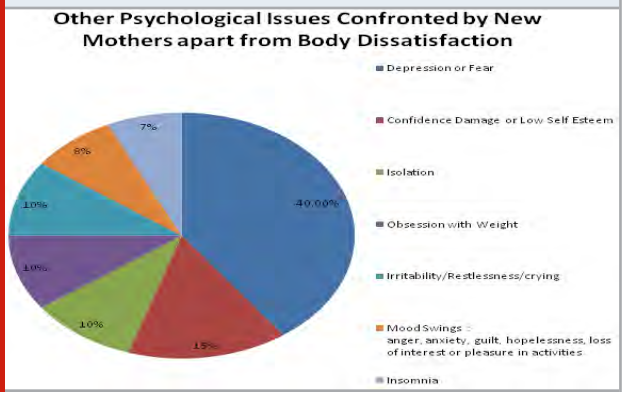
RESULTS AND DISCUSSION

In the light of the BMI measurements, 30% of the samples possessed a typical BMI, while 15% were undernourished; 45% were overweight and 10% were large. The investigation indicated that 81.35% of young ladies were disappointed with their self-perception. Table 1 shows the dissemination of self-perception discontentment based on the determined BMI of the members. It shows that 43% of the members with typical BMI were also disappointed with their appearance and wanted to either increase or reduce their body weight. In the underweight class, 74.5% wanted to increase their weight, and 8.5% still wanted to reduce their weight, further demonstrating the growing demand for having a dainty appearance. Higher BMI, higher financial status, sadness, expanded socio social strain to be flimsy, media impact, and low confidence were seen as essentially connected with self-perception discontentment (Saarikko et al., 2021).

Among 100 new mothers who took an interest in the present examination, 71% of them had already attempted some kind of weight control measures because they are under the intense pressure of either being body-shamed or in the grip of a fear of being body shamed. Among the studied cases, 18.65% were satisfied with their present

body size/ shape/ weight, but the fact is that 81.35% are developing a shoddy self-perception. Our examination additionally indicated that 65% of them managed to undergo at least one weight control measure. Multiple reasons were given by them, of which 75% gave the reason of improving their physical appearance and body shape as the main reason for going in for weight control measures and 25% said to look better in clothes (Saarikko et al., 2021).

Figure 1: Other Psychological Issues confronted Issue confronted by new mothers apart from body dissatisfaction.



Refraining from sufficient food intake is awful for your psychological and physical wellbeing. Weight variance can also bring a large group of related reactions that can hurt your physical wellbeing. Based on previous studies, it was found that many people feared that body fat has been connected to an expanded danger of cardiovascular sickness, Type 2 diabetes, and hypertension. The mental effect is similarly disturbing. When calorie counters are inclined, it leads to raised cortisol (the pressure hormone). Another investigation found that expanded worry and refraining from sufficient food intake may result in activated gorging (Lee 2019). Studies focusing on postpartum issues and fatness issues are very common, but body-shaming impact during postpartum are not widely discussed. In comparison to previous studies this work focuses on the health consequences of body shaming in new moms. Due to the fear of being body-shamed, most of them resort to some weight control measures. Some even develop severe psychological issues. Ultimately this article highlights contemporary culture's obsession with body-shaping practices and its harmful impacts on new moms (Saarikko et al.2021). Following pie chart is a pictorial representation of various psychological issues faced by new mothers apart from body dissatisfaction and eating disorders.

CONCLUSION

Body shaming is a powerful strategy asserted with a covert plan of controlling the population and dividing them based on discipline. This explains the growing trend of body shaming. It's high time to become alert of this victimization and bounce back with the slogan that you are beautiful in whatever size and shape you are in now. Based on this study, it is high time to become alert of the injustice steered on new moms in the form of body-shaming because 81.35% bear the psychological brunt of this cruelty (Rodriguez et al., 2019).

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Conflict of Interest: All authors contributed equally to this work with no conflicts.

Ethical Clearance Statement: The Current Research Work Was Ethically Approved by the Institutional Review Board (IRB) of Amrita Viswa Vidyapeetham University, Kochi Campus, India.

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Ecological Communication

Excessive Growth of *Euglena* sp. and its Effect on Shallow-Water Ponds

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ABSTRACT

The Bankura town of district Bankura, West Bengal, India is full of lentic water bodies like pond, reservoir and water tank etc. In these lentic water bodies, some are perennial. Most of the rest waterbodies are seasonal. Out of these, a large number of waterbodies are partly seasonal; that means, these waterbodies can retain the water to some extent during winter to summer, but the water level become low due to various causes. We referred this type of waterbodies as shallow-water pond. And the ultimate rest type of waterbodies is totally seasonal and get dried during winter to summer season. Our point of interest is to compare between the perennial and shallow-waterponds of Bankura town. The fish yield from the shallow-waterponds of Bankura town is quiet low. On the contrary, in the perennial ponds where the water depth is minimum 3-4 metre, do not face this problem at all. Till today it is said that, in Bankura town the pisciculture is less efficient due to various natural and anthropogenic causes. But the actual cause was unknown till date. After studying various physico-chemical and hydrobiological parameters following standard methods of APHA we have observed a remarkable change in these shallow-water ponds especially during that season. A rapid and exceptionally dense accumulation of algae (Euglena sp.) was observed floating over these shallow-water ponds. This floating algal bloom is hampering the pisciculture of Bankura town a lot by oxygen depletion. The correlation between dissolved oxygen and pH was not showing significant result in shallow-water ponds but, showed totally significant result in case of perennial ponds. The main aim of the study is to make the town dwellers as well as the pond owners of Bankura town aware about the cause of low yield in these shallow-water ponds, so that they can overcome the problem.

KEY WORDS: ALGAL BLOOM, EUGLENA, SHALLOW-WATER POND, PERENNIAL POND, BANKURA TOWN.

INTRODUCTION

About 71 percent of the Earth's surface is covered with water and the oceans hold about 96.5 percent of it. The rest 3.5 percent exists in the air as water vapour; in rivers and lakes as liquid; in icecaps, glaciers as solid and in the ground as soil moisture. Out of this 3.5 percent only 2% we found as fresh water in the form of surface and subsurface water bodies and are usable for both human consumption and aquaculture. Water is essential for the functioning of every single cell, tissue, organ and organ system in all animals as well as human being also. It is observed, during the last several decades the water quality of the Indian water bodies has been deteriorating, due to continuous discharge of industrial, agricultural and domestic sewage (Majumder and Dutta, 2014; López-Felices et al., 2020; Wato and Amare, 2020). Both lotic and lentic inland waterbodies of freshwater ecosystem are being subjected to constant environmental stress (Goswami et al., 2017; Borics et al., 2021).

Ponds are useful in many ways, as it is the most common source of open freshwater. The main nutritional cycles of an aquatic ecosystem are constituted by phytoplankton as these are the only primary food for many organisms, such as fish, crustaceans and shellfish (Goswami et al., 2017; Borics et al., 2021). These planktons can also act as indicators of trophic status of water bodies. Freshwater zooplankton is also an important component of an aquatic ecosystem and plays a crucial role for maintaining the chain of the ecosystem as they maintain the link between the producer and primary consumer. So, the distribution of both phyto as well as zooplanktons and the level of their abundance are useful for calculating the potentiality of a water body from fishery point of view (Majumder, 2020).

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It is known to all that, Bankura district is one of the backward districts of West Bengal. Still Bankura has ranked first in pisciculture (particularly in spawn production), as the whole Bankura district contains large number of waterbodies than the other districts of West Bengal (according to the Office of the Additional Director of Fisheries, Bankura, West Bengal, India; www.bankura.org.in/site/Fisheries.htm). But in case of Bankura town some ponds are perennial and most of the rest waterbodies are seasonal. Out of these seasonal ponds of Bankura town, a large number of waterbodies are partly seasonal; that means, these waterbodies can retain the water to some extent during winter to summer.

And the water level become low due to various causes. We referred this type of waterbodies as shallow-water pond. And the ultimate rest type of waterbodies is totally seasonal that get dried during winter to summer season. We compared the values of physico-chemical and hydrobiological parameters of perennial and shallow-water ponds of Bankura town to know the actual cause of low yield of fish production. This study has been done to make the town dwellers as well as the pond owners of Bankura town aware about the fact of low yield in these shallow-water ponds (Majumder et al., 2019b). For the rapid growth and development of different organs of fish, zooplanktons provide the necessary amount of protein; and the zooplanktons themselves get their nutrients from phytoplanktons of the water body (Islam and Bhuiyan, 2007; Anton-Pardo and Adamek 2015; Radhakrishnan et al., 2019). The Rotifers, one of the well-known zooplanktons that play a significant role in the food chain of pond ecosystem and became a pollution indicator or water quality monitor (Kamble and Meshram, 2005; Majumder et al., 2019b).

A eutrophic condition was observed due to excessive growth of *Euglena sanguinea*, a microalga that inhabits freshwater habitats throughout the world. In his species large amounts of the red xanthophyll astaxanthin (Ast) have been reported to occur (Frassanito et al., 2008; Wołowski, 2011; Guiry, 2017). By 2018 onwards report of dense growth of planktonic algae of one or few species came to know imparting a distinct colour to the water body, that is referred to as algal blooms. This universal phenomenon was termed as flowering of the water (Guiry, 2017; Majumder et al., 2019b).

Bloom formation was attributed to the algal genera belonging to the classes Bacillariophyceae, Chlorophyceae, Cyanophyceae, Dinophyceae, and Euglenophyceae. These water bodies were studied for different physico-chemical parameters and the plankton analyses were also conducted to study the dominance of Euglena in water bodies. Very high concentrations of organic matter and phosphates were the chief features of the pond's waters having Euglena blooms (Date et al., 2018). From 2019 onwards harmful algal bloom (HAB) namely red tide were reported to cause change in water colour of sea which has a serious impact on environment along the coast and aquatic ecosystem (Zohdi and Abbaspour, 2019; Gokul et al., 2020). Review article of 2020 disclose the methods of algal control (Fentie, 2020). But what happens to those pond (perennial and shallow-water) ecosystems, most importantly the changes taken place in physico-chemical and hydro-biological parameters are still unknown. And is there any relation between algal growth of pond and pisciculture is also unknown. This work may give some light to know the relation between algal growth and fish yield.

MATERIAL AND METHODS

Study Area: Figure 1 shows both Type I (perennial) and Type II (shallow-water) ponds of Bankura town of district Bankura, West Bengal, India. The total fieldwork was carried out consecutively for six months from January to June, 2020 at two different sites (Type I and Type II ponds) of Bankura town (Figure: 2 to 4). Figure 2 shows both these two sampling sites are located at two different sideds of Dwarkeswar river at Bankura. Actually, the Type I ponds are well managed for aquaculture and these ponds are perennial (water retains throughout the year up to 4 to 5 metre depth) in nature. On the other hand, the Type II ponds are not well managed for aquaculture purpose and retain shallow water depth up to one metre or so.

These Type II ponds are located throughout the Bankura town. Figure 3 shows pond Daser Bandh (located at 23°12'54"N and 87°2'12"E), Rajgram, Bankura [beside Bateswar Temple] and this is a Type I (pond with yellow marking in Figure 2 and 3) pond. Figure 4 shows pond Natun Bandh (located at 23°13'35"N and 87°3'4"E), Kankata, Bankura and this is a Type II (pond with red marking) pond. For the analysis of physico-chemical and hydrobiological parameters the water samples were collected in the morning between 6 to 7 AM from each of two collection sites. The hydrogen ion concentration (pH), dissolved oxygen, free and dissolved CO_2 were determined following standard methods of APHA-AWHA-WPCF (2005). The values were compared with standard values of BIS (Bureau of Indian Standards, 2003; Khanna and Bhutiani, 2008). A Celsius thermometer (scale ranging from 0°C to 100°C) was used to measure air and surface water temperature. For measuring intensity of light, the Digital Lux meter (HTC, Model No. LX-101A, Range 0 to 2,00,000) was used. pH of water was measured directly using a digital electrode pH meter (Systronics, Model No. SYS-335).

The planktons were collected with a modified nylon bolting silk plankton net (No. 25 mesh size 50µ) with a round metallic frame of 0.625 sq.m. area was used for collection of planktons. Collected samples were transferred to the labelled vials which contain 5% formalin solution. The plankton was observed and documented using Magnus Trinocular Microscope (Model MLX TR) attached with Nikon Coolpix Camera. During the laboratory experiment, most chemicals used (Merck, India) for analysis of the physico-chemical parameters were with highest purity available or of analytical AR grade. Statistical analysis was done by MS Excel, 2007.

Table 1. The physico-chemical and hydro-biological parameters of two different types of productive ponds of Bankura district of WB, India from January to June, 2020 are being summarized here. The values of different physico-chemical parameters are Mean ± S.E. where N=12.

Sampling sites → Parameters observed ↓	Type I Pond Daser bandh of Rajgram, Bankura (High productive)	Type II Pond Natun bandh, Kankata, Bankura (Less productive)	BSI standard
Average water depth (metre)	0.75-1.15	3.45-4.34	---
Latitude	23°12'54''N	23°13'35''N	---
Longitude	87°2'12''E	87°3'4''E	---
Air Temp (^{0}C)	29 ± 5.2	29 ± 3.4	---
Water Temp (^{0}C)	25 ± 3.6	22 ± 2.5	< 40^{0}C
Light intensity (Lux)	(349-943)x100	(768-1111)x100	---
pH	7.33 ± 0.71	6.14 ± 0.54	6.5 - 8.2
Dissolved O_2 (mg/L)	5.00 ± 0.25	2.64 ± 0.79	Upto 6.0
Free CO_2 (mg/L)	355 ± 26.5	372 ± 28.1	---
Dissolved CO_2 (mg/L)	330 ± 34.6	405 ± 38.6	---
Qualitative analysis of plankton	Planktons observedin moderate to high amount	Huge number of plankton (mainly Euglena sanguinea) observed	---

Table 2. Correlation matrix among the physico-chemical parameters of Type I pond water during January to June, 2020.

Parameters	pH	Dissolved O_2 (mg/L)	Free CO_2 (mg/L)
Water Temp (^{0}C)	-0.961** (p value 0.00001)	-0.929** (p value 0.000013)	0.912** (p value 0.000036)
pH	-	0.803** (p value 0.001661)	-0.746** (p value 0.005335)
Dissolved O_2 (mg/L)		-	-0.817** (p value 0.001178)

**= Correlation is significant at p<0.01 level, *= Correlation is significant at p<0.05 level, N=12

Table 3. Correlation matrix among the physico-chemical parameters of Type II Pond water during January to June, 2020.

Parameters	pH	Dissolved O_2 (mg/L)	Free CO_2 (mg/L)
WWater Temp (0C)	-0.705* (p value 0.010447)	-0.756** (p value 0.004445)	0.789** (p value 0.002283)
pH	-	0.819** (p value 0.001119)	0.647* (p value 0.022962)
Dissolved O2 (mg/L)		-	-0.619* (p value 0.031863)

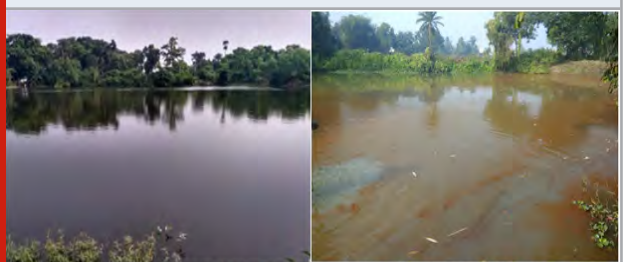
**= Correlation is significant at p<0.01 level, *= Correlation is significant at p<0.05 level, N=12

RESULTS AND DISCUSSION

The pH values were recorded here ranging from 6.14 to 7.33 (Table 1) at Type I and Type II ponds respectively; both the values are marginally acidic to neutral. The water temperature of Type I and II pond water showed negative correlation with water pH (r=-0.961, p<0.01 and r=-0.705, p<0.05 respectively) (Table 2 and 3). The water temperature of both the ponds water shows negative correlation with dissolved O2 (r=-0.929, p<0.01 and r=-0.756, p<0.01 respectively). But the water temperature of both the pond waters showed positive correlation with free CO_2 of water (r=0.912, p<0.01 and r=0.789, p<0.01). The pH values of both pond waters also showed positive correlation with dissolved O_2 (r=0.803, p<0.01 and r=0.819, p<0.01 respectively). These findings are comparable with several workers in their studies on different water bodies (Rai and Gary, 1980; Shardendu and Ambashth, 1988; Sinha, 1995; Zang et al., 2011; Liu et al., 2020).

It is also known that the dissolved oxygen (DO) plays a crucial role in sustaining flora and fauna in aquatic ecosystem. Among the two ponds, the pond of Daser bandh of Rajgram, Bankura showed the highest (5.00 ± 0.25 mg/L) level of DO in comparison to the other pond water (2.64 ± 0.79 mg/L) (Table 1). A moderate number of phyto as well as zooplankton were observed in Type I ponds but in Type II or the less productive ponds possess comparatively huge amount of Euglena sp (Figure 5, 7, 9 and 10). As the timing of water collection was between 6 to 7 AM; the level of DO was lower due to low intensity of sunlight. The value of light intensity was measured (from 349 to 943)x100 Lux in Type I ponds and (from 768 to 1111)x100 Lux in Type II ponds. The pond surroundings were full of trees in case of Type I ponds, whereas in Type II ponds the pond surroundings were devoid of plants. Furthermore, the algal bloom of the phytoplankton showed a blanketing effect on the Type II Pond, thereby preventing the penetration of sunlight into the pond water. It greatly affected the growth of beneficial algae by hampering photosynthesis. As a result, DO level was depleted in Type II ponds (Majumder et al., 2019a and Liu et al., 2020).

Figure 1: Two different types of ponds are observed at Bankura town



Type I (perennial) pond of Rajagram, Bankura | Type II (shallow-water) pond of Kankata, Bankura

Dissolved and free CO_2 in water plays an important role in maintaining the aquatic life. Main sources of carbon dioxide are respiration of aquatic organisms and also mixing of atmospheric CO_2 with the pond water. Due to the high affinity of CO_2 towards water, they can react to form carbonic acids and carbonates which alters the pH of water. The pH values of Type I and II pond water showed both negative and positive correlation with free CO_2 value of pond water (r=-0.746, p<0.01 and r=0.647, p<0.05 respectively) (Table 2 and 3). In this study no such remarkable change was observed (Table 1) in free and dissolved CO_2 values. But the DO value of Type I and II pond water showed negative correlation with free CO_2 of water (r=-0.817, p<0.01 and r=-0.619, p<0.05 respectively) (Liu et al., 2020).

Figure 2: Two sampling sites (marked red and yellow oval) at the two different sided of Dwarkeswar river of Bankura town. Type I (Yellow) and Type II (Red)

Figure 3: Daser Bandh (23012'54''N and 8702'12''E), Rajagram, Bankura [beside Bateswar Temple]

Type I (pond with yellow marking)

Figure 4: Natun Bandh (23013'35''N and 8703'4''E), Kankata, Bankura

Type II (pond with red marking)

Planktons Observed: The study of plankton is necessary in fisheries and aquaculture research as it provides food for fish in freshwater lakes and plays a major role in fish production. Zooplanktons are heterotrophic planktonic organisms floating in water. They are delicate aquatic

organisms and some of them are very weak swimmers. They contribute significantly to biological productivity of freshwater ecosystem. They serve as good indicators of change in water quality, because it is strongly affected by the environmental conditions and it quickly responds to change in environmental quality (Guy, 1992; Majumder et al., 2019b; Majumder, 2020). The freshwater zooplanktons constitute mainly Cladocera, Copepods, Rotifers, Ostracods, Protozoans and larva.

Figure 5: Four different ponds of Type I, their zooplanktons and huge fish yields

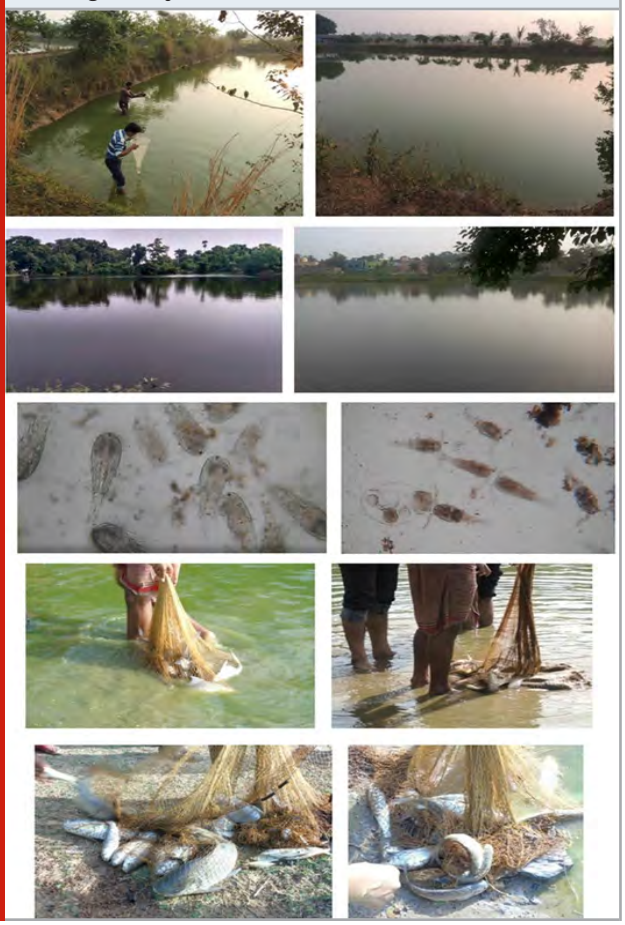


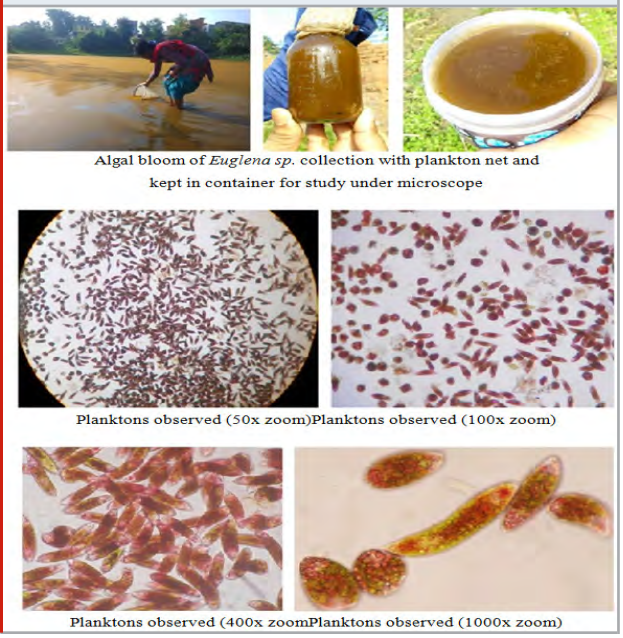
Figure 6: Eight different ponds of Bankura town of Type II along with algal bloom on the water surface

Among zooplanktons, the cladocera are the dominant group. This group is represented by *Daphnia* sp., *Moina* sp., *Ceriodaphnia* sp. and *Bosmina* sp. As per Murugan et. al., (1998) this group feeds on smaller zooplankton, bacterio-plankton and algae and they are highly responsive against pollutants. Cladocera are important food source for fry, fingerlings and adult of many economically important fish species. Cladocera were observed in both the two types of ponds but upto 13% in Type I ponds and only 2% in Type II ponds (Figure 9 and 10). Growth of Cladocera also been observed upto 18-25 % in Indian ponds (Majumder 2020; Sakhare and Chalak 2020). Copepoda comprises of the third most abundant group of zooplankton & this group is represented by *Cyclops* sp. and *Diaptomus* sp. Depending on the feeding habits there are three orders of copepods. Copepods of the order cyclopoida are the most important food items in freshwater aquaculture and their Nauplius is especially valuable for feeding fry (Wojciech et al., 2004). Cyclopoid copepods are commonly carnivorous who live on other zooplankton and fish larvae, though they also feed on algae, bacteria and detritus also. Copepoda were observed in both the two types of ponds but upto 51% in Type I and only 4% in Type II ponds (Figure 9 and 10). Increased level in number of Copepoda also been observed in ponds of Bankura (Majumder et al., 2019b; Majumder 2020).

Rotifers are the most important soft-bodied invertebrates having a very short life cycle among the zooplanktons. These are globally recognized as pollution indicator organisms in the aquatic environment (Kamble and Meshram, 2005). Rotifers occasionally become plenty when sufficient food is available and it can obtain

population density of over 5000 individual/L. Quantitative exploration during the period of study showed that the family Branchionidae exhibit maximum diversity of species (Joshi et al., 2015). Rotifers were observed in both the two types of ponds but upto 21% in Type I and only 7% in Type II ponds (Figure 9 and 10). The decreased number of Rotifers from perennial to shallow-water pond indicates the fact of low yield in pisciculture (Sakhare and Chalak 2020; Majumder 2020). The most dominant rotifer is Brachionus sp. It is illustrated by 4 species; among them *Brachionus bidentata* was found to be the predominant species. Brachionidae an important family of monogonont Rotifera and of the Rotifer fauna of India has received relatively more attention of the Indian workers relying on limnetic collections (Sharma and Sharma, 2014, Majumder 2020).

Figure 7: Planktons mainly found (*Euglena sanguinea*) in Type II ponds



Algal bloom of *Euglena sp.* collection with plankton net and kept in container for study under microscope

Planktons observed (50x zoom)Planktons observed (100x zoom)

Planktons observed (400x zoomPlanktons observed (1000x zoom)

Figure 8: Due to excessive growth of *Euglena sanguinea* fish yield decreases a lot in Type II ponds of Bankura town

Ostracods are small, poorly-segmented Crustacean in which the body parts are enclosed within a calcareous bivalve carapace. Ostracoda comprises of the least abundant group of zooplankton and this group is represented by *Cypris* sp. and *Heterocypris* sp. Ostracods are mainly bottom dwellers of lakes and live on detritus and dead phytoplankton. These organisms are food of fish and benthic macro-invertebrates (Chakrapani et. al., 1996). Ostracods were observed in both the two types of ponds but upto 13% in Type I and only 5% in Type II ponds (Figure 9 and 10). The decreased number of Ostracods from Type I to II pond indicates the fact of low yield in pisciculture (Majumder 2020). As ostracods occur in almost all aquatic habitats, the extreme antiquity of the group and the preservation of their valves in a wide variety of depositional environments make them an important tool in both palaeoecological and bio stratigraphic analysis (Armstrong and Brasier, 1980).

Figure 9: Planktons observed in Type I ponds

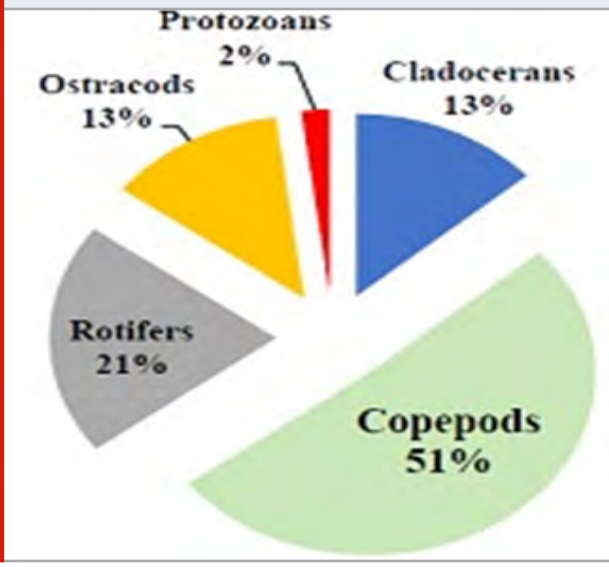
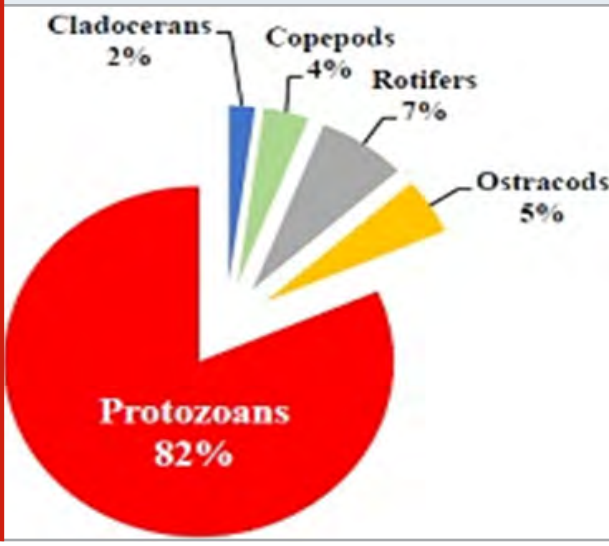


Figure 10: Planktons observed in Type II ponds



Some of the most common protozoa that can be found are *Euglena* sp., *Chlamydomonas* sp., *Phacus* sp. etc. Protozoa mainly *Euglena* sp. showed very dense population (82%) in Type II ponds whereas only 2% observed in Type I ponds. *Euglena sanguinea* is generally cylindrical with a blunt pointed end. This alga is found in fresh water

environments all over the planet. Like many other members of the algae (Protists subgroup) it seems to be capable of both sexual and asexual reproduction. It produces an interesting compound toxic to many fish species, and can also overgrow under certain conditions also leading to fish death (Grung and Liaaen-Jensen, 1993; Gerber and Hader, 1994; Paul et al, 2010). Understanding this microorganism is very important to fishing and fish-farming industries because under certain conditions these algae can have a detrimental impact on its surroundings (Zimba et al., 2004; Zohdi, 2019). They are capable of altering their shape, especially during motility: for example, they can extend their length up to ten times their width (Majumder, 2020).

They have a retractable flagellum, that remains mostly within the cell body, even when it is fully extended. They have also been noted to have granular eye spots of many varieties. They have been shown to reproduce asexually by mitosis. The photosynthetic algae are the autotrophs containing chlorophyll and an accessory pigment namely astaxanthin (a carotenoid). The accessory pigment prevents the cell's chloroplast from being overwhelmed under excessively bright conditions. The cells will appear red when utilizing this accessory pigment and green when these pigments are retracted into vesicles. This alga produces a compound known as euglenophycin that exhibits ichthyotoxic, herbicidal and anticancer activity at low ppm to ppb dosages (Paul et al., 2010). Actually, ichthyotoxic means toxic to fish. *Euglena sanguinea* a protist that is found in freshwater environments all over the world (Majumder, 2020).

Some researchers suggested that the toxin functions as a neurotoxin based upon the behavioural changes. Juvenile catfish exposed to cultures of the algal isolates died within 2h of exposure (Zimba et al., 2004). Some euglenophyte species have been reported to be red or to have the ability to become red with *Euglena sanguinea*. *Euglena sanguinea* is a microalga that inhabits eutrophic lentic freshwater habitats throughout the world (Wołowski, 2011; Guiry, 2017). It was originally described from a blood-coloured water sample from Silesia, although it was noted that cells were at first green and then red (Laza-Martínez et al., 2018). Present study revealed that *Euglena* sp. grows enormously in shallow-water ponds during winter to summer season. These algae produce a compound known as euglenophycin. The compound euglenophycin exhibits ichthyotoxic compound; means toxic to fish. *Euglena sanguinea* a protist that is found in freshwater environments all over the world (Laza-Martínez et al., 2018).

CONCLUSION

Present study revealed that Euglena grows enormously in shallow-water (not in perennial) ponds during winter to summer season which creates oxygen depletion leading to mass mortality of fish. Actually, Euglena cannot be mechanically or physically controlled, except by replacing the pond water which is not a practical option for most pond owners. Still, some of them are doing so along with aeration at night for several days. This may help to control the oxygen depletion. Rather the fish farmers can take necessary steps to reduce the algal growth in the pond by controlling the mixing of phosphate rich nutrients and effluents of agricultural land nearby the pond.

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Pharmaceutical
Communication

Green Synthesis, Characterization and Screening for Antibacterial Activity of Gold Nanoparticles Produced by *Salacia fruticosa* Leaf Extract

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ABSTRACT

In the present investigation, a detailed study on the synthesis, characterization and application of Gold Nanoparticles (GNPs) using medicinally important plant *Salacia fruticosa is* reported for the first time. The aqueous leaf extract of *S. fructicosa* was used as reducing agent for more rapid, facile, cost-effective and eco-friendly synthesis of metallic GNPs. The synthesis of gold nanoparticles was done by treating the different concentrations of aqueous Gold (III) chloride trihydrate(HAuCl4)solution with the plant leaf extract at physiological condition (pH 7.4). The formation of GNPs was studied by varying the metal salt concentrations in the reaction medium and was initially confirmed by UV-visible spectroscopy by measuring the peak between 400-700 nm. The GNPs synthesised at optimised gold salt concentration showed a peak at 545 nm. X-ray diffraction (XRD) analysis displayed Bragg's peak conferring the 310, 310, 330, 420 and 422 facets of the face centered cubic symmetry of nanoparticles suggesting that these nanoparticles were crystalline in nature. Possible interaction of phytochemicals in mediating and stabilization of nanoparticles was confirmed with Fourier transform infrared spectroscopy (FTIR). Size and shape of the nanoparticles was determined using Transmission electron microscopy (TEM) with size ranging from 20-50 nm. The plant *S. fructicosa* may found to be quite competent for the purpose of commercial gold nanoparticles production, since it is synthesized extracellular as well as rapidly. This work also presents a scientific support for the antibacterial activity of the gold nanoparticles against bacterial pathogens *Staphylococcus aureus* and *Pseudomonas aeruginosa* and consequently it may be used to discover the potential applications in the treatment of the infection caused by other microbial pathogens.

KEY WORDS: ANTIBACTERIAL ACTIVITY,GREEN SYNTHESIS, GOLD NANOPARTICLES, SALACIA FRUTICOSA, TRANSMISSION ELECTRON MICROSCOPY.

INTRODUCTION

Nanotechnology is emerging as a rapidly growing field and has received enormous amount of interest in recent years. This it developed as a part of material science which deals with the development of new improved materials in nanometre scale. Nanoparticles (1-100nm) being a fundamental building blocks of nanotechnology exhibits unique physicochemical properties compared to the bulk materials. Among noble metal nanoparticles, GNPs has an increasing interest and is considered an important area of research due to unique properties which made them use in divergent field of biological sciences (Vishwanatha et al., 2018; Yousaf et al., 2020).

GNPs have a wide application in drug delivery, tissue or tumour imaging, photo thermal therapy, diagnostic and in hyperthermia. Therefore, there is a lot of scope to develop eco-friendly process for the GNPs synthesis which has advantageous over physical and chemical methods of synthesis (Keshavamurthy et al., 2017). Biological synthesis of GNPs from plant materials, microorganisms and enzymes is simpler and eco-friendlier when compared to physical and chemical methods. Among the different synthetic methods in biological synthesis, phytochemicals mediated approach has been gaining significance in

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the recent years due to its renewable and eco-friendly nature (Nishanthi et al., 2019; Akintelu et al., 2020; Vaid et al.,2020).

The synthesis of GNPs involves the bioreduction of Au (III) to Au (0) by plant metabolites in aqueous medium with mild reaction conditions (Awad et al., 2019; Balasubramanian et al. 2020). Plant mediated nanoparticles synthesis is rapid and is cost effective in comparison to microbial mediated synthesis of nanoparticles which are slow rate of synthesis (Srinath and Ravishankar 2014). Biosynthesis of GNPs by plants such as *Azadirachta indica, Emblica officianalis, Aloe vera, Cinnamomum camphora, Magnolia kobus,Mangifera indica, Ocimum sanctum, Amaranthus spinosus, Garcinia combogia, Lantana camara, Pterocarpus santalinus, Terminalia bellerica* have been previously reported. However, standardization of experimental conditions is necessary to control the size, shape and dispersity of the nanoparticles (Shankar et al., 2004; Ankamwar et al., 2005; Chandran et al., 2006; Huang et al., 2007; Song et al., 2009; Philip et al., 2010; Daizyet et al., 2011; Ratul et al., 2012; Anish et al., 2014; Dash et al., 2014; Keshavamurthy et al., 2017; Kumar et al., 2018; Awad et al., 2019; Sherin et al., 2020).

Salacia fruticosa is an angiospermic herb belongs to the family Celastraceae. It is important medicinal plant which is traditionally being used in Ayurveda and is found in western ghats of India and mainly seen in the states Karnataka, Kerala and Tamil Nadu. Their leaves are simple, opposite, entire estipulate, 2-3 cm broad, 4-8 cm long and fruits are widely consumed by Kanis, the primitive indigenous tribal community residing in the Agasthyamala Biosphere Reserve of Southern Western Ghats (Saravanan et al., 2015; Subin et al., 2018). The research community in India has not given due emphasis to this plant due to limited literature on the diversity, distribution, phenology and its uses. Hence the present investigation was undertaken to explore the potential use of this plant in the synthesis and formation of gold nanoparticles deserves a special attention.

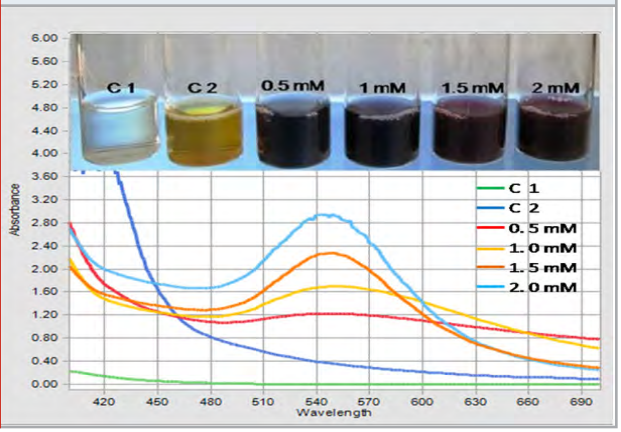
In the present study, we have reported simple, rapid, facile, stable, eco-friendly and cost-effective method for the biosynthesis of GNPs by using aqueous leaf extract of medicinally important plant *S. fructicosa* as a reducing and stabilizing agent. We also investigated the stability of GNPs, therefore carried out green synthesis of GNPs by treating the aqueous leaf extract with varied gold salt concentrations at physiological conditions (pH 7.4). The GNPs synthesised at optimised gold salt concentration was further characterized by using UV-visible spectroscopy, XRD, FTIR and TEM. The present study also emphasizes on the screening of GNPs against selected human pathogenic bacteria (Dudhane et al., 2019; Akintelu et al., 2020; Rautray and Rajananthini 2020).

MATERIAL AND METHODS

Gold (III) chloride trihydrate ($HAuCl_4.3H_2O$, 99.99%) and other components were purchased from Hi Media Laboratories Pvt. Ltd. and Sigma-Aldrich, Mysuru, India. Taxonomically authenticated fresh leaves of *Salacia fruticosa* was collected from Western Ghats of Subramanya (12°39′38″ North Latitude 75°36′32″ East Longitude) near Sakaleshpura, Karnataka, India. About 10g of fresh *S. fruticosa* leaves were weighed and washed twice with tap water and followed by distilled water to remove mud, dust particles and other pollutants. The leaves (Figure 1 a) were cut into small pieces and boiled with 90 mL of double distilled water and kept on boiling water bath (Keshavamurthy et al., 2017).

Figure 1: a. *Salacia fruticosa* plant b. Leaf extract

Figure 2: UV-Visible spectrum of Gold nanoparticles showing effect of different concentrations of HAuCl4 (0.5 mM to 2.0 mM) on GNPs biosynthesis. Inset shows the colloidal suspension of GNPs at different concentrations of $HAuCl_4$.

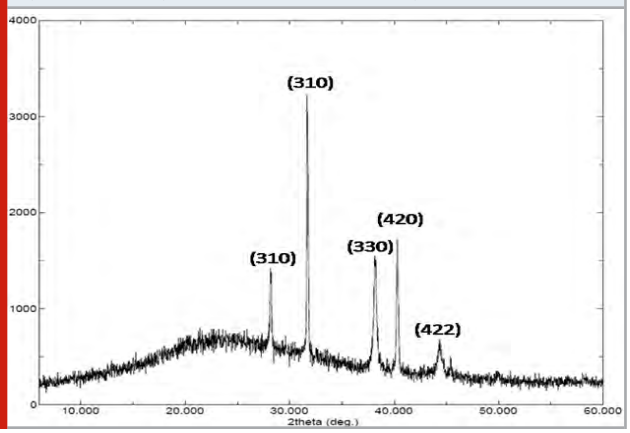


The temperature was maintained at 60°C for 15 min to facilitate the formation of aqueous leaf extract. The extract was filtered using What mann No. 1 filter paper. The filtrate (Figure 1 b) was stored at 4°C until further use (Keshavamurthy et al., 2017). The fresh leaf extract was used as a reducing and capping agent for the synthesis of GNPs. The green synthesis approach employed for the synthesis of GNPs is explained briefly as follows; 1mL of aqueous leaf extract was added to

4mL of gold salt solution (0.5 mM, 1 mM, 1.5 mM and 2 mM concentration) and the solutions were incubated at room temperature (28°C) under physiological condition (pH 7.4).

The bioreduction of the gold ions was monitored by measuring the solutions in UV-visible spectroscopy. Two controls were maintained at the same experimental conditions, one leaf extract without HAuCl4 and another control was gold salt without leaf extract. The UV-visible spectrophotometer (Thermo Scientific, Multiskan Spectrum) was used to record the colloidal suspension of GNPs in the range of 400-700 nm. The surface plasmon resonance peaks were assessed for size and distribution of synthesised GNPs. Deionised water was used as a blank. The crystalline nature of GNPs was studied by X-ray diffractometer (Rigakumini Flex 11) by operating at 30 kV and a current of 15 mA with Cu Kα radiation (λ= 1.5406 Å) and the 2θ scanning range was of 6-60° at 5° min^{-1}. The FT-IR measurements of the synthesized GNPs was used to analyze the presence of surface functional groups on GNPs.

Figure 3: X-ray diffraction pattern of gold nanoparticles synthesized by leaf extract of *Salacia fruticosa*



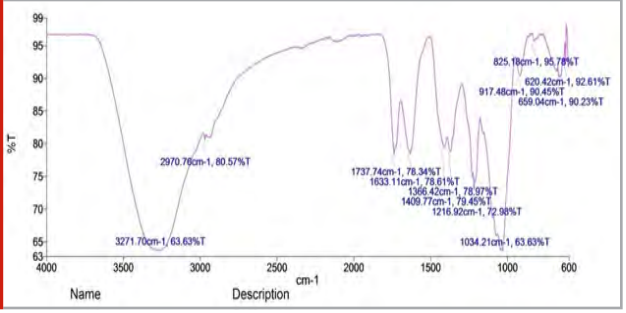
Samples were recorded in a Perkin Elmer spectrophotometer at room temperature by scanning it in the range of 4000 cm^{-1} to 400 cm^{-1} at a resolution of 4 cm^{-1}.TEM studies were performed to elucidate the size and distribution of the biosynthesised GNPs by using a TecnaiG2 spirit Bio TWIN, Netherland, operating at an accelerating voltage of 20-120 kV. For TEM analysis samples were prepared by placing a drop of colloidal suspension of GNPs on carbon coated copper grids. The films on the TEM grids were then allowed to dry at room temperature before analysis (Srinath and Ravishankar 2014; Keshavamurthy et al. 2017).The GNPs synthesized from S.fruticosa leaf extract was tested for its antibacterial activity using standard disc diffusion method against pathogenic bacteria such as gram-positive *Staphylococcus aureus* and gram negative *Psuedomonas aeruginosa* the overnight inoculated test bacterial cultures were swabbed uniformly on the freshly prepared Mueller-Hinton agar (MHA) plates using sterile cotton swab.

The 6 mm sterile discs were placed on solidified media and 50 µg/mL concentrations of GNPs were poured over the test discs. The control disc containing deionized water was also kept on the plate and incubated at 37 °C for 24 h. The antibacterial property of GNPs was determined by measuring the zone of inhibition around the discs in diameter (millimeter) after incubation. Chloramphenicol, a standard antibiotic of concentration 1mg/ml was used as positive control (Keshavamurthy et al., 2017; Al Saqr et al., 2021).

RESULTS AND DISCUSSION

Visual Observations: In the current study, the aqueous leaf extract of *S. fruticosa* was treated with different gold salt concentrations at physiological condition (pH 7.4) and the GNPs formation was indicated by a change in color from yellow to pinkish red or ruby red depending upon the size, shape and dispersity of GNPs in the colloidal suspension (Figure 2).The reaction took only two minutes for conversion of gold ions into gold nanoparticles. There was no color change in both the controls (C1 and C2) and therefore no nanoparticles synthesis occurred in control tubes. The color change remained stable for several months at 4°C. Previously, it wasreported that the wine red color of GNPs in aqueous solution is due to vibrations of Surface Plasmon exhibited by GNPs.Similar changes in color during were reported for the synthesis of metallic nanoparticles in previous studies (Shankar et al., 2004; Bonigala et al., 2018; Gomathi et al., 2019; Nayeem et al., 2020).

Figure 4: FTIR spectra of Gold nanoparticles synthesised by leaf extract of *S. fruticosa*

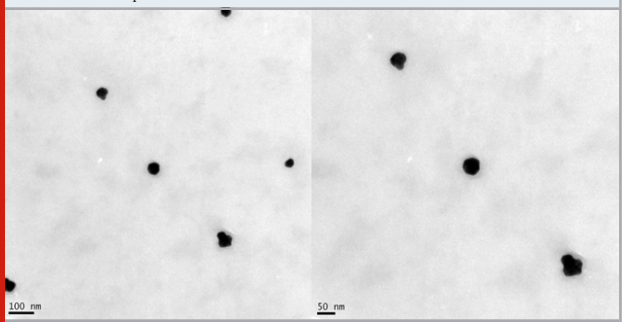


UV-visible spectroscopy: The bio reduction of gold ions to GNPs was preliminarily observed by appearance of red color and was confirmed by UV-visible spectroscopic studies. The UV-Visible spectrum of GNPs colloidal solution is shown in the Figure 2.The Surface Plasmon Resonance (SPR) bands centred between 545-560 nm confirmed the formation of GNPs in the solution. The salt control and the cell control tube did not show the peak characteristic for GNPs.

The height of the peak indicates the concentration of the GNPs produced and the shift of the peak towards higher wavelength indicates the larger size of the particles. Further, the sharp peaks and broader peaks indicate the mono dispersed and poly dispersed nature of GNPs

respectively. The spectrum of the reaction mixture containing 1.5 mM HAuCl4 showed a sharp peak in range of 545 nm with high intensity indicating that this concentration is optimum for GNPs synthesis. At concentrations of 0.5, 1.0 and 2 mM HAuCl4, broader peaks were observed at these salt concentrations with less intensity. With 1.5 mM gold salt, the peak was sharp and intensity was high indicating that this concentration was able to bring about a reduction of gold salt over almost the whole range. It is hypothesized that; this concentration was optimum for efficient activity of the bio molecules that were involved in the synthesis and stabilization of GNPs. The SPR of GNPs was attributed to the interaction between free electrons on the metal surface and incident light(Song et al. 2009; Pimprikar et al., 2009; Aromal et al., 2012; Francis et al., 2017; Nayem et al. 2020).

Figure 5: TEM image of gold nanoparticles formed at 1.5 mM $HAuCl_4$ by leaf extract of *S. fruticosa*.



X-ray diffraction analysis: The crystalline nature of GNPs synthesized was determined by using X-ray diffraction analysis. Figure 3 show XRD data of the synthesized GNPs. For GNPs sharp intense peaks observed at 2θ values of 28.19°, 31.70°, 38.19°, 40.30°, 44.37° correspond to the Braggs plane(310), (310), (330), (420) and (422), which confirmed that synthesized GNPs had a face-centered cubic structure. Thus, the XRD pattern indicates that the GNPs were crystalline in nature.The average crystallite size of the GNPs was found to be 25 nm and further confirmed by TEM analysis. The mean crystallite size was calculated by applying the Scherrer formula: $D=0.9\lambda/\beta_{1/2}\cos\theta$, where D is the average crystal size, λ is the X-ray wave length (λ=1.5406A°), θ is Bragg's angle (2θ), $\beta_{1/2}$ is full width at half maximum (FWHM) in radians. The unassigned peaks on the surface of GNPs in XRD analysis might come from the crystallization of bioorganic molecules (Borchertet al. 2005; Keshavamurthy et al., 2017; Vishwanath et al., 2018; Umamaheswari et al., 2018; Doan et al., 2020).

Fourier transform infrared spectroscopy (FTIR): spectroscopy was employed to deduce the possible bio molecules involved reduction of Au^{3+} to Au0 in the formation of GNPs. Figure3 shows the presence of different functional groups involved in leaf extract of *S. fruticosa* for the bio reduction of gold salt into biocompatible GNPs. The intense broad absorbance at 3271 cm-1 is the characteristic of the hydroxyl functional group in alcohols and phenolic compounds. The bands at 2970 $cm^{-1}$are corresponding to C-H group, 1737 cm^{-1} is C=O in esters1633 cm^{-1} corresponds to N-H bending modes and 1216 cm^{-1} is indicative of the presence of ester carbonyl and phenol.The bands at 1034 cm^{-1} corresponds to C-O stretch.This indicates the GNPs synthesized from *S. fruticosa* leaf extract are surrounded by some proteins and plant metabolites like terpenoids, flavonoids and phenolic compounds. The shifting and reduction in peak intensity of main absorbance band of GNPs revealed that biomolecules present in leaf extract were responsible for the reduction in gold salt which was reported in previous studies (Zhao et al. 2004; Ramamurthy et al., 2007; Naraginti et al., 2017; Ahmad et al., 2019).

TEM analysis: TEM analysis was employed to investigate the morphology, shape, and size of GNPs synthesized at optimized gold salt concentration (1.5 mM). TEM micrographs (Figure 5) depicted that GNPs were spherical in shape and uniformly distributed without significant agglomeration. Our result was in accordance with the previous studies done using different plant extract. The size of GNPs determined by TEM was in the range of 20 to 50 nm. Our results are un accordance with the previously published reports, where 13-26 nm, 22 nm size of GNPs were synthesised using different plant extract (Keshavamurthy et al. 2017; Mollick et al., 2019; Al Saqr et al., 2021).

Antibacterial activity: The antibacterial activity of GNPs was tested by disk diffusion method against the pathogenic bacteria, *S. aureus* and *P. aeruginosa*. Antibacterial property of GNPs reveals the diameter of zone of inhibition of 9 mm for *S.aureus* and 11 mm for *P.aueroginosa*. Positive control disc (Chloramphenicol) shows a zone of 15 mm. The tested bacterial strains were selected to represent different bacterial machineries that are present in different arsenals of virulence factors, besides their noticeable pathogeneses and high prevalence in human and animal life. The possible reason for the difference in the diameter of zone of inhibition observed might be due to their cell wall composition. Similar findings were found by GNPs prepared by aqueous extract of *Benincasa hispida*, against bacterial pathogens by well diffusion technique (Al Saqr et al., 2021).

This part of study is a preliminary time bound approach on the possible utilization of GNPs as an antibacterial agent. The results obtained in this investigation could be in support of developing an efficient, alternative, stable and biocompatible antibacterial agent from metal nanoparticles to combat drug resistance pathogens.The antibacterial mechanism exhibited by the GNPs is subject to the degree of susceptibility of microorganisms. The GNPs binds to cell wall of bacteria due to electrostatic interaction thereby penetrates inside the bacterial cell causes DNA damage and leakage of cell components is well documented by previous studies. Further it has also been reported that the interaction of various metal nanoparticles including GNPs with the amino acid

cysteine results in generation of reactive oxygen species, which also promotes bacterial cell death (Nikparast and Saliani 2018; Selvaraj et al., 2019; Akintelu et al.,2020).

CONCLUSION

The present investigation envisions the emerging role of plants for synthesis of metallic nanoparticles by employing a medicinally important plant *S. fruticosa*. The study highlights low cost, reproducible and rapid method to produces table GNPs at room temperature. The reaction rate achieved in the synthesis of nanoparticles is faster than microorganisms mediated synthesis. As no toxic reducing agents were used and the GNPs were synthesized at physiological conditions (pH 7.4) this method is environment friendly and the synthesized GNPs could be used in biomedical applications. The study also highlights effect of GNPs as antibacterial agent against bacterial pathogens conferring the emerging strategy to combat multidrug resistant microorganisms.

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Ecological
Communication

On the Diversity of Jumping Spiders of Maharashtra, India

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ABSTRACT

Family Salticidae (jumping spiders) is the largest family of spiders under order Araneae. Jumping spiders are ubiquitous in terrestrial ecosystems and are familiar to humans as they are also found in human dwellings and home gardens. They are well known for their complex vision-based behaviour, which include elaborate mating behaviours, stalking and capturing of prey species, araneophagy and mimicry. Jumping spiders are generally diurnal in habit and being predators exclusively, they have an important role in the terrestrial food webs. Being well-represented in agro ecosystems, they have a significant role in the biological control of pest species. Despite being a major arthropod group, not much is known regarding the diversity, distribution, taxonomy and behaviour of jumping spiders found in the various regions of India. Maharashtra is one of the largest states in India, however only a few records exist of the salticid fauna of Maharashtra. Hence, there was a need to ascertain the diversity of jumping spiders found in Maharashtra. The methodology which has been used to this purpose includes collection and identification of jumping spiders from different areas of Maharashtra and also the review of previously published reports. The jumping spiders of Maharashtra are represented by 29 species in 18 genera. This appears to be just a small portion of the salticid fauna actually found in Maharashtra and further work is required to thoroughly understand the diversity and biology of this group. This work highlights a neglected group of Arachnids, provides an up-to-date number of salticid species known from Maharashtra, and shall be of help to future researchers.

KEY WORDS: ANT-LIKE SPIDER, ARACHNIDA, BIOCONTROL, BIODIVERSITY, FAUNA, MYRMARACHNE.

INTRODUCTION

Currently 49,159 species of spiders in 4,207 genera of 128 families are known from the world. Taking into account the number of species, family Salticidae (jumping spiders) is the largest family with 6334 species in 659 genera (World Spider Catalog 2021). Indian jumping spider fauna consists of 181 species in 62 genera (Siliwal et al., 2005). It is difficult to estimate the actual number of jumping spider species found in India as this group is very diverse but has not been thoroughly studied in India. Jumping spiders are unique in the animal kingdom as they are known for their intricate vision-based behaviour during encounters with prey and conspecific individuals. This is achieved using eyes specialized for discerning fine detail (Cerveira et al., 2019).

Jumping spiders have a pair of large forward facing anterior-median eyes, which are the principal eyes (Fig. 1). They also have three pairs of smaller eyes called secondary eyes, which include one pair each of anterior-lateral, posterior-median and posterior-lateral eyes. The secondary eyes are highly proficient motion detectors. The salticid eyes provide a near 360° field of view and forward-looking spatial resolution surpassing that of all insects and even some mammals (Menda et al., 2014).

Among arachnids, jumping spiders are agile and dexterous jumpers and have a semi hydraulic system of locomotion (Brandt et al., 2021). Diverse predatory strategies have evolved in jumping spiders, including araneophagy, aggressive mimicry, myrmecophagy, and prey-specific prey catching behaviour (Jackson and Pollard 1996; Brandt et al., 2021).

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Table 1. Jumping Spiders (Salticidae) of Maharashtra

S. No.	Species Name	References
1.	*Asemonea tenuipes* (O.P.- Cambridge, 1869)	Maheshwari et al., 2018
2.	*Carrhotus viduus* (C.L. Koch, 1846)	Deshmukh, 2017; Meshram, 2011; Vairale and Wagh, 2021; Warghat et al., 2011
3.	*Hasarius adansoni* (Audouin, 1826)	Gajbe, 2016, 2020; Maheshwari et al., 2018
4.	*Hyllus semicupreus* (Simon, 1885)	Ahmad and Satam, 2015; Deshmukh, 2017; Maheshwari et al., 2018; Meshram, 2011; Warghat et al., 2011
5.	*Marpissa kalapani* Tikader, 1977	Deshmukh, 2017
6.	*Menemerus bivittatus* (Dufour, 1831)	Gajbe, 2016, 2020; Maheshwari et al., 2018
7.	*Myrmaplata plataleoides* (O.P.-Cambridge, 1869)	Tikader, 1973; Warghat et al., 2011
8.	*Myrmarachne melanocephala* MacLeay, 1839	Bastawade, 2006; Deshmukh, 2017; Meshram, 2011; Warghat et al., 2011
9.	*Myrmarachne poonaensis* Tikader, 1973	Deshmukh, 2017; Tikader, 1973
10.	*Myrmarachne prava* (Karsch, 1880)	Warghat et al., 2011
11.	*Myrmarachne robusta* (Peckham and Peckham, 1892)	Tikader, 1973; Vairale and Wagh, 2021
12.	*Phidippus bengalensis* Tikader, 1977	Deshmukh, 2017; Meshram, 2011
13.	*Phidippus bhimrakshiti* Gajbe, 2004	Deshmukh, 2017; Meshram, 2011
14.	*Phintella vittata* (C.L. Koch, 1846)	Deshmukh and Tekade, 2019;
15.	*Phlegra dhakuriensis* (Tikader, 1974)	Maheshwari et al., 2018; Warghat et al., 2011
16.	*Plexippus andamanensis* (Tikader, 1977) Deshmukh, 2017; Meshram, 2011; Vairale and Wagh, 2021	Deshmukh, 2017; Meshram, 2011; Warghat et al., 2011
17.	*Plexippus insulanus* Thorell, 1881	Deshmukh and Tekade, 2019
18.	*Plexippus paykulli* (Audouin, 1826)	Bastawade and Khandal, 2006; Bhandarkar and Paliwal, 2019; Deshmukh, 2017; Deshmukh and Tekade, 2019; Gajbe, 2016, 2020; Maheshwari et al., 2018; Meshram, 2011; Warghat et al., 2011; Vairale and Wagh, 2021
19.	*Plexippus petersi* (Karsch, 1878)	Deshmukh and Tekade, 2019; Gajbe, 2016, 2020; Maheshwari et al., 2018
20.	*Proszynskia anusuae* (Tikader and Biswas, 1981)	Deshmukh, 2017; Meshram, 2011
21.	*Rhene decorata* Tikader, 1977	Warghat et al., 2011
22.	*Rhene flavigera* (C.L. Koch, 1846)	Maheshwari et al., 2018
23.	*Rhene indica* Tikader, 1973	Vairale and Wagh, 2021
24.	*Stenaelurillus jagannathae* Das, Malik and Vidhel, 2015	Maheshwari et al., 2018
25.	*Stenaelurillus lesserti* Reimoser, 1934	Maheshwari et al., 2018
26.	*Telamonia dimidiata* (Simon, 1899)	Ahmed et al., 2019; Bhandarkar and Paliwal, 2019; Deshmukh and Tekade, 2019; Gajbe, 2016, 2020; Maheshwari et al., 2018; Meshram, 2011; Vairale and Wagh, 2021
27.	*Telamonia elegans* (Thorell, 1887)	Bastawade and Khandal, 2006
28.	*Thiania bhamoensis* Thorell, 1887	Deshmukh, 2017; Meshram, 2011; Warghat et al., 2011
29.	*Thyene imperialis* (Rossi, 1846)	Maheshwari et al., 2018

Jumping spiders perform a set of responses in catching prey. These responses consist of three primary patterns, each of which is subdivided into discrete motor elements, namely, Orientation—alert, swivel, and alignment; Pursuit—follow, run, and stalk; and Capture—pre-crouch, crouch, and jump. *Portia* is a genus of specialized web-invading jumping spiders that use aggressive mimicry (Jackson 2009; Forster 2010).*Portia fimbriata* routinely includes web-building spiders and cursorial salticids in its diet, both of these types of prey being dangerous and unusual prey for a salticid. Jumping spiders are basically diurnal predators. However,certain species show vision-based discrimination under low ambient light levels previously associated with nocturnal species (Li and Jackson 1996; Cerveira et al., 2019). Species in the genus *Habronattus* have high warming tolerances, suggesting that these species should be robust to future increases in habitat temperature (Brandt et al. 2020).

Jumping spiders being active hunters do not build webs. However, they do use their silk in different ways. For salticids, dragline silk is critical for dynamic stability and prey-capture efficiency (Chen et al., 2013). Jumping spiders also use their silk for constructing various types of retreats or shelters. For example, *Myrmarachne* spiders construct elaborate tubular shelters with multiple entrances (Hurni-Cranston and Hill2018). Recently, an unidentified jumping spider (*Anarrhotus* sp.) from south-western India has been recorded as constructing planar orb-webs that serve as nocturnal retreats.

These webs are not inhabited during the daytime and do not appear to play a role in prey capture by these spiders (Hill et al., 2019). Jumping spiders display complex patterns of mating behaviour. For example, *Plexippus paykulli* males use different mating tactics depending on the female's maturity and location (courtship versatility): visual displays if the female is mature and away from her nest (Fig. 2), vibratory displays if she is mature and, in her nest, and cohabitation if she is a sub-adult in her nest (Jackson and Macnab 1989; Hill et al., 2019).

Male jumping spiders of the genus *Habronattus* court females using a combination of ornament and motion (dance) displays coordinated with vibrational songs. Jumping spiders constitute one of the most important groups of predaceous organisms in terrestrial ecosystems. They are ubiquitous in agroecosystems and are well-known as biological control agents. Most biocontrol studies reflect the emphasis on specialist (monophagous) predators and parasitoids. Spiders being prey generalists (polyphagous), have not received much attention in biocontrol studies. Recent research has highlighted widespread non-consumptive effects and complex intraguild interactions of spiders. A better understanding of these effects is needed to optimize biocontrol services by spiders in agro ecosystems (Riechert and Lockley 1984; Michalko et al., 2018, Rivera et al., 2021).

Compared to other predator groups, very few studies have been conducted to ascertain the impact of jumping spiders as biocontrol agents of agricultural pests. In one of such studies, 25 species of jumping spiders were found in two crops, namely yerba mate and tea with their densities being 2.21 and 2.47 individuals per square metre for the two crops, respectively (Rubio et al., 2019). Despite being rich in faunal diversity, there exist only a few records of the salticid fauna of Maharashtra. In order to augment the knowledge of the diversity of jumping spiders found in Maharashtra, an annotated checklist of the jumping spiders of Maharashtra has been provided here. This is the first work of its kind on the jumping spiders of Maharashtra. It provides an up-to-date number of jumping spider species recorded from Maharashtra and adds to the biodiversity of the State. It shall be of assistance to researchers interested in exploring the salticid fauna of Maharashtra.

MATERIAL AND METHODS

Covering an area of 307,713 sq km, Maharashtra is the third largest state in India. It spans from the Central to the Western region of India. Arabian Sea lies to the west of Maharashtra. The Western Ghats are located on the western fringe of the State (Wikipedia 2021). An annotated checklist of jumping spiders of Maharashtra has been prepared, which is based on the species collected and identified by the author from Nagpur, Mumbai, Ratnagiri, and Satara districts of Maharashtra during 2014 to 2019, as well as published reports on the diversity and distribution of jumping spiders of Maharashtra by other researchers.

The collected spiders were preserved in 70% ethyl alcohol and identified with the help of scientific literature (Sebastian and Peter 2009; Gajbe 2020). The species recorded in previous studies have been ascertained by collecting research papers, books and chapters in edited books available in print and/or online, pertaining to the salticid fauna of Maharashtra. References for each species recorded have been provided against their names in Table 1.The scientific names of jumping spiders are based on the World Spider Catalog (2021).

RESULTS AND DISCUSSION

The salticid fauna of Maharashtra is represented by 29 species in 18 genera (Table 1, Figs. 3-12). Species such as *Hasarius adansoni, Plexippus paykulli* and *Plexippus petersi* are commonly observed in human dwellings actively searching for prey on the walls of buildings. *Plexippus paykulli* is native to South-East Asia but is now found in different parts of the world. Other species such as *Asemonea tenuipes, Carrhotus viduus, Hyllus semicupreus, Phintella vittata* and *Thyene imperialis* are generally seen among foliage, where they can hide and stalk insect prey, and also build silken retreats. Menemerus bivittatus is a pantropical species that is often seen on the bark of trees or on the walls of buildings (Gajbe 2020).

Two-striped spider, *Telamonia dimidiata* is common in Maharashtra and found in foliage. A few species of ant-like spiders of the genus *Myrmarachne* have been

reported in one of the earliest works on the salticid fauna of Maharashtra (Tikader 1973). These ant-like jumping spiders are remarkable since they not only mimic the form of the arboreal ant, Oecophylla smaragdina, but also mimic its behaviour by making ant-like movements, waving their front legs as if they are antennae (Gajbe 2020).

Figure 1-2: (1) Front eyes of Plexippus paykulli (2) Plexippus paykulli male and female displaying mating behaviour Figs. 3-12: (3) *Carrhotus viduus* (4) *Hasarius adansoni* (5) *Hyllus semicupreus* (6) *Menemerus bivittatus* (7) *Myrmaplata plataleoides* (8) *Phintella vittata* (9) *Plexippus petersi* (10) *Telamonia dimidiata* (11) *Thiania bhamoensis* (12) Thyene imperialis preying on a Hymenopteran insect

Recently, the ant-like species plataleoides has been transferred from the genus *Myrmarachne* to *Myrmaplata* (Prószynski 2016). A few salticid species have been reported from some protected conservation areas of *Maharashtra* such as Tadoba Andhari Tiger Reserve, Sanjay Gandhi National Park, Toranmal Sanctuary, Umred Karhandla Sanctuary, Navegaon National Park, and Pench National Park (Bastawade 2006; Bastawade and Khandal 2006; Meshram 2011; Gajbe 2016; Bhandarkar and Paliwal 2018; Gajbe 2020).

Five species of jumping spiders have been recorded from Charghad River Basin in Amravati district (Deshmukh and Tekade 2019). Six species of jumping spiders have been recorded from tropical reserve forest of Amravati district (Vairale and Wagh 2021). Due to a paucity of research, the jumping spider records from Maharashtra are sketchy and probably represent only a small portion of the actual salticid fauna of Maharashtra. Consequently, more field surveys are required in different parts of the State to have a true picture of the salticid fauna of Maharashtra. This work is significant as it provides an up-to-date number of the jumping spiders species found in Maharashtra and adds to the arachnid biodiversity of Maharashtra. This will help researchers in proper identification and study of the jumping spiders of Maharashtra.

CONCLUSION

The jumping spider fauna of Maharashtra is represented by 29 species in 18 genera. Considering the large size of Maharashtra and the different types of terrestrial habitats found in the State, the actual number of jumping spider species found in the State could be much more. Considering their ubiquity and unique adaptations among arthropods, jumping spiders require more attention from researchers with respect to their diversity, distribution, biology, behaviour and biocontrol properties.

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Biomedical Communication

A Correlation Between Navicular Drop and Quadriceps Angle Amongst Normal and Overweight Middle-Aged Individuals

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ABSTRACT

Foot is a segment which is in contact with the ground and bears body weight, its posture can be affected by changes that may occur within the body itself or from the effect of external environment. Postural imbalance can be caused by changes in the feet, arising mainly from the different alignment of the body posture. A higher body mass index with weak musculature may lead to collapse of the foot arches due to extra forces on the arches and consequently weakness of the respective muscles. This notably changes the contiguity area of the arches of the feet with the ground surface. Also, another important factor is that the Quadriceps angle, which is the angle of pull of the quadriceps muscle, may get altered with faulty alignment of the lower limb arising from the biomechanical changes in flat feet. The aim of the present study is to disinter correlation between Navicular drop and Q- angle amongst normal & overweight middle-aged individuals. A total of 150 subjects were recruited with 75 subjects in each two groups formed on the basis of BMI indexes as group A (Normal with BMI score 17.5 – 22.99) and Group B (Overweight with BMI score 23-27.99). Quadriceps angle and Navicular Drop Test were measured using goniometer method and Brody's method respectively and correlation between Q angle and Navicular drop was evaluated thereafter in both the groups. The result of the study demonstrated that there is statistically significant positive correlation between navicular drop test and Quadriceps angle in both the groups with slightly stronger association in overweight group (r=0.82(rt) and 0.77 (lf) at p=0.05). The lower limbs joints work in a kinematic chain where malalignment at one joint affect other, has been a well-established fact. With our study we have tried to fill in the gap of knowledge regarding the effect of increasing body weight on the relationship between Q angle and Navicular drop. The future modifications in guidelines for management of important knee and ankle conditions like knee OA, ankle sprain etc should definitely use this relationship as an important assessment and management tool, especially for overweight individuals, to achieve maximal improvements in patients.

KEY WORDS: NAVICULAR DROP, OVERWEIGHT, QUADRICEPS ANGLE.

INTRODUCTION

Posture is defined as "the position in which someone holds their body upright against gravity while standing, sitting or lying down". Posture and its anomalies can be linked to different types of activities associated with mankind like walking and other activities of daily living, which can be affected by any abnormal changes in the body posture (Borges et al. 2013). As foot is a segment which is in contact with the ground and bears body weight, so foot posture can be affected by changes that may occur from the body itself or from the effect of external environment. Postural imbalance can be caused by changes in the feet, arising mainly from the different alignment of the body posture (Eldesoky and Abutaleb 2015 Raizada etal 2019).

Sedentary lifestyles, food habits with high content of fat and calories intake lead to increase in Body Mass Index (BMI). Stress level of the individual also lead to increase in body weight. With ageing, there is reduction in muscle mass and metabolism that may lead to gain in the body weight. A higher body mass index with weak musculature may lead to collapse of the foot arches due to extra forces on the arches and consequently weakness of the respective muscles. This notably changes the contiguity

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area of the arches of the feet with the ground surface (Chougala et al. 2015). Modifications in the arches of the feet can lead to different biomechanical alteration in the feet posture. It is characterized by valgus alignment of the calcaneum leading to tibial internal rotation with collapse of the medial longitudinal arch and forefoot moving into abduction.

Also, another important factor is that the Quadriceps angle, which is the quadriceps muscle's angle of pull, may get altered with faulty alignment of the lower limb arising from the biomechanical changes in flat feet (Letafatkar et al. 2013; Chougala et al. 2015). Exaggerated foot pronation led to internal rotation of the lower extremity and may result in knee valgus with increase in Q-angle (Heggannavar et al. 2016). As the feet are important weight bearing elements of the body, increase body weight can lead to changes in the arches of the feet structure and may affect another important biomechanical factor like Q-Angle (Omololu et al. 2009; Freedman et al. 2014; Heggannavar et al. 2016; Park and Park 2018 Raizada etal 2019). There is paucity of the study on Indian population about biomechanical alteration in the kinematic chain segments of the lower limb associated with navicular drop and Q-Angle with increase in body mass. The purpose of the study is to assess the relationship between Navicular drop and Q angle amongst different BMI indexes in middle aged individuals and also to analyse the effect of increasing body weight on their relationship.

MATERIAL AND METHODS

A Correlational study was conducted on 60 subjects which were divided in 2 groups with 30 subjects in each group formed on the basis of Asian classification of BMI as measurement index (BMI = Weight in Kg/Height in m2), Group A as Normal group (BMI score 17.5 to 22.99) and Group B as Overweight group (BMI score 23 to 27.99). Both males and females between 25-45 years were included in the study. Outcome measures used were Quadriceps angle and Navicular drop test. Potential subjects were apprised of the procedure and its benefits. Prior to testing, the subjects were familiarized with the testing procedure. Those fulfilling the criteria were explained in detail about the study and a written informed consent, in their preferred language, was obtained from the subjects willing to participate. Navicular drop test and Q-angle measurement were done through Brody's method and Goniometer method in standing respectively for all the subjects in two groups. Descriptive data were also taken for all the subjects (Park and Park 2018).

For navicular drop measurement the subtalar neutral position was maintained and the most prominent point of the navicular tubercle was identified and marked with a pen with the subject in a sitting position having their feet in contact with the ground. Subtalar neutral position was checked by palpating the talus bone and ensuring equal medial and lateral talar depressions. The index card was positioned, on the inner aspect of the hind foot, perpendicular to the floor crossing the navicular bone. The height of the most prominent point of the navicular tubercle was indicated on the card. The same procedure was repeated with the subjects in standing position maintaining the neutral position of the subtalar joint. At last, the difference between the original height of the navicular tubercle's most prominent point in sitting and standing positions was measured for both the foot with the help of measuring tape in millimetres as shown in figure 1.

Figure 1: Navicular Height

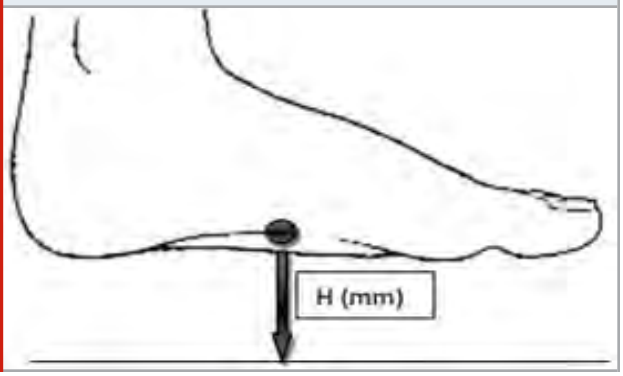
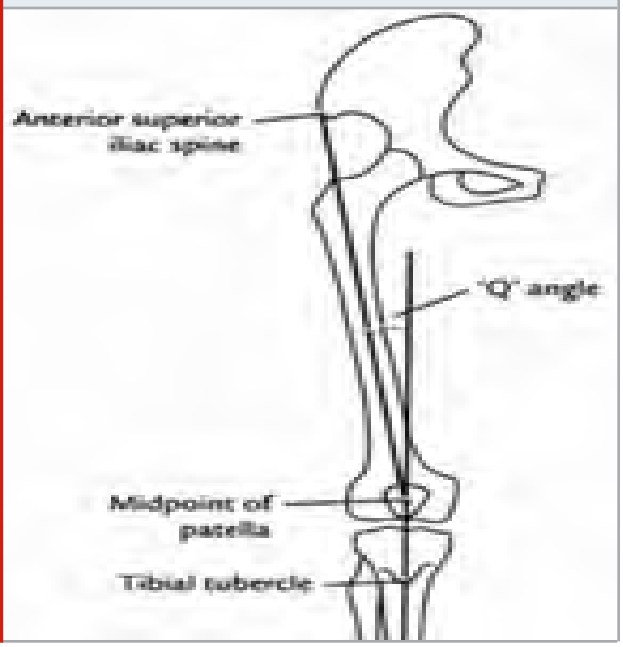


Figure 2: Surface landmarks for measuring Q-Angle



Navicular drop when measured less than 10 mm are categorized as normal and more that 10 mm are categorized as abnormal (Deng et al. 2010; Park and Park 2018).

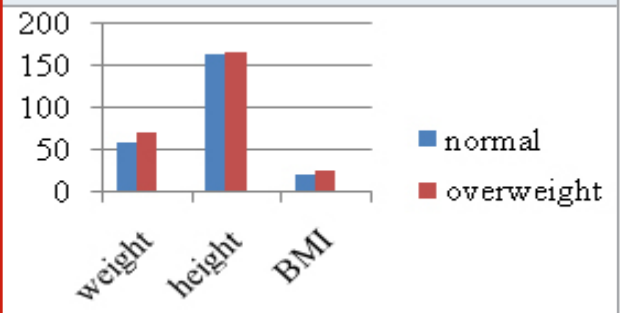
Q-angle was measured using a standard full circle goniometer. The right and left side angles were measured with subject in standing position. The ASISs, tibial tuberosity, and assessed midpoint of the patella as shown in figure 2 were palpated and marked prior to measurement with the help of removable adhesive stickers. The fulcrum of the goniometer was placed on

the centre of the patella. The bottom arm was directed towards tibial tuberosity. The upper arm was directed along the anterior superior iliac spine. The Q-angle in degree was thus measured on both sides (Raizada et al. 2019).

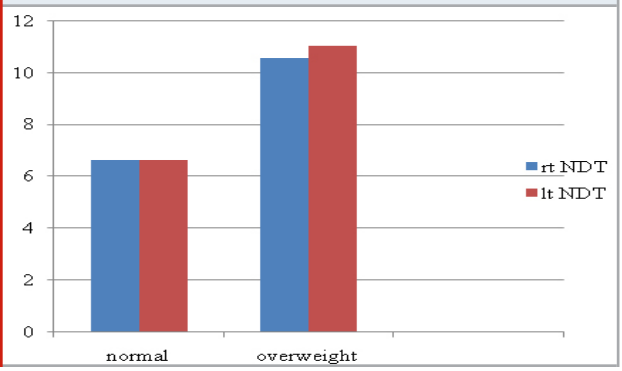
Table 1. Mean and Standard Deviation of variables in Normal & Overweight groups

	Age (Mean±SD)		
Normal Group	36.67±8.40		
Overweight Group	34.9±7.18		
	Weight (Mean±SD)	Height (Mean±SD)	BMI (Mean±SD)
Normal Group	60.5±7.87	164.23±9.81	22.39±1.93
Overweight Group	72.13±5.98	165.48±6.07	26.44±1.16
	rt NDT (Mean±SD)	lt NDT (Mean±SD)	
Normal Group	6.6±1.35	6.56±0.97	
Overweight Group	10.53±1.30	11±1.55	
	rt Q angle (Mean±SD)	lt Q angle (Mean±SD)	
Normal Group	14±1.91	13.93±1.63	
Overweight Group	16.8±1.84	16.86±1.97	

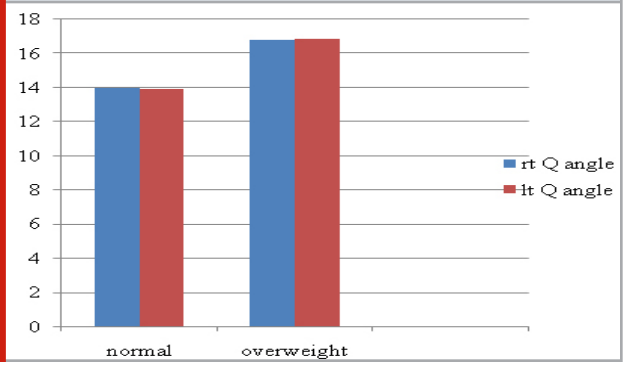
Graph 1: Mean comparison of weight, height & BMI in Normal & Overweight Groups



Graph 2: Mean Comparison of Right & Left NDT in Normal & Overweight Groups



Graph 3: Mean comparison of Right & Left Q-Angle in Normal & Overweight Groups



RESULTS AND DISCUSSION

The statistical analysis was done using SPSS (version 21). Mean and standard deviation were obtained for all dependent variables. Data was analysed by taking out mean, standard deviation of the Navicular Drop test and Q angle. Correlation of navicular drop and Q Angle of right and left was evaluated and then represented by scatter graphs for each group. Table 1 showing descriptive data of Normal group and Overweight groups respectively. Mean value of NDT and Q-Angles in both the foot was found to be greater in overweight group in comparison to normal group. Graph 1 showing the mean values of age, weight, height and BMI in both the groups. Graph 2 showing the mean values of Right & Left NDT in both the groups and Graph 3 showing the mean values comparison of Right & Left Q-Angles among normal and overweight individuals. Mean values of NDT were 6.6±1.35 (right) & 6.56±0.97 (left) in normal group and 10.53±1.30 (right) & 11±1.55 (left) in overweight group respectively. Mean values of Q-Angles right and left were 14±1.91& 13.93±1.63and 16.8±1.84& 16.86±1.97 in normal and overweight groups respectively.

Correlation Analysis
Group A (Normal)

Table 2 and 3 showed positive significant correlation between NDT and Q-Angle in both the foot with r value of 0.77 at $p<0.05$ (NDT Right & Q-Angle Right) and 0.67 at $p<0.05$ (NDT Left & Q-Angle Left) in Normal Group.

The correlation graphs 4 & 5 of the result showed a positive significant correlation between flat foot and Q-Angle in middle aged individuals with body weight categorised as normal, at p<0.05.

Group B (Overweight)

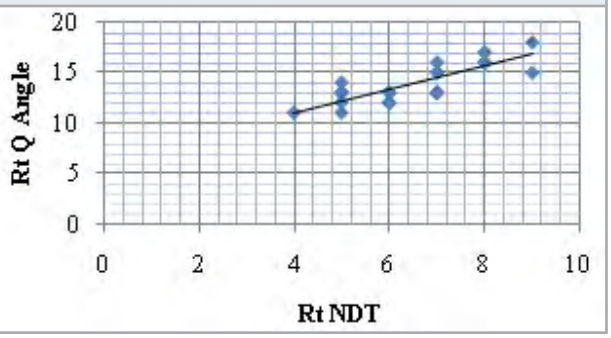
Table 2. Showing positive correlation between Right NDT and Right Q-Angle significant at p<0.05

		Rt Q angle
Rt NDT	Karl Pearson coefficient of Correlation (r)	0.77
	t value	<0.00001
	p value	<0.05

Table 3. Showing positive correlation between Left NDT and Left Q-Angle significant at p<0.05

		left Q angle
Left NDT	Karl Pearson coefficient of Correlation (r)	0.67
	t value	<0.00001
	p value	<0.05

Graph 4: Correlation between Right NDT & Right Q-Angle in Normal subjects



Graph 5: Correlation between Left NDT & Left Q-Angle in Normal subjects

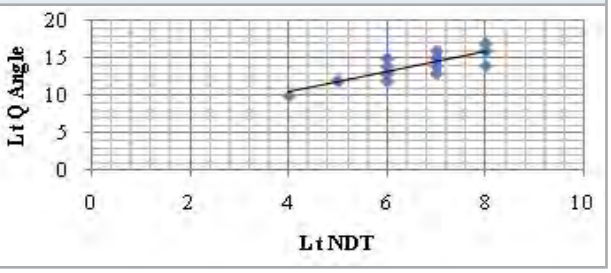


Table 4 and 5 showed positive significant correlation between NDT and Q-Angle in both the foot with r value of 0.82 at p<0.05 (NDT Right & Q-Angle Right) and 0.77 at p<0.05 (NDT Left & Q-Angle Left) in Overweight Group. There was slight stronger correlation in overweight individuals in comparison to normal group subjects.

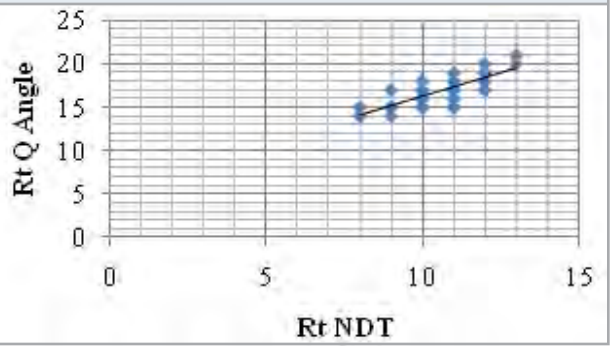
Table 4. Showing positive correlation between Right NDT and Right Q-Angle significant at p<0.05

		Rt Q angle
Rt NDT	Karl Pearson coefficient of Correlation (r)	0.82
	t value	<0.00001
	p value	<0.05

Table 5. Showing positive correlation between Right NDT and Right Q-Angle significant at p<0.05

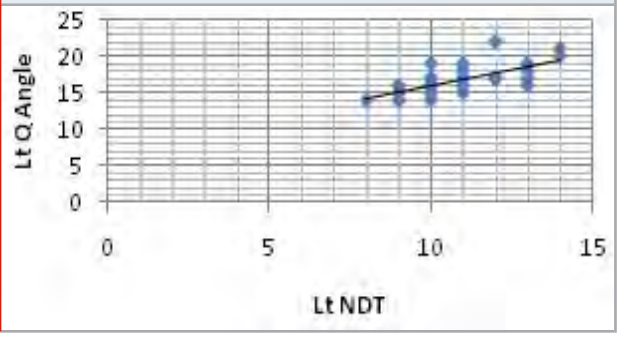
		left Q angle
Left NDT	Karl Pearson coefficient of Correlation (r)	0.67
	t value	<0.000046
	p value	<0.05

Graph 6: Correlation between Right NDT & Right Q-Angle in Overweight subjects



The correlation graphs 6 & 7 of the result showed a positive significant correlation between flat foot and Q-Angle in middle aged individuals with body weight categorised as overweight, at p<0.05. In addition, graphs 6 & 7 showed more stronger positive significant correlation between NDT and Q-Angle amongst overweight middle aged individuals, at p<0.05. So, the result exhibited that with increase in body weight, there was more navicular drop among overweight individuals leading to increase in Q-Angle also. Our inference about a greater Q-angle could be prognosticated by increased navicular drop was supported by preceding studies that delineated about association of an exaggerated pronation with internal rotation of the lower extremity and raised knee valgus and is therefore indicated to result in greater Q angle (Nguyen et al. 2009; Almeida et al. 2016).

Graph 7: Correlation between Left NDT&t Left Q-Angle in Overweight subjects



Our findings are in line with the result of these studies. There was statistically significant positive correlation in both the groups between Quadriceps angle and NDT with slightly stronger association found in overweight group. One possible explanation for slightly stronger relationship among variables in overweight group is that increase bodyweight give rise to elevated loading of the foot mechanically, thus leading to collapse of medial longitudinal arch of the foot, which is followed by out-toeing and increased foot pronation (Chougala et al. 2015) In addition, when foot is functioning biomechanically with persistent pronation, it result in excessive internal rotation of the entire lower limb (Almeida et al. 2016).

This stress due to excessive internal rotation of the leg may upshot to viable mechanical problems around the knee, including increase pull of quadriceps muscle laterally (Cote et al. 2005). Hamstra-Wright et al. (2015) supported the findings of the present study as they specified that a raised BMI and alteration in the posture of the foot must be taken into account in order to manage medial tibial stress syndrome. A study disclosed that there is significant positive correlation between BMI & Q-angle which arrives in accord with our study results. Therefore, there was a slightly stronger correlation between Q angle and NDT in overweight group and the level of arch drop and Q-angle was also greater in fatter individuals (Hamstra-Wright et al. 2015; Prakash et al. 2017).

These results are complimented by a study performed by Kim et al. (2010) showed that there exist significant correlations among navicular drop and quadriceps angle ($p<.05$), and internal rotation of hip ($p<.05$) and concluded that navicular drop has a viable effect on biomechanical arrangement of the lower limb. So, there is need to take into account the biomechanical alignment of the entire lower limb rather than a single factor, as there is potential for one mechanical factor to compensate for or affected by another (Kim et al. 2010; Prakash et al. 2017).

CONCLUSION

The lower limb joints work in a kinematic chain where mal-alignment at one joint affect other and has been a well-established fact. With our study we have tried to fill in the gap of knowledge regarding the effect of increasing body weight on the relationship between Q angle and Navicular drop. The future modifications in guidelines for management of important knee and ankle conditions like knee OA, ankle sprain etc should definitely use this relationship as an important assessment and management tool, especially for overweight individuals, to achieve maximal improvements in patients.

Conflict of Interests: There was no conflict in the interests of participating authors.

Ethical Statement: It is to inform you that on the recommendations of Departmental Research Committee Board of Study made on 19.08.2015, the Academic Council of this University in its meeting held on 24.12.2015 vide agenda item no. 17.3 has approved to register Mr. Manish Kumar S/o Mr. Pashupati Nath for Ph.D programme of this University w.e.f. 24.12.2015 in the broad area/ topic of research Relationship Between Pelvic Tilt, Body Mass Index And Changes In The Plantar Arch In Middle Aged Individual in the Faculty of Applied Sciences under the supe sion of Dr. Divya Sanghi, Asst. Prof. - FAS, MRIU. The final topic of research will have to t finalized at the time of pre-submission seminar of thesis. His registration no. is **15/Ph.D/002**.

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Biomedical Communication

Certain Hepatoprotective Effects of the Ajwa Date Phoenix dactylifera Seeds on Streptozotocin-Induced Diabetic Rats

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ABSTRACT

Diabetes mellitus is a lifelong metabolic condition resulting from chronic hyperglycemia. Non-alcoholic fatty liver and cirrhosis are among the most important complications of diabetes, which are associated with increased mortality.This study aimed to re-emphasize the protective effects of Ajwa seeds (Phoenix dactylifera seeds) against hepatic damage caused by diabetes, and to investigate the mechanisms underlying it, specifically the antioxidant, anti-inflammatory, and anti-apoptotic ones. Five groups (n = 5) of adults male Wistar rats were created. Group 1 was the control, group 2 was the control treated with Ajwa seeds (1g/kg), group 3 was the diabetes (STZ, 35 mg/kg), group 4 was the diabetes treated with Metformin (150 mg/kg), and group 5 was the diabetes treated with Ajwa seeds (1 g/kg). Metformin and Ajwa seeds suspension were administered orally using oral gavage six days a week, for four weeks. Ajwa seeds suspension significantly lowered STZ-induced hyperglycemia and increasing insulin secretion. It reduced the elevated liver enzymes (ALT, AST, ALP, and LDH), and improved liver tissue pathological features.Ajwa seeds increasedliver concentrations of enzymatic (SOD and CAT) and non-enzymatic (GSH) antioxidants and reduced levels of oxidative stress products (MDA and AGE). Ajwa seeds lowered the levels of inflammatory mediators (TNF-α and NF-κB) and NF-κBprotein expression. Moreover Ajwa seeds lowered the hepatic protein expression of pro-apoptotic marker, caspase 3. The antioxidant, anti-inflammatory, and antiapoptotic properties of Ajwa seeds can explain their hepatoprotective effects in this diabetes model.

KEY WORDS: AJWA SEEDS; DIABETES; ANTIOXIDANTS; ANTI-INFLAMMATORY; ANTIAPOPTOTI.

INTRODUCTION

Diabetes mellitus is a lifelong metabolic condition resulting from chronic hyperglycemia (Heindel et al., 2017). Diabetes mellitus affects roughly 425 million individuals globally, and it is expected to affect approximately 642 million people worldwide by 2040, (Meo et al., 2017, 2019). Many liver disorders, including non-alcoholic fatty liver and hepatobiliary ailments, are related to diabetes(Afrin et al., 2015). Similarly, unregulated glycogen accumulation in the liver can exacerbate insulin resistance, which, when combined with hyperglycemia, can damage the liver and contribute to higher morbidity and mortality among diabetes patients. Diabetes mellitus is most often linked with non-alcoholic steatohepatitis, alcoholic cirrhosis, chronic hepatitis C, and hemochromatosis (Dewidar et al., 2020; Garcia-Compean et al., 2009). The mortality rate from diabetes-induced cirrhosis is significantly higher than that of cardiovascular diseases (Harrison, 2006, Mohamed et al., 2016; Thobaiti and Abu Zeid, 2019 Naseri et al., 2020).

The common anti-diabetic medications are ineffective in preventing the progression of liver-related illness. Consequently, there is a real need for complementary and adjunctive medications to manage diabetes mellitus-related severe complications (Chaudhury et al., 2017, Al-Thobaiti and Abu Zeid, 2019) Herbal remedies and natural treatments are safer, more effective, more affordable, and less expensive alternatives to oral diabetes medications (Kooti et al., 2016 Al-Attar and Alsalmi, 2019). The date is one of many traditional supplements that is utilized in several developing nations' healthcare systems. Folk medicine providers widely use dates in country areas of many countries. The date palm tree is a member of the Arecaceae family (Angiosperms, monocotyledon), which includes over 2,500 species and 200 genera. *Phoenix*, which includes *Phoenix dactylifera* L., is one of the genera with nearly 14 species (Eoin, 2016).

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Phoenix dactylifera L. (var. Ajwa) is one of Southwest Asia's and North Africa's oldest and most important staple and historic plants (Al-Harrasi et al., 2014). Ajwa date is one of the most famous date varieties which recognized by Muslim communities for its therapeutic and religious importance (Nematallah et al., 2018). Ajwa fruits are rich in antioxidants, antibacterial, antifungal, and anti-proliferative effects, and have a greater nutritional and medicinal importance (Al-Alawi et al., 2017; Temitope Idowu et al., 2020). Ajwaseeds are traditionally dumped products of date fruit. It accounts for about 10% of the fruit's total weight. Dietary fiber, protein, carbohydrates, phenols, and minerals make up the bulk of the date seeds. Antioxidant and antimicrobial actions are parts of the numerous biological effects of these compounds (Mrabet et al., 2020).

A previous study has documented the antidiabetic potential of Ajwa date seeds extract in STZ-induced hyperglycemic model in rats; besides, it normalized the elevated liver transaminases in diabetic rats (Hasan and Mohieldein, 2016).Date seeds in an aqueous suspension may help to mitigate the early complications of diabetes, especially hepatic and renal problems (Abdelaziz et al., 2015). The aim of this study was to re-emphasize the protective properties of Ajwa seeds (*Phoenix dactylifera* seeds) against hepatic damage caused by diabetes, and to investigate the mechanisms underlying it, specifically the antioxidant, anti-inflammatory, and anti-apoptotic mechanism.

MATERIAL AND METHODS

Chemicals: Streptozotocin (STZ) S0130-1G was purchased from Sigma- Aldrich, (St. Louis, MO, USA); Metformin (Glucophage, 500 mg tablet, Merck Santé, France) was obtained from Alnahdi pharmacy, Jeddah, Saudi Arabia. Ajwa seeds used in this study were collected from Ajwa dates purchased from the Oasis Lina, Al-Madinah Al-Munawara, Saudi Arabia.

Preparation of Ajwa seeds suspension: The seeds were removed from the fruits, then washed to remove residues. Seeds were dried for 2-3 weeks at room temperature in a well-ventilated room. The dried Ajwa seeds were hammer-milled into a fine powder. A fresh aqueous suspension of Ajwa seeds was made by mixing 1 gram of powdered seeds with 10 mL of Tween-80 vehicle.

Animals: This study utilized 25adults male Wistar rats, their mean body weight ranged from150 ± 250 g. Rats were bought from King Fahad Research Centre, King Abdulaziz University, Jeddah, Saudi Arabia. After the one-week adaptation period, the experiment was performed under the standard laboratory conditions of temperature, moisture, and 12:12 h light/dark period. No limitations were applied to the rats on water and food. The Biomedical Ethics Research Council, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia, approved the study's procedure (346-19).

Induction of diabetes: The rats were injected once with intraperitoneal dose of STZ (35 mg/kg)(Meng et al., 2017). After 7 days fasting blood sugar concentrations were determined using ACCU-CHEK. Diabetic model rats were described as those with blood glucose levels greater than 200 mg/dL (Zhang et al., 2008).

Study Design: Rats were equally classifiedin5 groups (n = 5). The 1st group was the control group, the 2nd group was the control group treated with Ajwa seeds (1g/kg) (Khan et al., 2017), the 3rd group was the diabetes group, the 4th group was the diabetes group treated with Metformin (150 mg/kg) (El-Sayed et al., 2020), and the 5th group was the diabetes group treated with Ajwa seeds (1 g/kg). Metformin and Ajwa seeds were administered orally using oral gavage six days a week, for four weeks.

Sample collection: At the end of the experiment, rats were fasted before blood collection and dissection. All animals were euthanized using diethyl ether, then blood was collected from theorbital plexus sinus. The blood was centrifuged for 15min at 3500 rpm at -4°C to separate the serum which was kept in the freezer at -80°C. Using rodent guillotine rats ware decapitated and dissected. Liver's ware taken out and washed with normal saline then cut. For each organ, someof pieces were preserved in 10% formalin and some kept frozen at -80°C.

Measurement of serum indicators of hyperglycemia: Fasting serum glucose was measured by using the colorimetric kit of Reactivos GPL, Barcelona, Spain. Fasting serum glucose was measured by using the rat ELISA assay kit, Immunospec, CA.

Measurement of serum indicators of liver dysfunction: Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), Alkaline phosphatase (ALP), lactate dehydrogenase (LDH), and total protein (TP) were measured using the kits of Human, Germany for all the enzymes and Crescent Diagnostics kit, Saudi Arabia for the TP.

Measurement of serum indicators of liver oxidative stress: Hepatic concentration of reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), and malondialdehyde (MDA), were measured using the kits of Biodiagnostic, Egypt. and advanced glycation product (AGE)were measured using the kits of My BioSource, USA.

Measurement of serum indicators of liver inflammation: Hepatic concentration of tumor necrosis factor alpha (TNF-α) and nuclear factor kappa beta (NF-κB) were measured using the rat ELIZA kits of Abcam, USA.

Histopathological investigation of the liver tissue (hematoxylin and eosin (H & E) staining): Formalin-fixed liver specimens were packed with paraffin and cut into 4 µm segments. The segments were dyed with H & E and analyzed and photted utilizing light microscopy.

Immunohistochemistry investigation of apoptotic and inflammatory markers: An immunoperoxidase (peroxidase/antiperoxidase, PAP) protocol was utilized to stain theliversegments for NF-κB and caspase-3proteins. The antibodies (bought from LabVision, Fremont, CA) were diluted in 1:200 dilution. The segments were analyzed and photted, utilizing light microscopy.

Statistical analysis: The mean and SE were used to express the data. The data were analyzed statistically using ANOVA and Tukey's post-hoc test. A p 0.05 was chosen as the significant level. SPSS for Windows, version 22, Armonk, NY, was used to conduct the statistical analysis.

RESULTS AND DISCUSSION

Impact of Ajwa seeds on serum indicators of hyperglycemia: There was no significant difference in glucose levels between the Ajwa seeds rats and the control rats.The diabetes rats showed a significantly increased serum glucose contrasted to the control rats. In comparison to the diabetes rats, the diabetic rats who were given Metformin and Ajwa seeds had significantly lower serum glucose levels (Table 1). The insulin level between Ajwa seeds rats and the control rats showed no significant difference. The diabetes rats showed a significantly decreased serum insulin contrasted to the control rats. In comparison to the diabetes rats, treatment with Metformin and Ajwa seeds substantially increased serum insulin levels; however, Ajwa seeds was found to significantly increased serum insulin contrasted to the Metforminrats (Table 1).

Table 1. Impact of Ajwa seeds on serum indicators of hyperglycemia

Test Rats	Glucose (mg/dL)	Insulin (µIU/mL)
Control	134 ± 10	24 ± 0.36
Ajwaseeds	130 ± 5.0	19 ± 1.4
Diabetes	328 ± 1.0 ^{a***}	11 ± 0.26 ^{a***}
Diabetes + Metformin	203 ± 21 ^{b***}	22 ± 0.87 ^{b***}
Diabetes + Ajwaseeds	164 ± 29 ^{b***}	31 ± 2.40 $^{b***, c***}$

Findings were described as mean ± SE (n = 5). ^{a}Significant contrasted to the control rats. ^{b}Significant contrasted to the diabetes rats. ^{c}Significant contrasted to the Metformin rats.*** p<0.001.

Table 2. Impact of Ajwa seeds on liver tissueindicators of liver injury

Test Rats	ALT (U/L)	AST (U/L)	ALP (U/L)	LDH (U/L)	TP (g/dL)
Control	18.9 ± 2.8	19.6 ± 1.5	520 ± 30	250 ± 20	5.2 ± 0.04
Ajwaseeds	12.3 ± 1.9	13.3 ± 2.4	346± 19	249 ± 29	5.6 ± 0.12
Diabetes	81.4 ± 13.2^{a***}	122.3 ± 2.1^{a***}	1169 ± 108^{a***}	1496 ± 71^{a***}	5.4 ± 0.49
Diabetes + Metformin	67.1 ± 6.2	29.8 ± 1.5^{b***}	463 ± 39^{b***}	209 ± 15^{b***}	5.2 ± 0.06
Diabetes + Ajwaseeds	20.1 ± 1.1b$^{***, c***}$	44.9 ± 1.7b$^{***, c***}$	495 ± 95^{b***}	119 ± 9^{b***}	4.99 ± 0.31

Findings are described as mean ± SE (n = 5). ^{a}Significant contrasted to the control rats. ^{b}Significant contrasted to the diabetes rats. ^{c}Significant contrasted to the Metformin rats.***p<0.001.

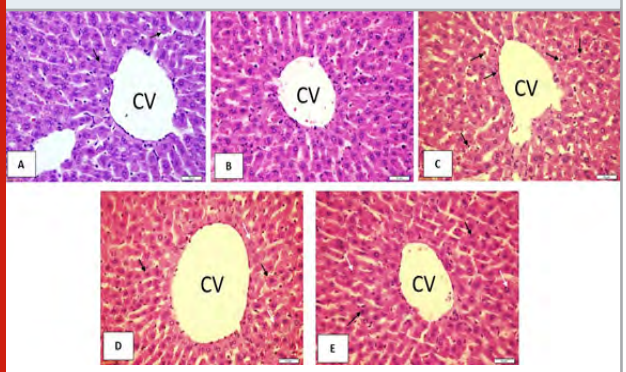
Impact of Ajwa seeds on serum indicators of liver injury: There was no significant difference in ALT, AST, ALP, and LDH levels between the Ajwa seeds rats and the control rats. The diabetes rats showed a significantly increased serum ALT, AST, ALP, and LDH contrasted to the control rats. Treatment of diabetes rats with Metformin and Ajwa seeds significantly decreased serum AST, ALP, and LDH contrasted to the diabetes rats. In comparison to the diabetes rats, Ajwa seed treatment substantially reduced serum ALT levels; however, Metformin therapy had no effect on serum ALT levels in diabetic rats. Ajwa seeds was found to significantly decreased serum insulin contrasted to the Metformin rats. There was no significant difference between serum TP in all the test rats (Table 2).

Impact of Ajwa seeds on liver tissue histopathology (H & E): The control and Ajwa seeds liver sections showed normal central vein (CV) and normal hepatocytes with rounded, active, and centrally located nuclei. The diabetes liver section showed numerous histological changes mainly in CV regions mainly dilated CV, hepatocytes necrosis, and degenerated nuclei. The liver sections of rats treated with Metformin and Ajwa seeds showed apparent preservation of the hepatocyte's morphology; besides, the CV was intact normal. More evident preservation was observed in the CV and hepatocytes of the Ajwa seeds ratscontrasted to the diabetes and Metformin rats (Figure 1).

Impact of Ajwa seeds on liver tissue indicators of oxidative stress: There was no significant difference in SOD, CAT, MDA, and AGE levels between the Ajwa seeds rats and the control rats. Ajwa seeds significantly increased liver GSH content contrasted to the control rats. The diabetes ratshad a significantly higherliver contents ofMDA and AGEand significantly lower liver contents of GSH, SOD, and CAT contrasted to the control rats. In

comparison to the diabetes rats, Metformin treatment significantly reduced MDA and AGE levels in the liver while significantly increasing SOD and CAT levels. In comparison to diabetes rats, Ajwa seeds treatment significantly lowered the liver content of MDA and AGE, whereas the liver content of SOD and CAT was significantly higher. Treatment of diabetes rats with Ajwa seeds significantly decreased liver content of AGE and significantly increased liver GSH, SOD, and CAT contrasted to the Metformin rats (Table 3).

Figure 1: Impact of Ajwa seeds on liver tissue histopathology (H & E)



A: Control; B: Ajwa seeds; C: Diabetes; D: Diabetes + Metformin; E: Diabetes + Ajwa seeds

Impact of Ajwa seeds on liver tissue indicators of inflammation: There was no significant difference in TNF-α and NF-κB levels between the Ajwa seeds rats and the control rats. The diabetes rats showed significantly increased liver contents of TNF-α and NF-κB contrasted to the control rats. In comparison to the diabetes rats, treatment with Metformin and Ajwa seeds substantially reduced the liver content of TNF- and NF-B. In comparison to Metformin rats, diabetes rats treated with Ajwa seeds had significantly lower TNF- levels in their livers (Table 4).

Impact of Ajwa seeds on liver tissue immune expression of NF-κB protein: The control and Ajwa seeds liver sections showed slight NF-κB protein expression. The diabetes liver section showed marked positive NF-κB immuno-stained cells contrasted to the control section. The liver sections of rats treated with Metformin and Ajwa seeds showed apparent decrease in the expression of NF-κB protein contrasted to the diabetes rats (Figure 2).

Table 3. Impact of Ajwa seeds on liver tissue indicators of oxidative stress

Test Rats	GSH (mg/g)	SOD (U/g)	CAT (U/g)	MDA (nmol/g)	AGE (ng/g)
Control	8.7 ± 0.05	1261 ± 58	7.7 ± 0.44	29 ± 1.9	73.9 ± 5.1
Ajwaseeds	10.1 ± 0.49^{a*}	1268 ± 43	7.0± 0.67	29 ± 1.4	78.6± 8.6
Diabetes	6.4 ± 0.20^{a***}	714 ± 8.0^{a***}	0.93 ± 0.16^{a***}	47.9 ± 1.2^{a***}	172 ± 8.2^{a***}
Diabetes + Metformin	7.22 ± 0.27	883 ± 24^{b*}	9.7 ± 0.29^{b***}	33.1 ± 1.6^{b***}	140.8 ± 7.8^{b*}
Diabetes + Ajwaseeds	10.83 ± 0.28^{b***}, ^{c***}	1077 ± 50^{b***}, ^{c*}	7.4 ± 0.46^{b***}, ^{c**}	27.1 ± 0.91^{b***}	86.1 ± 4.3^{b***}, ^{c***}

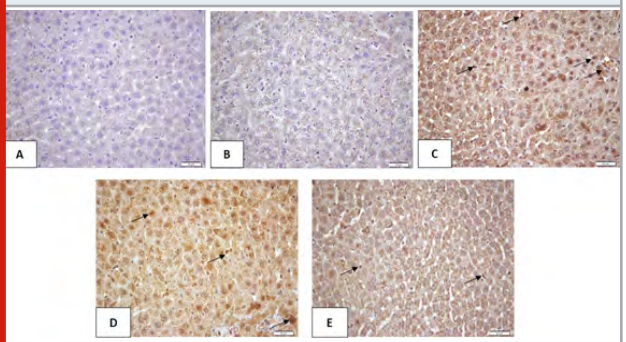
Findings are described as mean ± SE (n = 5).^{a}Significant contrasted to the control rats. ^{b}Significant contrasted to the diabetes rats.^{c}Significant contrasted to the Metformin rats.*p <0.05, **p <0.01, ***p<0.001.

Table 4. Effect of Ajwa seeds on liver tissueindicators of inflammation

Test Rats	TNF-α (ng/g)	NF-κB (µg/g)
Control	61 ± 7.2	1411 ± 109
Ajwaseeds	60 ± 2.1	1415 ± 53
Diabetes	152 ± 7.2^{a***}	6730 ± 72^{a***}
Diabetes + Metformin	125 ± 6.7^{b*}	1643 ± 90^{b***}
Diabetes + Ajwaseeds	77 ± 4.8^{b***},^{c***}	1488 ± 192^{b***}

Findings were described as mean ± SE (n = 5). ^{a}Significant contrasted to the control rats. ^{b}Significant contrasted to the diabetes rats. ^{c}Significant contrasted to the Metformin rats.*** p<0.001.

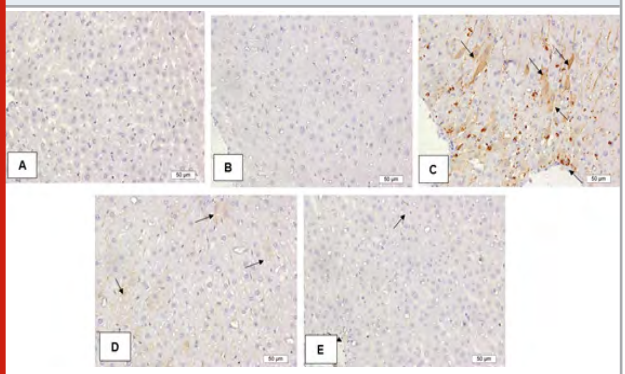
Figure 2. Impact of Ajwa seeds on liver tissue immuno expression of NF-κB protein



A: Control; B: Ajwa seeds; C: Diabetes; D: Diabetes + Metformin; E: Diabetes + Ajwa seeds

Impact of Ajwa seeds on liver tissue immune expression of caspase-3 protein: The control and Ajwa seeds liver sections showed slight caspase-3 protein expression. The diabetes liver section showed marked positive caspase-3 immuno-stained cells contrasted to the control section. The liver sections of rats treated with Metformin and Ajwa seeds showed apparent decrease in the expression of caspase-3protein contrasted to the diabetes rats. Ajwa seeds showed superior antiapoptotic effect contrasted to Metformin as shown in Figure 3.

Figure 3: Impact of Ajwa seeds on liver tissue immuno expression of caspase-3 protein



A: Control; B: Ajwa seeds; C: Diabetes; D: Diabetes + Metformin; E: Diabetes + Ajwa seeds

Diabetes is related to a number of liver disorders, including non-alcoholic fatty liver, acute liver failure, and elevated liver enzymes. Similarly, unregulated glycogen accumulation in the liver can exacerbate insulin resistance, which, when combined with hyperglycemia, can damage the liver and contribute to higher morbidity and mortality among diabetes patients (Mohamed et al., 2016). The results of the current study showed that consuming the Ajwa seed suspension protects rat's liver from diabetes-induced damage. The findings of this study bolstered the potential of Ajwa seeds to reduce the elevated levels of liver enzymes associated with diabetes, and to improve liver tissue pathological features. Similar results previously demonstrated that date seeds suspension improved liver enzymes (ALT and AST) and liver histopathology (H & E) in diabetic rats (Abdelaziz et al., 2015).Furthermore, in rats with alloxan-induced diabetes, Ajwa seed aqueous extract was found to normalize liver enzymes (ALT, AST, and ALP) (Sarfraz et al., 2017).

Hyperglycemia cause oxidative stress, which causes liver damage and metabolic changes (increased gluconeogenesis and ketogenesis) that provoke more oxidative stress and inflammation leading to a rise in serum liver enzymes (Mohamed et al., 2016; Sarfraz et al., 2017).The ability of Ajwa seeds to reverse the raised in the serum liver enzymes and to retain the histopathological features of the liver may be attributed to its ability to lower the elevated serum glucose level which in turn reverse the oxidative stress state and prevent the hyperglycemia linked metabolic changes, (Sarfraz et al., 2017). Numerous mechanisms are involved in hyperglycemia-induced tissue injuries including increased glucose flux through the polyol pathway, overproduction of intracellular AGE, increased AGE receptor expression, activation of protein kinase C isoforms, and induction of the hexosamine pathway. Several evidence suggest that all of these mechanisms are activated by a specific upstream incident, excess mitochondrial reactive oxygen species (ROS) formation (Brownlee, 2005; Giacco and Brownlee, 2010).

This study demonstrated the ability of Ajwa seeds to increase the concentrations of enzymatic (SOD and CAT) and non-enzymatic (GSH) antioxidants in the liver tissue of diabetic rats and their ability to reduce levels of oxidative stress products (MDA and AGE); these confirm their hepatoprotective action against the risk of destructive oxidative stress. By virtue of their antioxidant constituents, Ajwa seeds may be able to scavenge ROS and thus avoid diabetes-related hepatic oxidative stress, and liver damage (Habib et al., 2014; Habib and Ibrahim, 2011; Sarfraz et al., 2017). Several studies have shown that intracellular glucose overload reduces antioxidant enzyme activity (SOD and CAT) and increases lipid peroxidation (MDA) (Manna et al., 2010; Nain et al., 2012). The glycation of antioxidant enzymes may be the cause of this behavior (Yan and Harding, 1997). The current experiment showed that Ajwa seed-treated diabetic rats have a substantial increase in liver SOD and CAT levels. These results may be attributed to Ajwa seeds' ability to inhibit the glycation of the antioxidant enzymes and scavenge the ROS due to the plenty of powerful antioxidants, including flavonoids and phenolic active components (Habib et al., 2014).

According to many studies, Ajwa seeds contain many antioxidants as they constitute a wealthy source of total polyphenols (Al-Farsi et al., 2007; Habib et al., 2014). Ajwa seeds have a significant polyphenol content, equivalent to 51 g/kg, higher than in the fruit. Besides that, catechins and flavanones, among the polyphenolic phytochemicals present in Ajwa seeds, possess a good absorption. This confirms a perfect bioavailability of Ajwa seeds' polyphenols (Habib et al., 2014). TNF-α and other inflammatory cytokines are essential in the advancement of diabetes-induced liver inflammation(Chen et al., 2020; Soufi et al., 2012). Oxidative stress has also been shown to encourage the expression of NF-kB, which increases the production of pro inflammatory cytokines, including TNF-α (Arican et al., 2005; Chen et al., 2020).

Based on this evidence, targeted therapies that can tightly regulate blood glucose, minimize oxidative stress and inhibit pro-inflammatory cytokines are supposed to be effective in preventing diabetic complications (Chen et al., 2020).The present study findings demonstrated the ability of Ajwa seeds to decrease the concentrations of inflammatory mediators (TNF-α and NF-κB) and their ability to reduce the expression of NF-κBprotein in the hepatic tissue of hyperglycemic rats; these confirm their hepatoprotective action via inhibition of inflammation.

A recently published study suggested that Ajwa seeds' active constituents may prevent various inflammatory mediators (Bouhlali et al., 2020). Studies have suggested that Ajwa seeds' active constituents may prevent various inflammatory mediators. According to their research, rutin, quercetin, p-coumaric, and caffeic acids were the main polyphenols among the analyzed phenolic compounds abundant in Ajwa seeds. They found a significant correlation between the anti-inflammatory effect of Ajwa seeds and most of the fore mentioned polyphenols (Bouhlali et al., 2020). Ajwa seed extract was found to significantly lower the levels of many pro inflammatory cytokines, including TNF-α (Al-Rasheed et al., 2015; Saryono et al., 2019). Ajwa seeds can also suppress the pro inflammatory mediator (TNF-α) and inhibit NF-kB translocation (Saryono et al., 2019).

STZ-induced liver injury in diabetic animals has accompanied increased hepatocyte apoptosis, which is proven by enhanced biochemical factors implicated in both the extrinsic and intrinsic mechanisms of apoptosis, as shown by the up regulation of Fas/FasL/caspase-3 and Bax/Bcl2 proteins expression (Rodríguez et al., 2018). In STZ-nicotinamide induced hyperglycemic rats, the hepatic expression levels of anti-apoptotic markers, p-PI3K and Bcl-2 were down-regulated, while that of pro-apoptotic markers, cytochrome c, and cleaved caspase-3 were upregulated (Asokan et al., 2019).The present study results showed that Ajwa seeds can lowered the hepatic protein expression of pro-apoptotic marker, caspase 3, which might be another hepatoprotective mechanism of Ajwa seeds. The inhibitory effect of Ajwafruit extract on the hepatic apoptotic cell death may be related to their antioxidant activity and down-regulating the pro-apoptotic factor caspase-3 (Elsadek et al., 2017).

CONCLUSION

Ajwa seeds suspension significantly lowered STZ-induced hyperglycemia by increasing insulin secretion. Ajwa seeds suspension dramatically protects the liver against STZ-induced damage in diabetic rats and significantly increased antioxidants factors in the liver. Moreover, Ajwa seeds suspension successfully ameliorated STZ-induced liver damage by suppressing liver inflammation, as indicated by the decreased TNF and NFKB levels. It also lowered the expression of NFKB as well as the pro apoptotic marker, caspase-3 proteins.

Conflict of Interest: No conflict of interest has been reported.

Ethical Statement: The Biomedical Ethics Research Council, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia, approved the study's procedure (346-19).

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All data/tables/figures/Images (images must be submitted with the MS in high resolution which can be reproducible in print format without losing their quality.

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1. Original Research Articles
2. Critical Review Articles
3. Case Reports with Discussion
4. Rapid / Short Communications
5. Letters to the Editor/Editorials / Perspectives / Correspondence

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All articles under submission to Biosc Biotech Res Comm should aim for the development of technological concepts rather than merely recording the facts. Incomplete studies and manuscripts not in strict compliance with the journals policies will be strongly discouraged. Manuscript must be written in good English, typed using Times New Roman font size 12 double-spaced with one and half inch margin on all sides. All manuscripts must be accompanied by author declaration and ethical certificate signed by the corresponding author and all co -authors that they have seen and approved the final version of the manuscript and that the article has NOT been published or submitted to any other journal for publication. The corresponding author is responsible for obtaining permission from the copyright owner for the use of any copyrighted material in the submitted article.

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2. Methods
3. Results
4. Conclusion

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Results (Including Tables/Fig/Images)
Discussion
Conclusion
References
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Funding Statement and Acknowledgements (if any)

Introduction This section should provide a brief survey of literature, purpose of the study etc.; the rationale of the study undertaken should be given with proper clarity.

Material and Methods: This section consists of material/methods and procedures, its should be concise but detailed enough to enable the reader to reproduce the experiments / methodology. Commonly used procedures and methods in detail need not be described, but require a reference or citation to the original source.

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