BBRC

Bioscience Biotechnology Research Communications

VOLUME-13 NUMBER-1 (January-March 2020)

Print ISSN: 0974-6455

Online ISSN: 2321-4007

CODEN: BBRCBA

www.bbrc.in

University Grants Commission (UGC)
New Delhi, India Approved Journal

An International Peer Reviewed Open Access Journal For Rapid Publication

Published By:
Society for Science & Nature (SSN)
Bhopal India
Indexed by Thomson Reuters, Now Clarivate Analytics USA
ISI ESCI SJIF=4.186
Online Content Available: Every 3 Months at www.bbrc.in

SOCIETY
FOR SCIENCE AND NATURE
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Registered with the Registrar of Newspapers for India under Reg. No. 498/2007
Bioscience Biotechnology Research Communications
VOLUME-13 NUMBER-1 (JANUARY-MARCH 2020)

ISSN 0974-6455

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Bioscience Biotechnology Research Communications
(Abbreviation: Biosc. Biotech. Res. Comm.)
Web of Science (WoS ESCI Thomson Reuters, Clarivate Analytics USA & UGC India) Indexed Journal

BBRC
Bioscience Biotechnology
Research Communications

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Bioscience Biotechnology Research Communications, BBRC is a broad based internationally indexed official publication of Society for Science & Nature (SSN) since 2008. The international journal publishes peer reviewed original research papers, exciting reviews and short communications in basic and applied areas of life sciences and the upcoming state of the art technologies, including Biology and Medicine on a fast track. The young editorial team of *Biosc. Biotech. Res. Comm.* tries hard to provide a high quality flawless format of scientific communication for the popularization and advancement of science, worldwide. During these years hundreds of peer reviewed research papers of very high quality have been published in *Biosc. Biotech. Res. Comm.* and authors like Kiran Shaw Majumdar of Biocon, Bangalore have contributed to *Biosc. Biotech. Res. Comm.* helping it achieve high readership in a short span of time. Reviewing the published research articles, it becomes evident that on an average, about 7 papers out of 10 are subjected to healthy revisions in *Biosc. Biotech. Res. Comm.* making quality reading. We owe this achievement to our reverend reviewers! We hope the standards set by *Biosc. Biotech. Res. Comm.* will improve further making this international journal unique and easily accessible to the scientific fraternity across the globe. In its tenth year of successful existence as a scholarly publication, *Biosc. Biotech. Res. Comm.* has now become an open access Thomson Reuters ISI ESC Web of Science/Clarivate Analytics USA Indexed journal also approved by University Grants Commission (UGC) Ministry of Human Resource Development, Government of India, New Delhi and has a NAAS-2019, Government of India, Indian Council of Agricultural Research (ICAR) New Delhi rating of 4.38 and SJIF 4.196.

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Bioscience Biotechnology Research Communications
International Open Access Peer Reviewed Journal For Rapid Publication
(Indexed in Leading National and International Scientific Citation Agencies
Approved by University Grants Commission (UGC) New Delhi)
(NAAS 2020 Journal Score – 4.31 SJIF 2020-7.728)
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Bioscience Biotechnology Research Communications

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-Feb-March 2020 Pp- I-V

Corona Virus Disease-19: The New Challenge for Saving the Human Race

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The Prologue:

The worldwide research being carried out so dedicatedly by those who are affected: The US, China, Canada, UK and the rest of the world will certainly outpace and defeat the viral epidemic. There is light despite all the darkness!

Mankind has witnessed three epidemics recently – severe acute respiratory syndrome (SARS) in China in 2002-04, the Middle East respiratory syndrome (MERS) which started in Saudi Arabia in 2012, and the latest, Corona Viral Disease (Co-ViD19) which started in China too in Nov 2019. It has affected more than 8,00,000 people over 170 countries till date - the common thread lies in the fact that all of them belong to the same family, "Corona Viruses". The crown shaped viruses (hence the name Corona) measures approximately 60 - 140 nm in diameter, has a single long stranded RNA of a genome and a nucleocapsid of helical symmetry (genome size ranging from 27 - 34 kilobases in length). It has spike of glycoproteins on the envelope and is a group of related viruses that causes diseases in mammals and birds and recently is creating a havoc in humans too. Based on genetic and antigenic criteria, CoVs have been organised into four groups: α-CoVs, β-CoVs, γ-CoVs and Δ-CoVs and till date seven human CoVs (HCoVs: HCoV-OC43, and HCoV-HKU1 (beta CoVs of the A lineage); HCoV-229E, and HCoV-NL63 (alpha CoVs); SARS-CoV, SARS-CoV-2, and MERS-CoV (beta CoVs of the B and C lineage, respectively) capable of infecting humans have been identified (Chen, Liu and Guo, 2020; Chan et al., 2020).

Novel Covid-19 is the most dreadful among all types of Corona viruses so far. The first wave of coronavirus disease 2019 (COVID-19) pandemic is currently invading the world, and several countries are now struggling to fight it or trying to delay its start to help smooth its peak size for the purpose of lowering morbidity and mortality, and thereby reduce the overall tension on their health-care system, (Flahault, 2020). Since then, the virus has spread over 170 countries and WHO has declared it as global public health emergency on Jan 30th,2020. By Feb 9th, death toll in China surpassed that of 2002-2003 SARS epidemic with 811 deaths recorded and by Feb 12th 2020, corona virus cases started to spike in South Korea and by Feb 19th and 21st 2020 both in Iran and Italy respectively, the outbreak began on a horrible note. Since then after one month i.e on March 20 and 21st, Italy has reported its highest death toll of 627 and 793 respectively on a single day (Secon et al 2020).

Despite, the original source of the outbreak remains unknown and during the past three weeks, new major epidemic foci of coronavirus disease 2019 (COVID-19), some without traceable origin, have been identified and are rapidly expanding in Europe, North America, Asia, and the Middle East. On the basis of alarming levels of spread and severity, and by the alarming levels of inaction", on March 11, 2020, the Director-General of WHO characterised the COVID-19 situation as a pandemic. Currently, authorities have reported with 8,03,696 positive cases with about 5,92,192 being active and among ongoing cases, there are roughly, 1,72,434 recoveries and 39,070 deaths as per the report on 31st March, 2020 (Worldometers Info, 2020).

ARTICLE INFORMATION

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Received 31st March 2020 Accepted after revision 31st March 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

Clarivate Analytics crossref

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)
A Society of Science and Nature Publication,
Bhopal India 2020.
Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/1

Since the advent of SARS which affected more than 8000 cases around 26 countries in 2003, an upsurgence in the infectious disease is evident worldwide. MERS (Middle east Respiratory Syndrome) Viral outbreak in 2012 affected Saudi Arabia and other countries in the GCC whereas Ebola Virus (2013-2016) affected West Africa particularly the countries of Guinea, Liberia and Sierra Leone. Later in 2015-16, Zika Virus epidemic affected hundreds and thousands of people in Brazil and South and North America in 2015-16, (Marston et al., 2017). However, this time with Corona infection, which has taken the shape of a pandemic, the impact is devastating that has shattered businesses, stilled production, stopped travel, emptied public spaces, crashed economies and broken healthcare systems across the world, however the full blown impact is yet to be evaluated. The underlying fact across all these uprisings is to understand what makes the viruses potentially so threating and what needs to be done to curb them. Among all the potential causes, reported in large number of literature, one common factor that has been observed is that the interaction of the modern human beings with the environment around them has contributed to the success of dangerous viruses.

It is a well-known fact that any virus will replicate only when its been inside the cell of a living being, and spreads most efficiently when there is contact between two individuals. This one the COVID 19, hijacks human lung cells to produce more viruses and their copies, attaching by its spikes to the receptors on human respiratory cells. The viruses in suitable environments can replicate almost instantaneously and in huge numbers, contributing to high rate of mutations. Nevertheless, this invisible, very intelligent predator has been also known to adapt quite quickly to an adverse environment, such as the human immune system or drugs thereby allowing the virus to jump from an animal host to humans contributing to the spread of disease, which might have happened most likely in the case of the spread of Covid-19 and therefore has been seen as the most reliable explanation for the match of the corona virus genome of humans with that of bats and pangolins with 95% and 99% accuracy, (Burki 2020).

The impact of the global pandemic can be assessed from the fact that this virus took 67 days to infect the first 100,000 people while the next got infected in only 11 days. The number reached to 300,000 in another 4 days whereas the 400,000 figure touched in meagre 3 days. The number touched 5 lakhs in just 2.5 days and in the next 48 hours infected the next lakh taking the toll to 6 lakh people, a phenomenon quite unprecedented, (Business Today Report 28th March,2020).Among the various countries, on one hand the developed economies such as Italy, Spain, UK, Iran and now USA are facing their biggest humanitarian crisis, the developing nations in South Asia including India, Pakistan, Bangladesh and the Africa in the West which account for almost 14% of the world population are bracing for a head on collision with the corona virus and effects would be known only in next few weeks, (Kumar 2020; Signe and Fakim 2020).

Having said and done, the intricating questions are what defiance's lies ahead and what needs to be done? The challenges are multiple: Epidemiological, Social, Economical and Mental. As COVID 19 has become pandemic, epidemiological challenge lies in the fact how to halt its spread and prevent it to move to Stage 3 and above, (community transmission) of the disease. In the light of the fact, even the most effective and draconian containment strategies have only slowed the spread of Covid-19, all eyes have turned to the prospect of a vaccine, because only a vaccine can prevent people from getting sick. The critical issue remains here, how early the vaccine against Covid 19 can be developed and what are the bottlenecks. Rapid development of a vaccine against the deadly virus requires basic scientific understanding of the virus its structure, biology, including areas such as genomics. With prior experiences from SARS, H1N1 influenza, Ebola, Zika, the scientific community as well as the vaccine industry were asked to respond urgently to the epidemics. It was noticed that earlier with SARS and later with Zika, the epidemics ended before vaccine development was complete, (Laura 2020; WHO Report 2020).

Therefore, the funds allocated for vaccine development were diverted to other social projects, leaving manufacturers with huge financial losses thereby impeding other vaccine-development programs as well. However, during H1N1 influenza epidemic, the story was different. The vaccine manufacturers were able to be develop the antidote rather rapidly because the influenza-vaccine technology was already matured as well as the legislations regarding vaccine manufacturing using egg- and cell-based platforms for licensing as well as rules used for a strain change were pre-decided. For Corona vaccination, multiple DNA and RNA platforms including recombinant units are under development. The entire process is time-consuming with diversified checks and balances to prevent any mishaps. No RNA vaccines are approved till date however the vaccines have entered clinical trials with regulators reviewing the clinical trial applications and the time span might range from few months to almost a year, (Laura 2020).

Other potential problems likely would be commercial production of the vaccine. As soon as the vaccine would be approved, it will be required in vast quantities and many of the organisations in the COVID-19 vaccine race simply don't have the necessary production capacity. In business terms, vaccine development is a risky proposition. So, on one hand while virus biology and vaccines technology are potential limiting factors, the politics and economics surrounding it would far likely to be other key barriers to immunisation. The recent viral pandemic has posed gargantuan challenges to the world-wide health systems. The escalation of the scale of masses evident from numerous examples of Italy, Iran and now currently US points to the fact that irrespective

of the best of the healthcare available, inability of the national health systems to respond well in time and without adequate preparedness, the consequences can be devastating. On the contrary, China which is the epicentre of the pandemic has been able to counter the challenges effectively where other nations failed miserably irrespective of having developed economies. As more and more cases of COVID-19 have been reported since somewhere January 2020 in China, the entire public health machinery responded swiftly with immaculate planning and disciplined execution. Enormous health infrastructures have been raised, resources and manpower were mobilized for affected patients at lightning speed within a couple of days, a feat which no other country can achieve till now (Lai et al 2020).

The Chinese authorities have introduced numerous unprecedented measures including complete curb on people's movement in and out of Wuhan, the centre of the epidemic, as well as other 15 cities in the Hubei province, home to more than 60 million people. All means of transports were suspended including flights and trains as well as the roads were blocked. Soon after, disciplined enforcement of residential lockdown were extended to other cities with (~760 million people) roughly half of the country's population was asked to be confined to their homes for a period of around 2 months with the condition to venture out only to get food or for medical help. Besides this, rapid testing, rapid diagnosis, social distancing, quarantine of affected individuals as well as along with hospital preparedness to cater to the patients requiring both emergency respiratory care as well general medical care were key winning initiatives for the successful outcomes, (Chen et al 2020).

On Health Intelligence front, beforehand, epidemiologists in China using data and modelling estimated that probability of each corona infected person to pass the viral infections is to two people or more, making it highly contagious. Therefore, strict implementation of such measures helped to reverse the escalating cases. According to a published research paper, using multiple analyses model it was estimated that cutting off Wuhan delayed the spread of disease to other Chinese cities by roughly four days, whereas the ban on air travel stopped four of five cases from being exported from China to other countries for about two to three weeks. However, irrespective of all these measures taken, it was suggested that blocking travel could effectively only slow down the spread but cannot completely curb it. According to another recent study by Lai et al.,(2020), it was predicted that the combined effect of early detection and isolation along with drop in contact between people as well as ban on intercity travel prevented cases from increasing by 67-fold else China would had nearly received 8 million cases by the end of February alone, (Lai et al. 2020; Chen et al 2020).

It is worthwhile to cite the ELVIS (Edenborough and Lothians Viral Intervention Study) which showed that NaCl, sodium chloride can inhibit all types of viruses of common cold. The prevention of viruses is caused by the chloride component of salt, not the sodium, this evidence of chloride-ion dependent innate antiviral response in epithelial cells was used by an open pilot study to treat common colds by Ramalingam et al., (2019). Similar lessons on early detection paid off for both Singapore and South Korea where Diagnostic Testing, Quarantine and Social Distancing were made standard preventive models to curb the rising menace. These models are now being used as standard model for countries such as India, where aggressive social distancing can be one of the prevention methods of this viral epidemic as well, which has been implemented in a complete lockdown for 21 days from 25th March 2020. This has shown positive results so far, as the number of cases has not increased so dramatically. However, reports have also suggested that non-compliance to strict adherence to lockdown can result in humanitarian crisis with mass causalities as visible in Italy, Iran and slowly USA too which has reported the maximum number of cases now.

Though new cases of COVID-19 have slowed dramatically in China, but another fear that some epidemiologists suggest that once the country fully eases its control measures, the virus could start circulating again, which can turn out to be catastrophic, if proper measures are not taken, (Cyranoski 2020). On the social fronts, on one hand catering to the people need irrespective of their social strata is a major challenge, even distribution of the relief measures along with delivering monthly grains and lentil rations to the astonishing 800 million poor (60% of India's population) is a Herculean task, which at present is being taken care of. At a time of humanitarian crisis of such a magnitude, it becomes strangulating for the poor people and daily wagers to get both ends meet, especially in cases of lockdown.

However large scale voluntary work across the nations is taking shape and we look forward for a positive response. Reports suggest that the impact of lockdown on the masses especially the BPL(below poverty line) category people is devastating, as 1.8 million people in the country are homeless and almost 85% of the population work in the informal sector. Besides this, the other health challenges lie in the fact of relatively low numbers of testing levels. To add to these, the poor sanitary conditions in many isolation wards in the government hospitals might force some of those quarantined to escape these facilities. (Chaudhary and Prasad 2020).

Similarly, fake news on social media including misinformation and conspiracy theories as well as sensationalist reporting are key challenges on the information front and can dampen the efforts. To curb the menace of fake news and related information on health or other related information, WHO has recommended a "four-pronged approach," using its WHO Information Network for Epidemics platform to track misinformation in multiple languages and collaborating with social and digital companies such as Facebook, Weibo, and Twitter to rapidly filter out false information, (Ying et al. 2020, WHO 2020). The new coronavirus menace has proved to be an enormous stress test for globalization on the economic

front. As whole world is interconnected with business interests, production and customers of the various MNCs spread across the globe, the pandemic has disrupted the travel industry unprecedentedly both at international and national level, causing unexpected free fall in the global oil prices, affecting industrial production, supply chains been broken down, share markets crashing and henceforth the crisis is forcing a major re-evaluation of the interconnected global economy. The economic interrelationships between the various stakeholders are not only complex but intertwined. On one hand, while globalization has allowed for the rapid spread of contagious disease, at the same time it has fostered deep interdependence between firms and nations that makes them more vulnerable to unexpected shocks, (Bingham and Hariharan 2020).

The economic challenges thrown up have an important lesson for the world economic forum that globalization is fragile, irrespective of its benefits. For decades, the rigorous efforts of the corporate enterprises to eliminate redundancy has generated unprecedented wealth. But these relentless pushovers have also reduced the amount of unused resources referred to as "slack" in the globalized economy. The presence of too little slacks in this time of global crisis, points towards eliminating critical fail-safes causing supply chains to break down. With 26% of the world production been localized to China, majority of the countries have faced critical shortages ranging from medical equipment as simple as face masks or hand sanitizers to complex items such as electronics and other goods. This could be illustrated by the fact that partly as a result of supply chain problems, global production of laptops fell by as much as 50 percent in February, and production of smartphones could fall by 12 percent this coming quarter. Both products are built with components produced by specialized Asian manufacturers i.e China. With tens of millions of workers now in quarantine and parts in short supply, it is struggling to get economic activity back on track. Countries with well-honed crisis risk-management arrangements such as South Korea are faring better at slowing the spread of infection, although that does not make them immune to political and economic pressures (Cheung 2020).

As policymakers around the world struggle to deal with the new coronavirus and its aftermath, they will have to confront the fact that the global economy doesn't work as they thought it did. For developing economies like India, the economic impact from Corona pandemic is multiple. Post 2016, demonetization and GST implementation have made the Indian economy down, bringing the 8.5 % of GDP in 2016 to 4.5% in 2020. The countrywide implementation of lockdown as a result of corona pandemic is expected to bring the GDP to as low as 2.5% as predicted by Moody's Report (Business Today 2020). It is a known fact that even 1% loss in GDP results in loss of lakhs jobs, however there is always a better bounce back of economy in and after such crises, (Kannan and Raveendran 2019). Similarly, mass quarantines in cities or in border areas or stigmatize under lockdown, all these factors have contributed to increase mental health risks as people experience stress, anxiety and a sense of isolation and loss of control over their lives. Even for the affluent class, residential lockdown, travel bans, loss of jobs have resulted in a greater social vacuum. Similarly, suffering from already existing disease/spread of infection or coping with surging care needs, further reduces confidence in the competence level adding to predisposing stress level (Chaudhary and Prasad 2020).

The recent Chinese experience of combining non-pharmaceutical interventions to curb outbreak trends seems rather convincing. Although starting late in the process, authoritarian Chinese authorities succeeded in combining forced isolation of the population with all available social distancing interventions. To what extent, at which pace, and how should they start lifting their intervention and allow people to resume normal social and economic life is a matter of speculation, (Flahault, 2020).

Despite all the challenges, the silver linings lies in the fact that the Corona Infection would be short lived and very soon will be taken over by multitudes of humanitarian efforts. Since the human civilization has not come across a pandemic of such a scale in the last 70 – 80 years of human existence, therefore it has taken people by shock and awe. Nevertheless, Mans indomitable spirit, coupled with science and technology and his great struggle for survival will overcome this difficult challenge very soon. Hope is the only road forward.

REFERENCES

Bingham RS. And Hariharan K. (2020) This is the impact of the Coronavirus on business. World Economic Forum Report. Last assessed from https://www.weforum.org/agenda20/02/ 2020 why-is-coronavirus-a-global-business-risk/

Burki T.(2020) Outbreak of Coronavirus disease 2019. News Desk. The Lancet.com/infection;20(3) : 292 – 293.

Chan JF, Kok KH, Zhu Z, Chu H, To KK, Yuan S, Yuen KY. (2020) Genomic characterization of the 2019 novel human-pathogenic coronavirus isolated from a patient with atypical pneumonia after visiting Wuhan. Emerg Microbes Infect. 2020;9 (1):221-236

Chaudhary S. and Prasad SK. (30th March, 2020) How India plans to put 1.3 billion people on a coronavirus lockdown. The Washington Post. Last assessed from https://www.washingtonpost.com/politics/2020/03/30/how-india-plans-put-13-billion-people-coronavirus-lockdown/)

Chen W, Wang Q, Li YQ, et al.(2020) Early containment strategies and core measures for prevention and control of novel coronavirus pneumonia in China. Chinese Journal of Preventive Medicine; 54(3): 1-6. Assessed from doi: 10.3760/cma.j.issn.0253- 9624.2020.03.003

Chen Y, Liu Q, Guo D.(2020) Emerging coronaviruses:

Genome structure, replication, and pathogenesis. J. Med. Virol., 92(4):418-423.

Cheung MC. (Feb 26th, 2020).Corona virus's impact on consumers and businesses in China. E-Marketer. Last assessed from https://www.emarketer.com/content/coronavirus-china-us-covid-19-impact-retail-travel

Cyranoski D. (2020) What China's coronavirus response can teach the rest of the world. Nature; 579(3) : 479 – 480. (Assessed from https://www.nature.com/articles/d41586-020-00741-x)

Flahault A. (2020) COVID-19 cacophony: is there any orchestra conductor? The Lancet March 18, 2020 https://doi.org/10.1016/ S0140-6736(20)30675-9

Hellewell J, Abbott S, Gimma A, et al.(2020) Feasibility of controlling COVID-19 outbreaks by isolation of cases and contacts. The Lancet Global Health. doi: 28 10.1016/S2214-109X(20)30074-7.

Jaya Kumar PB.,(March 28, 2020) Coronavirus fury: 1 lakh cases globally in 67 days; 5 lakh in just 22 days Business Today Report. Last assessed from https://www.businesstoday.in/current/world/coronavirus-fury-1-lakh-cases-globally-in-67-days-5-lakh-in-just-22-days/story/399492.html).

Kannan KP and Raveendran G. (2019) From Jobless to Job-loss Growth Gainers and Losers during 2012–18. Economics and Political Weekly; 44 : 38 – 44.

Lai S., Ruktanonchai NW., Zhou L., Prosper O., Luo W., Wesolowski A., Zhang C., Du X., Y H. and Tajem AJ (2020) Effect of non-pharmaceutical interventions for containing the COVID 19 outbreak. Worldpop University of South Hampton: 1-44. (Assessed from https://www.worldpop.org/events/COVID_NPI)

Laura S. (2020). Coronavirus vaccine: when will it be ready? The Guardian (Last assessed on 31st March https://www.theguardian.com/world/2020/mar/31/coronavirus-vaccine-when-will-it-be-ready)

Marston HD, Paules CI, Fauci AS (2017) The critical role of biomedical research in pandemic preparedness. JAMA ;318:1757-1758.

Moody's Report (27th March 2020) Coronavirus: Moody's cuts India's 2020 GDP growth forecast to 2.5%. Business Today. Last assessed from https://www.businesstoday.in/current/economy-politics/coronavirus-moodys-cuts-india-2020-gdp-growth-forecast-to-2-point-5-percent/story/399377.html

Ramalingam S, Catriona Graham, Jenny Dove, Lynn Morrice and Aziz Sheikh (2019) A pilot, open labelled, randomised controlled trial of hypertonic saline nasal irrigation and gargling for the common cold Scientific Reports Volume 9, Article number: 1015

Secon H.,Wood Ward A., Mosher D (March 27,2020) A comprehensive timeline of the new coronavirus pandemic, from China's first COVID-19 case to the present. Business Insider Report (Last assessed on 31st March, 2020 from https://www.businessinsider.com/coronavirus-pandemic-timeline-history-major-events-2020-3)

Signe L. and Fakim AG.(March 26, 2020) Africa Is Bracing for a Head-On Collision With Coronavirus. Last Assessed on 31st March, 2020 from https://foreignpolicy.com/2020/03/26/africa-coronavirus-pandemic-economic-crisis/)

WHO Report (2020) A coordinated global research roadmap. Last accessed on 20th March, 2020 https://www.who.int/blueprint/priority-diseases/key-action/Roadmap-version-FINAL-for-WEB.pdf?ua=1

World Health Organization (2020) Coronavirus Disease (COVID-19) Press Conference, 21 February 2020. 2020. https://www.who.int/docs/default

Worldometers (31st March, 2020) Last assessed from https://www.worldometers.info/coronavirus/

Ying S, Li F, Geng X, et al. (2020) Spread and control of COVID-19 in China and their associations with population movement, public health emergency measures, and medical resources. medRxiv : 2020.02.24.20027623. doi: 10.1101/2020.02.24.20027623

Biotechnological
Communication

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp-01-09

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A Novel Long Noncoding RNA, AC092834.1, Regulates the Adipogenic Differentiation of Human Adipose-Derived Mesenchymal Stem Cells Via the DKK1/Wnt/B-Catenin Signaling Pathway

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ABSTRACT

Long noncoding RNAs (lncRNAs) have been implicated in a range of developmental processes and diseases, but the roles and mechanisms by which they act in adipogenic differentiation and adipose tissue biology are still unknown. By comparing the different expression patterns of lncRNAs before and after the adipocyte differentiation of human adipose-derived mesenchymal stem cells (hADSCs), we characterized a novel lncRNA, AC092834.1, which is significantly increased in preadipocytes. By gain- and loss-of-function experiments, we demonstrated that lncRNA AC092834.1 potentiated adipogenic differentiation through directly increasing the level of expression of DKK1, which competitively binds with LRP5 to inhibit the Wnt-β-catenin pathway and reduce the inhibition of adipogenesis by Wnt signaling. This finding provides novel mechanistic insights into a critical role for lncRNA AC092834.1 as a regulator of adipogenic differentiation, which expands our knowledge about the molecular mechanisms of obesity and other adipogenic differentiation-related disorders.

KEY WORDS: LONG NONCODING RNA; ADIPOGENIC DIFFERENTIATION; DKK1; HADSCS.

ARTICLE INFORMATION

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Received 7th Jan 2020 Accepted after revision 20th March 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)
A Society of Science and Nature Publication, Bhopal India

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/2

INTRODUCTION

Obesity has attracted increasing attention worldwide, particularly because obesity is a major risk factor for diabetes, hyperlipidemia, atherosclerosis, fatty liver and chronic inflammation, (Martenstyn, et al., 2020; Sun and Karin, 2012; Shulman, et al., 2014). The rising incidence of obesity has become a serious health challenge with the current prevalence predicted to triple by 2030 according to the World Health Organization (WHO), (Corrales, et al.,2018). Adipocytes, the main component of adipose tissue, play crucial roles in systemic metabolism and energy homeostasis. In response to abnormal adipogenesis and as adipocytes accumulate excessively, a host of metabolic problems emerge, (Cohen, et al., 2016). Human adipose-derived mesenchymal stem cells (hADSCs) are major sources of adipocyte generation and have the capacity of self-renewal and multi-lineage differentiation potential such as commitment into preadipocytes. The underlying pathophysiology of obesity remains ill-defined and further understanding of the adipogenesis mechanism is of great significance for the prevention and therapy of obesity-related diseases. The mechanisms governing hADSCs adipogenesis are quite complex, and a great deal of investigation has focused on regulating adipocyte lineage commitment by modulating cell signaling pathways or numerous transcription factors.

A range of transcription factors such as peroxisome proliferator-activated receptor-gamma (PPARγ) and several members of the CCAAT/enhancer-binding proteins (C/EBPs, specifically C/EBPα/β) have been demonstrated to be critical for the differentiation of hADSCs into adipocytes, (Hadrich and Sayadi, 2018). Importantly, PPARγ can promote adipogenesis in the absence of C/EBPα, while C/EBPα does not function similarly in PPARγ-deficient cells, (Lee, et al., 2019). In terms of signaling pathways in adipogenesis, multiple extracellular and intracellular signaling pathways such as Wnt/β-catenin, TGF-β, MAPK, and PI3K signaling are vitally important for adipogenesis and are well-studied, (Chen, et al., 2016). On the other hand, epigenetic regulation, including DNA methylation, histone modification, and noncoding RNA modulation also exert important roles in the regulation of adipogenic differentiation of hADSCs. Recently, large-scale sequencing initiatives demonstrated that noncoding RNAs play significant roles in the control of transcription and epigenetic regulators, and, crucially, help establish lineage specification through dependent or independent mechanisms.Long noncoding RNAs (lncRNAs) are a subset of noncoding RNAs that are longer than 200 nt and are characterized by only rarely encoding proteins; instead, lncRNAs are involved in the regulation of gene expression at the epigenetic, transcriptional, and posttranscriptional levels(Kopp and Mendell, 2018).

A growing number of researchers have indicated that lncRNAs are extensively involved in the regulation of numerous cellular processes, including stem cell pluripotency cell differentiation and human disease pathogenesis(Fico, et al., 2019).Recent studies have prompted us to hypothesize that lncRNAs (such as ADINR, slincRAD and H19) are implicated in adipocytes' fate decisions of hADSCs through the control of master adipogenic transcriptional factors, including PPARγ and C/EBPα,(Xiao, et al., 2015;; Schmidt, et al., 2018 Yi, et al., 2019). Despite progress in identifying and functionally dissecting lncRNAs involved in adipogenic differentiation and adipose tissue biology, our understanding of how lncRNAs control hADSCs commitment into preadipocytes differentiation and function in particular remains elusive.

To gain molecular insights into these processes, we here performed RNA-sequencing (RNA-Seq) of hADSCs and preadipocytes and characterized a novel adipocyte-specific lncRNA potentiating adipogenic differentiation. This novel lncRNA, named AC092834.1, was significantly increased in preadipocytes and played a positive role in adipogenesis of hADSCs. By gain- and loss-of-function experiments, for the first time, we demonstrated that overexpression of AC092834.1 directly promoted an increase in the level of DKK1, which competitively binds to LRP5 to inhibit the Wnt-β-catenin pathway and reduced inhibition of adipogenesis by Wnt signaling. Our findings provide novel mechanistic insights into a critical role for lncRNA AC092834.1 as a regulator of adipogenic differentiation, which expands our knowledge about molecular mechanisms of obesity and other adipogenic differentiation related disorders.

MATERIALS AND METHODS

Induced adipocyte differentiation and Oil Red O staining: When the hADSCs were 90% confluent, the culture medium was replaced with adipogenesis induction medium (AM), which consists of high-glucose Dulbecco's modified Eagle medium (DMEM) containing 10% FBS, 0.1 mM ascorbic acid, 1 μM dexamethasone and 0.5 mM 3-isobutyl-1-methylxanthine. The AM was changed every 3 days until the intracellular lipid droplets were big and round. Oil Red O staining was used to monitor the progress of the lipid droplets. Briefly, cells were washed twice with PBS and fixed with 4% paraformaldehyde for 5 min. Then, the paraformaldehyde was washed away with distilled water and the cells were stained with filtered 0.6 mg/mL Oil Red O solution for 30 min at room temperature. After staining, excess stain was removed by rinsing with distilled water and the results were visualized by light microscopy. RNA extraction and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) Total RNA was extracted from cultured cells with TRIzol Reagent and treated with DNase I (Ambion, USA) at 37°C for 30 min. cDNAs were synthesized using a high-capacity cDNA reverse transcription kit (Applied Biosystems, USA) and 100 ng of cDNA was used in each sample. Primers used are listed in the Supplementary Table S1. The relative expression level of mRNA was evaluated using the 2-ΔΔCt method and was normalized to the GAPDH expression level.

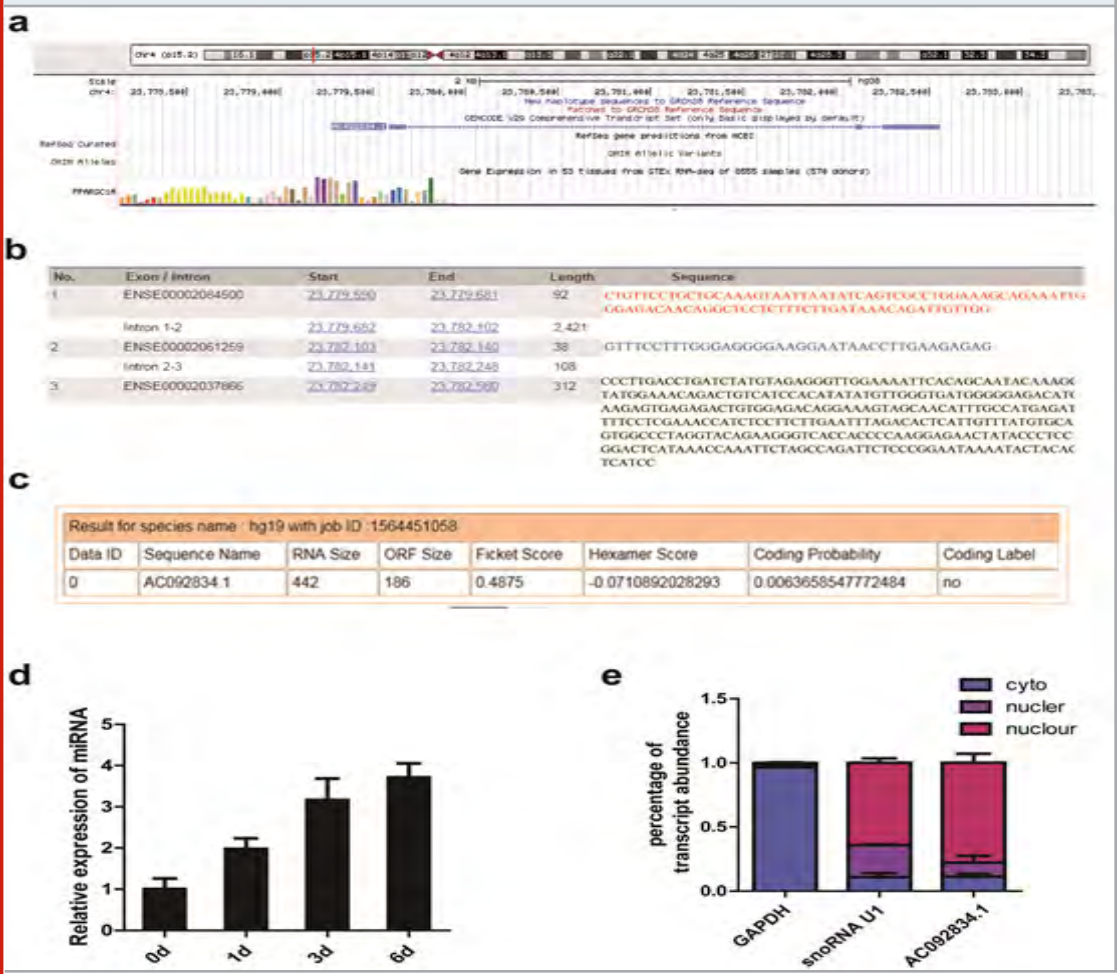
Cytoplasmic and Nuclear RNA Extraction: The extraction was performed as according to the manufacturer's

protocol of the NE-PER™ Nuclear and Cytoplasmic Extraction Reagent kit (Thermo Scientific, USA). First, harvested cells were washed with ice-cold PBS and centrifuged to remove the supernatant. Then, 100 µL ice-cold CER I was added to each 10 µL cell precipitation and vortexed for 15 sec at high speed and then incubated on ice for 10 min. We repeated this step 2 times to fully lyse the cells. Last, 5.5 µL of ice-cold CER II was added to each 10 µL cell precipitation and vortexed for 5 sec on the highest setting, and then incubated on ice for 2 min. We repeated this step 2 times and the supernatant was collected as the cytoplasmic fraction while the precipitate was the nuclear fraction. For RNA extraction, both fractions were first incubated with Proteinase K (10 mg/mL) at 37 for 20 min and then RNA was extracted by TRIzol.

ShRNA transfection and lentiviral vector infection: lncRNA shRNA or the negative control (RiboBio, China) at a concentration of 50 nM was transfected into cells using Lipofectamine 3000 (Invitrogen, USA) and Opti-MEM (Gibco, USA) culture medium, according to the manufacturer's instructions. Then, 48 h after transfection, cells were harvested and the interference efficiency of the treatments were detected. The lncRNA AC092834.1 sequence was cloned into the LV5-EF1-a-EGFP-Puro vector plasmid, and a vector that expressed a scrambled RNA was used as a negative control. The lentiviruses were produced through the transient transfection of 293T cells using Lipofectamine Plus. Lentivirus vectors and plasmid vectors were prepared according to the protocol from GenePharma (GenePharma, China). The lentiviruses were named lenti-AC092834.1 and lenti-NC. After infection with lentivirus (MOI=10) and selection with puromycin, almost all of the cells were GFP-expressing, indicating stable expression of the lentiviruses.

Western blot analysis: Total protein was extracted using RIPA lysis buffer with 1 mM PMSF, centrifuged and we quantified the supernatant using a BCA Protein Assay Kit (Beyotime, China), and then the proteins were denatured for 10 min in boiling water. A sample of 20

Figure 1. Characterization and expression pattern analysis of lncRNA AC092834.1.
a. The genome location locus of AC092834.1 checked in the UCSC database system. b. AC092834.1 exon sequence information shown in the Ensemble database. c. Prediction of protein coding probability of lncRNA AC092834.1 by Coding Potential Assessment Tool (CPAT). d. qRT-PCR analyses detected the expression levels of lncRNA AC092834.1 e. The expression level of AC092834.1 in cytoplasmic and nuclear extracts of hADSCs followed by qRT-PCR analysis. GAPDH served as a cytoplasmic mRNA control and snoRNA U1 served as a nuclear RNA control.



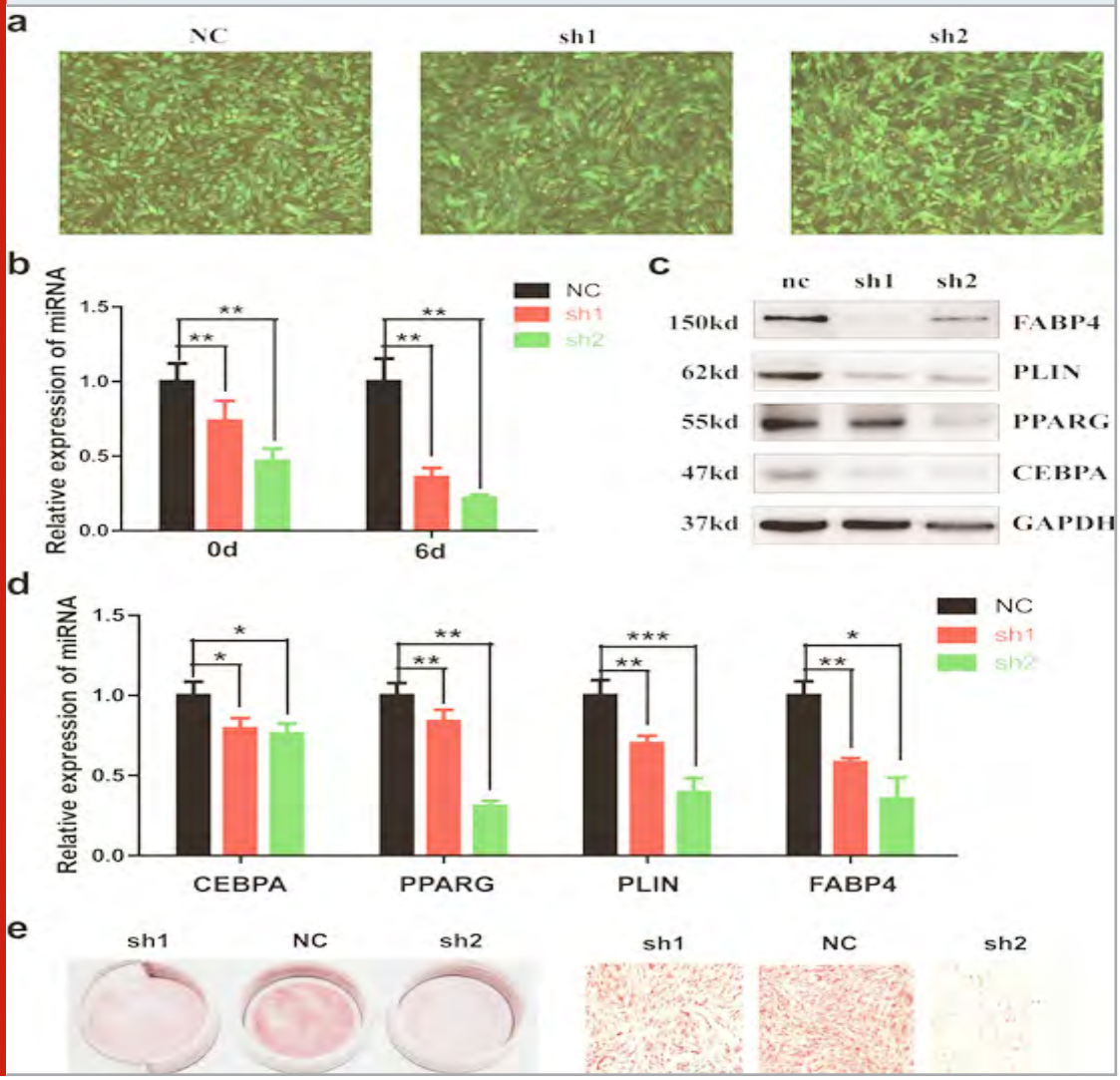
μg of protein from each lysate was separated by SDS-PAGE and transferred onto a PVDF membrane (0.22 μm, Millipore). The PVDF membrane was blocked in 5% nonfat milk in TBST for 1 h and then incubated with primary antibodies at 4 overnight, followed by incubation with horseradish peroxidase (HRP)-coupled second antibodies. The specific primary antibodies used were as follows: CEBPA polyclonal antibody (Abcam, #ab2295), PPARG polyclonal antibody (Cell Signaling Technology, #2443), PLIN polyclonal antibody (Abcam, #ab172907), FABP4 polyclonal antibody (Abcam, #ab92501), DKK1 polyclonal antibody (Abcam, #ab61275), β-catenin polyclonal antibody (Abcam, # ab32572) and GAPDH polyclonal antibody (Proteintech, # 60008-1-Ig).

Statistical analysis: All of the staining and western blot images were conducted in triplicate. GAPDH was used as an internal control in the qRT-PCR and western blot analyses. All of the numerical results are presented as the means± S.D from more than three experiments. The significant differences between groups were analyzed using Student's t-tests (two-tailed) and among multiple groups were assessed by one-way ANOVA. $P < 0.05$ was considered statistically significant, as indicated by asterisks (*P < 0.05, **P< 0.01, ***P< 0.001).

RESULTS AND DISCUSSION

lncRNA AC092834.1 was significantly upregulated during the adipogenic differentiation of hADSCs: HADSCs were cultured and characterized (Fig.S1). RNA-sequencing analyses and qRT-PCR analyses found the lncRNA

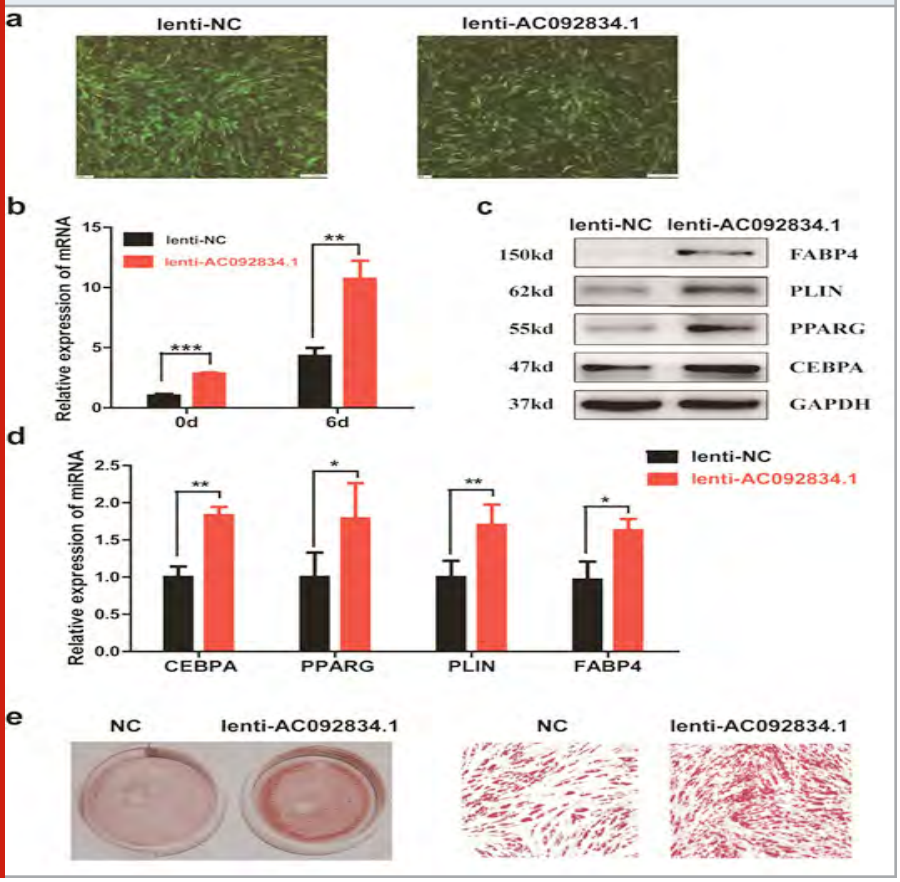
Figure 2: AC092834.1 knockdown inhibited the adipogenic differentiation of hADSCs.
a. lncRNA AC092834.1 was silenced using two independent shRNAs (sh1 and sh2). The knockdown efficiency was verified by qRT-PCR compared with the negative control (NC) on day 0 and day 6 of adipogenesis after lncRNA AC092834.1 knockdown. b., c. qRT-PCR and western blot analyses detected the mRNA and protein levels of adipogenesis transcription factors and marker genes CEBPA, PPARG, PLIN, and FABP4 after lncRNA AC092834.1 knockdown. d. Oil Red O staining was performed on day 12 of adipogenic differentiation to evaluate the efficiency of adipogenesis after lncRNA AC092834.1 knockdown.



AC092834.1 (UCSC ID: uc062vpi.1, chr4:23,779,590-23,782,560) was significantly upregulated during adipogenic differentiation of hADSCs.To identify the basic genome information of AC092834.1, the AC092834.1 genomic locus was checked by UCSC (http://genome.ucsc.edu) (Fig. 1a) and Ensemble database (http://www.ensembl.org/index.html) was utilized to obtain its transcriptional variants sequences (Fig. 1b). To further exclude its coding potential, the sequences of lncRNA AC092834.1 were input into the Coding Potential Assessment Tool (CPAT) (http://lilab.research.bcm.edu/cpat/) program to predict its potential to code proteins and the results showed that AC092834.1 has no protein coding probability (Fig. 1c). qRT-PCR analyses detected the expression levels of lncRNA AC092834.1 was significantly upregulated during the adipogenic differentiation of hADSCs (Fig. 1d). Since subcellular localization and cell fractionation are essential for understanding the function and mechanism of lncRNAs, we separated the cytoplasm and nuclear RNA by extraction and detected the AC092834.1 expression level by qRT-PCR analysis. The results showed that AC092834.1 largely displayed a nuclear distribution (>90%), which indicated that AC092834.1 might function at the transcription level (Fig. 1e).

Downregulation of lncRNA AC092834.1 inhibited the adipogenic differentiation of hADSCs: To study the function of lncRNA AC092834.1 during adipogenesis, we inhibited the endogenous expression of lncRNA AC092834.1 in hADSCs by two shRNA lentiviral vectors (sh1, sh2) and a negative control (NC) (Fig. 2a). Then, the qRT-PCR analysis confirmed that the intracellular lncRNA AC092834.1 mRNA levels were significantly downregulated on day 0 and day 6 of adipogenesis (Fig. 2b). qRT-PCR and western blot analyses revealed that when lncRNA AC092834.1 was downregulated, adipogenic transcription factors and marker genes CEBPA, PPARG, PLIN, and FABP4 were markedly decreased at both the mRNA and protein levels (Fig. 2c, 2d). Consistently, we also found knockdown of lncRNA AC092834.1 resulted in an adipogenesis delay, as shown by the decreased number of Oil Red O staining positive cells (Fig. 2e). These results suggested that lncRNA AC092834.1 plays an important role in adipogenesis of hADSCs.

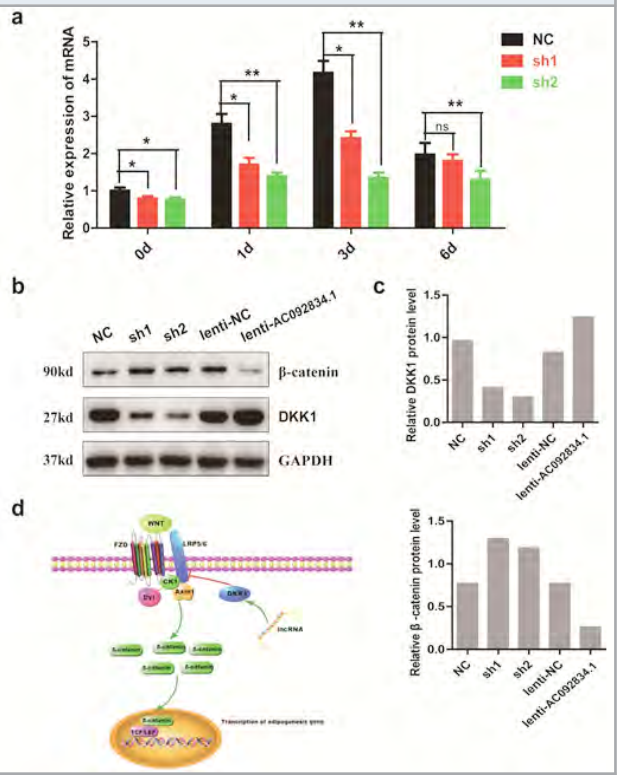
Figure 3: AC092834.1 promoted the adipogenic differentiation of hADSCs
a. LncRNA AC092834.1 overexpression lentiviral vectors (lenti-AC092834.1) were used to upregulate AC092834.1 expression. Images show GFP-positive cells under a fluorescence microscope, indicating that the cells were stably transfected. b. qRT-PCR analysis detected the mRNA level of lncRNA AC092834.1. c., d. qRT-PCR and western blot analysis detected the mRNA and protein levels of CEBPA, PPARG, PLIN, and FABP4. e. Oil Red O staining was performed to view the efficiency of adipogenesis.



Overexpression of lncRNA AC092834.1 facilitates adipogenesis of hADSCs: To further confirm the role of AC092834.1 in the differentiation of hADSCs to adipocytes, we overexpressed full length AC092834.1 in hADSCs using a lentivirus vector (Fig. 3a), and AC092834.1 expression was thus significantly upregulated on day 0 and day 6 of adipogenesis (Fig. 3b). Compared with the control, ectopic expression of AC092834.1 significantly impaired the expression of the adipogenic transcription factors and marker genes CEBPA, PPARG, PLIN, and FABP4, as verified by qRT-PCR and western blot analyses (Fig. 3c, 3d). Moreover, Oil Red O staining indicated that there were more lipid droplets after the treatments with the lentivirus vector, indicating more mature adipocytes and that the adipogenesis of hADSCs was accelerated (Fig. 3e). Altogether, these data demonstrate that AC092834.1 is a positive regulator in the differentiation of hADSCs towards adipocytes.

lncRNA AC092834.1 might induce DKK1 expression and antagonize the Wnt/β-catenin pathway: The observations from the AC092834.1 loss-of-function and gain-of-function studies suggested that AC092834.1 plays an important role in adipogenesis gene regulation. Wnt/β-catenin signaling is an important pathway that regulates adipogenesis, and by analyzing changes in important molecules, we found that DKK1 mRNA expression was significantly decreased during adipocytes differentiated from the AC092834.1 knockdown (Fig. 4a). DKK1 antagonizes WNT signaling by binding as high-affinity antagonists to LRP5/6 co-receptors that results in the degradation of cytosolic β-catenin. The results showed that the protein level of DKK1 was reduced while β-catenin was increased in AC092834.1 knockdown cells, and the contrary results were seen in AC092834.1 overexpression cells (Fig. 4b, 4c). These results demonstrated that AC092834.1 might promote DKK1 expression and thus more DKK1 was secreted extracellularly to competitively combine with LRP5/6 and block WNTs binding, subsequently inducing decreases in the β-catenin protein level. Suppression of the Wnt/β-catenin pathway promoted the transcription of adipogenic factors and thus facilitated the differentiation of hADSCs into adipocytes (Fig. 4d).

Figure 4: LncRNA AC092834.1 regulated the expression of DKK1 and β-catenin of the Wnt pathway.
a. qRT-PCR analysis of DKK1 mRNA expression in hADSCs that were transfected with siRNA or infected by lentiviral vectors. b. Western blot analysis of the protein level of DKK1 and β-catenin in hADSCs that were transfected with siRNA or infected by lentiviral vectors. c. Protein quantitative analysis of DKK1 and β-catenin by ImageJ software. d. Schematic diagram of the regulatory effects of AC092834.1 on hADSCs adipogenic differentiation.



Mesenchymal stem cells (MSCs) are multipotent cells that can differentiate into ectodermal, mesodermal and endodermal lineage cells, and have immune modulatory properties that could benefit patients with autoimmune diseases, (Ullah,et al., 2015). The multipotential capacities and therapeutic potential of hADSCs have prompted numerous clinical studies based on MSC with encouraging results reported to date[Le, et al., 2008]. MSCs have received increasing attention as a new target for obesity therapy, and the results suggested that obesity could be prevented by controlling MSC adipogenesis, (Matsushita and Dzau, 2017). However, understanding of the relationship between MSCs and obesity and its potential clinical implications remains ill defined, and further studies are necessary of the mechanisms, regulation and outcomes of adipogenesis crucial for MSC-based treatments for obesity. Recently, some studies proved that long noncoding RNAs have a significant and crucial role in the maintenance, commitment and differentiation of MSCs to adipocyte lineages by regulating the principal adipogenic transcription factors and signaling pathways, (Cai, et al., 2018). LncRNA ADINR has been proven to transcriptionally activate C/EBPα by specifically binding to PA1 and recruiting MLL3/4 histone methyl-transferase complexes to increase H3K4me3 and decrease H3K27me3 histone modification, (Xiao, et al., 2015).

The PPARγ -activator RBM14-associated lncRNA (Paral1) is restricted to adipocytes and is decreased in humans with an increased body mass index and in diet-induced or genetic mouse models of obesity. LncRNA Paral1 favors adipocyte differentiation and coactivates the master adipogenic regulator PPARγ, and thus it is considered an obesity-sensitive regulator of adipocyte differentiation and function, (Firmin, et al., 2017). To explore more lncRNAs that play important roles in adipogenic differentiation, we compared the lncRNA expression profiles of preadipocytes and hADSCs through high-throughput RNA-sequencing

and selected several lncRNAs that were significantly upregulated in preadipocytes. Among these lncRNAs, a novel lncRNA with the gene number AC092834.1 that was mainly located in the nucleus was found to have significantly upregulated expression. Further study suggested that AC092834.1 may be a positive regulator of adipogenesis progression and knockdown of AC092834.1 could significantly inhibit the expression of adipogenesis transcription factors CEBPα and PPARγ and adipocyte marker genes PLIN and FABP4, thus strongly repressing the adipogenic hADSCs process.

The Wnt/β-catenin signaling pathway is not only featured in multiple biological processes, such as embryonic development, inflammation, and stem cell differentiation (Marchetti,et al., 2020), but also recently emerged as an attractive negative regulator of adipocytes differentiation,(Jeon,et al.,2016). Studies demonstrated that activation of Wnt/β-catenin signaling prevents the induction of C/EBPα, C/EBPβ, and PPARγ transcription factors, resulting in the suppression of adipogenesis of hADSCs; however, blocking endogenous Wnt signaling promotes adipogenic differentiation, suggesting that Wnt acts as a brake for adipogenesis, (Chung,et al.,2012). The canonical Wnt ligands bind with specific cell surface Frizzled (FZD) receptors and low-density lipoprotein-receptor-related protein-5 or -6 (LRP5/6) coreceptors, and then combines with intracellular degradation complex Dishevelled (Dvl), Axin 1 and casein kinase I (CK1), which results in hypophosphorylation of β-catenin and its translocation to the nucleus where it binds to the lymphoid-enhancer-binding factor/T-cell-specific transcription factor (LEF/TCF) family of transcription factors to activate the PPARγ and CEBPα genes, (Nusse and Clevers, 2017).

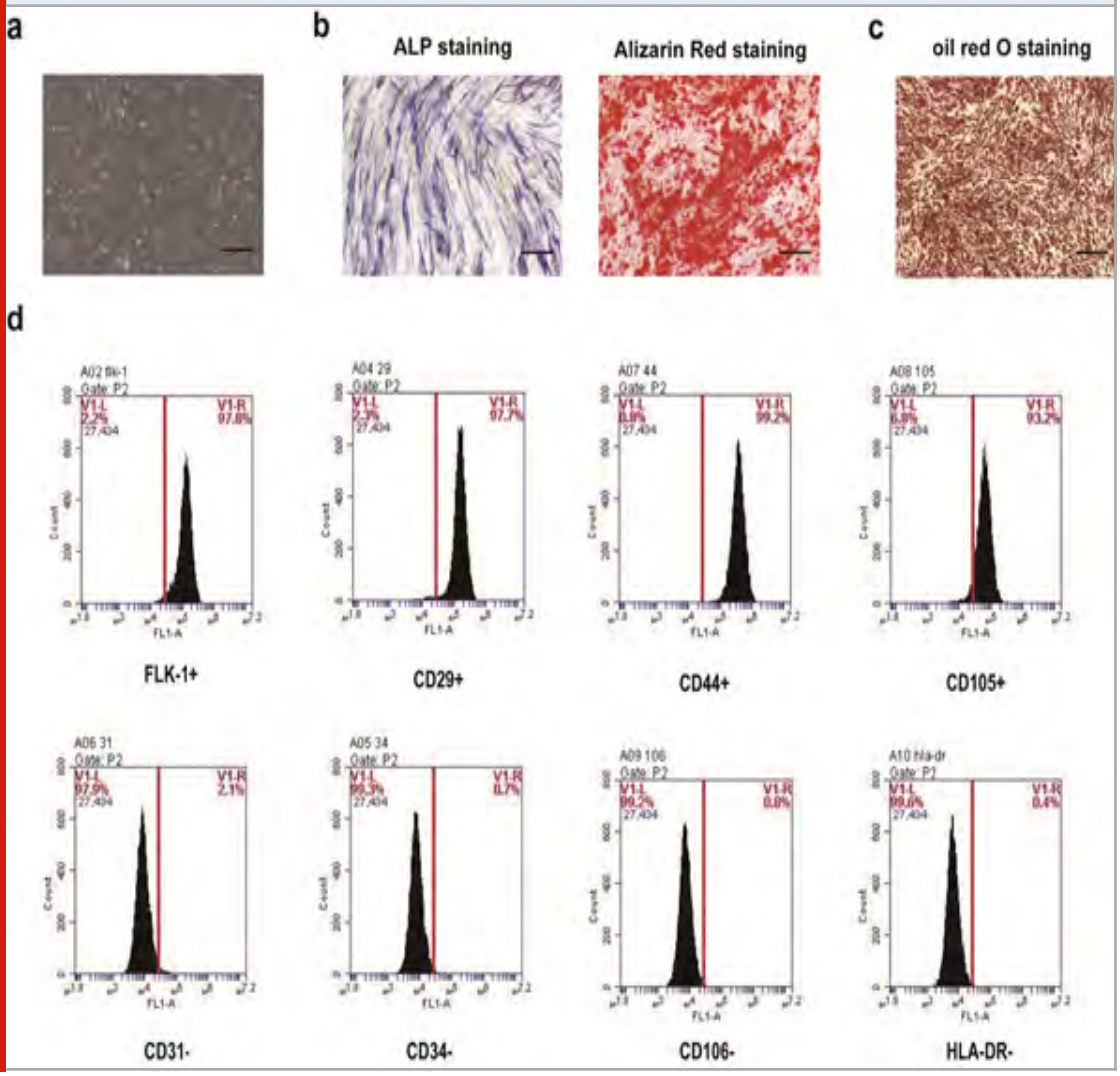


Figure S1: Identification and characterization of hADSCs.
a. The morphology of passage 3 of hADSCs under a light microscope. b. The early osteogenic differentiation was identified by ALP staining and Alizarin Red staining. c. The adipogenic differentiation of the hADSCs was confirmed by Oil Red O staining to detect the formation of lipid droplets in the cells. d. Flow cytometry assay detected cell surface marker expression of hADSCs. Scale bar: 100 μm.

Wnt signaling is modulated by several extracellular antagonists such as dickkopf-1 (DKK1), a newly recognized secreted glycoprotein, that has been demonstrated to inhibit WNT signaling by binding as a high-affinity antagonist to LRP5/6 co-receptors, resulting in the degradation of cytosolic β-catenin,(Rachner and Göbel, 2014). Thus, DKK-1 serves as a potential therapeutic target in many diseases such as cancer, diabetic nephropathy, and osteoporosis[Chae,et al.,2019]. Recently, DKK-1 has been found to promote early adipogenesis during human adipogenesis. Studies found that increasing DKK-1 expression during the early stage of adipogenesis and exogenous rhDKK-1 exposure accelerates differentiation by upregulating PPAR-γ and C/EBPα, (Lu, et al.,2016; Park,et al., 2008). In our study, we found that AC092834.1 could promote DKK1 expression and thus more DKK1 was secreted extracellularly to competitively combine with LRP5/6 and block Wnt binding, subsequently inducing β-catenin degradation leading to a decreased β-catenin protein level. This resulted in suppression of the Wnt/β-catenin pathway and facilitated adipogenic differentiation of hADSCs, which is consistent with the findings of previous studies.

In this study, we identified a novel lncRNA with functions in adipocyte physiology by comparing the expression patterns of lncRNAs before and after the adipocytes differentiation of hADSCs. We found that the expression of lncRNA AC092834.1 was strongly increased during adipogenesis, and for the first time, we demonstrated that lncRNA AC092834.1 acts as a positive regulator of adipogenic differentiation of hADSCs. Importantly, mechanistic analysis revealed that AC092834.1 increased DDK1 expression and subsequently competitively combined with LRP5/6 to accelerate β-catenin degradation, which suppressed the Wnt/β-catenin pathway and, thereby, impacted genes involved in the adipogenesis of hADSCs. This finding adds a new piece to the puzzle of the contribution of lncRNAs to the adipogenesis of hADSCs, and further identification of functional lncRNAs and cell type-specific signaling in adipogenesis and obesity regulation will help to expand the therapeutic repertoire to combat obesity and other adipogenic differentiation-related disorders.

ACKNOWLEDGEMENTS

The authors thank American Journal Experts for providing English language editing of the manuscript.

Conflicts of Interest: None

REFERENCES

Cai R, Sun Y, Qimuge N, Wang G, Wang Y, Chu G, Yu T, Yang G, Pang W. (2018) Adiponectin AS lncRNA inhibits adipogenesis by transferring from nucleus to cytoplasm and attenuating Adiponectin mRNA translation. Biochim Biophys Acta Mol Cell Biol Lipids. Apr;1863(4):420-432.

Chae WJ, Bothwell ALM. Dickkopf (2019) An immunomodulatory ligand and Wnt antagonist in pathological inflammation. Differentiation. Jun 12. pii: S0301-4681(19)30006-4

Chen Q, Shou P, Zheng C, Jiang M, Cao G, Yang Q, Cao J, Xie N, Velletri T, Zhang X, Xu C, Zhang L1, Yang H, Hou J, Wang Y, Shi Y. (2016) Fate decision of mesenchymal stem cells: adipocytes or osteoblasts? Cell Death Differ. 23(7):1128-1139.

Chung SS, Lee JS, Kim M, Ahn BY, Jung HS, Lee HM, Kim JW, Park KS. (2012) Regulation of Wnt/β-catenin signaling by CCAAT/enhancer binding protein β during adipogenesis,Obesity (Silver Spring). Mar;20(3):482-487.

Cohen P, Spiegelman BM. (2018) Cell biology of fat storage. Mol Biol Cell. 2016; 27,2523–2527.

Corrales P, Vidal-Puig A, Medina-Gómez G. PPARs and Metabolic Disorders Associated withChallenged Adipose Tissue Plasticity, Int J Mol Sci. 19, 2124-2140.

Fico A, Fiorenzano A, Pascale E, Patriarca EJ, Minchiotti G. (2019) Long non-coding RNA in stem cell pluripotency and lineage commitment: functions and evolutionary conservation. Cell Mol Life Sci. 76(8):1459–1471. doi:10.1007/s00018-018-3000-z

Firmin FF, Oger F, Gheeraert C, Dubois-Chevalier J, Vercoutter-Edouart AS, Alzaid F, Mazuy C1, Dehondt H, Alexandre J, Derudas B, Dhalluin Q, Ploton M, Berthier A, Woitrain E, Lefebvre T, Venteclef N, Pattou F, Staels B, Eeckhoute J, Lefebvre P. (2017) The RBM14/CoAA-interacting, long intergenic non-coding RNA Paral1 regulates adipogenesis and coactivates the nuclear receptor PPARγ. Sci Rep. Oct 26;7(1):14087-14102.

Hadrich F, Sayadi S. (2018) Apigetrin inhibits adipogenesis in 3T3-L1 cells by downregulating PPARγ and CEBP-α. Lipids Health Dis. 25;17(1):95

Jeon M, Rahman N, Kim YS. (2016) Wnt/β-catenin signaling plays a distinct role in methyl gallate-mediated inhibition of adipogenesis.BiochemBiophys Res Commun. Oct 7;479(1):22-7

Kopp F, Mendell J.T.. (2018) Functional Classification and Experimental Dissection of Long Noncoding RNAs. Cell. Jan 25;172(3):393-407.

Le Blanc K, Frassoni F, Ball L, Locatelli F, Roelofs H, Lewis I, Lanino E, Sundberg B, Bernardo ME, Remberger M, Dini G, Egeler RM, Bacigalupo A, Fibbe W, Ringdén O. (2008) Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. Lancet. May 10;371(9624):1579-1586.

Lu H, Li X, Mu P, Qian B, Jiang W, Zeng L. (2016) Dickkopf-1 promotes the differentiation and adipocytokines secretion via canonical Wnt signaling pathway in primary cultured human preadipocytes.Obes Res Clin Pract. 10(4):454-464.

Marchetti B, Tirolo C, L'Episcopo F, et al. (2020) Parkinson's disease, aging and adult neurogenesis: Wnt/β-catenin signalling as the key to unlock the mystery of endogenous brain repair [published online ahead of print, 2020 Feb 12]. Aging Cell. e13101. doi:10.1111/

acel.13101.
Martenstyn J, King M, R, (2020) Impact of weight loss interventions on patient-reported outcomes in overweight and obese adults with type 2 diabetes: a systematic review.J Behav Med. Feb 14. doi: 10.1007/s10865-020-00140-7
Nusse R, Clevers H. (2017) Wnt/β-Catenin Signaling, Disease, and Emerging Therapeutic Modalities,Cell. Jun 1;169(6):985-999.
Park JR, Jung JW, Lee YS, Kang KS. (2008) The roles of Wnt antagonists Dkk1 and sFRP4 during adipogenesis of human adipose tissue-derived mesenchymal stem cells,Cell Prolif. Dec;41(6):859-874.
Rachner TD, Göbel A2, Benad-Mehner P, Hofbauer LC, Rauner M. (2014) Dickkopf-1 as a mediator and novel target in malignant bone disease.Cancer Lett. May 1;346(2):172-177.
Rosen ED, Hsu CH, Wang X, Sakai S, Freeman MW, Gonzalez FJ, Spiegelman BM. (2002) C/EBPalpha induces adipogenesis through PPARgamma: a unified pathway. Genes Dev.,1;16(1):22-26.
Schmidt E, Dhaouadi I, Gaziano I, Oliverio M, Klemm P, Awazawa M, Mitterer G, Fernandez-Rebollo E, Pradas-Juni M, Wagner W, Hammerschmidt P, Loureiro R, Kiefer C, Hansmeier NR, Khani S, Bergami M, Heine M, Ntini E, Frommolt P, Zentis P, Ørom UA, Heeren J, Blüher M, Bilban M, Kornfeld JW. Linc (2018) RNA H19 protects from dietary obesity by constraining expression of monoallelic genes in brown fat. Nat Commun. Sep 6;9(1):3622-3637
Shulman, G.I. (2014) Ectopic fat in insulin resistance, dyslipidemia,and cardiometabolic disease. N Engl J Med, 371,1131–1141.
Sun, B., and Karin M.. Obesity, inflammation, and liver cancer. J Hepatol, (2012). 56, 704–713.
Ullah I, Subbarao RB, Rho GJ. (2015) Human mesenchymal stem cells – current trends and future prospective.Biosci Rep. Apr 28;35(2). pii: e00191. doi: 10.1042/BSR20150025.
Xiao T, Liu L, Li H, Sun Y, Luo H, Li T, Wang S, Dalton S, Zhao RC, Chen R. Long (2015) Noncoding RNA ADINR Regulates Adipogenesis by Transcriptionally Activating C/EBPα. Stem Cell Reports. Nov 10;5(5):856-865.
Yi F, Zhang P, Wang Y, Xu Y, Zhang Z, Ma W, Xu B, Xia Q, Du Q. (2019) Long non-coding RNA slincRAD functions in methylation regulation during the early stage of mouse adipogenesis. RNA Biol. Jun 19:1-13

Pharmaceutical Communication

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March Pp-10-14

In-vitro Anti-inflammatory and *in-silico* Anti-aging Properties of *Psidium guajava* Leaves

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ABSTRACT

The present study has been aimed to evaluate the anti-inflammatory property of *P. guajava* leaves by *in-vitro* using HRBC membrane stabilization method and anti-aging potential by *in-silico* method using AutoDock. The anti-inflammatory and anti-aging activity of leaf extracts of *Psidium guajava* collected from North Chennai region, India were evaluated in the present study. The *in-vitro* method showed significant anti-inflammatory property and anti-aging potential by binding with the target. The maximum membrane stabilization depicting the anti-inflammatory activity of *P. guajava* extracts was found to be 50% at a dose of 750 ug/ml. The effect of ascorbic acid from *P. guajava* leaves extract for preventing skin aging showing minimal binding energy for binding ligand (ascorbic acid) with the target protein (AP-1) was observed.

KEY WORDS: *PSIDIUM GUAJAVA*, ANTI-INFLAMMATORY, HRBC MEMBRANE STABILIZATION, ANTI-AGING, DOCKING.

INTRODUCTION

Medicinal plants have a key role in combating human health issues since the Stone Age. They act as restorative, defensive and supportive agents for human body. The World Health Organization (WHO) reports revealed that 80% of populations in Asian and African countries rely on traditional medicines for primary health care necessities (Kim et al., 2012). A pivotal role of plants in the health scenario is attributed to bioactive compounds, which could delay or inhibit the inception of degenerative diseases and increase life expectancy (Jagadish et al., 200, Lakkadi et al 2018, Korkina et al., 2018 Aleksandra et al 2020). Antioxidant medicinal plants, including phenolic and flavonoid are considered beneficial because of their protective actions in diseases as cancer. Phenol and flavonoids have been showed a wide range of biological activities (Bravo et al., 1998), including anticarcinogenic actions. Most of the beneficial health effects of flavonoids are attributed to their antioxidant and chelating abilities. Over product ion of reactive oxygen species (ROS) has shown to have detrimental effects on human health leading to cell/tissue damage and degenerative disorders such as inflammation, cardiovascular and neurogenic diseases, cancer, and aging related disorders.

ARTICLE INFORMATION

*Corresponding Author: jerrine.jj@gmail.com
Received 13th Jan 2020 Accepted after revision 16th March 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)

*Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/3*

Many reports suggest that ROS are principal mediators of apoptosis (Simbula et al., 2007, Korkina, et al., 2018 Aleksandra et al 2020). Antioxidants are added to food to slow the rate of oxidation and, if used properly, they can extend the length life of the food. ROS are produced by mitochondrial electron transfer processes and cytochrome P450 systems in hepatocytes (Robertaet al., 2005). Human hepatoma cell line (HepG2) is quite suitable for cytotoxicity evaluation due to the quality and stability of its enzymes and metabolic background (Ossenieet al., 2000). Many biological, chemical, and physical agents can generate inflammation with increased danger of human cancers (Nadia et al., 2016) many studies are currently going to develop inhibitors from medicinal plants to prevent or cure chronic inflammatory conditions for minimal side effects (Ashraf et al.,2016).

Among the numerous traditional medicinal herbs, *Psidium guajava* L. (Myrtaceae), commonly known as guava, has long been used in folk medicines as a therapeutic agent for the treatment of a number of diseases (Venkatachalam et al., 2012). The main constituents of *Psidium guajava* leaf extract are a variety of polyphenolics, flavonoids and triterpenoids, (Korkina et al., 2018 Aleksandra et al 2020). Plants have long been used in the cosmetic industry as amongst others, skin lighteners and sun-screen agents. Dietary and topical ascorbic acid have beneficial effects on skin cells, and some studies have shown that vitamin C may help prevent and treat ultraviolet (UV)-induced photo damage (Gulluce et al.,2007). The present study aimed to evaluate the traditional anti-inflammatory, anti-oxidant and anti-aging potential of this species.

MATERIALS AND METHODS

Sample collection and extraction: The *Psidium guajava* L. (Myrtaceae) plant leaves were collected from North Chennai region, India and were shade dried for 24 hours. The dried leaves were powdered and 25gm of the powdered leaves were subjected to soxhlet extraction using ethanol as the solvent.

Phytochemical screening: The ethanol extract and its fractions were tested by the *Liberman Burchard*, Lead acetate, Ferric chloride, Magnesium tracings, Vanillin sulphuric acid, Dragandroff's reagent, Millon's reagent and Liquid ammonia tests to determine the presence of steroids, phenolic compounds, tannin, flavonoids, saponins, alkaloids, proteins and anthraquinones respectively (Korkina et al., 2018).

Purification using thin layer chromatography: TLC plates were prepared by the application of a uniform layer of adsorbent (silica gel) on to 25mmX75mm glass slide. The plates were heated at 100 for 15 minutes to activate the silica gel. The sample is loaded on the plates leaving 1.5 cm from the bottom of the plates. The plates were inserted into the beaker containing the mobile phase (ethyl acetate and hexane). After the development of the chromatogram, the compounds were located and the retention factor for each compound was calculated using the following formula

Rf value = distance travelled by the sample/ distance travelled by the solvent.

Total Phenol Content: Total phenolic content was estimated by FolinCiocalteu's method. One milliliter of aliquots and standard gallic acid (100, 200, 300 µg/ml) was positioned into the test tubes and 5 ml of distilled water and 0.5 ml of Folin Ciocalteu's reagent was mixed and shaken. After 5 minutes, 1.5 ml of 20 % sodium carbonate was added and volume made up to 10 ml with distilled water. It was allowed to incubate for 2 hours at room temperature. Intense blue color was developed. After incubation, absorbance was measured at 750nm spectrophotometer. The blank was performed using reagent blank with solvent. Gallic acid was used as standard. The data for total phenolic contents of polyherbal formulation were expressed as mg of gallic acid equivalent weight (GAE) per 100gram of dry mass.

Antimicrobial Activity: Antibacterial activity was carried out by the disc diffusion method (Aliero et al., 2006). First, the different extracts of plant parts tested were dissolved in DMSO at a concentration of 100 mg/mL and filtered through 0.45 µm sterile filter membranes. Then, 100µL of bacterial inoculums containing 108 CFU/ml were spread over plates containing Mueller Hinton agar, and discs (6 mm in diameter) impregnated with 10 µL of the extracts (1 mg/disc) were placed on the surface of the media. Two control discs were used containing DMSO and Gentamicin (10 µg/ disc) as negative and positive controls, respectively. The plates were incubated for 24 h at 37 °C, and the experiments were performed in duplicate. The diameters of inhibition zones were measured and antibacterial activity was considered for diameters of inhibition zone greater than 9 mm. Antibacterial and Antifungal activities were determined using agar diffusion methods against gram positive bacteria (*Bacillus subtilis*), gram negative bacteria (*Escherichia coli*) and a fungal species *Aspergillus niger*. Nutrient agar medium was prepared and the organisms were separately inoculated in the respective petri plates. Different concentration of the sample ranging from 20-80µl were added to the disc prepared from Whatman filter paper. It was incubated 24 hrs for bacterial pathogens and 48hrs for fungal pathogen and the results were observed. The diameter of the zone of inhibition was measured (Dharmanda et al., 2003).

Anti-inflammatory Activity: The blood was collected from healthy human volunteers and mixed with equal volume of Alsever's solution (2% dextrose, 0.8% sodium citrate, 0.5% citric acid and 0.42% NaCl) and were centrifuged at 3,000 rpm. The packed cells were washed with iso saline and a 10% suspension was made. Various concentrations of extracts were prepared (250, 500 and 750 mcg/ml) using distilled water. To each concentration 1 ml of phosphate buffer, 2 ml hyposaline and 0.5 ml of HRBC suspension were added. It was incubated at 37°C

for 30min and centrifuged at 3,000rpm for 20min. The haemoglobin content of the supernatant was estimated spectrophotometrically at 560 nm. Diclofenac (50 mcg/ml) was used as reference standard and a control was prepared by omitting the extracts. The percentage inhibition of lysis was calculated as follows:% hemolysis= (OD of test sample/ OD of control) X100

% protection= 100 - [(OD of test sample/ OD of control) X 100]

Antioxidant Activity: The antioxidant activity of ethanol leaves extract was measured in terms of hydrogen donation or radical scavenging activity using the stable radical DPPH (2,2-diphenyl-1- picrylhydrazyl) assay. DPPH radical scavenging activity of the samples was estimated according to the methods of (Venkatachalam et al., 2012). Different concentration of samples (100 and 200 µl) and DPPH solution (200 µM) were prepared using methanol. DPPH solution was mixed with sample, and the reaction mixture was left to stand for 30 min at room temperature in the dark. The scavenging activity of samples was estimated by measuring the absorption of the mixture at 515nm, which reflects the amount of DPPH radical remaining in the solution. The percentage of antioxidant activity was calculated using the following formula.

% of antioxidant activity = [Abs (control) – abs (sample)] x 100/ Abs (control)

Anti-aging activity: 3D Structure of the target protein, AP-1 was retrieved from the protein data bank (PDB), with PDB ID of 1FOS. The DNA bound with the transcription factor was removed to prevent the interference during binding site prediction using Chimera software. The 3D structure of the active ingredient (Ascorbic Acid) are obtained from Pubchem in the SDF file format (*.sdf). A part from the active ingredient from natural source, 3D structure of SP100030, and a synthetic AP-1 inhibitor also retrieved which was used as a control. AutoDock is a suite of automated docking tools. It is predicted to design how small molecules, such as substrates or drug candidates, bind to a receptor of known 3D structure.

RESULTS AND DISCUSSION

Phytochemical methods could provide the needed preliminary observations necessary to select among crude extracts, those with potentially useful properties for further chemical and pharmacological investigations (Joseph et al., 2010). A large variety of phytochemicals that have been reported from natural product research has been proven successful as anticancerous agents (Androutsopoulo et al., 2008). Elucidation of the chemical structures of these compounds can lead to the synthesis of more potent drugs with minimal toxicity. Plant parts that contain tannins are astringent in nature and have important roles as stable and potent antioxidants (Díaz-de-Cerio et al., 2016).The present results of the phytochemical screening of the leaves of *Psidium guajava* L. revealed the presence of tannin, saponin, protein, steroids and phenol by positive reaction. Similarly, tannin, saponins, alkaloids, phenols, saponin, cardiac glycosides and carbohydrates found in the leaves of *Psidium guajava L* (Garode et al.,2014).

High performance liquid chromatography method has been validated to compare the ascorbic acid content in ethanolic extract of *Psidium guajava* leaves with the standard. The retention time is 4.728 min proved that ascorbic acid presence in the *P.guajava* leaves extracts. (Figure 1). Similarly (Rahmanet al., 2018) reported that, HPLC analysis of *P. guajava* leaves exhibited the presence of gallic acid, in a high amount.The phenolic content was estimated as 49 mg of gallic acid equivalent/ g of dry material at 200ml concentration of the sample. Similarly, (Weni et al.,2011) reported that the ethanol extract of *P. guajava* leaves showed 201 mg/g of phenolic content.

The extracts of Psidium guajava leaves showed potent antimicrobial activity against gram positive strains than gram negative strain and considerable activity against the fungal strain. As the concentration of the sample was increased, the radius of the zone of inhibition also increased (Table 1). Similarly,Harbone et al., (1984) showed the antibacterial activity of leaves extract of chloroform and ethanol of *Psidium guajava* L. against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and

Figure 1: HPLC Analysis of Sample (A) and Ascorbic acid (B)

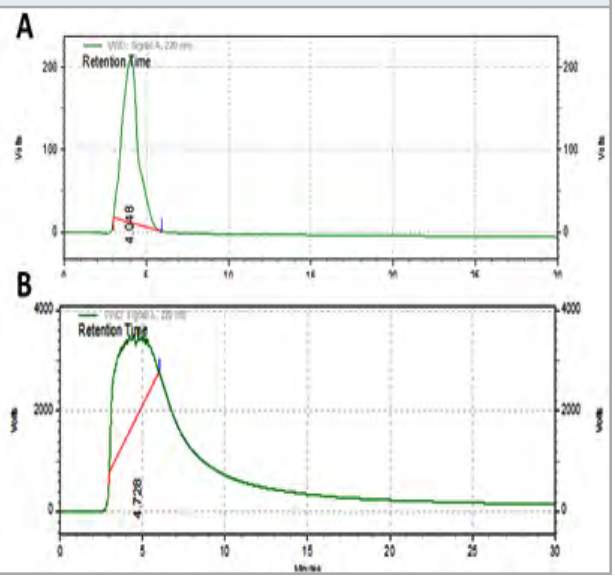


Table 1. Antimicrobial activity of *Psidium guajava* leaves

Concentration (µg/ml)	zone of inhibition (millimeter in diameter)		
	B. subtilis	*A. niger*	*E. coli*
20	7.5	7	5
40	12	15	8
60	15	30	12

Salmonella typhi. It was found that ethanol extract showed maximum activity against *S. typhi* and lowest activity against *S. aureus*. Inflammation is a normal protective response to tissue injury caused by physical trauma, noxious chemicals or microbiologic agents. Inflammation is body's response to inactivate or destroy the invading organisms, remove irritants and set stage for tissue repair.

Inflammation is triggered by the release of chemical mediators from the injured tissues and migrating cells. The specific chemical mediators vary with the type of inflammatory process and include amines such as histamine, serotonin, and lipids such as prostaglandins and small peptides such as kinins (Jiménez-Escriget al., 2001). The anti-inflammatory activity of ethanolic extract of Psidium guajava leaves carried out by HRBC stabilization method using diclofenac sodium as a standard revealed that the percentage of hemolysis decreases and the percentage of protection increases as the concentration of the sample increases (Table 2). Similarly, *P. guajava* extract reduced inhibitory percentage activities by 40.81, 55.45 and 43.61% ($p< 0.05$) respectively (SubbaRao et al., 2018). DPPH assay is considered as a valid method to evaluate scavenging activity of antioxidants, since the radical compound is very stable and do not have to generate as in other radical assays. DPPH radicals react with suitable reducing agents and then electrons become paired off and the solutions loses colourstoichiometrically with the number of electrons taken up such reactivity has been widely used to test the ability of plant extract to act as free radical scavengers. DPPH assay of ethanolic extract showed a dose dependent increase in the percentage of inhibition of free radicals (Lakkadi et al., 2013; Siwarungson et al., 2013).

Table 2: Anti-inflammatory activity

Concentration (µg/ml)	Hemolysis (%)	Protection (%)
250	76	24
500	56	44
750	50	50
Diclofenac (100)	8.8	91.2

Figure 2: DPPH free radical scavenging activity of ethanolic extract of *Psidiumguajava* leaves

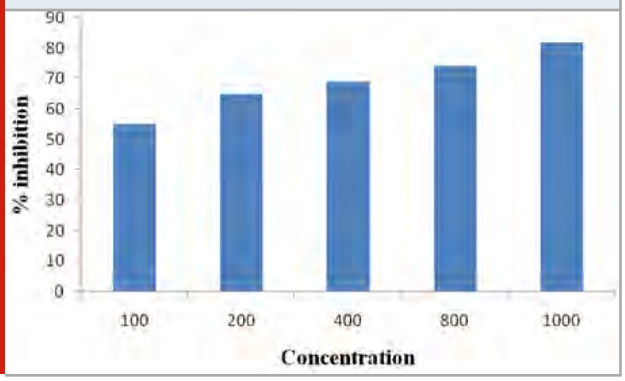
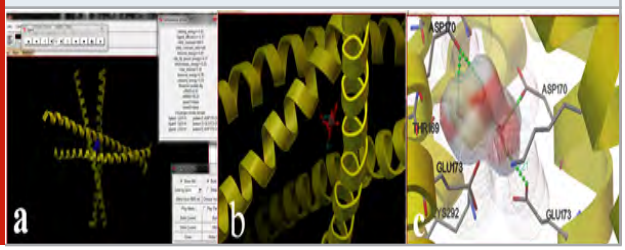


Figure 3: Binding of ascorbic acid with AP-1 (a) Closer view of binding (b) and Hydrogen bonding with amino acids (c)



The ethanolic extract fraction was found to show a good antioxidant potential.The extract of *Psidiumguajava* leaves exhibited strong antioxidant activities and it was observed that as the concentration of the sample increases, the OD value decreases which indicates the increase in the antioxidant activity (Figure 2). Antioxidants and anti-melanoagents with redox properties can prevent or delay skin pigmentation by scavenging reactive oxygen species and reactive nitrogen species, known to induce melanin synthesis. They can also reduce o-quinones and other intermediates in the melanin biosynthesis thus delaying oxidative polymerization. Antioxidants in particular can prevent skin aging and degeneration of cells.

It has often been noted that combinations of antioxidants and antimelanogents are more effective than one another acting independently. Thus high levels and quality of antioxidants and anti-melanogents, extracted from our suggested varieties of Thai Guava can not only be used as food supplements but also in cosmetic and pharmaceutical industries for developing products and drugs preventing skin aging and deterioration (Siwarungson et al., 2013). The docking procedure was carried out with the ligand ascorbic acid and the target protein AP-1. 10 conformations were obtained out of which the binding energy was found to be the minimum for 10th conformation. The binding energy was found to be -4.43 (Fig 8, 9, 10). Similarly Lakkadi et al., (2018) reported that docking results reviled that molecules 12, 14 and 15 are the best active antioxidants for Poly (ADP- ribose) polymerase.

CONCLUSION

Psidiumguajava leaves are easily available and very cheap with abundant medicinal values. Their medicinal property such as antimicrobial and anti-inflammatory property was increases as the sample concentration increases. It exhibits strong antioxidant property by scavenging the free radicals generated by UV exposure, oxidative stress, environmental conditions etc., since it exhibits antioxidant property, it may invariable have antiaging property in which it maintains the healthy and normal integrity of the skin there by protecting the collagen network and reducing photo aging damages.

Authors Contributions: All authors have equal contribution in bringing out this research work.

Conflict of Interest: None.

REFERENCES

Aleksandra, Aleksandrova , Mariia Nesterkina , Svitlana Gvozdii , Iryna Kravchenko (2020) Phytochemical analysis and anti-inflammatory activity of *Cladophora aegagropila* extract J Herbmed Pharmacol. 9(1): 81-85

Aliero, A.A. and Afolayan, A.J. (2006).Antimicrobial activity of *Solanum tomentosum*. African Journal of Biotechnology, 5(4), 369-372.

Androutsopoulos, V., Arroo, R.R., Hall, J.F., Surichan, S. and Potter, G.A. (2008).Antiproliferative and cytostatic effects of the natural product eupatorin on MDA-MB-468 human breast cancer cells due to CYP1-mediated metabolism.Breast Cancer Research, 10(3), R39.

Ashraf, A., Sarfraz, R.A., Rashid, M.A., Mahmood, A., Shahid, M. and Noor, N. (2016). Chemical composition, antioxidant, antitumor, anticancer and cytotoxic effects of Psidium guajava leaf extracts.
Pharmaceutical biology, 54(10), 1971-1981.

Bravo, L. (1998), Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. Nutrition Reviews, 56 (11), 317-333.

Dharmanda, S., (2003).Gallnuts and the uses of tannins in Chinese Medicine-A paper presented at the Institute for Traditional Medicine.Portlant, Oregon, 3, pp.941-945.

Díaz-de-Cerio, E., Gómez-Caravaca, A.M., Verardo, V., Fernández-Gutiérrez, A. and Segura-Carretero, A. (2016). Determination of guava (*Psidium guajava* L.) leaf phenolic compounds using HPLC-DAD-QTOF-MS. Journal of functional foods, 22, pp.376-388.

Garode,AM and Waghode, S.M. (2014) Antibacterial activity of guava leaves extracts against S. mutans. Int. J. Bioassays, 3 (2), 1794-1796.

Gulluce, M., Sahin, F., Sokmen, et al., (2007). Antimicrobial and antioxidant properties of the essential oils and methanol extract from *Mentha longifolia* L. *ssp. longifolia*. Food chemistry, 103(4), 3370 -3372

Harbone, JB., (1984) Phytochemical method-a guide to modern technique of plant analysis IInd ed. Champman and Hall, New York.:3-1

Jagadish, L.K., Krishnan, V.V., Shenbhagaraman, R. and Kaviyarasan, V. (2009).Comparitive study on the antioxidant, anticancer and antimicrobial property of *Agaricus bisporus* (JE Lange) Imbach before and after boiling.African Journal of Biotechnology, 8(4).654-661, 18.

Jimenez-Escrig, A., Rincon, M., Pulido, R. and Saura-Calixto, F. (2001).Guava fruit (*Psidium guajava* L.) as a new source of antioxidant dietary fiber. Journal of Agricultural and food Chemistry, 49(11), 5489-5493.

Joseph, B. and Priya, R.M. (2010). Preliminary phytochemicals of *Psidium guajava* L. leaf of methanol extract and its cytotoxic study on HeLa cell lines. Inventi Rapid: Ethnopharmacology, 1(2),1121-15

Kim, J.H., Gupta, S.C., Park, B., Yadav, V.R. and Aggarwal, B.B. (2012). Turmeric (Curcuma longa) inhibits inflammatory nuclear factor (NF)-kB and NF-kB-regulated gene products and induces death receptors leading to suppressed proliferation, induced chemosensitization, and suppressed osteoclastogenesis. Molecular nutrition & food research, 56(3),454-465.

Korkina, L., Kostyuk, V., Potapovich, A., Mayer, W., Talib, N. and De Luca, C. (2018). Secondary Plant Metabolites for Sun Protective Cosmetics: From Pre-Selection to Product Formulation. Cosmetics, 5(2), 32.

Lakkadi A, Vuppala S, Tigulla P. (2013) Novelin vitro antioxidant estimation of phenolic compounds and molecular modeling studies. International research journal of pharmacy 2. 148-152

Lakkadi, A., Vuppala, S. and Tigulla, P.(2018) Novel in vitro antioxidant estimation of phenolic compounds and molecular modeling studies.International Research Journal of Pharmacy. 4(9), 148-152.

Nadia L, Giovanna M. The molecular events behind ferulic acid mediated modulation of IL-6 expression in LPS-activated Raw 264.7 cells Immunobiology 2016; 221 (3). 486-493

Osseni, P. Rat, A. Bogdan, J.M. Warnet, Y. Touitou Evidence of prooxidant and antioxidant action of melatonin on human liver cell line HepG2 Life Sci., 68 (2000), pp. 387-399

Rahman, M.M., Zaman, S., Mamun, F., Gias, Z.T., Alam, M.N., Ulla, A., Hossain, M.H., Reza, H.M. and Alam, M.A. (2018). Phenolic content analysis in *Psidium guajava* leaves powder by HPLC-DAD system and in vivo renoprotective and antioxidant activities in fludrocortisone acetate-induced rats. Journal of food biochemistry, 42(6), 12687.

Roberta M, Roberta Di B, Rosaria V, Carmela F, Claudio G. Novel mechanisms of natural antioxidant compounds in biological systems: involvement of glutathione and glutathione-related enzymes The Journal of Nutritional Biochemistry.2005; 16(10) 577-586

Simbula G., Columbano A., Ledda-Columbano G.M., Sanna L., Deidda M., Diana A., Pibiri M. (2007).Increased ROS generation and p53 activation in α-lipoic acid-induced apoptosis of hepatoma cells. Apoptosis.12:113–123

Siwarungson, N., Ali, I. and Damsud, T. (2013).Comparative analysis of antioxidant and antimelanogenesis properties of three local guava (*Psidium guajava* L.) varieties of Thailand, via different extraction solvents. Journal of Food Measurement and Characterization, 7(4), 207-214.

SubbaRao Ch, Arun Kumar S, Javvad Ali, Priyadarshini P, Hinduja M, Keerthi R, Nivedita L, Priyanka D. In vitro antioxidant activity of *Psidium guajava* Linn.by using ethanolic extract fraction of leaves and bark. Adv Cell Sci Tissue Cult. 2018, 2 (1), 19-22.

Venkatachalam, R.N., Singh, K. and Marar, T. (2012). Phytochemical screening in vitro antioxidant activity of *Psidium guajava*.Free Radicals and Antioxidants, 2(1), pp.31-36.

Weni, L., Harliansyah, H. and Widayanti, W. (2011). Anti-inflammatory activity of the extract of guava leaves (*Psidium guajava* L) in the rat (*Rattus norvegicus* L). Indonesian Journal of Cancer Chemoprevention, 2(1), pp.169-172.

Ecological
Communication

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March Pp-15-22

BBRC
Bioscience Biotechnology
Research Communications

Productivity of Galega (*Galega orientalis*) in Single-Species and Binary Crops with Sainfoin (*Onobrychis arenaria*): A Case Study of Forest-Steppe of European Russia

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ABSTRACT

The results of field experiments on the cultivation of Eastern galega (*Galega orientalis Lam.*) in pure form and in binary sowings together with Hungarian sainfoin (*Onobrychis arenaria* (Kit.) DC) on eroded black soils of the south of European Russia were given. It is established that the culture of *O. arenaria* does not cause great suppression of *G. orientalis*. By year 4 of cultivation the aboveground dry phytomass of *G. orientalis* was 1012.3±58.1 g m-2 in binary sowings and did not differ significantly (at P=0.95) from phytomass in single-crop sowings (1078.5±114.0 g m-2). The phytocenosis of binary sowing of G. orientalis and O. arenaria has formed a more stable and productive phytocenosis for 2 cuts in total as compared to single-crop sowings, its component crops. On average for 2012-2015 a binary sowing of *G. orientalis + O. arenaria* had productivity of dry substance of aboveground phytomass of 1270.4 g m-2 (V=9.4%), a single-crop sowing of *G. orientalis* was 985.4 g m-2 (V=17.0%), and a single-crop sowing of *O. arenaria* 1121.0 g m-2 (V=21.7%). Binary sowings form aboveground phytomass in the first 2 years of the use by means of *O. arenaria*. At this time, its share in the phytocenosis is about 80.0%. Starting from the third year of life, the above-ground phytomass of phytocenosis is formed by *G. orientalis* whose share increases to 60.3% and reaches more than 80% by year 4 of the use (fifth year of life). It is noted that the weeds are most developed in single-crop sowings of *G. orientalis* during in the first 2 years of grass stands use. At this time, a share of weeds reaches 79.2-59.9% in the phytocenosis. Binary sowings are clogged much less. A share of weeds in the phytocenosis is within the range of 4.2-9.3% during the entire period of studies. It has been concluded that there is a need to cultivate *G. orientalis* in binary sowings together with Hungarian sainfoin in order to have success in the development of highly productive grass stands of this agricultural crop on the eroded black soils in the southern part of the European Russia.

KEY WORDS: *GALEGA ORIENTALIS, ONOBRYCHIS ARENARIA*, SLOPE ECOSYSTEMS, BINARY CROPS, OVER GROUND PHYTOMASS, PRODUCTIVITY, WEEDINESS CROPS.

ARTICLE INFORMATION

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Received 15th Feb 2020 Accepted after revision 28th March 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)
A Society of Science and Nature Publication, Bhopal India

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/4

INTRODUCTION

From ecological considerations, it is necessary to expand the species composition of cultivated legumes, including non-traditional culture for many regions of the world of *G. orientalis* (Adamovich, 2001; Baležentiene et al., 2011; Guo et al., 2013; Bushuyeva, 2014a). The culture of *G. orientalis* is of great scientific and practical interest. The main advantages of this crop are high longevity and productivity and high feed properties (Saloniemi and Kallela, 1993; Faireych et al., 2000; Skorko-Sajko et al., 2005; Wang et al., 2012; Bushuyeva, 2014b; Rymuza, 2017). In regions with developed animal husbandry under changing climatic conditions, it is necessary to adjust and revise the species composition of perennial grass sowings in order to stabilize grass cultivation productivity and to create uninterrupted green and raw material conveyor (Dumacheva et al., 2018; Cherniavskih et al., 2019 a,b). In addition, it is a honey plant. However, a deficiency of *G. orientalis* is also found, which is its slow development in the first years of life.

The sowings do not have required projective cover; therefore, there are conditions for the introduction of weed species, which reduces the quality of feed and its profitability (Bardule et al., 2013; Meripõld et al., 2017). One of the ways to overcome this disadvantage is to use common sowings of *G. orientalis* jointly with cereals and legumes (Bekuzarova et al., 2013; Rancane et al., 2014).There are studies conducted in relation to common sowings of *G. orientalis* together with timothy grass (*Phleum pratense L.*), smooth brome (*Bromopsis inermis Holub.*), meadow fescue (*Festuca pratensis Huds.*), reed fescue (*Festuca arundinacea Schreb.*) and cocksfoot grass (*Dactylis glomerata L.*). There is data available on the cultivation of it as part of a mixture with the following legumes: alfalfa *(Mediago)* and clover *(Trifolium)* are reflected in a number of publications (Bull et al., 2011; Povilaitis et al., 2016; Meripõld et al., 2017).

However, most studies are conducted in regions with sufficient rainfall, where there are favourable conditions for the development of grasses. There it is expedient to use cereal-legume mixtures with *G. orientalis*.The conditions of the south of the Central Russian Upland are characterized by unstable humidification, and with close occurrence of Cretaceous rocks (in stony and rocky substratum), plant communities are formed in association with xerophytic plants of stressed conditions (Arora and Meena, 2016). Varieties of perennial grasses are poorer under such conditions; cereal grasses do not have high productivity and suffer from droughts. At the end of the 20th and beginning of the 21st centuries, a slight negative trend in precipitation changes is noted in the region (Novikova et al., 2017). The modern regional climate system is characterized by considerable variability in annual precipitation (V=21%), which fits into the rhythmic of 11-year cycles, which is determined by helio-climatic relations (Ivanov and Lisetskii, 1995). Climatic rhythm is refracted in the specifics of vegetation periods and forms the features of interannual variation in plant productivity (Lisetskii, 2007). Over the past 15 years, the overage rainfall factor was 0.87-0.94, which made it possible to regard the climatic situation as slightly arid.However, on eroded slopes, especially in the south-facing part, the deficiency of soil moisture is as a rule clearly manifested when compared with smooth lands, (Lebedeva et al., 2019).

As a forage and nectariferous crop the sainfoin is widespread in culture, especially on eroded slopes. Slopes with inclines of more than 5o occupy 8% of the area of the Central Chernozem region, a significant part of which is occupied by the Central Russian Upland. However, the Belgorod region has a more intense relief because slopes with 5 degrees or more cover 13.4% of the territory with soil cover erosion being about 60%. Agrogenic transformation of soils with the active development of erosion significantly changes the soil climate (Lisetskii, 2008). A significant proportion of slope soils occupied by pastures are heavily eroded due to previous use in ploughing. Therefore, post-agrogenic long-term fallow land has its own specifics both in the manifestation of autogenic succession (Lisetskii, 1998), and in soil evolution (Lisetskii et al., 2013), which distinguishes them from virgin slope soils.In the conditions of development of ecological agriculture for the breeding of large and small cattle (especially in goat husbandry) sainfoin has been again gaining importance in the modern world as a forage crop (James and PittsSinger, 2008; Dzyubenko and Abdushaeva, 2012; Vazhov et al., 2013; Dzyubenko, 2015).

There are no studies in joint sainfoin and galega sowings described in the literature, although it is of significant scientific and practical interest for several reasons:increased productive longevity of grass stands due to *G. orientalis* culture;possible high productivity per 1 year of grass stands use due to sainfoin culture;nearly the same quality of herbage (both cultures cause no distention in animals, are well eaten by the same species of animals, and being high-protein crops have roughly the same stem coarseness);simultaneous onset of mowing ripeness.However, it remains unclear whether it is possible to cultivate and to grow them jointly. The aim of the present studies is to look into formation dynamics of aboveground phytomass of Eastern galega in binary sowing with Hungarian sainfoin in comparison with pure sowings of these crops and to assess the potential productivity of these agrophytocenoses in the eroded black soils of southern part of Russia. The objectives of the study included an assessment of accumulation dynamics of aboveground phytomass dry substance, weed infestation of crops and evaluation of productivity of agrophytocenoses in general.

MATERIALS AND METHODS

Study area: South-western slope of the Central Russian Upland, which occupies about 14% of the area of the European part of Russia. The research was carried out in the Belgorod region, in the Krasnoyruzhsky district (47920 ha). The climatic conditions of the study area are characterized by the following indicators: sunshine

duration – about 1800 hours, total solar radiation reaches about 4000 MJ m-2, the average annual air temperature is 5.8 °C (in summer 18.4 °C, in winter -6.5 °C), frost-free period on average 157 days. The average annual rainfall is 560 mm, summer humidity averages 70%.

Weather conditions during the years of research (2012-2015) were at the level of long-term average.The studies were carried out as a stationary field experiment. Slope northeast exposure, slope 5-7°. The soil is typical moderately eroded black soil. Humus content – 3.9 %, P_2O_5 – 108 mg kg^{-1} (by Chirikov), K_2O – 106 mg kg^{-1} (by Chirikov), the content of easily hydrolyzable nitrogen before laying experience 140 mg kg^{-1}, pH HCl 6.0. The experiment investigated Eastern galega *(Galega orientalis Lam.)*, Gale variety, and Hungarian sainfoin *(Onobrychis arenaria (Kit.)* DC), Zernogradskiy grade.

Experiment options

1. Pure sowing of sainfoin (sowing rate of 5 million germinating seeds per 1 ha).
2. Pure sowing of galega (sowing rate of 2 million germinating seeds per 1 ha).
3. Binary sowing sainfoin and galega (sowing rate of sainfoin 5 million viable seeds per 1 ha, galega – 2 million germinating seeds per 1 ha).

The method of experiment establishment – organized replications. Total plot area 12 m2. The replication is sixfold. The crops were sown in spring 2011 under the cover of mustard seeds (mustard sowing rate of 6 kg per 1 ha). The planting method - line one with 25 cm inter-row spacing. A combined seeding of galega and sainfoin was carried out crosswise at an angle of 15 cm. Mixed samples for soil analyses were taken by a sampler at 10 places (0-10 cm) on the plot and then prepared a combined sample. Mixed samples were prepared from each of the six plots. The soil was brought to air-dried condition, ground down and analysed. The biological repetition for all indicators is 2-fold and the total one is 12-fold.The productivity of aboveground phytomass (in a green form) was determined by mowing method 2 times a season during the budding phase. Land plot 4 m2. The replication is 6-fold.The proportion of each component and weed vegetation was determined using the method of sheaf analysis. Cut off the aerial mass from an area of 1 m2 and separately weighed the stems of crops and weeds. To determine the content of absolutely dry substance a 1.5-2 kg sample was taken from the total mass and then brought to air-dried condition in gauze bags. The air-dry mass was completely crushed to powder in the mill. From the mass obtained, 50-60 g samples were taken out on a four-time repeated basis and dried completely in a thermostat at a temperature of 105-106 °C within 8 hours.

The dry matter content (percentage) was calculated for each repetition and the average value was determined. The results were statistically processed using single-factor and two-factor analysis-of-variance methods for field experiments (Dospekhov, 1985; Lakin, 1990). We determined the reliability of differences, the least significant difference and the share of influence by the studied factors on the resultant feature. To estimate how close the relations were a pair correlation coefficient was used.

RESULTS AND DISCUSSION

The theoretical basics of the formation process of the combined crops productivity in comparison with single-crop sowings were justified by the H.G. Tooming works (1972, 1984). The principles of time sequence of growth, component development and maximum use of photosynthetically active radiation (PAR) make the basis for sustainable growth and development of combined plantings. Consistent co-growth and stability of phytocenosis can be achieved subject to concurrent fulfilment of two conditions. Firstly, phytocenosis should consist of species, which have different specific radiation intensity (SRI) (SRI means flux density PAR to achieve maximum gas exchange efficiency factor (CP)). Secondly, the maximum efficiency factors of these species should be close (Tooming, 1984).Thus, to ensure a high degree of use of solar radiation by the entire planting, the crops, which have been included in the grass mixture with similar biological and abiotic planting factors, should be different in SRI and have very close efficiency factors (Tooming, 1984).

As regard, our experience of *G. orientalis* Hungarian sainfoin can be the most complementary related culture in grass mixtures. This conclusion is based on four arguments. First, the architectonics of sainfoin leaves provides for significant penetration of photosynthetic active radiation deep into the grass stand, which can greatly reduce competition for the light. Secondly, sainfoin generates up to 80% of its first mowing yield. This makes lighted any crop planted in the second half of the summer, which practically excludes competition for the light. Thirdly, sainfoin has a tap-root system, which is located in deeper soil horizons as compared to G. orientalis's sprouted surface root system, which can diminish competition for nutrients and root secretions allelopathy. Fourthly, sainfoin can form primary above-ground phytomass in 1 to 2 years of the use of grass stands. In subsequent years, it can dramatically decrease of productivity, which significantly reduces its competitiveness.

Architectonics and structure of the *G. orientalis* bush can be considered ideal from the point of view of the rational use of photosynthetic active radiation since the upper leaves of the plant are almost vertical and they gradually move to a horizontal position at the bottom of the stem (Tammers, 1970). Sainfoin plants have similar leaf arrangement. Thus, one can be assume that these two species will minimally compete for the light, and due to this, the intensity of the main form of competition for photosynthetic active radiation will be lower for mixed sowing.The result should be ended in phytocenosis with sainfoin being dominated at stage 1 and without strong suppression of galega. By the time when less longeval

sainfoin plant has fallen out of grass stand, the galega will have already reached its maximum development and further form its own multi-year grass stand.

The key efficiency indicator for the use of the environment by plants is their ability to form phytomass. The higher competition the species face from their neighbours, the lower their ability to accumulate the above-ground phyto mass. The research results have shown that in single-crop sowings with significant level of probability being equal to P=0.95 in the first 3 years of vegetation the G. orientalis plants exceeded in terms of above-ground phytomass amount the first mowing of plants grown in a mixture with Hungarian sainfoin. By year, four of the use of grass stands the differences used to become unreliable. In the second mowing significant differences in the productivity of galega in pure form and in a mixture with sainfoin were established at the probability level of P=0.95 for years 2 and 3 of the use of grass stands. No differences were found by the fourth year of use (Table 1).

Table 1: Accumulation of aerial phytomass of *G. orientalis* culture in single-species and binary crops with two mowing of grass stands, g m^{-2} (absolutely dry matter)

Mowing	Sowing Method	Year of research				
		2012	2013	2014	2015	2012-2015
First	single-species	145.0	235.8	512.5	639.0	383.1
	binary	124.6	193.1	402.9	609.7	332.6
	average	134.8*	214.4*	457.7*	624.4	357.8*
	LSD_{05}	15.4	24.8	41.7	42.6	38.6
Second	single-species	27.8	90.2	313.5	439.5	217.8
	binary	24.7	74.9	260.4	402.6	190.6
	average	26.3	82.6*	287.0*	421.1	204.2*
	LSD_{05}	4.4	11.4	33.6	55.2	26.2
Just two mowing	single-species	172.8	326.0	826.0	1078.5	600.8
	binary	149.3	268.0	663.3	1012.3	523.2
	average	161.1*	297.0*	744.7*	1045.4	562.0*
	LSD_{05}	6.9	23.8	47.3	86.0	41.0

Note: * differences between single-species and binary crops are significant at P=0.95.

In general, a similar tendency was noted for two mowing. The reliability of differences at the level of P=0.95 was ascertained in the course of the first 3 years of research. By year 4 of life, the differences were bridged.The productivity of the second mowing of *G. orientalis*, when cultivated in pure form, was 19.1% of the value of the first mowing in 2012 and 68% in 2015. When cultivated in binary sowing, its value was 19% in 2012 and 66.0% in 2015 respectively. The reverse trend was observed for sainfoin sowings. The largest aboveground phytomass was formed in the first 2 years of grass strand use with a sharp decrease by year 3 and 4 of life. The sainfoin plants had much higher decreased in their productivity for binary sowings in competition with to galega. Except for the first mowing of year 1 of life (2012), in all other cases a significant decrease in the productivity of this crop was established for binary sowings at the level of P=0.95 as compared with single-crop planting (Table 2).

Table 2. Accumulation of aerial phytomass of *O. arenaria* culture in single-species and binary crops with two mowing of grass stands, g m^{-2} (absolutely dry matter)

Mowing	Sowing Method	Year of research				
		2012	2013	2014	2015	2012-2015
First	single-species	750.6	818.3	349.3	293.5	552.9
	binary	711.4	650.8	220.7	130.4	428.3
	average	731.0	734.6*	285.0*	212.0*	490.6*
	LSD_{05}	41.8	38.9	29.8	15.1	30.2
Second	single-species	467.9	527.7	145.0	112.8	313.4
	binary	382.6	430.0	113.8	48.1	243.6
	average	425.3*	478.9*	129.4*	80.5*	278.5*
	LSD_{05}	40.9	32.2	23.4	19.5	29.1
Just two mowing	single-species	1218.5	1346.0	494.3	406.3	866.3
	binary	1094.0	1080.8	334.5	178.5	671.9
	average	1156.3*	1213.4*	414.4*	292.4*	769.1*
	LSD_{05}	49.1	37.8	27.1	27.2	35.2

Note: * differences between single-species and binary crops are significant at P=0.95.

Table 3. The share of the influence of the studied factors on the total variability of the productive trait "magnitude of the aboveground phytomass" of *G. orientalis* and *O. arenaria* when cultivated in clean and binary crops (for a total of two mowings), %

Source of variation	Years of research			
	2012	2013	2014	2015
Reiteration	0.48	0.42	5.21	2.13
Random errors	0.35	0.08	1.28	0.99
Options, including	99.17*	99.50*	93.51*	96.88*
Factor A – "Crop"	98.90*	95.33*	78.40*	92.30*
Factor B – "method of sowing"	0.25	2.96	14.10*	3.52
Factor AB - Interaction	0.02	1.21	1.01	1.06

Note: * differences are significant at P=0.95.

To study the extent of influence of such key organized factors as A – "crop" *(G. orientalis* and *O. arenaria)*, B – "method of sowing" (single-crop and binary) and the "yearly conditions" factor in the total variation of resultant variable "overground phytomass size" we used practical two-way analysis of variance. An analysis of the data obtained over 4 years of research showed that the share of influence by the studied factors in the total variability is from 89.2% in 2014, up to 99.2% in 2012. The share of influence in the total variability for the resultant variable "aboveground phytomass size" was equal to 78.4% in 2014, up to 98.9 in 2012 for two mowing by the key factor A – "crop", and it has always been reliable at the level of P=0.95. The share of influence by the factor B – "method of sowing" varied from 0.25% in 2012, up to 14.1% in 2014, and it has not been always reliable at the level of P=0.95. The share of influence by interrelated factors AB is unreliable (Table 3).

The Table 3 shows that the year of 2014 is noted for significant share of influence by factor B – "method of sowing". This is year 3 of grass stands use when the maximum level of mutual competition among sainfoin, galega and weeds was observed. At that, time the sainfoin crop already started to fall out of the grass stand and the galega crop did not have sufficient mass yet. There was competition emerged both inter-crops and between the weeds and the crop.An analysis of the share of influence by the studied factors in the total variability of the resultant variable "aboveground phytomass size" (by 2014) has showed that in the first mowing the studied factors made 89.25%, and the share of repetitions was 10.36%. In the total variance of variants the share of the studied factor A "crop" was reliably (at the level of P=0.95) equal to 60.36%, and the share of the factor B – "method of sowing" was 28.7%. The share of interaction was negligible. This goes to prove that to the first cut 3 years of life the processes of the most intense competition in the genotype-environment system occurred when the influence of the environment became significantly high. y the second mowing, the process was stabilized.

The share of influence by the factor decreased to 5.87% and the factor's A share increased up to 82.3%. Subsequently, the galega crop finally began to dominate in joint sowings and dynamically increased its vegetative mass.An analysis of the total vegetative mass of phytocenoses showed that the binary sowing formed a more stable phytocenosis over two mowing in total as compared to single-crop sowings. In the first 2 years of the use of grass stands a single-crop sowing of *O. arenaria* and a binary sowing of *G. orientalis + O. arenaria* significantly exceeded a single-crop sowing of *G. orientalis* at 456.9-620.4 g m-2 in terms of above-ground phytomass, thus not differing significantly from each other by this indicator (Table 4).

The galega sowings and a binary planting under the conditions of year 3 and 4 of the use significantly exceeded the Hungaring sainfoin sowings in terms amount of dry substance of above-ground phytomass. The coefficient of variation of above-ground phytomass of binary phytocenosis of *G. orientalis + O. arenaria* was 9.4% on the average for the research years and that of pure *G. orientalis* – 17.0%, and *O. arenaria* – 21.7%. Over an average of 4 years of studies that monospecific sainfoin sowings and binary plantings are significantly superior to the single-crop sowing of *G. orientalis* by above-ground phytomass size. The amount of aboveground phytomass of monospecific galega sowing and its binary planting together with sainfoin by year 4 of the use did not differ significantly.The share of the main phytocenosis components in the total phytocenosis productivity was analysed (Table 5). In general, based on the experience available it has been found that the share of Galega tends to increase in grass stands and its sowings, both single-crop and binary ones tend to be less infested as the grass stand becomes older.There was a tendency found towards decreased productivity of sainfoin and its share in the phytocenosis as the grass stand became older and towards increased sainfoin infestation.

Table 4: Accumulation of aboveground phytomass by *G. orientalis* and *O. arenaria* phytocenoses in (taking into account weed vegetation) in single-species and binary crops with two mowing of grass stands, g m^{-2} (absolutely dry matter)

Crops	Years of research				
	2012	2013	2014	2015	2012–2015
G. orientalis	832.2*	812.5*	1092.1	1204.6	985.4*
O. arenaria	1289.1	1428.7	863.7*	902.5*	1121.0*
G. orientalis + O. arenaria	1305.6	1432.9	1099.8	1243.1	1270.4
LSD_{05}	38.7	21.5	38.1	49.3	38.6

Note: * differences are significant at P=0.95.

During the entire research period the binary sowings of *G. orientalis* and *O. arenaria* were significantly infested to a lower extent as compared to the pure sowings of G. orientalis at the level of P=0.95. In the first 2 years single-crop sowings of sainfoin did not differ significantly by the level of infestation from binary plantings (P=0.95). Starting from year 3 of life the sowings became more infested as the main crop was gradually failed and the proportion of weeds reached 42.8-55.0%.

We have established that any mixed sowing consistently tends to have a reduced share of sainfoin in the aboveground phytomass from 83.8 to 14.4% and an increased share of galega from 11.4 to 81.4% over 4 years of joint cultivation against the background of a stable proportion of weeds being at the level of 4.2-9.3%. One can observe an actual succession change of plant community dominant when one legume crop gradually replaces another, and the share of the legume component (cultural component of the community) remains stable at 90.7-95% (V=2.1%).

Table 5. Percentage of participation of various components in the phytomass structure of phytocenoses of single-species and binary crops of *G. orientalis* and *O. arenaria* (an average of two mowing), %

Crops	Phytocenosis components	Years of research				
		2012	2013	2014	2015	2012-2015
G. orientalis	crop	20.8*	40.1*	75.6	89.5	56.5
	weeds	79.2*	59.9*	24.4	10.5	43.5
O. arenaria	crop	94.5	94.2	57.2*	45.0*	72.7
	weeds	5.5	5.8	42.8*	55.0*	27.3
G. orientalis + *O. arenaria*	crop of everything, including:	95.2	94.1	90.7	94.8	94.0
	G. orientalis	11.4	18.7	60.3*	81.4*	43.0
	O. arenaria	83.8*	75.4*	30.4	14.4	51.0
	weeds	4.8	5.9	9.3	4.2	6.0
LSD_{05}	crop	2.2	3.0	3.6	3.1	3.0
	G. orientalis	1.2	1.4	1.8	3.0	1.9
	O. arenaria	2.2	3.8	2.4	1.8	2.5
	weeds	1.3	1.3	1.9	1.7	1.6

Note: * differences are significant at P=0.95.

The coefficient of variation of the cultural component in the single species crops of *G. orientalis* and *O. arenaria* was higher, 61.0% and 48.4%, respectively. In general, based on the experiment available we have found a weak negative correlation between the value of absolutely dry substance in *G. orientalis* phytomass and the value of absolutely dry substance in *O. arenaria* phytomass as regards binary sowings (r= – 0.318±0.128), there is also moderate negative correlation between the value of *G. orientalis* phytomass and the value of phytomass of weeds' absolutely dry substance in plantings (r= – 0.534±0.130).

CONCLUSION

To create of sustainable high-yielding grass stands with *G. orientalis* in the south of European Russia it is necessary to have its binary sowings with *O. arenaria*. In joint sowings, the *O. arenaria* crop causes no strong suppression of *G. orientalis*. In binary sowings, the productivity of *G. orientalis* above-ground phytomass has weak dependence on the value of *O. arenaria* aboveground phytomass, which results in favourable conditions for their joint growth in grass mixtures. When being used on a double-cutting basis the binary sowings of *G. orientalis* and *O. arenaria,* tend to form more productive and stable phytocenosis as compared to single-crop plantings. Binary sowings can ensure stable generation of above-ground phytomass in the first two years of the use by means of O. arenaria. During this period, it is part of the cultural part of 83.0% phytocenosis. Starting from year 3 of life the above-ground phytomass of phytocenosis is formed by *G. orientalis* whose share increases up to 60.3% and reaches > 80% by year 4 of the use (fifth year of life). It is noted that the weeds are most developed in single-crop sowings of *G. orientalis* during in the first 2 years of grass stands use. At this time, a share of weeds reaches 79.2-59.9% in the phytocenosis. Binary sowings are clogged much less. A share of weeds in the phytocenosis is within the range of 4.2-9.3% during the entire period of studies. In single-crop, sowings of *O. arenaria* weeds have been noted to spread most widely in year 3 to 4 of the use of grass stands and is 42.8-55.0%.

ACKNOWLEDGEMENTS

The study was supported within the intra-university grant of Belgorod State National Research University to support the creation and development of scientific departments – centres of excellence.

REFERENCES

Adamovich, A.M. (2001). Productivity and yield quality of fodder galega (*Galega orientalis Lam.*). Grass mixed swards. In: Horst, W.J. et al. (eds). Plant Nutrition. Developments in Plant and Soil Sciences, 92: 1008–1009.

Arora, S. and Meena, S. (2016). Morphological screening of endangered medicinal plants of milkweed family from Thar Desert, Rajasthan, India. Bioscience Biotechnology Research Communications, 9(3): 406–414. https://doi.org/10.21786/bbrc/9.3/10

Baležentiene, L. and Spruogis, V. (2011). Experience of

fodder galega *(Galega orientalis Lam.)* and traditional fodder grasses use for forage production in organic farm. Veterinarija ir Zootechnika, 56(78): 19–26.

Bardule, A., Rancane, S., Gutmane, I., Berzins, P., Stesele, V., Lazdina, D. and Bardulis, A. (2013). The effect of fertiliser type on hybrid aspen increment and seed yield of perennial grass cultivated in the agroforestry system. Agronomy Research, 11(1): 13–24.

Bekuzarova, S.A., Gassiev, V.I., Sokolova, L.B. and Tzugkieva, V.B. (2013). Binary Mixtures Of Galega orientalis. News of the Gorsky State Agrarian University, 50(2): 21–26.

Bull, I., Gienapp, C., Wiedow, D. and Burgstaler, J. (2011). View Correspondence (jump link) *Galega orientalis* – A new persistant plant as fodder crop and substrate for biogas production. Journal fur Kulturpflanzen, 63(12): 423–429.

Bushuyeva, V.I. (2014a). Ecological Consequences of Increasing Crop Productivity: Plant Breeding and Biotic Diversity. Ecological Consequences of Increasing, 277–286.

Bushuyeva, V.I. (2014b). The effectiveness of inoculation of the seeds of *Galega orientalis* with microbial agents Vogal and Rhizophos. Chemistry and Chemical Biology: Methodologies and Applications, 173–193.

Cherniavskih, V.I., Dumacheva, E.V., Sidelnikov, N.I., Lisetskii, F.N. and Gagieva, L.Ch. (2019 a). Use of Hissopus officinalis L. Culture for Phytomelioration of Carbonate Outcrops of Anthropogenic Origin the South of European Russia. Indian Journal of Ecology, 46(2). URL: http://dspace.bsu.edu.ru/handle/123456789/27386

Cherniavskih, V.I., Sidelnikov, N.I., Dumacheva, E.V., Borodaeva, Zh.A., Glubsheva, T.N., Gorbacheva, A.A., Vorobyova, O.V. and Korolkova, S. (2019 b). Biological resources of natural forage grassland of the cretaceous south of the European Russia. EurAsian Journal of BioSciences, 13: 845–849.

Dospekhov, B.A. (1985). Methodology of field experience: (With the basics of statistical processing of research results). Moscow, Kolos.

Dumacheva, E.V., Cherniavskih, V.I., Gorbacheva, A.A., Vorobyova, O.V., Borodaeva, Z.A., Bespalova, N.E. and Ermakova, L.R. (2018). Biological resources of the Fabaceae family in the Cretaceous south of Russia as a source of starting material for drought-resistance selection. International Journal of Green Pharmacy, 12(2): 354–358.

Dzyubenko, N.I. (2015). Genetic resources of cultivated plants as the basis for Russia's food and environmental security. Herald of the Russian Academy of Sciences, 85(1): 15–19.

Dzyubenko, N.I. and Abdushaeva, Ya.İ. (2012). Adaptation of the American Ecotypes of Onobrychis arenaria (Kit.). Ser. in the Novgorod Region. Agricultural Biology, 47(4): 106–112.

Fairey, N.A., Lefkovitch, L.P., Coulman, B.E., Fairey, D.T., Kunelius, T., McKenzie, D.B., Michaud, R. and Thomas, W.G. (2000). Cross-Canada comparison of the productivity of fodder galega (*Galega orientalis Lam.*) with traditional herbage legumes. Can. J. Plant Sci., 80: 793–800.

Ivanov, I.V. and Lisetskii, F.N. (1995). Manycentury periodicity of solar-activity and soil formation. Biofizika, 40(4): 905–910.

James, R.R. and PittsSinger, T.L. (2008). Bee pollination in agricultural ecosystems. New York, Oxford University Press.

Lakin, G.F. (1990). Biometry. Moskow, Higher School.

Lebedeva, M.G., Lupo, A.R., Chendev, Y.G., Krymskaya, O.V. and Solovyev, A.B. (2019). Changes in the atmospheric circulation conditions and regional climatic characteristics in two remote regions since the mid-20th century. Atmosphere, 10(1): 11.

Lisetskii, F.N. (2008). Agrogenic transformation of soils in the dry steppe zone under the impact of antique and recent land management practices. Eurasian Soil Science, 41(8): 805–817.

Lisetskii, F.N. (1998). Autogenic succession of steppe vegetation in postantique landscapes. Russian Journal of Ecology, 29(4): 217–219.

Lisetskii, F.N. (2007). Interannual variation in productivity of steppe pastures as related to climatic changes. Russian Journal of Ecology, 38(5): 311–316.

Lisetskii, F.N., Stolba, V.F., Ergina, E.I., Rodionova, M.E. and Terekhin, E.A. (2013). Post-agrogenic evolution of soils in ancient Greek land use areas in the Herakleian Peninsula, southwestern Crimea. The Holocene, 23(4): 504–514.

Meripõld, H., Tamm, U., Tamm, S., Võsa, T. and Edesi, L. (2017). Fodder galega (*Galega orientalis Lam)* grass potential as a forage and bioenergy crop. Agronomy Research, 15(4): 1693–1699.

Novikova, E.P., Grigoryev, G.N., Vagurin, I.Yu. and Chumeykina, A.S. (2017). Variations of the hydrothermal regime in the Chernozemiye over the last 30 years on the background of global climate change. Nauch Ved Belgoro En, 39(11): 105–113.

Povilaitis, V., Šlepetys, J., Lazauskas, S., Tilvikiene, V., Feiziene, D., Kadžiuliene, Ž., Kukujevas, A., Šlepetiene, A., Amaleviciute, K., Liaudanskiene, I., Ceseviciene, J. and Feiza, V. (2016). The Productivity And Energy Potential Of Alfalfa, Fodder Galega And Maize Plants Under The Conditions Of The Nemoral Zone. Acta Agriculturae Scandinavica. Section B: Soil and Plant Science, 66(3): 259–266.

Rancane, S., Makovskis, K., Lazdina, D., Daugaviete,

M., Gutmane, I. and Berzins, P. (2014). Analysis of economical, social and environmental aspects of agroforestry systems of trees and perennial herbaceous plants. Agronomy Research, 12(2): 589–602.

Rymuza, K. (2017). Application of logistic function function to describe the growth of fodder galega. Journal of Ecological Engineering, 18(1):125–131.

Saloniemi, H., Kallela, K. and Saastamoinen, I. (1993). Study of the phytoestrogen content of goat's rue (Galega orientalis), alfalfa (*Medicago sativa*) and white clover (*Trifolium repens*). Agricultural and Food Science, 2(6):517–524.

Skórko-Sajko, H., Tywonczuk, J., Skomiał, J., Pajak, J.J., Minakowski, D. and Sajko, J. (2005). Nutritional value of galega (*Galega orientalis Lam.*) forage and silage for ruminants. Journal of Animal and Feed Sciences, 14(1): 311–314.

Tammers, T.Kh. (1980). Transmission of integral solar radiation by grass sowing. Meteorology and Hydrology, 3: 111–113.

Tooming, H.G. (1984). Ecological principles of maximum crop productivity. Leningrad, Gidrometeoizdat.

Tooming, H.G. (1977). Solar radiation and harvest. Leningrad, Gidrometeoizdat.

Vazhov, V.M., Kozil, V.N. and Odintsev, A.V. (2013). General Methods of Buckwheat Cultivation in Altai region. World Applied Sciences Journal, 23(9): 1157–1162.

Wang, Z., Wang, J.-E., Wang, X.-M., Gao, H.-W., Dzyubenko, N.I. and Chapurin, V.F. (2012). Assessment Of Genetic Diversity In *Galega officinalis L.* Using Issr And Srap Markers. Genetic Resources and Crop Evolution, 59(5): 865–873.

Biotechnological
Communication

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp-23-29

Feasibility of Edible Coating, Storage Temperature and Packaging for Rancidity and Proteolytic Activity of Dry-Salted Snakeskin Gourami, *Trichopodus pectoralis*

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ABSTRACT

Snakeskin gourami (*Trichopodus pectoralis*) has been considered as a valuable and suitable species for breeding in fresh and brackish water regions in Vietnam. It has a high meat yield and favouritely consumed as dry-salted fish. However the dry-salted snakeskin gourami quality has gone down very fast owing to rancidity and proteolytic activity because it has high fat content and protease in its abdomen. It's necessary to have an appropriate processing and preserving approach to accelerate its commercial value in local and international markets. Edible coating supports a natural cover on the product surface to control weight loss, oil rancidity and solute movements. Biodegradability, edibility and barrier attributes are some benefits of edible coating superior to plastic bag. Storage temperature and packaging procedure affected the rancidity and proteolytic activity in the dry-salted snakeskin gourami (*Trichopodus pectoralis*). Purpose of this research demonstrated the efficacy of carrageenan coating on storage of dry-salted snakeskin gourami (*Trichopodus pectoralis*). The dried snakeskin gouramis were coated by various concentrations of carrageenan (0.5%, 0.75%, 1.0%, 1.25%, 1.5%). The efficacy of carrageenan coating was defined via quality indicators of dry-salted snakeskin gouramis such as fat rancidity: Peroxide value (mEq02/ kg), Thiobarbituric acid (mg maloaldehyde/ kg); proteolytic changes: total volatile base (TVB-N, mg N/100 g) nitrogen content and trimethylamine (TMA, mg N/100 g). In 3 month-interval, all coated samples were periodically analyzed during 12 months of preservation at ambient condition. Results showed that the carrageenan coating of 1.25% w/w, packed in vacuum bag and stored at 4oC could avoid microbial decomposition and fat oxidation of the dry-salted snakeskin gouramis. From this investigation, the dry-salted snakeskin gouramis had shelf-life at ambient storage for 12 months without deterioration. The research implied that the edible coating would be ideal to extend the stability of this valuable oil fish by inhibition of proteolytic reaction, reduction of fat rancidity and enhancement of overall acceptance.

KEY WORDS: *TRICHOPODUS PECTORALIS*, CARRAGEENAN, PROTEOLYTIC REACTION, RANCIDITY, STABILITY.

ARTICLE INFORMATION

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Received 13th Jan 2020 Accepted after revision 29th Feb 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)

*Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/5*

INTRODUCTION

Snakeskin gourami (Trichogaster pectoralis) is one of the most common fish in paddy fields and rivers of Vietnam. It lives in waters at low dissolved oxygen and high organic accumulation and feeds on zooplankton, crustaceans and insect larvae (Jafaryan et al., 2014). It achieves good maturity stages after 3 months of rearing. It has a high meat yield and is consumed as a dried fish in Vietnam. Farming area of snakeskin gourami has been opened dramatically in recent years offering attractive income for local farmers (Minh et al., 2019). The aquaculture sector contributes an important source of nutritious food for human consumption. Fish and fishery products are among the most important agricultural commodities (Bharda et al., 2017). This species is considered as an alternative species to shrimp farming in rainy season. They are good sources of proteins, macro-nutrients, minerals and some vitamins. The mineral elemental levels and vitamins of this species is a function of the availability preferential accumulation. Aquaculture of P. elongatus in the Ca Mau province (Vietnam) has developed rapidly during the past decade due to relatively high demand and high marketable value (Minh et al., 2019).

At death, the pH value in fish muscle begins to decrease due to formation of lactic acid from glycogen via a series of enzymatic reactions in the muscles. Enzymes from spoilage microorganisms produce different volatile compounds causing bad smell. The combination of ammonia (NH3) and TMA in fish is accounted for the total volatile base (TVB-N) nitrogen content of the fish and is normally considered as an indicator of quality decomposition. With the proliferation of spoilage bacteria after death in fish, a subsequent increase in TMAO reduction to TMA happens. On the other hand, the increase in the TVB-N is mainly created by the formation of TMA, which is prevalent in spoiled fish that have TMAO (mainly in marine pelagic fish) and is the most common cause of fishy odor. Aeromonas spp., psychrotolerant Enterobacteriaceae, Photobacterium phosphoreum, Shewanella putrefaciens-like organisms and Vibrio spp. are the bacteria that are able to reduce TMAO to TMA (Heising et al., 2014).

Rancidity is a matter in fatty fish related to the dried preservation. Indeed, the shelf-life of dried fatty fish usually ends with the onset of rancid flavors. The speed of hydroperoxide formation strongly relates to fat oxidation in its early stages. Aldehydes, ketones and similar compounds are the secondary products which form as the hydroperoxides react. The reactions lead to aldehydes and other products that can be evaluated using the thiobarbituric acid (TBARS) test (Turienzo et al., 2011). There were several notable researches mentioned to application of edible coating in fishes to control oxidation and proteolytic change. Whey proteinbased coatings delayed lipid oxidation of salmon fillets (Turienzo et al., 2011). The chitosan coatings and packaging might have been sufficient to retard lipid oxidation in lingcod (Ophiodon elongates) fillets (Duan et al., 2010). The effects of chitosan and different organic acid on fresh Japanese sea bass fillets were studied (Qiu et al., 2014). Quality characteristics of Japanese sea bass (Lateolabrax japonicus) during refrigerated storage were affected by e-polylysine, sodium alginate and e-polylysine/sodium alginate (Cai et al., 2015). The effectiveness of edible chitosan coating on the quality changes of Indian oil sardines (Sardinella longiceps) was studied by Mohan et al. (2102).

Several studies mentioned to the processing and preservation of snakeskin gourami. The effect of various salt concentrations and other soluble elements on the moisture content and water activity (aw) of dried snakeskin fish was studied (Muoi et al., 2008). The influence of sorbitol and ethanol on the water activity and quality changes of dried snakeskin fish was examined (Truc et al., 2009). Ethanol treatment to eliminate fishy ordor; addition of salt, sorbitol, as well as dry temperature that affected to to water activity (aw), microbial load (coliform, cfu/g), sensory score of dried snakeskin gourami (Trichogaster pectoralis) were thoroughly investigated. Shelf-life of the dried product was also evaluated during preservation (Minh et al., 2019).

In Vietnam, the snakeskin gourami P. elongatus is a high value species and has high potential for aquaculture in the Mekong Delta. P. elongatus aquaculture has developed rapidly to supply the high demand of domestic consumers. However there was not any research mentioned to the investigation of carrageenan coating to the dry-salted snakeskin gouramis (*Trichopodus pectoralis*) as well as the effect of storage temperature and packing procedure to oil rancidity and proteolytic activity. Purpose of this research was to demonstrate the feasibility of carrageenan coating, storage temperature and packaging procedure in prolonging product quality of dried snakeskin gouramis (*T. pectoralis*). Coatings can be considered as a barrier to protect against discoloration, degradation and oxidative rancidity.

MATERIAL AND METHODS

Material: Snakeskin gourami (*Trichopodus pectoralis*) were harvested from Ca Mau province, Vietnam. After harvesting, they were cooled below 4oC and moved to laboratory as soon as possible for further experiments. They were washed and sanitized under washing tank having 20 ppm per acetic acid with air bubble blowing to remove foreign matters. Besides snakeskin gourami we also used other material during the research such as per acetic, salt, carrageenan. Lab utensils and equipment included digital weight balance, Rotronic, stomacher, incubator, colony counter, and dry oven. Oil rancidity of dry-salted snakeskin gouramis was carried out by carrageenan coating during storage: They were soaked in 20 minutes with solution containing 4.0% salt, 1.0% sugar, 0.1% monosodium glutamate, 0.5% garlic extract, 0.5% honey. After that they were dried at 50°C to 18% moisture content. Various carrageenan concentrations were verified (0.5%, 0.75%, 1.0%, 1.25%, 1.5%) on

the dry-salted snakeskin gouramis. Oil rancidity was evaluated by Peroxide value (mEqO_2/ kg), Thiobarbituric acid (mg maloaldehyde/ kg). All coated samples were periodically taken in 3 month-interval during 12 months of preservation at ambient temperature.

Proteolytic decomposition of dry-salted snakeskin gouramis by carrageenan coating during storage: Raw snakeskin gouramis (*Trichopodus pectoralis*) were soaked in 20 minutes with solution containing 4.0% salt, 1.0% sugar, 0.1% monosodium glutamate, 0.5% garlic extract, 0.5% honey. After that they were dried at 50oC to 18% moisture content. Various carrageenan concentrations were verified (0.5%, 0.75%, 1.0%, 1.25%, 1.5%) on the dry-salted snakeskin gouramis. Proteolytic decomposition was evaluated by the total volatile base (TVB-N, mg N/100 g) nitrogen content and trimethylamine (TMA, mg N/100 g). All coated samples were periodically taken in 3 month-interval during 12 months of preservation at ambient temperature.

Oil rancidity and proteolytic decomposition of dry-salted snakeskin gouramis was done by packaging during preservation: Raw snakeskin gouramis (*T. pectoralis*) were soaked in 20 minutes with solution containing 4.0% salt, 1.0% sugar, 0.1% monosodium glutamate, 0.5% garlic extract, 0.5% honey. After that they were dried at 50oC to 18% moisture content. 1.25% carrageenan coating and 4oC of storage were applied for all samples. Two packaging types (zipper, vacuum) were examined on the dry-salted snakeskin gouramis. Oil rancidity was estimated by Peroxide value (mEqO_2/ kg), Thiobarbituric acid (mg maloaldehyde/ kg). Proteolytic decomposition was evaluated by the total volatile base (TVB-N, mg N/100 g) nitrogen content and trimethylamine (TMA, mg N/100 g). All coated samples were periodically taken in 3 month-interval during 12 months of preservation at ambient temperature. Chemical evaluation of dry-salted shrimp during storage: Peroxide value (mEqO_2/ kg) was evaluated by the CDR Food Lab® instrument. Thiobarbituric acid (mg maloaldehyde/ kg) was evaluated by 1,1,3,3-tetraethoxypropane (Torres-Arreola et al., 2007). Standard methods recommended by Food and Agriculture Organization of the United Nations (FAO, 1986) were utilized to determine of total volatile base (TVB-N, mg N/100 g) and trimethylamine (TMA, mg N/100 g). Statistical analysis: The experiments were run in triplicate with three different lots of samples. Statistical analysis was performed by the Statgraphics Centurion XVI.

RESULTS AND DISCUSSION

Oil rancidity of dry-salted *T. pectoralis* was carried out by carrageenan coating during storage. Seafood was highly perishable and has a short shelf-life. During storage many reactions occurred leading to changes in quality such as endogenous chemical and enzymatic reactions. The safety and shelf-life were related to the presence of food spoilage and pathogenic microorganisms. Edible coatings could improve the quality of fresh and frozen products by retarding microbial growth, reducing lipid oxidation and moisture loss, and functioning as a carrier of food additives (Dehghani et al., 2018). A barrier against both moisture and oxygen migration could be beneficial for seafood (Arfat et al., 2015; Li et al., 2013). Coatings could be used to provide physical protection to protect food products from mechanical damage, and from physical, chemical and microbiological activities (Min et al., 2005). Carrageenan was generally very hydrophilic, an anionic linear polysaccharide that is derived from red seaweed (Bourtoom, 2008). There were three kinds of carrageenan such as kappa, iota and lambda with different numbers and positions of sulfate groups on the galactose dimer (Lin et al., 2018).Carrageenan was formed by gelation through a process of moderate drying. After evaporation of solvent, the polysaccharide double helices will form a three-dimensional network, which subsequently forms a solid film (Karbowiak et al., 2007). It retarded moisture loss from the food products by adding additional moisture on the surface.

Table 1. Peroxide value (mEqO2/ kg), Thiobarbituric acid (mg maloaldehyde/ kg), Total volatile base (TVB-N, mg N/100 g) nitrogen content and Trimethylamine (TMA, mg N/100 g) in dry-salted *Trichopodus pectoralis* by various carrageenan concentrations of coating (0.5%, 0.75%, 1.0%, 1.25%, 1.5%) after 3 months of storage

Carrageenan concentration	Peroxide value (mEqO_2/ kg)	Thiobarbituric acid (mg maloaldehyde/ kg)	TVB-N (mg N/100 g)	TMA (mg N/100 g)
Control	1.65±0.00^{a}	0.97±0.00^{a}	49.83±0.02^{a}	42.09±0.02^{a}
0.5%	0.89±0.02^{b}	0.54±0.01^{b}	37.69±0.01^{b}	31.65±0.01^{b}
0.75%	0.64±0.01^{bc}	0.41±0.03^{bc}	34.74±0.03^{bc}	28.74±0.00^{bc}
1.0%	0.51±0.03^{c}	0.32±0.02^{c}	30.48±0.02^{c}	26.57±0.03^{c}
1.25%	0.43±0.01^{cd}	0.29±0.00^{cd}	28.65±0.01^{cd}	24.12±0.00^{cd}
1.5%	0.38±0.00^{d}	0.25±0.03^{d}	27.17±0.00^{d}	22.04±0.02^{d}

Note: the values were expressed as the mean of three repetitions; the same characters (denoted above), the difference between them was not significant (α = 5%).

In this research, raw snakeskin gouramis were soaked in 20 minutes with solution containing 4.0% salt, 1.0% sugar, 0.1% monosodium glutamate, 0.5% garlic extract, 0.5% honey. After that they were dried at 50oC to 18% moisture content. Various carrageenan concentrations were verified (0.5%, 0.75%, 1.0%, 1.25%, 1.5%) on the dry-salted snakeskin gouramis. Oil rancidity was evaluated by Peroxide value (mEqO_2/ kg), Thiobarbituric acid (mg maloaldehyde/ kg). All coated samples were periodically taken in 3 month-interval during 12 months of preservation at ambient temperature. Results from table 1 and 2 emphasize that 1.25% carrageenan coating would be adequate to assure oil rancidity in dry-salted snakeskin gouramis at the lowest level during preservation.

Proteolytic decomposition of dry-salted snakeskin gouramis by carrageenan coating during storage: Owing to the reaction of internal enzymes existing in fish products or microbial activities, nitrogen compounds such as trimethylamine-N-oxide (TMAO) are decomposed to ammonia, formaldehyde and trimethylamine (measured as TMA-N). These may cause protein aggregation, thus reducing the proteins' ability to bind water (Barraza et al., 2015). Raw snakeskin gouramis were soaked in 20 minutes with solution containing 4.0% salt, 1.0% sugar, 0.1% monosodium glutamate, 0.5% garlic extract, 0.5% honey. After that they were dried at 50oC to 18% moisture content. Various carrageenan concentrations were verified (0.5%, 0.75%, 1.0%, 1.25%, 1.5%) on the dry-salted snakeskin gouramis. Proteolytic decomposition was evaluated by the total volatile base (TVB-N, mg N/100 g) nitrogen content and trimethylamine (TMA, mg N/100 g). All coated samples were periodically taken in 3 month-interval during 12 months of preservation at ambient temperature.

Results from table 3 and 4 emphasized that storage at temperature 4oC could slow down the oil rancidity in dry-salted snakeskin gouramis to utmost level during 12 months of preservation. Minh et al. (2019) proved that 40% ethanol at ratio 20:80 for primary treatment; 2.0% of salt soaking; 1.0% of sorbitol addition; 46oC of drying were appropriate to maintain water activity (aw=0.65).

Oil rancidity and proteolytic decomposition of dry-salted *T. pectoralis* by packaging procedure during preservation: The edible muscle tissues of fish are liable to react with O2 in the presence of air. An increase in free fatty acid (FFA) lipolysis resulting from the enzymatic hydrolysis of esterified lipids also occurred in fish tissue post-mortem (Dehghani et al., 2018). Raw snakeskin gouramis (*T. pectoralis*) were soaked in 20 minutes with solution containing 4.0% salt, 1.0% sugar, 0.1% monosodium glutamate, 0.5% garlic extract, 0.5% honey. After that they were dried at 50oC to 18% moisture content. 1.25% carrageenan coating and 4oC of storage were applied for all samples. Two packaging types (zipper, vacuum) were examined on the dry-salted snakeskin gouramis. Oil rancidity was estimated by Peroxide value (mEqO_2/ kg), Thiobarbituric acid (mg maloaldehyde/ kg). Proteolytic decomposition was evaluated by the total volatile base (TVB-N, mg N/100 g) nitrogen content and trimethylamine (TMA, mg N/100 g). All coated samples were periodically taken in 3 month-interval during 12 months of preservation at ambient temperature. Results from table 5 and 6 strongly emphasized that vacuum packaging could effectively limit the oil rancidity and proteolytic decomposition in dry-salted snakeskin gouramis during 12 month storage.

Drying enhanced fish quality by inactivating enzymes and decrease water activity to prevent bacterial and mold proliferation. Fatty fish cannot be dehydrated by normal drying procedure, and is not possible to preserve it in the normal way. Fat rancidity was one of the most obstacles in the dry-salted fish (Minh et al., 2018). This phenomenon negative affected the taste, odor and color of dry-salted fish. One method to prolonging the stability and quality of dry-salted fish was vacuum packaging. It is an effective strategy to slow down the lipid oxidation by limiting oxygen molecule (Taheri and Motellabi 2012). By preserving under vacuum in PA bag, the dry-salted snakeskin gourami still extended the product shelf-life for 12 months without any deterioration (Minh et al., 2019).

Table 2. Peroxide value (mEqO2/ kg), Thiobarbituric acid (mg maloaldehyde/ kg), Total volatile base (TVB-N, mg N/100 g) nitrogen content and Trimethylamine (TMA, mg N/100 g) in dry-salted *T. pectoralis* by 1.255% carrageenan coating during 12 months of preservation

Storage (months)	Peroxide value (mEqO2/ kg)	Thiobarbituric acid (mg maloaldehyde/ kg)	TVB-N (mg N/100 g)	TMA (mg N/100 g)
3	0.43±0.01^{b}	0.29±0.00^{b}	28.65±0.01^{b}	24.12±0.00^{b}
6	0.49±0.02^{ab}	0.32±0.03^{ab}	29.04±0.00^{ab}	24.38±0.01^{ab}
9	0.53±0.03^{ab}	0.35±0.02^{a}	29.11±0.02^{ab}	24.43±0.03^{ab}
12	0.58±0.01^{a}	0.36±0.01^{a}	29.13±0.01^{a}	24.45±0.02^{a}

Note: the values were expressed as the mean of three repetitions; the same characters (denoted above), the difference between them was not significant (α = 5%).

Table 3. Peroxide value (mEqO2/ kg), Thiobarbituric acid (mg maloaldehyde/ kg), Total volatile base (TVB-N, mg N/100 g) nitrogen content and Trimethylamine (TMA, mg N/100 g) in dry-salted *T. pectoralis* by different concentration of preservation temperature (4°C, 12°C, 20°C, 28°C) after 3 months of preservation

Preservation temperature (°C)	Peroxide value ($mEqO_2$/ kg)	Thiobarbituric acid (mg maloaldehyde/ kg)	TVB-N (mg N/100 g)	TMA (mg N/100 g)
4	0.04 ± 0.00^{c}	0.05 ± 0.02^{c}	19.83 ± 0.03^{c}	17.63 ± 0.02^{c}
12	0.12 ± 0.01^{b}	0.14 ± 0.01^{b}	27.46 ± 0.02^{b}	21.08 ± 0.03^{b}
20	0.37 ± 0.03^{ab}	0.21 ± 0.00^{ab}	27.94 ± 0.01^{ab}	23.79 ± 0.01^{ab}
28	0.43 ± 0.01^{a}	0.29 ± 0.00^{a}	28.65 ± 0.01^{a}	24.12 ± 0.00^{a}

Note: the values were expressed as the mean of three repetitions; the same characters (denoted above), the difference between them was not significant (α = 5%).

Table 4. Peroxide value (mEqO2/ kg), Thiobarbituric acid (mg maloaldehyde/ kg), Total volatile base (TVB-N, mg N/100 g) nitrogen content and Trimethylamine (TMA, mg N/100 g) in dry-salted *T. pectoralis* by preservation temperature (4°C) during 12 months of preservation

Storage (months)	Peroxide value (mEqO2/ kg)	Thiobarbituric acid (mg maloaldehyde/ kg)	TVB-N (mg N/100 g)	TMA (mg N/100 g)
3	0.04 ± 0.00^{a}	0.05 ± 0.02^{c}	19.83 ± 0.03^{c}	17.63 ± 0.02^{c}
6	0.07 ± 0.03^{ab}	0.09 ± 0.01^{b}	20.09 ± 0.01^{bc}	17.94 ± 0.00^{bc}
9	0.11 ± 0.02^{ab}	0.16 ± 0.03^{ab}	20.57 ± 0.03^{b}	18.15 ± 0.03^{b}
12	0.15 ± 0.01^{b}	0.22 ± 0.02^{a}	21.45 ± 0.00^{a}	19.49 ± 0.01^{a}

Note: the values were expressed as the mean of three repetitions; the same characters (denoted above), the difference between them was not significant (α = 5%).

Table 5. Peroxide value (mEqO2/ kg), Thiobarbituric acid (mg maloaldehyde/ kg), Total volatile base (TVB-N, mg N/100 g) nitrogen content and Trimethylamine (TMA, mg N/100 g) in dry-salted *T. pectoralis* by two packaging procedure (zipper and vaccum) after 3 months of preservation

Packaging procedure	Peroxide value ($mEqO_2$/ kg)	Thiobarbituric acid (mg maloaldehyde/ kg)	TVB-N (mg N/100 g)	TMA (mg N/100 g)
Zipper	0.04 ± 0.00^{a}	0.05 ± 0.02^{a}	19.83 ± 0.03^{a}	17.63 ± 0.02^{a}
Vaccum	0.01 ± 0.03^{a}	0.03 ± 0.00^{a}	14.32 ± 0.01^{b}	13.04 ± 0.03^{b}

Note: the values were expressed as the mean of three repetitions; the same characters (denoted above), the difference between them was not significant (α = 5%).

Table 6. Peroxide value (mEqO_2/ kg), Thiobarbituric acid (mg maloaldehyde/ kg), Total volatile base (TVB-N, mg N/100 g) nitrogen content and Trimethylamine (TMA, mg N/100 g) in dry-salted *T. pectoralis* by vaccum during 12 months of preservation

Storage (months)	Peroxide value (mEqO2/ kg)	Thiobarbituric acid (mg maloaldehyde/ kg)	TVB-N (mg N/100 g)	TMA (mg N/100 g)
3	0.01±0.03b	0.03±0.00b	14.32±0.01b	13.04±0.03b
6	0.03±0.01ab	0.07±0.03ab	14.94±0.02ab	13.49±0.00ab
9	0.09±0.03ab	0.11±0.00ab	15.08±0.01ab	14.83±0.03ab
12	0.14±0.02a	0.16±0.01a	15.21±0.00a	14.91±0.02a

Note: the values were expressed as the mean of three repetitions; the same characters (denoted above), the difference between them was not significant (α = 5%).

CONCLUSION

The snakeskin gourami has a high commercial meat yield and favouritely consumed as dried fish in Vietnam. Commercial farming of snakeskin gourami has been developed rapidly in recent years. Fat rancidity is responsible development of off-flavors, and the loss of fat-soluble vitamins and pigments especially in dry-salted snakeskin gourami fish. The reduction of oxygen to a low concentration can decrease oxidation. Our research demonstrated that fat oxidation and proteolytic decomposition create major changes in snakeskin gourami (*Trichopodus pectoralis*) quality. Carrageenan as edible coating will be an ideal approach to preserve dry-salted fish because it creates a good barrier against enzymatic decomposition, spoilage microbial as well as auto oxidation. The coating attributes prolong stability against physicochemical changes such as color, texture, and moisture. Our research successfully proved that 1.25% w/w carrageenan coating and 4oC in vaccum packaging was adequate for the dry-salted snakeskin gouramis for 12 months of preservation. Carrgeenan as edible coating should be verified on other valuable dry-salted fishes to improve their commercial value during distribution.

REFERENCES

Arfat, Y. A., Benjakul, S., Vongkamjan, K., Sumpavapol, P., & Yarnpakdee, S. (2015). Shelf-life extension of refrigerated sea bass slices wrapped with fish protein isolate/fish skin gelatin-ZnO nanocomposite film incorporated with basil leaf essential oil. Journal of Food Science and Technology 52: 6182–6193.

Barraza, F. A. A., León, R. A. Q., & Álvarez, P. X. L. (2015). Kinetics of protein and textural changes in Atlantic salmon under frozen storage. Food Chemistry 182: 120–127.

Bharda S, AY Desai, Tandel Rutvik Kumar, P Tandel, Jitendra Kumar T

(2017). Production of different type of dry fish and its yield measurement at Veraval, Gujarat, India. Research Journal of Recent Sciences 6(7): 28-32.

Bourtoom, T. (2008). Edible films and coatings: characteristics and properties. International Food Research Journal 15: 237–248.

Cai, L., Cao, A., Bai, F., & Li, J. (2015). Effect of e-polylysine in combination with alginate coating treatment on physicochemical and microbial characteristics of Japanese sea bass (Lateolabrax japonicus) during refrigerated storage. LWT- Food Science and Technology 62: 1053–1059.

Dehghani S, Seyed Vali Hosseini, Joe M. Regenstein (2018). Edible films and coatings in seafood preservation: A review. Food Chemistry 240: 505–513

Duan, J., Jiang, Y., Cherian, G., & Zhao, Y. (2010). Effect of combined chitosan-krill oil coating and modified atmosphere packaging on the storability of cold-stored lingcod (Ophiodon elongates) fillets. Food Chemistry 122: 1035–1042.

Heising, J. K., Van Boekel, M. A. J. S., & Dekker, M. (2014). Mathematical models for the trimethylamine (TMA) formation on packed cod fish fillets at different temperatures. Food Research International 56: 272–278.

Jafaryan H Javad Sahandi; Javad Bagheri Dorbadam (2014). Growth and length-weight relationships of Trichopodus trichopterus (Pallas, 1770) fed a supplemented diet with different concentrations of probiotic. Croatian Journal of Fisheries , 72: 118–122.

Karbowiak, T., Debeaufort, F., & Voilley, A. (2007). Influence of thermal process on structure and functional properties of emulsion-based edible films. Food Hydrocolloids 21: 879–888.

Li, T., Li, J., Hu, W., & Li, X. (2013). Quality enhancement in refrigerated red drum (Sciaenops ocellatus) fillets using chitosan coatings containing natural preservatives. Food Chemistry 138: 821–826.

Mee Gie Lin, Ola Lasekan, Nazamid Saari & Siti

Khairunniza-Bejo (2018). Effect of chitosan and carrageenan-based edible coatings on post-harvested longan (Dimocarpus longan) fruits. CyTA - Journal of Food 16: 490-497.

Min, S., Harris, L. J., & Krochta, J. M. (2005). Listeria monocytogenes inhibition by whey protein films and coatings incorporating the lactoperoxidase system. Journal of Food Science 70: 317–324.

Minh, Nguyen Phuoc Minh, Pham Xuan Mai and Nguyen Thi Van Linh (2019). Physical aspects influencing to the production of dry-salted snakeskin gourami (Trichogaster pectoralis). Oriental Journal of Chemistry 35: 773-777.

Minh, Nguyen Phuoc Lam Phuong Hang, Nguyen Quoc Thong, Nguyen Thi Van Linh (2018). Technical factors affecting to production of dry-salted snakeskin gourami (*Trichopodus pectoralis*). Journal of Global Pharma Technology 10(08): 344-352.

Mohan, C. O., Ravishankar, C. N., Lalitha, K. V., & Gopal, T. S. (2012). Effect of chitosan edible coating on the quality of double filleted Indian oil sardine (Sardinella longiceps) during chilled storage. Food Hydrocolloids 26: 167–174.

Nguyen Van Muoi; Tran Thanh Truc (2008). Effect of different solutes on water activity of dried snakeskin fish (Trichogaster pectoralis Regan). Can Tho University Scientific Journal 10: 151-160.

Qiu, X., Chen, S., Liu, G., & Yang, Q. (2014). Quality enhancement in the Japanese sea bass (Lateolabrax japonicus) fillets stored at 4oC by chitosan coating incorporated with citric acid or licorice extract. Food Chemistry 162: 156–160.

Rodriguez-Turienzo, L., Cobos, A., Moreno, V., Caride, A., Vieites, J. M., & Diaz, O. (2011). Whey protein-based coatings on frozen Atlantic salmon (Salmo salar): Influence of the plasticiser and the moment of coating on quality preservation. Food Chemistry 128: 187–194.

Taheri S, and Motallebi AA. (2012) Influence of vauum packaging and long term storage on some quality parameters of cobia (Rachycentron canadum) fillets during frozen storage. American-Eurasian J. Agric. & Environ Sci. 12(4): 541-547.

Torres-Arreola, W., Soto-Valdez, H., Peralta, E., Cardenas-López, J. L., & Ezquerra-Brauer, J. M. (2007). Effect of a low-density polyethylene film containing butylated hydroxytoluene on lipid oxidation and protein quality of Sierra fish (Scomberomorus sierra) muscle during dried salted storage. Journal of Agricultural and Food Chemistry 55(15): 6140-6146.

Truc TT; Do Thi Khanh Doan; Nguyen Van Muoi (2009). Effect of sorbitol and ethanol on the aw and quality changes of dried snakeskin fish. Can Tho University Scientific Journal 11: 317-326.

Environmental Communication

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March Pp-30-33

Analysis of Microbial Pollution in the Seawater of the Aqaba Gulf, Haql, Saudi Arabia

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ABSTRACT

Seashores are vital areas for countries in various areas and are centers of attraction for tourists and residents to spend the times of tranquility and entertainment. The present study is aimed to ensure the presence of microbial pollution in the Gulf of Aqaba waters adjacent to the city of Haql, Saudi Arabia. The study was carried out during the period between February and May 2018. Four sites were sampled from the study area. Elements were found in the seawater of the Aqaba Gulf of the Red Sea such as Ammonia, Nitrate, Sulfate, and Chloride. Moreover, total dissolved solids were 38100, pH 8.62 and turbidity 0.25. The Biochemical oxygen demand was found to be 28 mg/L. Moreover, chemical oxygen demand (Dichromate) was 3040 mg/L. The Total Kjeldahl Nitrogen was <1.00 mg/L. Bacteria, fungi, and coliform were present in all the collected samples from the four sites. *Escherichia coli* was found to be 2 CFU/mL. This study demonstrates that there was microbial pollution in the seawater showing E. coli isolated from all the samples. .

KEY WORDS: *E.COLI*, GULF OF AQABA, NITRATE, POLLUTION, SEAWATER.

INTRODUCTION

Seashores are exposed to various anthropogenic activities such as tourist visits, surfing, fishing, industries, etc. which adversely affect the quality of coastal waters. Beaches are contaminated by many pollutants, which creates two main types of pollutions, the first type is natural pollution, and appears in the change of water temperature, or increase its salinity, or the increase of suspended substances. The second type is chemical pollution, caused by wastewater, oil spill, agricultural residues including pesticides and fertilizers. The Red Sea has two large gulfs, and the Aqaba Gulf (24 km wide, 800 to 1800 m deep) is one of them, located to the east of the Sinai Peninsula and west of the Saudi Arabia (Suggett et al., 2009). Oligotrophic oceanic waters of the Gulf is recognized as a deep winter mixing and intense summer stratification; consequently, these waters experience strong seasonal changes of the predominant limiting factor for microbial growth (Mackey et al., 2007; 2009; Overmans and Agusti, 2019).

The main elements for nutrients in the Gulf are changed in the winter to the nitrogen: phosphorus ratio (N:P) close to the 'Redfield ratio' (N:P = 16) (Hase et al., 2006; Asmala et al., 2017). These conditions will affect eukaryotic algae to be dominated but growth is limited by light availability with deep mixing (Al-Najjar et al., 2007).Microbial contamination of water means the presence of organisms visible or invisible to the naked eye, whether plant or animal in the aquatic environment. Water pollution is often caused by certain pathogenic microorganisms, such as bacteria, viruses, parasites, algae, and protozoa,

ARTICLE INFORMATION

*Corresponding Author: m.alsolami@ut.edu.sa
Received 10th Jan 2020 Accepted after revision 20th March 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)

*Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/6*

or by aquatic plants and animals. Contaminants from pathogens are often caused by the mixing of human and animal waste with water, either by direct discharge into water bodies, or indirectly by mixing with sewage. The presence of this type of pollution leads to many diseases. Therefore, this water should not be used for washing or drinking, unless it has been treated with various sterilizers, such as chlorine, ozone, mechanical filtering, and other treatment systems. However, northeastern portion of the Gulf of Aqaba contains high concentrations of metal pollutants discharged into the water of the Gulf from permanent sources of pollution (Abu-Hilal and Badran, 1990). Abu-Hilal and Badran (1990) also reported that the most polluted sites in a port area and near sewage outlets, the surface and vertical distributions of trace elements are similar to the distributions of organic carbon, total phosphate-phorous and fluoride which were derived over the past three decades from local release from deposited phosphate rock particles, raw sewage, old barges, boat and ship activities in addition to the recent industrial discharges in the southern section of the study area (Abu-Hilal and Badran, 1990; Crosby, 2017).

Many pathogenic microbes have been documented in the seawater, which are often associated with water contamination of sewage products, the most important of which are *Salmonella typhi, Salmonella typhimurium*, and *Escherichia coli*, which are responsible for many human poisonings (EPA, 2011). There are also many animal protozoans and some viruses. Several bodies are interested in such tests that determine the validity or pollution of water, including the World Health Organization (WHO), the International Organization for Standardization and Metrology (ISO) and the Saudi Arabian Gulf Standards Organization (SASO). Other researches in Agaba Gulf reported different types of microorganisms such as *Escherichia coli*, fecal *streptococci*, and *staphylococcus aureus* (Evans 1977; Yoshpe-Purer and Golderman, 1987, EL-Shenawy, 2005). The present study was carried out to ensure the presence of microbial pollution and to test the quality of thw water of the Gulf of Aqaba adjacent to the city of Haql, Saudi Arabia.

MATERIALS AND METHODS

Sample collection: The study was carried out during the period between February and May 2018 in the northern Gulf of Aqaba from the Saudi Arabian site in the City of Haql. During the cruise, 8 sites were visited, but we sampled only 4 sites of a transect across the Gulf of Aqaba (Fig. 1). Each site was visited at least twice. Water was collected with 1 L polyethylene (PE) flasks for further measurements.

Seawater analysis: The water samples were used for the analyses of various quality attributes. Total Kjeldahl nitrogen (TKN) was estimated by colorimetric method (EPA, 1978). Ammonia (NH_3) was determined using Nessler method as described by Koch and McMeekin (1924), and nitrate (NO^{3-}) was estimated according to Rodger et al. (2017) using cadmium reduction method. Fluoride was estimated by SPADNS method adapted from standard methods for the examination of water and wastewater (APHA, 1998). Total phosphorus (P) and total chlorine residual, sodium, chloride, and iron (Fe) were analyzed using standard methods as described in method 8048-Hach, 8167-Hach, P05-012A, 8225-Hach, and 8008-Hach, respectively (APHA, 1995). These tests were carried out in Kingdom laboratories (Jeddah, Saudi Arabia).

Hydrographical analysis: Hydrographical parameters including water temperature (oC), salinity (S%), and pH were measured at each sites using CTD (YSI-6000).

Figure 1: The study sites in Haql city on the Gulf of Aqaba

Table 1. Elemental analysis in the water of the Gulf of Aqaba, Haql, Saudi Arabia

Elements	Method	Result (mg/L)
Ammonia Total as –N	8038 -Hach	11.24
$NO3^-$	8039 -Hach	2.4
Sulfate	8051 -Hach	2175
Fluoride	8029 -Hach	1.78
Total phosphorus	8048 -Hach	0.06
Chlorine residual total	8167 -Hach	0.03
Sodium	P05-012A	15000
Chloride	8225 -Hach	22400
Iron	8008 -Hach	0.05

Each value in mean of four replicates.

Microbial analysis: Samples for enumerating bacteria were fixed with 2% formalin. Enumeration was done by epifluorescence microscopy according to Porter and Feig (1980). Within 2 h of sampling, 2 to 5 ml were stained with DAPI (4,6-diamidinophenylindole) for 3 to 5 min, filtered through 0.2 µm black Nuclepore filters, and stored frozen at –20 °C until enumeration in the laboratory 6 mo later. Bacterial numbers were determined at 1000× magnification with an epifluores.

Table 2: Total dissolved solids, pH and turbidity in the water of the Gulf of Aqaba, Haql, Saudi Arabia

Parameters	*Method*	*Result*
Total dissolved solids (mg/L)	Conductivity meter	38100
pH	pH meter	8.62
Turbidity (NTU)	Nephelometer	0.25

Each value in mean of four replicates

Table 3. Biochemical oxygen demand, chemical oxygen demand, total Kjeldahl nitrogen in the water of the Gulf of Aqaba, Haql, Saudi Arabia

Parameters	Method	Result (mg/L)
Biochemical Oxygen Demand	10099-Hach	28
Chemical Oxygen Demand	Dichromate	3040
Total Kejldahl Nitrogen	EPA- 351.3	<1.00

Each value in mean of four replicates.

RESULTS AND DISCUSSION

The data from table show that NH_3 as N, and NO^{3-} were 11.24 mg/L, and 2.4 mg/L, respectively. The concentration of sulfate, fluoride and total P were 2175 mg/L, 1.78 mg/L, 0.06 mg/L, respectively. Whereas, Chlorine Residual total was wa0.03 mg/L, Sodium was 15000 mg/L, Chloride was 22400 mg/L, and Iron 0.05 mg/L. For total dissolved solids in seawater which was found to be 38100 mg/L (Table 2). In the four samples taken. The p7H found the results as 8.62. The turbidity of water in the sample areas was 0.25 NTU. The Biochemical oxygen demand was found to be 28 mg/L (Table 3). Moreover, chemical oxygen demand (Dichromate) was 3040 mg/L. The total Kjeldahl nitrogen was <1.00 mg/L. The microbiology test was found that bacteria were present in all samples (Table 3). The bacteria count were 1000 CFU/mL and total Coliform were 100 CFU/mL.

The *Escherichia coli* were found to be 2 CFU/mL. Moreover, fungi were in the range of 3000 CFU/mL (Table 4). This is the first study on the elemental analysis, hydrographical analysis, and microbial analysis of the seawater of the Aqaba Gulf of the Red sea at the Saudi Arabian side. The elements found in the seawater were in the average showing NH_3 as total as N 11.24 mg/L, NO^{3-} 2.4 mg/L, sulfate 2175 mg/L, fluoride mg/L, total Phosphorus 0.06 mg/L, chlorine residual total 0.03 mg/L, sodium 15000 mg/L, Chloride 22400 mg/L, and Iron 0.05 mg/L. Sawatrai (et al., 1996) found similar results when they determined the multielement of trace elements in coastal seawater by ICPMS and ICP-AES after aluminum coprecipitation associated with magnesium.

In this study, the total dissolved solids were found in the range of 38100 mg/L and the pH was 8.62. Moreover, the turbidity of the water sample was 0.25 NTU. Furthermore. the biochemical oxygen demand was found to be 28 mg/L, chemical oxygen demand was 3040 mg/L and the total Kjeldahl nitrogen was <1.00 mg/L. These results were different from what Batayneh et al. (2014) found in the Aqaba Gulf of the Red sea on the Saudi Arabia when they tested the pH and found it 7.02 to 7.82. This difference could be to the place of the sample taken and the time because the Batayneh et al. (2014) collected the samples in 2014 and our samples were taken in 2018. Moreover, our samples were taken close to the cost and this could be another reason.

Table 4. Total bacteria count, Total coliform, E.coli and fungi in the water of the Gulf of Aqaba, Haql, Saudi Arabia

Microbial populations	Result (CFU/mL)
Total bacteria count	1000
Total coliform	100
Escherichia coli	2
Fungi	3000

Each value in mean of four replicates.

The microbiological test exhibited that bacteria were present in all samples (Table 4). The bacteria count were 1000 CFU/mL and total coliform were 100 CFU/mL. The *E. coli* were found to be 2 CFU/mL in all samples. Moreover, fungi were found in the range of 3000 CFU/mL. Several researchers found that seawater contains bacteria and fungi and coliform and other microorganisms (Samarasekera and Abeygunawardena, 2017; Park et al., 2003; Roslan et al., 2016; Chernikova et al., 2018; Yoon and Ryu, 2019). In Aqaba Gulf, bacteria and other microorganisms were isolated from different areas other than Saudi Arabian side (Grossart and Simon, 2002; El-Shenawy 2005; Suggett et al., 2009; Hussien et al., 2019). This study is the first study to report that *E. coli* and other bacteria and fungi and coliform were isolated from the Aqaba Gulf on the Saudi Arabian side. The occurrence of *E. coli* might be due to the contamination of sewage coming to the sea. Moreover, sewage could change the pH and this will stimulate growth of *E. coli*. Therefore, it can be concluded that there was microbial pollution in the seawater on the Aqaba Gulf of the Red Sea at the Saudi Arabia side.

ACKNOWLEDGMENTS

The author is thankful to the Dean, College of Haql, for providing necessary facilities.

Funding: Nil

REFERENCES

Abu-Hilal, A.H., Badran, M.M., (1990). Effect of pollution source on metal concentration in sediment cores from the Gulf of Aqaba. Marine Pollution Bulletin., 21, 190–197.

Al-Najjar, T., Badran, M. I., Richter, C., Meyerhoefer, M., Sommer, U., (2007). Seasonal dynamics of phytoplankton in the Gulf of Aqaba, Red Sea. Hydrobiologia., 579, 69–83.

APHA, (1995). Standard Methods for the Examination of Water and Wastewater, 19th Edition, American Public Health Association Inc., New York.

APHA, (1998). Standard Methods for the Examination of Water and Wastewater, 20th Edition, American Public Health Association Inc., Washington.

Asmala, E., Carstensen, J., Conley, D.J., Slomp, C.P., Stadmark, J., Voss, M., (2017). Efficiency of the coastal filter: Nitrogen and phosphorus removal in the Baltic Sea. Limnology and Oceanography., 62, S222-S238.

Batayneh, A., Zaman, H., Zumlot, T., Ghrefat, H., Mogren, S., Nazzal, Y., Elawadi, E., Qaisy, S., Bahkaly, I., Al-Taani, A., (2014). Hydrochemical faciès and ionic ratios of the coastal groundwater aquifer of Saudi Gulf of Aqaba: Implication for seawater intrusion. Journal of Coastal Research., 30, 75-87.

Chernikova, T.N., Kyrpides, N., Bargiela, R., Woyke, T., Shapiro, N., Whitman, W.B., Golyshin, P.N., (2018). Draft genome sequence of *Monaibacterium marinum* C7T, isolated from seawater from the Menai Straits, Wales, United Kingdom. Genome Announcements., 6, e01444-17.

Crosby, C., (2017). Calcium Phosphate Mineralization as a Nexus of Geosphere - Biosphere Interactions. University of Minnesota, ProQuest Dissertations Publishing.

EL-Shenawy, M., (2005). *Staphylococcus aureus* and fecal indicators in Egyptian coastal waters of Aqaba Gulf, Suez Gulf, and Red Sea. Egyptian Journal of Aquatic Research., 31, 113-124.

EPA (1978), Nitrogen, Kjeldahl, Total (Colorimetric, Automated Phenate) by Autoanalyzer, Method 351.1, U.S. Environmental Protection Agency, Washington, D.C.

EPA (2011), Environmental Protection Agency, Decontamination Research and Development Conference. U.S. Environmental Protection Agency, Washington.

Evans, J.B., (1977). Coagulase positive staphylococci as indicators of potential health hazards from water. In: Hoadley, A.W., Dutka, B.J., (Editors), Bacterial indicators/ health hazards associated with water. American Society for Testing and Materials.

Grossart, H-P., Simon, M., (2002). Bacterioplankton dynamics in the Gulf of Aqaba and the northern Red Sea in early spring. Marine Ecology Progress., 239, 263–276.

Hase, C., Al-Qutob, M., Dubinsky, Z., Ibrahim, E.A., Lazar, B., Stambler, N., Tilzer, M.M., (2006). A system in balance? Implications of deep vertical mixing for the nitrogen budget in the northern Red Sea, including the Gulf of Aqaba (Eilat). Biogeosciences Discussions., 3, 383–408.

Hussien, E., Juhmani, A-S., AlMasri, R., Al-Horani, F., Al-Saghir, M., (2019). Metagenomic analysis of microbial community associated with oral mucus from the Gulf of Aqaba. Heliyon, 5, e02876.

Koch, F.C., McMeekin, T.L., (1924). A new direct nesslerization micro-kjeldahl method and a modification of the nessler-folin reagent for ammonia. Journal of the American Chemical Society., 46, 2066–2069.

Mackey, K.R.M., Labiosa, R.G., Calhoun, M., Street, J.H., Post, A.F., Paytan, A., (2007). Phosphorus availability, phytoplankton community dynamics, and taxon-specific phosphorus status in the Gulf of Aqaba, Red Sea. Limnology and Oceanography., 52, 873–885.

Mackey, K.R.M., Paytan, A., Rivlin, T., Post, A.F., (2009). Nutrient and light regimes during a bloom. Marine Biology., 156, 1531–1546.

Overmans, S., Agusti, S., (2019). Latitudinal gradient of uv attenuation along the highly transparent Red Sea Basin. Photochemistry and Photobiology., 95,1267–1279.

Park, J.C., Lee, M.S., Lee D.H., Park, B.J., Han, D.W., Uzawa, M., Takatori, K., (2003). Inactivation of bacteria in seawater by low-amperage electric current. Applied and Environmental Microbiology., 69, 2405–2408.

Porter, K.G., Feig, Y.S., (1980). The use of DAPI for identifying and counting aquatic microflora. Limnology and Oceanography., 25, 943–948.

Rodger, B.B., Andrew, D.E., Eugene, W.R., (2017). Standard Methods for the Examination of Water and Wastewater, 23rd Edition, APHA, Washington, DC.

Roslan, N.N., Sabri, S., Oslan, S.N., Baharum, S.N., Leow, T.C., (2016). Complete genome sequence of Photobacterium sp. strain J15, isolated from seawater of southwestern Johor, Malaysia. Genome Announcements., 4, 739–16.

Samarasekera, K., Abeygunawardena, S.I., (2017). Microbiology of seawater and sand in a selected bathing site of Sri Lanka – A study towards microbial quality assessment. Frontiers in Environmental Microbiology., 3, 9-18.

Sawatrai, H., Hayashi, T., Fujimori, E., Hirose, A., Haraguchi H., (1996). Multielement determination of trace elements in coastal seawater by ICP-MS and ICP-AES after aluminum coprecipitation associated with magnesium. Bulletin of the Chemical Society of Japan., 69, 1925-1931.

Suggett, D.J., Stambler, N., Prášil, O., Kolber, Z., Quigg, A., Vázquez-Dominguez, E., Zohary, T., Berman, T., Iluz, D., Levitan, O., Lawson, T., Meeder, E., Lazar, B., Bar-Zeev, E., Medova, H., Berman-Frank, I., (2009). Nitrogen and phosphorus limitation of oceanic microbial growth during spring in the Gulf of Aqaba. Aquatic Microbial Ecology., 56, 227-239.

Yoon, J., Ryu, J., (2019). *Altererythrobacter rubellus* sp. nov., a marine alphaproteobacterium isolated from seawater. FEMS Microbiology Letters., 366, fnz131.

Yoshpe-Purer, Y., Golderman, S., (1987). Occurrence of *Staphylococcus aureus* and *Pseudomonas aeruginosa* in Israeli coastal water. Applied and Environmental Microbiology., 53 (5), pp: 1138-1141.

Biomedical
Communication

BBRC
Bioscience Biotechnology
Research Communications

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp-34-42

Effect of Non-Ionizing Electromagnetic Field on Peripheral Nerve and its Functional Disorders – A Review

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ABSTRACT

As the revolution in technologies and industries continues in the modern world, there is a diverse process of evolution in electromagnetic field (EMF) induced by appliances that include laptops, mobiles, cellular base station, etc. The electromagnetic field has more negative effects on the living things, but can also be used in regenerating the nerve. Radiation is reported to influence isolated nerve preparations, the central nervous system, chemistry and histology of the brain, and the blood-brain barrier. Peripheral nerve injury occurs due to nerve crushing and are the most common lesions within the nerve injury. The low frequency non-ionizing EMFs vibrates are able to modify the tissues structures of the nerves due to their thermal effects. The effects of pulsating EMFs on nerves has been a subject of research in humans and animal by studying their behavior and nerve electrical properties. This review gives a brief introduction to types of EMFs and addressess the biologic consequences of electromagnetic field on the nervous system with special focusing on the peripheral nerves. In this review recovery characteristics of soft electromagnetism currents in nerve injury and regeneration of nerve, the therapeutic process associated with it has been discussed.

KEY WORDS: ELECTROMAGNETIC FIELD; BIOTIC EFFECT; NERVOUS SYSTEM; PERIPHERAL NERVE; NERVE REGENERATION.

ARTICLE INFORMATION

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Received 15th Feb 2020 Accepted after revision 28th March 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)
A Society of Science and Nature Publication, Bhopal India

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/6

INTRODUCTION

Electromagnetic field (EMF) consists of oscillating electric and magnetic field perpendicular to each other and perpendicular to direction of propagation. The energy of the electromagnetic waves are quantized by quanta called Photons. The electromagnetic spectrum consist of various regions frequency of photons ranging from 101 Hz to 1024 Hz. The region of frequency 4x1014 to 7.5x1014 Hz is called visible regions and others are invisible, Fig1. Visible and invisible EMF exist everywhere in our surroundings in the environment due to manmade and natural sources. High frequency EMF consist of photons of high energy, they ionize the materials that they pass through. Low frequency EMF are non-ionizing emanates from many man-made electrical and industrial devices. Furthermore, based on frequencies these EMF are classified as extremely low-frequency electromagnetic fields (ELEMF) frequency ranging from 1 to 300 Hz, intermediate frequency EMF from 300 Hz to 100 kHz, commonly called as Low-frequency EMF.

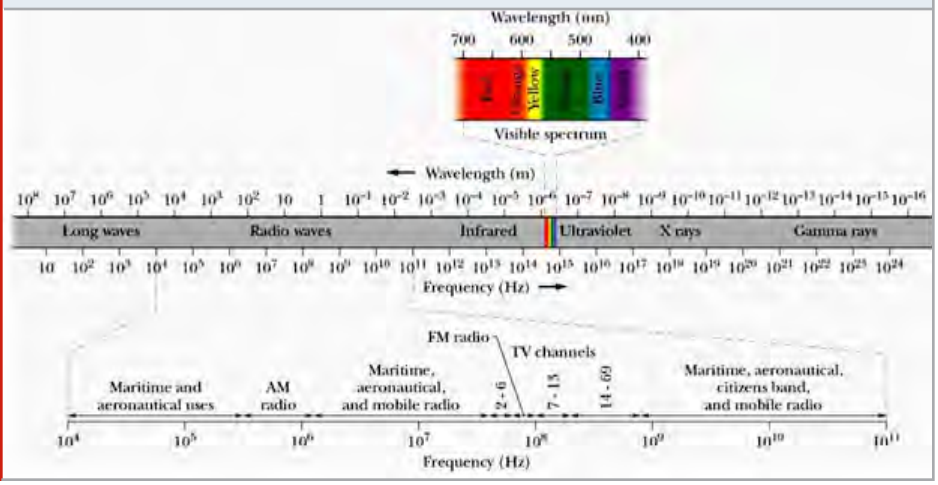
Further the high frequency but non-ionizing EMF can be classified as Radio frequency EMF frequency ranging 100 kHz- 300 MHz, Micro waves 300 MHz -30 GHz, millimeter waves 30 GHZ -300 GHz, and Terahertz waves frequency ranging from 300 GHz to 10 or 30 THz. EM radiations from different regions of EM spectrum leaves different effect of absorbing materials. When atoms or molecules absorb the electromagnetic energy from terahertz regions, then they are transferred to higher energy levels. The electrons are promoted to higher orbital by visible or ultra violet radiations, vibrations are excited by infrared radiation and rotations are excited by microwaves. The atomic absorption spectroscopy measures the concentration of an element in a sample; whereas atomic emission spectroscopy measures the concentration of elements in samples (Ahmad, 2010; Ahmad 2014, Ju 2015; Terzi 2016, Ross 2019, Lin 2020).

General public is mainly exposed to the low frequency EMFs in microwave regions from 300 MHz-3 GHz due to indoor electrified appliances particularly microwave oven, TVs, smart phones, computers, science games, induction cooking heats vessels, and fluorescent tubes, etc. The magnetizing vibrates releases frequency from the mentioned instruments ranges from Low to very high frequency domains. Electromagnetic vibrates is an extra area of energy created by electrical devices. Several research groups are giving attention to the possible effects of the EMFs with biological relationships, particularly in the light of recent study proposing that EMFs may contribute to degenerative nerve illness. Several searches have shown the EMF influences on human health but still there is no clear cut evidence to relationship effects caused by EMF exposure. EMFs have improved in human daily life and this are useful even in medical field for diagnostic and treatment purposed. The harmful effects of EMF exposure are most commonly skin problems including ruddiness, tickling and burning feel as well as nervous breakdown indications in a mode of fatigue, concentricity lose, whirling sensation, and motion troubles. Large number of experiments done on the EMF impacts on nerve system and associated sensory apparatus. It is reported that EMF can lead to chemical, morphological, and electrical changes in the nerve system (WHO, 2002; Terzi, 2016; Peter Lyttkens 2018 Lin 2020).

The biotic effects of contact with Low frequency EMF (LEMF) results from such devices rely on the distance of the servant from them. Prolong exposure of LEMF by electronic gadgets such as micro wave devices and outdoor sources power bases, high-voltage overhead and underground power lines used for the electricity traffic cause Electro-hypersensitivity (EHS) called microwave syndrome. Some of the symptoms of EHS are headache, fatigue, insomnia, tinnitus, photophobia, loss of memory, sense of cognitive dysfunction, irritability, pain at different sites and sometimes cardiovascular abnormalities (Liakouris, 1998; Khurana, 2010; WHO, 2013; Jhonson, 2015; Carpenter, 2015;.Dekun Gao et al 2018).

Biological Effects of Low Frequency EMF: When an Low

Figure 1. Electromagnetic spectrum; (Ahmad 2010)



frequency electromagnetic radiation is applied to metal it will be reflected, where as it get attenuated and fall off exponentially in biological subjects. The effects of these electromagnetic forces on the biological systems have been primarily associated to thermal and non-thermal attributes. An EMF in microwave region on biological subject can considered as imposing time-varying forces on charged ions, molecules, regions of bonds between molecule having dipoles and monopoles in tissue by virtue of its oscillating electric field.

Thermal Effects: All electromagnetic forces possibly transformed into thermal performance during interactions with materials; thus, all electromagnetic force has thermal effects on living systems. The frequency of electromagnetic pulsing have thermal consequences as it affects the permeation into biological systems. At first, it has been thought that low frequency EMFs did not have sufficient power to make significant heating, and could not lead to any potential alterations in the biological complexes. Recently many studies have shown that electromagnetic vibrates are able to modify living tissues structures due to its thermal effects (Binhi, 2002; Gajšek, 2016). An endothelium of a large blood vessel or tissue boundary of a mucous membrane of the stomach has an electrically polarized cell membranes with a regular coulomb structure, the orientation of the membrane dipoles are completely randomly oriented in the absence of EMF. But when an EMF is applied coherent microwave beam producing a standing E-field wave, this radiation wave pattern can diffract stirring charged particles into highly organized patterns. When charges are rotated a torque will acts on them in phase with the EMF, the work done is converted in thermal energy (Nairz, 2001; Williams, 2016; Ahmad, 2018).

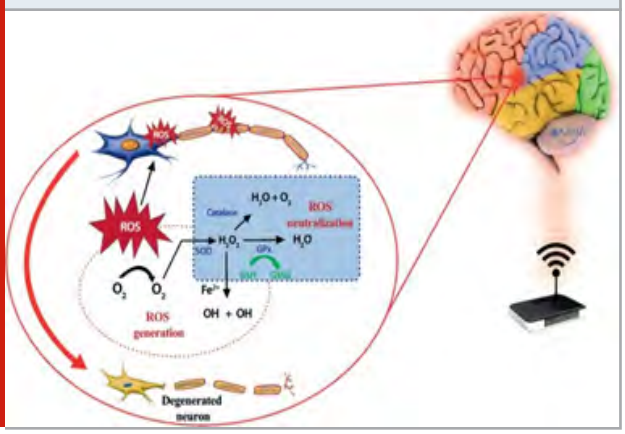
Non-Thermal Effects: Non-thermal effects are not caused by temperature changes but by some other changes in the tissues as the magnetizing force is absorbed into the body. The leading mechanisms of the interaction between electromagnets and biological complexes at non-thermal levels are still unclear (Binhi, 2002; Gajšek, 2016). Many studies have reported that both thermal and non-thermal effects of EMF can affect nervous system and peripheral nerves.

EMF And Nervous System: The Peripheral nerve in human body has been proved to be an ultimate electromagnetic system. It works by releasing different chemicals and producing heat that creates electromagnetic fields in the body. Different exposure conditions can been used to study the effect of EMF on biological and immune system. Radiation that allowed to expose on the nerve includes continuous-wave (CW) or pulsed-wave RF radiation of different frequencies and with power densities having different specific absorption rates (SARs) in biological material. Researchers use continuous and/or intermittent pulse exposures, some groups argue that the intermittent exposures are more efficient in causing biological effects, (Rannug, 1994) while, some other suggest peaked EMF exposures. The comparison of homogeneous and gradient static EMF exposures indicates that the gradient EMF is more biologically effective (Hirose, 2003).

Some studies suggest that EMF and Radio Frequency radiation will have no direct effect on DNA, some conditions exert a biological effect reminiscent of heat shock and/or stress. This effect is weak but much dependent on the state of cell homeostasis prior to radiation exposure. Studies suggest that the effects of EMF exposures are mild in comparison to other ionizing radiation, heat shock, nutrient deprivation, etc. and unpredictable. The effects of electromagnetic pulsing on nerves have been a subject for research since alterations in animal behavior and nerve electrical properties were first reported in the end of the nineteenth century. Radiation is reported to influence isolated nerve preparations, the central nervous system, chemistry and histology of the brain, and the blood-brain barrier (National Research Council (US), 1993). The transmission of signals in biological systems occurs through complex electrochemical events. The biological effects of EMF is more pronounced in pathway of the plasma membrane, through which the sinusoidal EMF signal induces a voltage change. The signal exerts forces on free ions which are present on either sides of the plasma membrane leading it to travel across the cell surface through trans membrane proteins.

These free ions often generate an intracellular vibration which is responsible for the influx of extracellular Ca^{2+} ions and the binding affinity of calmodulin (CaM), which is the basic pathway to the secondary messengers, cAMP and cGMP, that have been found to influence inflammatory pain (Christina, 2016). The nature of the electrical communication in humans and animals suggests a potential interactive target for influences from external EMFs. Some research groups described the thermal nature of MW energy absorption, some other groups associate non-thermal or specific MW effects at the molecular and cellular level. The description of the effect of MW energy of 3 to 30 GHz in the centimeter range on the conditional response activity of animals was experimentally studied by Gordon et al. (Gordon, 1955; Martin 2016).

Figure 2. Neuron Histology - Degeneration and Regeneration of nerve (Grinsell, 2014)



In subsequent years, the study of non-thermal effects of MWs gradually taken the main role in electrophysiological studies by many researchers. It is reported that single and repeated exposures of MW of power density 50 to 150 W/m2 to rats, led to weakening the excitation process and decreased the functional mobility of cells in the cerebral cortex of rats. Edematous changes due to aqueous accumulations were found throughout the cortex. The greatest number of altered cells was noted with repeated exposures at 150 W/m2 (Yakovleva, 1968; Novitskiy, 1971). Studies have made it is clear that the amount of energy absorbed due to exposure of MW depends on factors such as frequency and wavelength, body shape and size, and orientation in the fields. On the whole, the evidence for differential nervous system or behavioral responses to continuous or pulsed-wave MWs is fairly weak (Frey, 1975; Durney, 1986; Gandhi, 1990; Rocha 2015). Furthermore, some research groups studied the comparison of pulsed and CW EMF in MW region and can be found that exposure of MWs can cause conditioned reflex activity and functional changes in the activity of CNS which is reversible. In a study by some other group, proliferative reaction of glial cells shows that, even at high peak powers and wideband exposures of MWs, no evidence was observed of differences between CW and PW (Sherry, 1995).

EMF and Peripheral Nerves: Biological stimulation of nerve by electromagnetic fields can greatly modify the functions of nervous tissue. It accelerate the regenerative capacity of the tissue. The Peripheral nervous scheme consist of sensory neurons and motor, the neurons are composed of cell bodies in the spinal cord and axons. The axons are the sensory argons, grouped together in a set of spatially sensory bundles called fascicles. A groups of fascicles are enclosed within a peripheral nerve encircling a connective tissue layer called epineurium. The internal epineurium separates fascicles, while external epineurium surrounds all the fascicles in the nerve. The epineurium is sutured in nerve repair and nerve grafting and contributes about half of the total cross sectional area of a peripheral nerve. Exterior of this layer consists the mesoneurium, providing the blood supply to the nerve. A fine but fragile network of capillaries exists at the endoneurial level which can be easily disrupted due to tension or trauma at the nerve repair (Sunderland, 1948; Flores, 2000; Siemionow, 2009;). The Pulsed electromagnetic field (PEMF) is widely used as a non-invasive procedure and efficacious treatment for resuscitation of peripheral nerve, it has been proved to be promoting extension of neurites in vitro. Thus it can be taken into account as a novel pretreatment modality for crush injury cases. Numerous studies have been carried out by various research groups on the electromagnetic effects on the peripheral nerve mechanism and functionality. This literature review is a brief outlines within the context of certain published writings of correlated studies.

A study done by Nari Seo and et al. to assess the effects of the Pulsed EMF on mesenchymal stem cells (MSC) multiplication and on nerve resuscitation. Result of pre-therapy with Pulsed EMF showed an increase in cell multiplication process along with increasing in Glial fibrillary acidic protein expression. Additionally, it was found to promote the release of growth factors like NGE and BDNF were observed. In addition to this Histological investigation showed an increase in total of axon number and density, suggesting an axonal regeneration (Seo, 2018). Boise et-al applied pulse EMF stimulation on human bone marrow MSC cultured on a substrate of nano-structured TiO2 to study the effect of surface nano-topography with exposure of low-frequency Pulse EMF on cells differentiation, with a special focus on behavior of Ca^{2+} related cell metabolism. It is reported that the osteogenic differentiation of hBM-MSCs occurs in complex manner, it is not the simple sum of each isolated effect. Surface nanostructure, OM treatment and PEMF stimulation have been confirmed to alter cellular calcium homoeostasis, the overall effect of an integrated treatment is strongly non-summative (Aubin, 2001; Bloise, 2018; Unal, 2018, Ross 2019).

A second study by Jensen and et al applied Bipolar electromagnetic pulsing stimulation applied to the brain (T-PEMF) therapy to improve function of fine motor skills of Parkinson's disease patient, reduced muscle rigidity, lower muscle spasms and tingle, and less tiredness through the time of T-PEMF therapy. They reported improvement in fine motor skills functioning, and acknowledged T-PEMF therapeutic as a potential long-term therapy (Jensen, 2018). The study of pulse EMF on microcirculation and angiogenesis by Pan Y et-al using a model of acute hind limb ischemia in diabetic rats showed that the pulse EMF has increased the acute hind limb ischemia-related perfusion and angiogenesis, which is associated with up-regulating FGF-2 expression and activating the ERK1/2 pathway in diabetic rats. It concluded the pulse EMF might be important for the treatment of diabetic patients with ischemic injury (Pan, 2013; Ross 2019). Wei-Hong Hei., et al. (Hei, 2016) conducted a study on immortalized schwann cells derived from rat to assess the effects of exposure frequency of pulse EMF on neuro regeneration. The study concluded that the irradiation of 50 Hz EMF in pulse mode for 1 hour led to enhancement of peripheral nerve resuscitation. This enhancement has attributed to Schwann cell multiplication along with increase of expression rate in S100 gene in neurotrophic factors level (Hei, 2016). Studies demonstrated that the exposure of pulse EMF has increased iSCs mRNA expression of S100 and brain-derived neurotrophic factor (BDNF).

Further studies showed that pulse EMF exposure improved BDNF expression in vivo both in dorsal root gangion (DRG) and nerve segment. EMF increased in vitro and in vivo angiogenesis via endothelial release of FGF-2. It is reported that the Pulsed EMF enhances BDNF expression through L-type voltage-gated Ca2+ channel-and Erk-dependent signal pathways in rats dorsal root ganglion neurons (Kim, 2014; Tepper, 2004; Li, 2014). Furthermore investigation carried out by Kolosova to evaluate the recovery characteristics of soft electromagnetism currents in nerve injury by using the

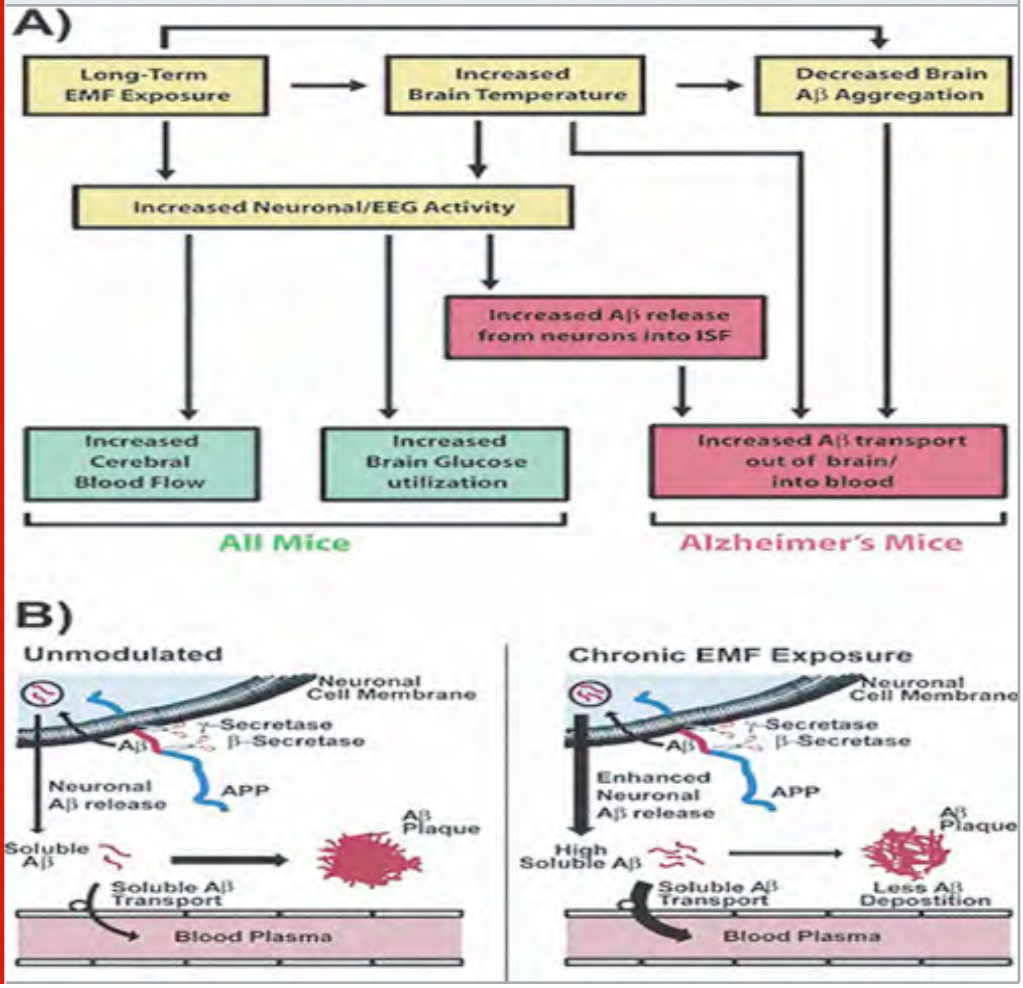
popular model rat sciatic nerve. These study have shown that electromagnetism currents had an impetus effects on recovery performance of operated rats (Kolosova, 1996; Lin, 2020).

In contrast, Michael Kelleher and his group carried a study on adult sheep to illustrate the impacts of introducing stable magnetized domain on sensory nerves medicament after nerve injury to these nerves and repair. The study concluded introduction to fixed magnetized domains make no improvement move of peripheral nerves (Kelleher, (2006; Grinsell 2014). Behavioral studies on EMFs reported no significant effects on cochlear and brainstem auditory operation, these study revealed cross result on involuntary and elicit brain electrical action. The conflicting conclusion could be false positive for different evaluations and thus appropriate study design and data analysis considering various comparisons and effect size are needed to minimize controversy in this important area of research (Kwon, 2011; Joan 2019).

A research conducted to determine the thermal marks concerning electromagnetic radiation emitted from mobiles of the seventh Cranial Nerve (CN VII) and surrounding soft-tissue. The study concluded that The electromagnetic radiation arising out of mobiles could be a matter for unstable disorders of Cranial Nerve VII through heat erratic increasing on the framing soft-tissue of the CN VII (Acar,2009). Results of recent studies have been achieved to identify the preliminary effects of the Electromagnetic Fields on Peripheral Nerve and its functions, previous studies have yielded inconsistent findings. Most of the previous research have shown that EM pulsing has the potential to be recommended as an appropriate and effectual therapeutic for peripheral nerve cure in clinical applications. Some studies did not show any recovery pattern for electromagnetic pulsing in peripheral nerve injure. Furthermore, it is essential rating lowest frequencies of magnetized fields proved to be efficient for injured nerve recovery. Moreover studies should consist of morphological and Ultra structural properties by the molecular techniques to determine the exact effects (Lei, 2013; Terzi, 2016).

Regeneration of nerve: Non-ionizing EMF are being used for regeneration of nerves. Some studies have used the crushed sciatic nerve in rat are used as model to study the functional, biochemical and morphological properties. An improvement in the regeneration was observed after exposure of LFMF sinusoidal waves of magnetic flux

Figure 3. Mechanism of action for the intellectual effect of long exposure period to EMF in standard and AD Tg mice (Arendash 2012)



densities of about 0.5 mT (Rusovan,1991; Rusovan, 1992; Bervar, 2005). A rotating magnetic field using Helm-holtz coils has been used to delivered variable magnetic flux densities on the animals, depending on the position of exposure coils. Various flux densities, all at frequency 40 Hz, have been used in the study, and the highest interval (150–300 μT) showed the largest improvement to regeneration compared to control condition. An Improved regeneration of the muscle and nerve in mice due to crush injury of the upper part of thigh (Pulse EMF about 50 Hz) has been reported. A positive effects on hemi-sectioned spinal cord in rats due to sinusoidal 50 Hz wave of magnetic flux 17.96 μT have also been observed (Suszy ski, 2014; Das, 2012; Stölting, 2016). Survey of literature reveals studies considering the effect of Pulse EMF on spinal cord regeneration was covering both in vivo as well as in vitro studies. It observed that the regenerative effect depends on the signal that have used initially reduce inflammation, then regeneration proceed. These positive effects are reported with Pulse EMF at frequencies under 100 Hz and flux densities below 5 mT (Ross, 2017).

Literature reveals the Pulse EMF promote peripheral nerve regeneration to an degree comparable to that with hormones, conditioning lesions, and growth factors. Exposure of pulsed EMF prior to treatment on crush injury has resulted in acceleration of axonal regrowth, and was in consistent with a spur of regenerative neurite outgrowth increased outcomes like walking behavior, promotes neutrite growth invitro (Greenebaum, 2007; Baptisa, 2008; Walker 1994). But in some studies it has been observed that prolonged Pulse EMF treatment resulted to delayed histological peripheral nerve regeneration and increased oxidative stress but no loss of function recovery (Baptisa, 2009; Zang, 2019). These contradictory results could be due to procedural differences. A study by Minoo Shadel et-al reveals the exposure of pulse EMF on the whole body of wistar rats could speed up functional recovery after nerve allografting in sciatic nerve (De Lahunta, 2009; Minoo, 2017; Alvites, 2018). This may have clinical implications for the surgical management of patients after nerve transection. In this study rats were divided in to normal, allograft, and PEMF treated group. In the allograft group the left sciatic nerve was exposed through a gluteal muscle, while for PEMF group the whole body was exposed to pulse EMF of 0.3 mT and frequency of 2Hz for 4h/day within 1-5 days (Khan, 2014; Faroni, 2015; Lin, 2020).

CONCLUSIONS & RECOMMENDATIONS

EM waves exists anywhere in the environment around us. Due to the use of household appliances and outdoor electromagnet paths and towers that induce non-ionizing pulsating electromagnetic field has increased markedly all over the world. The electromagnetic field has more negative effects on the living things, but can also be used in regenerating the nerve. Peripheral nerve injury occurs due to nerve crushing and are the most common lesions within the nerve injury. It could represent the limit between the lesions inclined to spontaneous regeneration and those that require essential surgical intervention for regeneration to occur. Peripheral nerve crush lesions generally occur linked with compressive forces and fractures and could affect the neighboring tissues, leading to a difficult conditions. The important factor for nerve regeneration in is the concern of exuberant inflammatory reactions, the adhesions of the nerve with surrounding tissues, occurrences of axonal misdirecting and failures in demyelization. Pulsed EMF and used in regeneration of nerve. There are some positive sides of these pulses. Studies shows that the pulsed EMF have successfully used in authentic clinical scopes to improve the regeneration performances and to supply sustainable health conditions of peripheral nerves.

Many of the preceding research have shown that EM pulsing has the potential to be recommended as an appropriate and effectual therapeutic for peripheral nerve cure in clinical applications. On the contrary, some studies did not display any recovery pattern for electromagnetic pulsing in peripheral nerve injure. Thus, a proper investigation design and appropriate data interpretation are Key factors that determine the exact effects of these therapy approach. Furthermore, it is essential rating lowest frequencies of magnetized fields efficient for injured nerve recovery. Moreover studies should consist of morphological and Ultra structural properties by the molecular techniques to determine the exact effects. All previous data suggest further research are highly required to judge the role and function of pulse and continuous EMF in therapeutics in clinical scopes. Clinical application of Low frequency Electromagnetic fields are emerging multidisciplinary field. Tissue engineering is the process of developing methods that associated with nerve regeneration by applications of EMFs. The peripheral nerve regeneration involves basic Science research inorder to address the issues such as nerve growth factor of selected nerve, release kinetics related to regeneration etc.

REFERENCES

Acar, G.O; Yener, H.M; Savarun,F.K (2009). Thermal effects of mobile phones on facial nerves and surrounding soft tissue. The Laryngoscope. Vol. 119. No.3. Pages. 559-562.

Ahmad, S.I. (2018). Fundamentals of Applied Engineering Physics Applied Engineering Physics for Scientist and Engineers. Isbn: ISBN13: 978-613-9-93127-9. Publisher: LAP Lambert Academic Publisher.

Ahmad, S.I; Syed I A, Ravi P. P. Ahmad A.(2014). Quantitation of urea in urine by Fourier transforms infrared spectroscopy. Der Pharma Chem., Vol 6 (1). Pages 90-96.

Ahmad, S.I. Studies on some biophysical aspects of human renal excretory fluid, Ph.D. dissertation, Department of Physics, Jawaharlal Nehru Technological University, Hyderabad, India, 2010.

Alvites, R; Caseiro, A. R; Pedrosa, S.S; Branquinho, M.V; Ronchi, G; Geuna, S; Varejão, A.S.P; and Maurício, A.C.

(2018), Peripheral nerve injury and axonotmesis: State of the art and recent advances. Alvites et al., Cogent Medicine. Vol.5: 1466404 https://doi.org/10.1080/2331205X.2018.1466404.

Arendash, G. W; Mori,T; Dorsey,M; et-al. Electromagnetic Treatment to Old Alzheimer's Mice Reverses β-Amyloid Deposition, Modifies Cerebral Blood Flow, and Provides Selected Cognitive Benefit. (2012). PLoS One. 7(4): e35751. doi: 10.1371/journal.pone.0035751

Aubin, J. E. (2001). Regulation of Osteoblast Formation and Function. Rev Endocr Metab Disord. Kluwer Academic Publishers. Vol. 2. Pages. 81–94. https://doi.org/10.1023/A:1010011209064

Baptista, A.F; Gomes, J.R; Oliveira, J.T; Santos, S.M; Vannier Santos, M.A. et al. (2008) High- and low-frequency transcutaneous electrical nerve stimulation delay sciatic nerve regeneration after crush lesion in the mouse. J Peripher Nerv Syst. Vol. 13. No.1. pages. 71-80.

Baptista, A.F; Goes, B.T; Menezes, D; Gomes, F.C.A; Zugaib, J; Stipursky, J; Gomes, J.R.S; Oleveria, J.T; Vannier-Santos, M.A and Martinez, A.M.B. (2009). PEMF fails to enhance nerve regeneration after sciatic nerve crush lesion. J. Peripheral Nerv. System. Vol. 14. No 4. Pages 285-293. https://doi.org/10.1111/j.1529-8027.2009.00240.x

Binhi, V.N., Repiev, A and Edelev, M. (2002). Magnetobiology: Underlying Physical Problems.San Diego: Academic Press. Pages. 1-16.

Bervar M. (2005). Effect of weak, interrupted sinusoidal low frequency magnetic field on neural regeneration in rats : functional evaluation. Bioelectromagnetics. Vol.26. Pages. 351–356. doi:10.1002/bem.20108

Bloise, N; Petecchia, L; Ceccarelli, G; Fassina, L; Usai, C; Bertoglio, F. et al. (2018) The effect of pulsed electromagnetic field exposure on osteoinduction of human mesenchymal stem cells cultured on nano-TiO2 surfaces. PLoS ONE. Vol. 13. No.6: e0199046. https://doi.org/10.1371/journal.pone.0199046

Carpenter, D.O. (2015). The microwave syndrome or electro-hypersensitivity: historical background. Rev. Environmental Health, 30(4), Pages 217–222. DOI: https://doi.org/10.1515/reveh-2015-0016.

Christina L. R; Teli, T; Harrison, B.S. (2016). Electromagnetic Field Devices and Their Effects on Nociception and Peripheral Inflammatory Pain Mechanisms. Alternative Therapies. Vol. 22. N0. 3: Pages 34-47.

Das, S; Kumar, S; Jain, S; Avelev, V.D and Mathur, R. (2012). Exposure to ELFmagnetic field promotes restoration of sensori-motor functions in adult rats with hemisection of thoracic spinal cord. Electromagn Biol Med. Vol.31. No.3. Pages180–194. doi:10.3109/15368378.2012.695706

De Lahunta, A; Glass, E; and Kent, M. (2009). Lower motor neuron: Spinal nerve, general somatic efferent system. Veterinary

Dekun Gao , Hui Sun , Jin Zhu, Yinda Tang, Shiting Li, (2018) CXCL12 induces migration of Schwann cells via p38 MAPK and autocrine of CXCL12 by the CXCR4 receptor. Int. J. Clin. Exp. Pathol;11(6):3119-3125

Durney C.H; Massoudi H and Iskander M.F. (1986). Radiofrequency Radiation Dosimetry Handbook, 4th ed., Report USAFSAM-TR-85-73, USAF School of Aerospace Medicine, Brooks AFB, TX.

Faroni, A; Mobasseri, S. A; Kingham, P. J and Reid, A. J. (2015). Peripheral nerve regeneration: Experimental strategies and future perspectives. Advanced Drug Delivery Reviews, 82–83, 160–167. doi:10.1016/j.addr.2014.11.010

Flores,A.J; Lavernia, C.J and and Owens, P.W. (2000). Anatomy and physiology of peripheral nerve injury and repair. The American Journal of Orthopedics Vol. 29, No. 3. Pages 167–173.

Frey, A.H and Feld, S.R. (1975). Avoidance by rats of illumination with low-power nonionizing electromagnetic energy, J. Comp. Physio. Psych. Vol.89. Pages.183–188.

Gajšek, P; Ravazzani, P; Grellier, J; Samaras, T; Bakos, J and Thuróczy, G. (2016) Review of Studies Concerning Electromagnetic Field (EMF) Exposure Assessment in Europe: Low Frequency Fields (50 Hz–100 kHz), Int. J. Environ. Res. Public Health Vol.13. Pages 875-887.

Gandhi O.P. (1990). Electromagnetic energy absorption in humans and animals, in: Biological Effects and Medical Applications of Electromagnetic Energy, Gandhi O.P., Ed., Prentice Hall, Englewood Cliffs, NJ. Pages. 174–195.

Gordon, Z.V; Lobanova, Y.A and Tolgskaya, M.S. (1955). Some data on the effect of centimeter waves (experimental studies), Gig. Sanit. (USSR) 12:16.

Greenebaum, B and Sisken, B.F. (2007) Does direction of induced electric field or current provides a test of mechanism involved in nerve regeneration? Bioelectromagnetics. Vol 28. No 6. Pages 488-492.

Grinsell, D; Keating, C.P. Peripheral Nerve Reconstruction after Injury: A Review of Clinical and Experimental Therapies. (2014). Biomedical Res. 698256. doi: 10.1155/2014/698256

Hirose, H., Nakahara, T., Zhang, Q.M., Yonei, S., and Miyakoshi, J. (2003). Static magnetic field with a strong magnetic field gradient (41.7 T/m) induces c-Jun expression in HL-60 cells, In Vitro Cell Dev. Biol. Anim. Vol. 39 (8–9). Pages. 348–352.

Hei; Wei-Hong; Soo-Hwan Byun; Jong-Sik Kim; Soochan Kim; Young-Kwon Seo; Joo-Cheol Park; Soung-Min Kim; Jahng, J,W. and Lee, J.H. (2016). Effects of electromagnetic field (PEMF) exposure at different frequency and duration on the peripheral nerve regeneration: in vitro and in vivo study. International Journal of Neuroscience. Vol. 126. No. 8. Pages 739-748.

Johansson, O. (2015) Electro hypersensitivity: a functional impairment due to an inaccessible

environment. Rev Environ Health. Vol.30. No 4. Pages 311-321. doi:10.1515/reveh-2015-0018.

Joan M. S; Oury, M; Farnaz, A; Chantel T. D. Transcranial Magnetic and Direct Current Stimulation (TMS/tDCS) for the Treatment of Headache: A Systematic Review. (2019). Headache. doi: 10.1111/head.13479

Jensen, B.R; Malling, A.S.B; Morberg, B.M; Gredal O; Beach, P and Wermuth L. (2018). Effects of Long-Term Treatment with T-PEMF on Forearm Muscle Activation and Motor Function in Parkinson's Disease. Case Rep. Neurol. Vol. 10. Pages 242–251.

Ju, D.T; Liao, H.E; Shibu MA, Ho T.J; Padma, VV, et al. (2015) Nerve Regeneration Potential of Protocatechuic Acid in RSC96 Schwann Cells by Induction of Cellular Proliferation and Migration through IGF-IRPI3K-Akt Signaling. Chin J Physiol 58(6): 412-419.

Khan, A. A; Faruqi, N. A and & Ansari, M. S. (2014). Effects of hydrocortisone on the sciatic nerve crush injury in adult rat-a light microscopic study. Current Neurobiology, Vol.5. No.1.

Khurana, V.G; Hardell, L, Everaert, J; Bortkiewicz, A; Carlberg, M and Ahonen, M. (2010). Epidemiological evidence for a health risk from mobile phone base stations. Int. J. Occup. Environ Health. Vol. 16. Pages 263-267.

Kim Y.T; Hei W.H; Kim, S. et al. (2014). Co-treatment Effect of Pulsed Electromagnetic Field (PEMF) with Human Dental Pulp Stromal Cells and FK506 on the Regeneration of Crush Injured Rat Sciatic Nerve. International Journal of Neuroscience. Vol. 125. No. 10. Pages. 1-27.

Kolosova, L. I., et al. (1996). Effect of low-intensity millimeter wave electromagnetic radiation on regeneration of the sciatic nerve in rats. Bioelectromagnetics: Journal of the Bioelectromagnetics Society, The Society for Physical Regulation in Biology and Medicine, The European Bioelectromagnetics Association. Vol. 17. No.1. Pages. 44-47.

Kelleher, M. O; Al-Abri, R. K; Lenihan, D. V and Glasby, M. A. (2006). Use of a static magnetic field to promote recovery after peripheral nerve injury. Journal of neurosurgery. Vol.105. No.4. Pages 610-615.

Kwon; Myoung Soo and Heikki Hämäläinen. (2011). Effects of mobile phone electromagnetic fields: critical evaluation of behavioral an neurophysiological studies. Bioelectromagnetics. Vol. 32. No.4. Pages253-272.

Liakouris, J.A.G. (1998) Radiofrequency (RF) sickness in the Lilienfeld study: an effect of modulated microwaves? Arch Environ Health 53:226-228.

Lin QM, Tang XH, Lin SR, Chen BD, Chen F. Bone marrow-derived mesenchymal stem cell transplantation attenuates overexpression of inflammatory mediators in rat brain after cardiopulmonary resuscitation. (2020). Neural Regen Res;15(3) Pages: 24-31. DOI: 10.4103/1673-5374.265563

Li, Y; Yan, X; Liu, J. et al. (2014). Pulsed electromagnetic field enhances brain-derived neurotrophic factor expression through L-type voltage-gated calcium channel-and Erk-dependent signaling pathways in neonatal rat dorsal root ganglion neurons. Neurochemistry international. Vol. 75. Pages. 96-104.

Lei, T; Jing, D; Xie, K; Jiang, M; Li, F; Cai, J and Guo, W. (2013). Therapeutic effects of 15 Hz pulsed electromagnetic field on diabetic peripheral neuropathy in streptozotocin-treated rats. PLoS One, 8(4), e61414.

Martin, L.P. Microwave frequency electromagnetic fields (EMFs) produce widespread neuropsychiatric effects including depression. (2016) J. Chem. Neuroanatomy,B.7. Pages 43-51. https://doi.org/10.1016/j.jchemneu.2015.08.001

Minoo, S; Payman, T and Rahim, M. (2017). Effects of Pulsed Electromagnetic Fields on Peripheral Nerve Regeneration Using Allografts in Sciatic Nerve: An Animal Model Study. Biomed J Sci & Tech Res. Vol. 1. No.6. MS.ID.000509. DOI : 10.26717/BJSTR.2017.01.000509.

Nairz, O., Brezger, B., Arndt, M. and Zeilinger, A. (2001). Diffraction of Complex Molecules by Structures Made of Light. Physical Review Letters. Vol. 87. No.16. 401.1 - 401.4 (online article #160401).

National Research Council (US) Committee on Assessment of the Possible Health Effects of Ground Wave Emergency Network (GWEN). (1993). Assessment of the Possible Health Effects of Ground Wave Emergency Network. Washington (DC): National Academies Press (US); 1993. 6, Effects of Electromagnetic Fields on Organs and Tissues. Available from: https://www.ncbi.nlm.nih.gov/books/NBK208983/

Novitskiy, Y.I; Gordon Z.V; Presman A.S and Kholodov, Y.A. (1971). Radio Frequencies and Microwaves, Magnetic and Electrical Fields, National Aeronautics and Space Administration (NASA TT F-14.021), Washington, D.C.

Pan, Y; Dong, Y; Hou, W; Ji, Z; Zhi, K; Yin, Z. et al. (2013). Effects of PEMF on microcirculation and angiogenesis in a model of acute hindlimb ischemia in diabetic rats. Bioelectromagnetics. Vol.34. No.3. Pages. 180–188.

Peter Lyttkens. Electromagnetic field and neurological disorders Alzheimer´s disease, why the problem is difficult and how to solve it. (2018). Upasala University, PhD Thesis. Brain - Functions, Diseases and Mysteries I, 7.5 ECTS credits.

Rannug, A., Holmberg, B., Ekstrom, T., Mild, K.H., Gimenez-Conti, I., and Slaga, T.J. (1994). Intermittent 50 Hz magnetic field and skin tumor promotion in SENCAR mice, Carcinogenesis. Vol.15. No.2. Pages 153–157.

Rocha, S; Melo, L; Boudoux, C; Foerster, Á;Araújo, D; Monte-Silva K. (2015). Transcranial direct current stimulation in the prophylactic treatment of migraine based on interictal visual cortex excitability abnormalities: A pilot randomized controlled trial. J. Neurol Sci.349 (1-2):33-39

Ross, C.L; Syed, I; Smith, T.L and Harrison, B.S. (2017)

The regenerative effects of electromagnetic field on spinal cord injury. Electromagn Biol Med. Vol. 36. No. 1. Pages. 74–87. doi:10.3109/15368378.2016.1160408

Ross, C.L; Ang, D.C; Almedia-Porado, G. Targeting Mesenchymal Stromal Cells/Pericytes (MSCs) With Pulsed Electromagnetic Field (PEMF) Has the Potential to Treat Rheumatoid Arthritis.(2019). Front. Immunol. 10: 266. . doi: 10.3389/fimmu.2019.00266. eCollection 2019

Rusovan, A and Kanje M. (1991). Stimulation of regeneration of the rat sciatic nerve by 50 Hz sinusoidal magnetic fields. Exp Neurol. Vol.112, No.3. Pages. 312–316. doi:10.1016/0014-4886(91)90132-V

Rusovan, A; Kanje, M and Mild, K.H. (1992). The stimulatory effect of magnetic fields on regeneration of the rat sciatic nerve is frequency dependent. Exp Neurol. Vol.117. No.1. Pages.81–84. doi:10.1016/0014-4886(92)90113-5

Sherry, C.J; Blick, D.W; Walters, T.J; Brown, G.C and Murphy, M.R (1995). Lack of behavioral effects in non-human primates after exposure to ultra wide band electromagnetic radiation in the microwave frequency range, Radiat. Res. Vol.143. Pages. 93–97

Siemionow, M and Brzezicki,G (2009). Chapter 8-Current techniques and concepts in peripheral nerve repair. International Review of Neurobiology, Vol. 87, No. C, Pages. 141–172.

Sunderland, S and Ray, L.J. (1948). The intraneural topography of the sciatic nerve and its popliteal divisions in man. Brain. Vol. 71, part 3, Page. 242–273.

Seo, N.R; Lee, S.H; JU, K.W; Woo, J.M; Kim, B.J; Kim, S.M; Jahng, J.W and Lee, J.H. (2018). Low-frequency pulsed electromagnetic field pretreated bone marrow-derived mesenchymal stem cells promote the regeneration of crush-injured rat mental nerve. Neural Regen. Res. Vol. 13.N0. 1. Page.145–153.

Suszynski, K; Marcol, W; Szajkowski, S. et al. (2014). Variable spatial magnetic field influences peripheral nerves regeneration in rats. Electromagn Biol Med. Vol. 33. No.3. Pages.198–205. doi:10.3109/15368378.2013.801351

Stölting, M.N.L; Arnold, A.S; Haralampieva, D; Handschin, C; Sulser, T and Eberli, D. (2016). Magnetic stimulation supports muscle and nerve regeneration after trauma in mice. Muscle Nerve. Vol.53. No.4. Pages 598– 607. doi:10.1002/mus.24780

Terzi,M; Ozberk,B; Deniz, O,G and Kaplan,S. (2016) The role of electromagnetic fields in neurological disorders. Journal of Chemical Neuroanatomy. Vol 75-B. Pages 77-87. http://dx.doi.org/10.1016/j.jchemneu.2016.04.003.

Tepper, O.M; Callaghan, M.J; Chang, E.I. et al. (2004). Electromagnetic fields increase in vitro and in vivo angiogenesis through endothelial release of FGF-2. The FASEB journal. Vol.18. No.11. Pages. 1231-3.

Terzi, Murat, et al. (2016). The role of electromagnetic fields in neurological disorders. Journal of chemical neuroanatomy. Vol. 75. Pages. 77-84.

Unal, M; Creecy, A and Nyman, J.S. (2018). The Role of Matrix Composition in the Mechanical Behavior of Bone. Curr Osteoporos Rep. https://doi.org/10.1007/s11914-018-0433-0 PMID: 29611037

Walker, J.L; Evans, J.M; Resig, P; Guarnieri, S; Meade, P, et al. (1994) Enhancement of functional recovery following a crush lesion to the rat sciatic nerve by exposure to pulsed electromagnetic fields. Exp Neurol. Vol.125. No.2. pages 302-305.

Williams, J.M. (2016). Biological Effects of Microwaves: Thermal and Nonthermal Mechanisms. Biological Thermal and Nonthermal Mechanisms. Vol. 4.7.

WHO (2002). Radiation and environmental health department of protection of the human environment. Establishing a dialogue on risks from electromagnetic fields. Geneva WHO. Electromagnetic Field and public Health.

WHO (2013). Electromagnetic fields and public health: Electromagnetic hypersensitivity. http://www.who.int/peh-emf/publications/facts/fs296/en/.

Yakovleva, M.I; Shlyafer I.P and Tsvetkova I.P. (1968). On the question of condition cardiac reflexes, the functional and morphological state of cortical neurons under the effect of superhigh-frequency electromagnetic fields, Zh. Vyssh. Nervn. Deyat. (USSR) 18:973.

Zhang, P.X; Han, N; Kou, Y.H; et-al. Tissue engineering for the repair of peripheral nerve injury. (2019). Neural Regen. Res;14:Pages 51-8. DOI: 10.4103/1673-5374.243701

Dental
Communication

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp-43-47

Influence of Hydrofluoric Acid Etching, Resin Bonding and Resin Removal Treatments on the Surface Roughness of Ceramics

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ABSTRACT

The aim was to investigate the surface roughness of de-bonded ceramic surfaces with residual resin when treated with different resin removal treatment protocols. Sixty disc specimens of 5mm diameter and 3mm height of Lithium disilicate ceramic (IPS Emax Press) were fabricated. All specimens were treated with Hydrofluoric (HF) acid (9.5%) for 30 secs. Except 10 specimens as controls, all received silane treatment and a resin cement build-up (3mm x 2mm). All build-ups were sheared with universal testing machine and residual resin was removed using 5 protocols. Group 1: No treatment; Group 2: Bur treatment; Group 3: Heat treatment at 650° C (1min); Group 4: Heat treatment at 750° C (7 min); Group 5: Sandblasting (Al2O3) (2 min). The surface roughness (Ra) of all specimens was assessed using a non-contact laser surface profilometer. Data was assessed using ANOVA and multiple comparisons test. The highest roughness value was observed in the ceramic specimens exposed to sandblasting (Gp 5), which was 9.027 (1.362) µm. However the lowest Ra was observed for Gp 1 specimens [5.092 (0847) µm]. Overall, the difference among the study groups for surface roughness values was statistically significant ($p<0.05$). Presence of residual resin on de-bonded ceramic surfaces, compromises the surface roughness. Removal of residual resin with heat treatment at 650° C (1min) and sandblasting significantly improved and restored the surface roughness of de-bonded ceramics in comparison to Hydrofluoric acid etched ceramic surface.

KEY WORDS: CERAMIC, DE-BONDING, RESIN REMOVAL, HF ACID, HEAT TREATMENT, SANDBLASTING.

ARTICLE INFORMATION

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Received 17th Jan 2020 Accepted after revision 18th March 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)
A Society of Science and Nature Publication, Bhopal India

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/7

INTRODUCTION

Adhesive dentistry is the mainstay for esthetic restorations in oral rehabilitations. Resin and ceramic based materials are frequently employed for replacement of lost or damaged tooth structure and esthetic rejuvenations (Patil & Shetty, 2009). Esthetic ceramics are used in the form of veneers or crowns, however require surface conditioning of both the tooth and ceramic for predictable adhesive bonding (Farias et al., 2019). Mechanisms used to secure ceramics to tooth structure include, mechanical retention with ceramic surface treatments for improved surface energy and micro-rough surface (Saker et al., 2019). In addition, application of a chemical silane bifunctional molecule, connecting silica to hydroxyl ions in resin, increasing surface energy of ceramic allowing for penetration of luting agents (Murillo-Gómez et al., 2019). Ceramic surface etching with Hydrofluoric acid (HF acid) with varying concentration and duration is standard treatment for developing mechanically retentive surface. It is suggested that increasing the duration of HF acid application improves the surface pores, roughness and wettability (Zogheib et al., 2011).

Resin cemented ceramic veneers are employed to improve tooth shade, shape, size and form. However debonding of intact veneers followed by re-bonding is a frequent phenomenon due to inadequacies in clinical procedure (Granell Ruíz et al., 2014). Re-bonding is a challenging procedure, as it requires complete removal of luting resin from the ceramic surface without damage (Blatz et al., 2003). It is considered critical to expose the ceramic after removing the luting resin from the surface pores to re-create the desired surface topography of ceramics as displayed after acid etching (Magne et al., 2006; Martins et al., 2012). It is reported that contamination of etched ceramic surface results in compromised ceramic resin bond (Magne et al., 2006; Martins et al., 2012). Contaminants of the etched ceramic surface reduce surface pore size and numbers, potentially altering the surface topography. Therefore a compromised surface microstructure of the ceramic will jeopardize micromechanical retentive properties of ceramic, reducing the potential for ceramic bonding to tooth.

Surface roughness of ceramics is critical in producing an effective micromechanical bond. Increased surface roughness improves micro and nano-scale pores increasing surface area of the bondable ceramic (Ho & Matinlinna, 2011). In addition, increased roughness of ceramic surface further improves the wettability by reducing the contact angle facilitating the penetration of resin for ceramic bonding (Xiaoping et al., 2014; Colares et al, 2013). In the study by Román-Rodríguez et al., HF acid etched ceramics covered with resin were treated for removal of resin (Román-Rodríguez et al., 2015).They concluded that placement of ceramic in furnace at 650 °C for 1 minute pyrolized the resin and left a clean and retentive surface for re-bonding (Román-Rodríguez et al., 2015). However they did not assess the surface roughness of the heated ceramic surface. In addition, the influence of other treatments employed for the removal of resin from debonded ceramic surfaces including diamond bur, sandblasting and ceramic glaze treatment still need to be investigated. It is hypothesized that use of different methods, including diamond bur, heat treatments in furnace (650 °C and glaze treatment) and sandblasting will significantly influence the surface roughness of de-bonded ceramic surface. Therefore the aim of the study was to investigate the surface roughness of de-bonded ceramic surfaces with residual resin when treated with different resin removal treatment protocols.

MATERIAL AND METHODS

Sixty disc specimens of 5mm diameter and 3mm height of Lithium disilicate ceramic (IPS Emax Press, Ivoclar Vivadent, NY, USA), were fabricated using the Hot-Press technique. Discs were initially prepared using inlay wax (Inlay casting wax- Kerr-CA, USA) and a putty mold was produced using it. The wax discs were invested using investment material (IPS PressVest, Ivoclar Vivadent, NY, USA). Ceramic ingots (IPS Emax Press, Ivoclar Vivadent, NY, USA) of light translucency (LT) were hot pressed in a Press furnace (Programat EP 5010) at 5 bar and 925 °C. The divested ceramic discs were sandblasted with glass beads to remove the reaction layer and ultrasonic cleaned in distilled water for 5 minutes. Ceramic discs were polished with 600 grit silicon carbide paper on a slow speed wheel with continuous water. All ceramic specimens were treated with Hydrofluoric (HF) acid (Porcelain Etchant, 9.5%, Bisco-IL.USA) for 30 secs, followed by washing with water (20 sec) and ultrasonic cleaning in distilled water for 5 min. 10 specimens were kept as controls with no further treatment (positive control) (Gp C). The remaining fifty specimens were treated with silane (1 min) (Monobond Plus- Ivoclar Vivadent. NY, USA). It was followed by a build-up of dual cure resin cement (Nexus 3rd Gen- Kerr CA, USA) (2mm diameter and 3mm height) on the ceramic surface using a putty mold and a glass slide. Excess cement was removed and resin cement was light cured (Bluephase, Ivoclar, Vivadent) for 40 sec from top and 40 sec after removal of mold.

All cement build-ups were sheared from the ceramic discs using a chisel placed parallel to the interface between the cement and ceramic. A controlled force was applied using a universal testing machine (Instron-5965) until fracture. The fifty specimens were further divided into 5 subgroups (n=10) based on the surface treatment for removal of residual resin on the ceramic surface.

Gp 1: No removal of resin (negative control)
Gp 2: Diamond bur on slow speed handpiece to remove the resin cement.
Gp3: Place ceramic in furnace for 1 min at 650 ° C.
Gp 4: Place ceramic in furnace and use glazing heat treatment procedure to pylorize (6+1 min at 750 ° C)
Gp 5: Removal of resin ceramic with sandblasting for 2 minutes (Aluminum oxide).

After ceramic surface treatments, surface profilometry was performed to assess the surface roughness (Ra) using

a 3D non-contact optical surface profiler (Contour Gt-K1 optical profiler, Bruker Tucson, AZ, USA). The surface profilometer produced scale independent outcomes and produced sub-nanometer vertical resolutions. The profiler used a strong Vision 64 interface for image production and scanning. The specimens did not need any preparation and prior to each measurement the profilometer was calibrated. An average of upto nine measurements were performed in parallel, oblique and perpendicular planes. The value of Ra in µm, presents an average of the surface traced by the profiler. The data obtained was assessed for normality and compared for roughness among the study groups using ANOVA and Tukey Multiple comparisons test.

RESULTS AND DISCUSSION

All data obtained from profilometer assessments was normally distributed. The surface roughness after HF acid etching- control group (Gp C) was 7.683 (0.609) µm. With resin coating of the etched ceramic surface (Gp 1) the surface roughness reduced to 5.092 (0847) µm. Four resin removal treatments were assessed in the present study. The surface of ceramic specimens placed in the furnaces i.e. group 3 and group 4, showed roughness (Ra) values of 8.274 (0.652) µm and 7.381 (0.721) µm respectively. The observed roughness values among ceramic specimens treated with diamond bur (Gp 2) were 8.187 (1.539) µm. The highest roughness value was observed in the ceramic specimens exposed to sandblasting (Gp 5), which was 9.027 (1.362) µm (Table 1). Overall, the difference among the study groups for surface roughness values was statistically significant ($p<0.05$) using ANOVA. Using multiple comparisons test, study groups were statistically compared (Table 2).

The groups with HF acid treatment (Gp C-positive control) [7.683 (0.609) µm] showed significantly higher Ra compared to resin treated group [5.092 (0847) µm] (Gp 1- negative control) specimens ($p<0.05$) and significantly lower Ra to sandblasted group specimens (Gp 5) [9.027 (1.362) µm] ($p<0.05$). Specimens with resin treated ceramics without removal treatment (Group 1) showed significantly lower surface roughness [5.092 (0847) µm] compared to all groups ($p<0.05$). Ra among groups 2 [8.187 (1.539) µm], 3 [8.274 (0.652) µm] and 4 [7.381 (0.721) µm] specimens were statistically comparable ($p>0.05$). Group 5 ceramic specimens (sandblasted) [9.027 (1.362) µm] exhibited significantly higher Ra values compared to Group C [7.683 (0.609) µm] and Group 1 specimens [5.092 (0847) µm] ($p<0.05$) (Figure 1). In addition, surface roughness of sandblasted specimens [9.027 (1.362) µm] was also significantly higher than group 4 specimens [7.381 (0.721) µm] ($p<0.05$) (Figure 2).

The present study was based on the hypothesis that use of different methods, including diamond bur, heat treatments in furnace (650 °C and glaze treatment) and sandblasting will significantly influence the surface roughness of de-bonded ceramic surface. In the present study, specimens treated for resin removal using heat treatment, sandblasting and burs, all showed a significant change in ceramic surface roughness in comparison to untreated specimens. Therefore the proposed hypothesis was accepted. A myriad of explanations could support the outcomes in the present study, including chemical properties of resin, high speed impact of sandblasting and and physical properties of resin and ceramics. Surface roughness of Lithium disilicate (LD) ceramics was assessed as they are widely used in dentistry for oral rehabilitations. They are comprised of a silica glass matrix and lithium oxide crystals, which act as flux. LD ceramics show higher flexural and fracture resistance to conventional glass ceramics, due to the randomly arranged needle like crystals acting as crack stoppers (Awad et al., 2019).

When ceramic veneers are debonded, to improve surface finish, glaze procedure is often repeated; therefore heat treatment of resin-covered ceramics was investigated in the present study. In addition, diamond or carbide burs on a slow speed handpiece are the most common methods of resin removal for clinicians, chairside. Moreover, sandblasting with aluminum oxide particles is commonly employed in laboratories to remove material remnants and improve surface roughness of metals and ceramics (Barutcigil et al, 2019). “Ra” as a parameter of surface profile is widely used in material and ceramic research reports (Blunt et al, 2008). It is recommended that Ra is measured over “a number of consecutive sampling lengths”, and average values to be reported, as observed in the present study (Leach , 2010). In the present study, HF acid etched surface was considered the standard (control), which showed higher Ra compared to resin treated specimens. The resin cement tends to occupy the surface pores produced by etching, covering the etched surface resulting in low roughness outcomes. Re-bonding of such surfaces does not allow penetration of bonding agent into the micro-porosities, hence resin covered surfaces were treated for resin removal.

Table 3. Mean and standard deviations of surface roughness (Ra) among study groups.

Study Group	Group C HF Acid (+ve control)	Group 1 No Treatment (-ve control)	Group 2 Bur Treatment	Group 3 650 °C (1 min)	Group 4 750° C (7 min)	Group 5 SB (1 min)
Surface Roughness (Ra)	7.683 (0.609)	5.092 (0.847)	8.187 (1.539)	8.274 (0.652)	7.381 (0.729)	9.027 (1.362)

Table 2. p values among the study using Tukey HSD Post-hoc Test * Indicate statistical significant difference among study groups

	HF Acid	Group 1 No Treatment	Group 2 Bur Treatment	Group 3 650 °C	Group 4 750° C	Group 5 SB
HF Acid	0	0.001*	0.6268	0.4518	0.9368	0.001*
Group 1 No Treatment		0	0.001*	0.001*	0.001*	0.001*
Group 2 Bur Treatment			0	0.999	0.134	0.105
Group 3 650 °C				0	0.0711	0.1909
Group 4 750° C					0	0.001*
Group 5 SB						0

Figure 1. Profilometry Micrograph For surface roughness (Ra) of a group 1 specimen

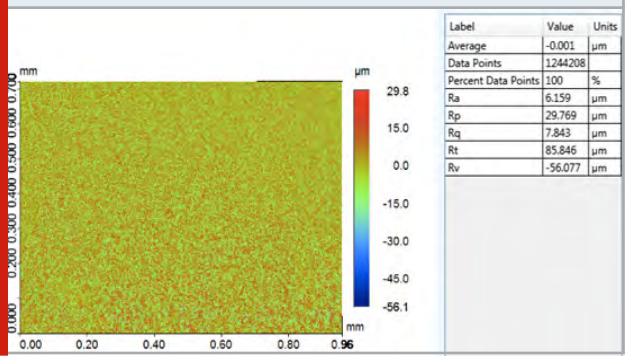
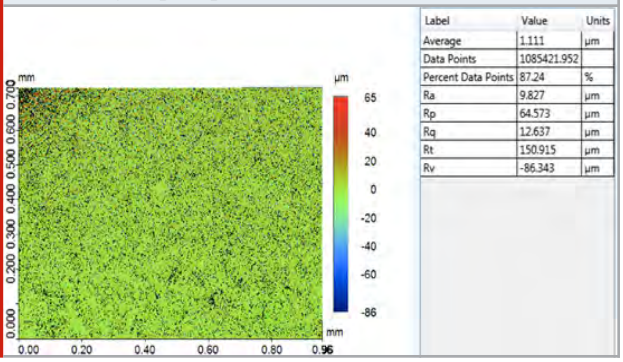


Figure 2: Profilometry Micrograph For surface roughness (Ra) of a group 5 specimen



The use of diamond bur (group 2) in the present study resulted in a significantly higher Ra then group1 specimens (no resin removal). It has been revealed in previous studies that diamond bur and disc treatments improve adhesive resistance of ceramics (Guler et al., 2005). Interestingly, the standard deviations of the bur treatment were wide (1.539), reflecting the operator dependency and error prone nature of this treatment modality. We assessed the influence of heat treatments at 650° C and 750° C in an attempt to remove resin and expose the etched ceramic surface. It was shown that both heat treatments exhibited significant removal of resin and the Ra improved significantly from group 1. A possible explanation for this finding lies in the boiling point of resins, which is lower than the temperatures applied by the furnace in glaze cycles.

In a study by Román-Rodríguez et al., (Román-Rodríguez et al., 2015) it was concluded that placement of ceramic veneer in the furnace at 650° C for 1 minute would burn out the resin on ceramic surface. It is now revealed that the ceramic surface roughness will be re-acquired as a result of heat treatment procedure. In addition the Ra for ceramics heated to 750° C (7 min) was lower (7.381) compared to the Ra for ceramics heated at 650° C (1 min) (8.274). This may be attributed to the melting effect of high temperature heating for silica matrix within the lithium disilicate ceramics (Shenoy and Shenoy, 2010). This results in loss of depth width and size of micro-porosities produced by acid etch (Shenoy and Shenoy, 2010).

It was observed in the present study that resin covered ceramic surface when sandblasted, resulted in the highest surface roughness among all groups. The application of aluminum oxide particles at high speed creates impacts resulting in removal of resin on ceramic surface. However the increased surface roughness does not only represent the resin removal but merely shows the topographical effects of the high-speed particle impacts on the ceramic surface. In addition, the sandblasted specimens also showed a wide standard deviation for Ra (1.362), indicating a resin removal method with less reliability and possible untoward effects on ceramics (Zhang et al., 2004).

Therefore although the surface roughness was significantly improved by sandblasting a standard protocol for its use in resin removal should be devised. The outcomes indicate that the removal of resin from ceramic surface using heat treatment at 650° C (1 min) or sandblasting can result in a rough ceramic surface similar to an etched ceramic surface. However a possible limitation of the study is the lack of adhesive

bond strength assessment which would determine the effect of the resin removal on the re-bond of ceramic to resin cements. In addition, it is critical to identify the remaining resin on the ceramic surface after resin removal treatments. Therefore further studies assessing the amount of residual resin and its influence on bond strength after resin removal from de-bonded ceramic surfaces are recommended.

CONCLUSION

Presence of residual resin on de-bonded ceramic surfaces, compromises the surface roughness. Removal of residual resin with heat treatment at 650° C (1min) and sandblasting significantly improved and restored the surface roughness of de-bonded ceramics in comparison to Hydrofluoric acid etched ceramic surface.

Conflict of Interest: The author declare no conflict of interest

REFERENCES

Awad, M.M., Al Jeaidi, Z.A., Almutairi, N., Vohra, F., Özcan, M. and Alrahlah, A., 2019. Effect of self-etching ceramic primer on bond strength of zirconia-reinforced lithium silicate ceramics. Journal of Adhesion Science and Technology, pp.1-11.

Barutcigil, K., Barutcigil, Ç., Kul, E., Özarslan, M.M. and Buyukkaplan, U.S., 2019. Effect of different surface treatments on bond strength of resin cement to a CAD/CAM restorative material. Journal of Prosthodontics, 28(1), pp.71-78.

Blatz, M.B., Sadan, A. and Kern, M., 2003. Resin-ceramic bonding: a review of the literature. The Journal of prosthetic dentistry, 89(3), pp.268-274.

Blunt, L., Jiang, X., Leach, R., Harris, P. and Scott, P., 2008. The development of user-friendly software measurement standards for surface topography software assessment. Wear,264(5-6), pp.389-393.

Colares, R.C.R., Neri, J.R., Souza, A.M.B.D., Pontes, K.M.D.F., Mendonca, J.S. and Santiago, S.L., 2013. Effect of surface pretreatments on the microtensile bond strength of lithium-disilicate ceramic repaired with composite resin.Brazilian dental journal, 24(4), pp.349-352.

Farias, D.C.S., Gonçalves, L.M., Walter, R., Chung, Y. and Blatz, M.B., 2019. Bond strengths of various resin cements to different ceramics. Brazilian oral research, 33.

Granell-Ruíz, M., Agustín-Panadero, R., Fons-Font, A., Román-Rodríguez, J.L. and Solá-Ruíz, M.F., 2014. Influence of bruxism on survival of porcelain laminate veneers. Medicina oral, patologia oral y cirugia bucal, 19(5), p.e426.

Guler, A.U., Yilmaz, F., Ural, C. and Guler, E., 2005. Evaluation of 24-hour shear bond strength of resin composite to porcelain according to surface treatment. The Journal of Prosthetic Dentistry, 94(6), p.538.

Ho, G.W. and Matinlinna, J.P., 2011. Insights on ceramics as dental materials. Part II: chemical surface treatments. Silicon,3(3), p.117.

Leach, R.K, 2010. Fundamental principles of engineering nanometrology. Elsevier. Micro & nano technologies series. Elsevier, William Andrew, Amsterdam, 1. ed edition,. OCLC: 837259110

Magne, P. and Cascione, D., 2006. Influence of post-etching cleaning and connecting porcelain on the microtensile bond strength of composite resin to feldspathic porcelain. The Journal of prosthetic dentistry, 96(5), pp.354-361.

Martins, M.E., Leite, F.P., Queiroz, J.R., Vanderlei, A.D., Reskalla, H.N. and Ozcan, M., 2012. Does the ultrasonic cleaning medium affect the adhesion of resin cement to feldspathic ceramic. J Adhes Dent, 14(6), pp.507-509.

Murillo-Gómez, F., Wanderley, R.B. and De Goes, M.F., 2019. Impact of Silane-containing Universal Adhesive on the Biaxial Flexural Strength of a Resin Cement/Glass-ceramic System.Operative dentistry, 44(2), pp.200-209.

Patil, R. and Shetty, O., 2019. Full mouth rehabilitation performed using customized Broadrick's flag to restore mandibular posterior teeth-A Case Report. Clinical Dentistry (0974-3979), 13(2).

Román-Rodríguez, J.L., Alonso-Pérez-Barquero, J., Bruguera-Álvarez, A., Agustín-Panadero, R. and Fons-Font, A., 2015. Cleaning and retreatment protocol for a debonded ceramic restoration. Journal of clinical and experimental dentistry, 7(1), p.e60.

Saker, S., Özcan, M. And Al-zordk, W., 2019. The impact of etching time and material on bond strength of self-adhesive resin cement to eroded enamel. Dental materials journal, pp.2018-126.

Shenoy, A. and Shenoy, N., 2010. Dental ceramics: An update. Journal of conservative dentistry: JCD, 13(4), p.195.

Xiaoping, L., Dongfeng, R. and Silikas, N., 2014. Effect of etching time and resin bond on the flexural strength of IPS e. max Press glass ceramic. Dental Materials, 30(12), pp.e330-e336.

Zhang, Y., Lawn, B.R., Rekow, E.D. and Thompson, V.P., 2004. Effect of sandblasting on the long-term performance of dental ceramics. Journal of Biomedical Materials Research Part B: Applied Biomaterials:, 71(2), pp.381-386.

Zogheib, L.V., Bona, A.D., Kimpara, E.T. and Mccabe, J.F., 2011. Effect of hydrofluoric acid etching duration on the roughness and flexural strength of a lithium disilicate-based glass ceramic. Brazilian dental journal, 22(1), pp.45-50.

Biotechnological Communication

BBRC Bioscience Biotechnology Research Communications

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp- 48-52

Isolation and Identification of Potential Ligand Molecules from *Glycyrrhiza glabra* Against EGFR by Molecular Docking Approach

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ABSTRACT

Lung cancer is uncontrolled cell growth in tissues of the lung. Most cancers that start in the lung, known as primary lung cancer, are carcinomas. *Glycyrrhiza glabra* produced bioactive compounds were isolated through GCMS. Thus in the present investigation, bioactive compounds from *G. glabra*, were screened and identified through molecular docking. EGFR is important lung cancer progression, invasion and metastasis. So it is used as one of the most potent pharmacological target for lung cancer. 3D structure of target protein EGFR retrieved from Swiss-Prot database. Molecular docking studies revealed that bioactive compounds from *G. glabra* had very good interactions with EGFR protein molecule. Out of 19 bioactive compounds top two 2,2'-dioxospirilloxanthinand Pregn-5-en-20-one were identified as potential drug molecules against EGFR. These compounds had acceptable binding energy values and could be used for further in-vitro validation. In conclusion, the present study explained traditional phyto constituents from *G.glabra* could be further promoted as potential lead molecule against EGFR to control ovarian cancer. More photochemistry and molecular studies required to reaffirm the work

.KEY WORDS: LUNG CANCER, EGFR, *GLYCYRRHIZA GLABRA*, GCMS, MOLECULAR DOCKING, HEX 8.0.

INTRODUCTION

Lung cancer is the most frequently diagnosed of all cancers and occurred in 1.8 million people in worldwide in 2012 and responsible for 1.8 million deaths (Ferlay et al.,2012). There are two main types of lung cancers: small-cell lung cancer (SLCL) and Non-small-cell lung cancer (NSCLC). The most common form of lung cancer is NSCLC and first-line of treatment often involves platinum-based combination chemotherapy (Zappa and Mousa, 2016). Epidermal growth factor receptor (EGFR) is a protein on the surface of cells. It normally helps the cells grow and divide. Some NSCLC cells have many EGFR, which regulates cell proliferation, apoptosis, angiogenesis, and tumor invasion. Drugs called *EGFR* inhibitors can block the signal from EGFR that helps the cells to grow, (Herbstet al., 2008). Now a days many drugs can be used alone without chemotherapy to treat NSCLC such as Erlotinib (Tarceva), Afatinib (Gilotrif), Gefitinib (Iressa), Osimertinib (Tagrisso) and Dacomitinib (Vizimpro).However the most common side effects of all

ARTICLE INFORMATION

*Corresponding Author: menakamallika@gmail.com
Received 12th Feb 2020 Accepted after revision 25th March 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)
A Society of Science and Nature Publication, Bhopal India

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/9

EGFR inhibitors were include skin problems, diarrhoea, mouth sores and loss of appetite. Therefore, there is an urgent need for development of new improved drugs to avoid concern to the patients, hence researchers are looking alternative medicines from the natural sources using computer aided drug designing programmes (Newman and Cragg, 2010; Hoelderet al., 2012).

Almost 60 percent of drugs approved for cancer treatment are of natural origin. *Glycyrrhiza glabra* Linn is one of the most widely used herbs from the ancient medical history of Ayurveda, both as a medicine also as a flavouring herb. This plant is also pharmacologically studied for its anti-ulcerogenic,antioxidant, antimicrobial, and anti-inflammatory properties,(Pastorinoet al., 2018). *Glycyrrhiza* roots are useful for treating cough also effective against anaemia, gout, sore throat, tonsillitis, flatulence, sexual debility, hyperdypsia, fever, skin diseases and swellings. *G. glabra* is considered as one of the best remedies for relieving pain (Parvaizet al., 2014). Thus in the present investigation, bioactive compounds from *G.glabra*, were screened and were attempted to identify the potential molecule which can act as EGFR inhibitor using molecular docking methods.

MATERIALS AND METHODS

Collection of plant materials: Fresh roots of *G. glabra* was collected from the Madurai, Tamil Nadu.The taxonomic identity of the species was authenticated by comparison with herbarium voucher specimens deposited in the Herbarium Sri Paramakalyani Centre for Environmental Sciences Manonmaniam Sundaranar University Alwarkurichi-627412 Tamil Nadu. The leaves and roots were washed under running tap-water, shade-dried, powdered mechanically in a mixer grinder, sieved and stored in an airtight container for plant extract preparation.

Plant extract Preparation: The powdered plant materials were subjected to successive solvent extractions as follows. The powder was placed in a 1-L thimble of a Soxhlet apparatus and extracted with petroleum ether (40–60°) (Merck, Mumbai, India) for 72 hours in four batches of 250 g each. Before each extraction, it was dried at room temperature. All the extracts were filtered and concentrated in a vacuum with rotary flash evaporator (Büchi, Flawil, Switzerland). Leftover solvent was completely removed in a water bath, and the extracts were dried in a desiccator. The crude extracts obtained from each solvent were labelled and weighed, and the percentage yield was recorded. The crude petroleum ether extract of *Glycyrrhiza glabra* was then subjected to qualitative tests to determine the bioactive constituents.

Isolation and characterization of bioactive compounds: The powdered sample were soaked and dissolved in methanol for 24 h. Then the filtrates were collected by evaporated under liquid nitrogen. The samples were injected in split mode as 10:1. Mass Spectral scan range was set at 45-450 (mhz).GC-MS analysis was done on a Thermo Trace GC Ultra coupled with Polaris Q MS and TriPlus auto-sampler using a DB-5 (0.25 mm × 30 m × 0.22 µm) column in which helium was used as carrier gas. GC-MS analysis was performed as per mehtod described by Ivanova et al., 2005.Shortly described here, the temperature was set between 50°C to 250°C at a rate of 10°C min-1. The initial temperature was held for 2 min and final temperature of 250°C was held for 10 min. The GC flow rate was 1 ml min–1 and the total run time was 32 min. MS was performed at scan mode between 0 – 600 m/z with an Ion trap EI+.

The instrument was set to an initial temperature of 110°C, and maintained at this temperature for 2 min. At the end of this period, the oven temperature was raised up to 280°C, at the rate of an increase of 5°C/min, and maintained for 9 min. Injection port temperature was ensured as 250°C and Helium flow rate as 1 ml/min. The ionization voltage was 70 eV. The samples were injected in split mode as 10:1. Mass Spectral scan range was set at 45-450 (mhz). The chemical constituents were identified by GC-MS.The fragmentation patterns of mass spectra were compared with those stored in the spectrometer database using National Institute of Standards and Technology Mass Spectral database (NIST-MS). The percentage of each component was calculated from relative peak area of each component in the chromatogram.

Molecular docking studies: EGFR enzyme plays a crucial role in causing lung cancer. Thus, it is considered as potential target protein and EGFR is retrieved from UniProtKB/Swiss-Prot database (http://www.uniprot.org/). A list of bioactive compounds was retrieved from *G. glabra* using Chem spider database (http://www.chemspider.com/) and further these compounds were considered as ligands. Two dimensional structures of these ligands were converted into 3D structure using swiss pdb viewer (http:// www.spdbv.vital-it.ch/). The Protein-ligand interaction plays an important role in structural based drug designing. In this study molecular docking analysis between target protein (EGFR) and ligand molecules from *G.glabra* was carried out by Hex8.0

Figure 1a. 2D Structure of 2,2'-dioxospirilloxanth in

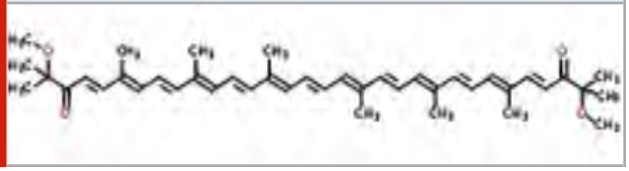
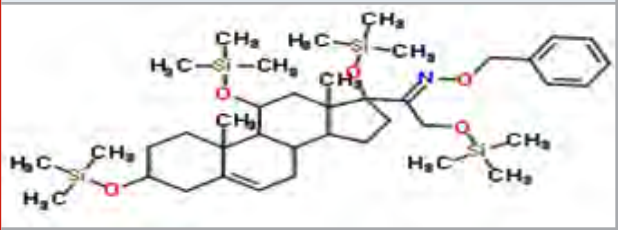


Figure 1b. 2D Structure of Pregn-5-en-20-one



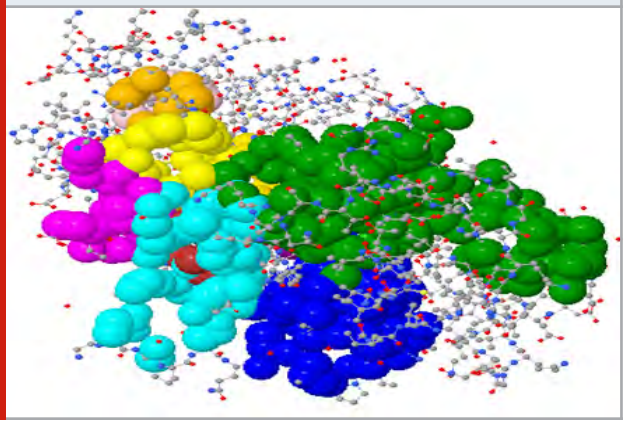
docking program (http://www.hex.loria.fr/dist50/). It was performed by adjusting appropriate parameters such as twist range-360, receptor range-180,ligand range-180, FFT mode-3D fast lite, grid dimension-0.6 and distance range-40. The obtained scores of binding energy was tabulated and analysed.

RESULTS AND DISCUSSION

In herbal medicine literature, G. glabra plays an important part in Ayurveda and Siddha arrangement of drug acting as ulcer protective, demulcent, expectorant, anti-tussive and purgative the utilized for therapeutic purposes and it is additionally pre-clinically and clinically the most focused one (Amarowiczet al., 2004; Chin et al., 2007). Thus, in the present investigation we attempted to analyze the bioactive compounds of *G.glabra* compare drug interaction properties with EGFR, which is potent lung cancer causing receptor. In this study, alcoholic extracts of *G. glabra* were analyzed and isolated 11 new phenolic compounds, glycybridins A–K (1–11), along with 47 known compounds.The chemical structures of extracted compounds were elucidated on the basis of extensive NMR and MS analyses as well as experimental and computed ECD data.

Figure 2. 3-D structure of EGFR kinase

Figure 3. 3-D structure of EGFR kinase showing ligand binding sites (shown indifferent colours)



Bioactive compounds of *G.glabra*: In the present study we isolated 19 bioactive compunds were follows, 2,4,6-Decatrienoic acid, Bulleyanin, 2,2'-dioxospirilloxanthin (Fig.`a), Dihydroagathic acid, Bruceantine, Hydrate, Diacétate de (3α,5ξ,9ξ,18β)-lup-20(29)-ène-3,28-diyle, Acetic acid, cyclopenta[a]phenanthren-3-yl (2Z)-2-methyl-2-butenoate, Betamethasone acetate, Taxine B, 1,7-Dioxa-4,10-diazacyclododecane-4,10-diethanol, Di-tungsten, tris(cyclooctatetraene), Pregn-5-en-20-one (Fig.1b), Sarsasapogenin, 5-Germaspiro, Molybdenum, 1-Monolinoleoylglyceroltrimethylsilyl ether and Ethyl oleate. In literatures, the bioactivities of individual compounds present in *G. glabra* showed anticancer effects on the lung cancer cell line (Mayo and Donner, 2002). Duke et al., (2004) reported administration of licorice administration to patients prevented various cancers. Kataria (2012) described the antitumor activity of compound isolate from *G. glabra*. For *in silico* studies, all 19 bioactive compound structures were retrieved from Chemspider database.

EGFR: The epidermal growth factor receptor (EGFR) belongs to the ErbB family of receptor tyrosine kinases (RTK). These trans-membrane proteins are activated following binding with peptide growth factors of the EGF-family of proteins. Evidence suggests that the EGFR is involved in the pathogenesis and progression of different carcinoma types including lung cancer (Normannoet al.,2006).So, in the present study UniProtKB/Swiss-Prot database was used to retrieve EGFR and considered as

Figure 4. Molecular docking of EGFR kinase with 2,2'-dioxospirilloxanthin

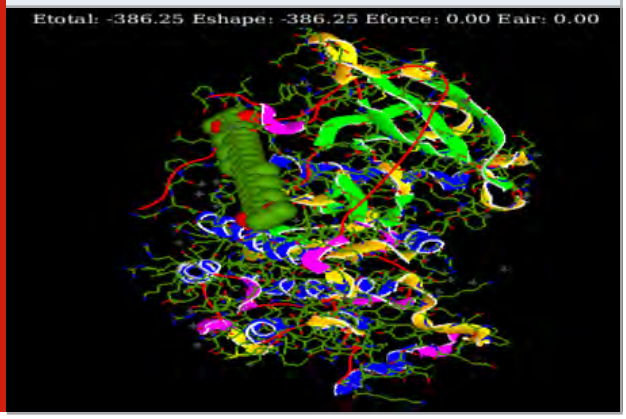
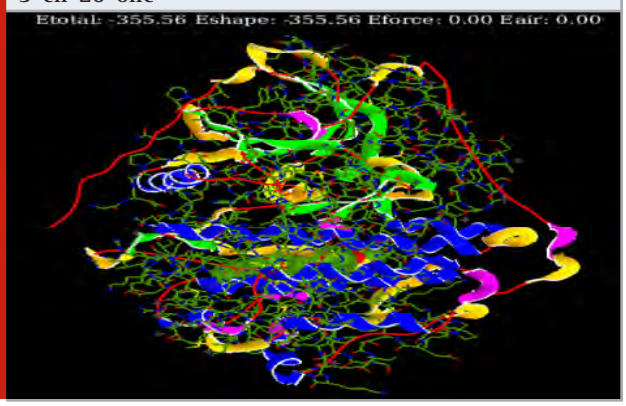


Figure 5. Molecular docking of EGFR kinase with Pregn-5-en-20-one



target molecule (antigen). EGFR was formed by a single polypeptide chain with 327 amino acids. The polypeptide is composed of 352 sheets connected by 198 H-bonds with 1182 helices in its structure (Fig.2). This receptor is also an effective antigen of many cancer types, which induces lung cancer. EGFR is having 10 ligand binding sites on its surface. One of the binding sites suitable for a particular ligand acts as the active binding site by which an energetic molecular docking takes place (Fig.3). The area of the 10 possible ligand binding sites is ranging from 73.1 to 1228.9 Sq Å and the volume from 51.8 to 2055.7 Cu Å.

Prediction of binding sites: In order to find out the most effective drug among the 19 bioactive compounds extracted from *G. glabra*, molecular docking was carried out against EGFR kinase using Hex 8.0 software. The docking scores were ranging from -231.83 KJ/mol to -386.25KJ/mol (Table.1). 2,2'-dioxospirilloxanthin (Fig.4) and Pregn-5-en-20-one(Fig.5) were selected as they showed a strong interaction and binding with target protein having best docking scores when compared to other tested ligands. 1,7-Dioxa-4,10-diazacyclododecane-4,10-diethanol have scored low energy value -231.83. Thakur and Raj (2017) reported that pharmacological activities, characterized secondary metabolites and traditional uses of *G. glabra* warrants its potential therapeutic uses. However, further scientific validations along with evidence-based studies are still required in support to its traditional uses, to serve as "lead" for development of novel agents and a better drug candidate in future. There is currently no clinically proven hormonal therapy for lung cancer. However, there is several estrogen modulating treatments that are widely used in breast cancer which could be explored in lung cancer therapy (Li et al., 2017). Hence the present study will helpful to understand the potential drug candidate to effectively bind and inhibit EGFR.

Table 1. Shown molecular docking of EGFR Kinase with bioactive compounds of *Glycyrrhiza glabra*

S. No	Name of the bioactive compounds	Molecular formula	Molecular mass	Energy value (e-value)
1	2,4,6-Decatrienoic acid	$C_{35}H_{46}O_8$	594.735 Da	-336.51
2	Bulleyanin	$C_{28}H_{38}O_{10}$	534.595 Da	-266.83
3	2,2'-dioxospirilloxanthin	$C_{42}H_{56}O_4$	624.892 Da	-386.25
4	Dihydroagathic acid	$C_{20}H_{32}O_4$	336.466 Da	-249.89
5	Bruceantine	$C_{28}H_{36}O_{11}$	548.579 Da	-289.93
6	Hydrate	$C_{19}H_{28}ClNO_4$	369.883 Da	-266.47
7	Diacétate de (3α,5ξ,9ξ,18β)-lup-20(29)-ène-3,28-diyle	$C_{34}H_{54}O_4$	526.790 Da	-290.75
8	Acetic acid	$C_{29}H_{38}O_3S$	466.675 Da	-281.88
9	cyclopenta[a]phenanthren-3-yl	$C_{32}H_{52}O_2$	468.754 Da	-292.14
10	(2Z)-2-methyl-2-butenoate Betamethasone acetate	$C_{24}H_{31}FO_6$	434.498 Da	-297.00
11	Taxine B	$C_{28}H_{38}O_{10}$	534.595 Da	-270.05
12	1,7-Dioxa-4,10-diazacyclododecane-4,10-diethanol	$C_{12}H_{26}N_2O_4$	262.346 Da	-231.83
13	Di-tungsten, tris(cyclooctatetraene)	$C_{24}H_{24}W_2$	680.141 Da	-328.80
14	Pregn-5-en-20-one	$C_{40}H_{71}NO_5Si_4$	758.337 Da	-355.56
15	Sarsasapogenin	$C_{27}H_{44}O_3$	416.637 Da	-316.01
16	5-Germaspiro	$C_{44}H_{50}B_2Ge$	673.130 Da	-294.30
17	Molybdenum	$C_{30}H_{42}Mo_2O_4$	658.535 Da	-247.46
18	1-Monolinoleoylglycerol trimethylsilyl ether	$C_{27}H_{54}O_4Si_2$	498.886 Da	-329.91
19	Ethyl oleate	$C_{20}H_{38}O_2$	310.514 Da	-292.13

CONCLUSION

In conclusion, from the list of 19 bioactive compounds retrieved from the data bank of *G. glabra*, two, 2,2'-dioxospirilloxanthinand Pregn-5-en-20-one were identified by selecting the suitable ligands for EGFR protein receptor for controlling lung cancer. A complex between 2,2'-dioxospirilloxanthin and EGFR molecular docking studies reveals best dock score-386.25 KJ/mol. Thus, we concluded that 2,2'-dioxospirilloxanthin

could inhibit EGFR protein and consider as potential drug candidate against treating lung cancer and further in vitro studies are required to confirm these results. The molecular docking in the current study was concrete enough to discover the binding mechanism and interaction between the 19 different compounds, which are the ligands and EGFR. The results obtained in this study shall be useful for future drug designing and development of novel compounds with higher inhibitory activity against EGFR. However, it is mandatory need to validate these compounds in in-vitro and in-vivo studies for establishing them as potential novel drug candidates.

REFERENCES

Amarowicz R Pegg RB Rahimi-Moghad-dam P Barl B Weil JA (2004) Free-radical scavenging capacity and antioxidant activity of selected plant species from the Canadian prairies Food Chem 84:551-62

ChinYW Jung HA LiuY Su BN Castoro JA Keller WJ Pereira MA and Kinghorn D (2007). Anti-oxidant Constituents of the Roots and Stolons of Licorice (Glycyrrhiza glabra) J. Agric. Food Chem 55 4691-4697

Duke JA Godwin MB Cellier J and Duke PA (2004) CRC Hand Book of Medicinal Spices. CRC Hand Book Boca Raton London New York Washington p.p 174-181

Ferlay J Soerjomataram I Ervik M et al., (2012) GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11

Herbst RS1 Heymach JV Lippman SM (2008) Lung cancer N Engl J Med Sep 25 359(13)1367-80 doi: 10.1056/NEJMra0802714

Hoelder S1 Clarke PA Workman P (2012) Discovery of small molecule cancer drugs successes challenges and opportunities MolOncol 6(2)155-76 doi: 10.1016/j.molonc.2012.02.004

KatariaR Hemraj G Singh and Avneet Gupta (2013) Pharmacological activities on glycyrrhizaglabra–a review Asian J Pharm Clin Res Vol 6 Suppl1: 5-7

Li K1, Ji S1, Song W1, Kuang Y1, Lin Y1, Tang S1, Cui Z1, Qiao X1, Yu S1, Ye M1 (2017) Glycybridins A-K, Bioactive Phenolic Compounds from Glycyrrhiza glabra J Nat Prod 80(2):334-346. doi: 10.1021/acs.jnatprod.6b00783

Mayo LD and Donner DB (2002) The PTEN, MDM2, p53 tumor suppressor-oncoprotein network. Trends BiochemSci 27: 462-467.

Newman DJ Cragg GM(2012) Natural products as sources of new drugs over the 30 years from 1981 to 2010 J Nat Prod 75(3) 311-335

Normanno N de Luca A Bianco C et al. (2006) Epidermal growth factor receptor (EGFR) signaling in cancer Gene366(1):2-16.

ParvaizM K Hussain S Khalid N Hussnain N Iram Z Hussain and M A Ali (2014) bA Review: Medicinal Importance of Glycyrrhiza glabra L. (Fabaceae Family) Global Journal of Pharmacology 8 (1) 08-13 2014ISSN 1992-0075© IDOSI Publications, DOI: 10.5829/idosi.gjp.2014.8.1.81179

Pastorino GL Cornara S Soares F Rodrigues M Beatriz and P Oliveira (2018) Liquorice (Glycyrrhiza glabra) A phytochemical and pharmacological review Phytotherapy research 32(12) 2323-2339

Thakur AK, Raj P (2017) Pharmacological Perspective of Glycyrrhiza glabra Linn.: a Mini-Review J Anal Pharm Res 5(5): 00156 DOI: 10.15406/japlr.2017.05.00156

Zappa C Mousa S A (2016) Non-small cell lung cancer: current treatment and future advances. Translational lung cancer research5 (3) 288–300 doi:10.21037/tlcr.2016.06.07

Web addresses http://www.uniprot.org/,http://www.chemspider.com/,http:// www.spdbv.vital-it.ch/ http://www.hex.loria.fr/dist50/

Biomedical
Communication

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp-53-59

Factors Effecting Level of Job Satisfaction Among Prosthodontist Working In Kingdom of Saudi Arabia: A Cross Sectional Study Design

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ABSTRACT

The present study has evaluated the overall job satisfaction among prosthodontists, highlighting the significance of intrinsic and extrinsic factors and their satisfaction level with patients, facilities and workplace when working in Kingdom of Saudi Arabia.A questionnaire consisting of 38 questions in seven domains related to socio-demographic characteristics, academic, professional qualifications, satisfaction as a prosthodontist, workplace environment facilities and their satisfaction with patients were sent using survey monkey instrument to prosthodontists from the office of Saudi dental Society. Out of the total 150 emails were sent to the prosthodontists. 78 (52%) responses were attained. Standard deviation (SD), percentages and means were calculated. Comparison between the demographic, academic, professional qualification variables and other domains were explored by comparing mean scores by applying ANOVA. Demographic factors exhibited that 91.0% (71) were Saudi nationals, whereas, 8.9% (7) were non-Saudis. Gender analysis indicated 53 respondents (67.9%) were male, whereas 25 (32%) were females. Majority 38 (48.7%) of the participants fall within professional experience of 3–8 years and 37 (47.4%) participants were engaged in academics-based practice. Majority of the respondents, 48 (61.6%), reported “challenging profession” as the main reason for choosing prosthodontic. Moreover, 70.3% stated that prosthodontic was their primary choice among all dental specialities. Furthermore, eighty four percent of the participants affirmed that they are content with working environment within the practice team because it was conducive. Approximately, 90% of the prosthodontist were of the view that they have good relations with their patients. The findings concluded that prosthodontists working in KSA are content and satisfied with their job. Moreover, improvements in administrative responsibilities of prosthodontists should be enhanced to further boost their professional capabilities.

KEY WORDS: JOB SATISFACTION, PROSTHODONTIST, INTERNAL AND EXTERNAL FACTORS, CROSS SECTIONAL SURVEY.

ARTICLE INFORMATION

*Corresponding Author: nalabban@ksu.edu.sa
Received 15th Jan 2020 Accepted after revision 26th Feb 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)
A Society of Science and Nature Publication, Bhopal India

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/9

INTRODUCTION

Sustained high stress levels with poor job satisfaction causes burn out syndrome in dental care professionals (DCP) (Myers and Myers, 2004). Poor job satisfaction reduces dentist's commitment and dedication to organization and increases intention to leave the job (Kay and Scarrott, 1997). Evidence suggests dentists experience highest stress levels (Moore and Brødsgaard, 2001; Stack, 2001). It is important to understand job satisfaction among dentists and how work environment, facilities, staff have an impact on it. Studies have revealed that lowered job satisfaction among healthcare workers is directly linked to increased stress level (Cooper et al., 1988).Measuring job satisfaction is important as it may influence physical and mental health and may affect job related behavior and performance (Bergström et al., 2010). Job satisfaction in dentistry motivates a dentist, provides improvement, satisfaction and pleasure at work, increasing the efficiency, (Puriene et al., 2007, Lo Sasso et al., 2015a, Alqahtani et al., 2018, Nash and Benting 2019).

This indirectly improves patient care and satisfaction, benefiting dental auxiliaries, patient and dentists. Henceforth, enhancing the success and progress of oral health care and practice (Judge et al., 2001).High suicidal rate associated with poor job satisfaction makes dentistry a hazardous profession (Stack, 2001). However, studies have reported that job satisfaction in dentists can be improved due to flexible working hours, better salary packages, medical insurance and appreciable attitudes of senior faculty (Judge et al., 2001; Lo Sasso et al., 2015a). There are ample job satisfaction surveys done already on dentists, (Goetz et al., 2012; Wells and Winter, 1999) but satisfaction surveys on dental specialities such as prosthodontists, orthodontics, periodontists are very limited and scarce. Recently, job satisfaction survey on orthodontists have been performed by Alqahtani et al., (2018) in Saudi Arabia yielding high satisfaction rate in the region. Similarly, a study Al-Mudaf et al., (2003) conducted survey among three different dental specialities oral surgery, periodontics and fixed prosthodontics in city of Kuwait claiming all specialities to have high job and patient satisfaction.

To our knowledge from indexed literature, evidence related to job satisfaction and motivation factors among prosthodontist working in the Kingdom of Saudi Arabia (KSA) is not available. Therefore, the aim of the present study, was to evaluate overall job satisfaction among prosthodontist highlighting the significance of intrinsic and extrinsic factors and their satisfaction level with patients, facilities and workplace when working in KSA. The data gathered can give a glimpse of quality of life (QoL) of prosthodontist working in KSA.

MATERIAL AND METHODS

The study was approved by the ethical committee of King Saud University Riyadh Saudi Arabia under ethical number (E18-3360). The study was in accordance to STROBE statement of reporting cross sectional surveys. The duration of the study was three months i.e. from April 2019 to July 2019. A literature review was performed to validate the present cross-sectional survey, which revealed that there was no available data on assessment of job satisfaction amongst prosthodontists. Contact details of registered prosthodontists were requested from the office of Saudi Dental Society. A questionnaire was formulated consisting of total 38 questions in seven domains. Research team of statistician along with authors reviewed the content of each question to make sure that the survey reflected appropriate phrasing and understanding and validation. A total sample size of 100 prosthodontists were well-thought-out but since dropouts, invalid responses were anticipated so a sample size of 150 prosthodontists were considered appropriate. Using power calculation sample size was assessed from a study by Alqahtani et al., (2018) A link containing details of the questionnaire using survey monkey instrument was sent to prosthodontists from the office of Saudi dental Society. Reminder email were sent periodically to improve response rate.

The seven domains of the cross-sectional survey consisted of questions related to socio-demographic characteristics, academic, professional qualifications, motivation behind choice of prosthodontist as a specialty, satisfaction as a prosthodontist, satisfaction with the workplace environment facilities and staff and their satisfaction with their relationship with patients. The questions of the survey were sourced from a study by Alqahtani et al., (2018) and were included in the study with some modification. The responses were measured using a five-point Likert Scale coded as, 1 strongly disagree; 2 disagree; 3, neutral; 4 agree; 5 strongly agree. Out of the total 150 emails sent to the prosthodontists. 78 (52%) responses were attained. All the responses were evaluated by a single investigator to minimize bias. Statistical Package for the Social Sciences (SPSS Inc., software version 21 Chicago, IL, USA) was used for tabulation of descriptive analysis. Standard deviation (SD), percentages and means were calculated. Comparison between the demographic, academic, professional qualification variables and other domains were explored by comparing mean scores by applying ANOVA.

RESULTS AND DISCUSSION

The results of 78 respondents revealed highly significant difference among responses to different included questions ($p < 0.05$). Socio-demographic characteristics, academic and professional qualifications and other demographic factors exhibited that 91.0% (71) were Saudi national. Whereas, 8.9% (7) were non-Saudis. Gender analysis indicated 53 respondents (67.9%) were male, whereas 25 (32%) were females. Forty-two (53.8%) of the participants had age between 31-40 years. (Table 1)

Table 1. Sociodemographic Characteristics of respondents

Characteristics	Frequency	Percent (%)	Significance
Age of the respondents			
< 30 years	10	12.8	
31-40 years	42	53.8	
41- 50 years	20	25.6	
51-60 years	2	2.5	
> 60 years	4	5.12	
Gender of the respondents			
Male	53	67.9	<0.001
Female	25	32.0	
Nationality of the respondents			
Non-Saudi	7	8.97	
Saudi	71	91.0	

Table 2 presents the academic qualifications of the respondents. 38 (48.7%) of the dentists had their qualification from Saudi commission of health sciences, by completing the Prosthodontic Saudi board specialist training and examination. While, 15 (19.2%) of the responders completed a master's degree and certificate in prosthodontic; and only 11(14.1%) had a doctorate degree. The results further revealed that amongst the total respondents, 47 (60.2%) completed their residency program within Kingdom of Saudi Arabia (KSA) while, 28 approximately (35.8%) completed their residency program in a foreign country. Details regarding length of work experience and place of work is enumerated in Table 3.

Majority 38 (48.7%) of the participants fall within professional experience of 3–8 years. Followed by 9 respondents who possessed 9-15 years of work experience and only 5 participants had professional experience between 16–25 years. Moreover, amongst 78 respondents, 37 (47.4%) participants were engaged in academics-based practice, followed by 14 respondents (17.9%) employed by the Ministry of Health (MoH) and 15 (19.2%) were working in a private dental practice. Motivation for choosing prosthodontic as a speciality:Majority of the respondents, 48 (61.6%), reported "challenging profession" as the main reason for choosing prosthodontic. This was followed by "professional growth," "financial gains," "prestigious specialty," and "family influence." (Table 4)

Table. 2: Academic qualifications and training of respondents

Education and Training	Frequency	Percent (%)	Significance
Qualifications of the respondents			
Certificate in endodontics	14	17.9	<0.001
M.Sc. & Certificate endodontics	15	19.2	
Ph.D.	11	14.1	
Board	38	48.7	
Where did you attend a residency program?			
Saudi Arabia	47	60.2	<0.001
Europe	7	8.9	
North America	21	26.9	
(USA & Canada)			
Other	3	3.8	

Table 3: Respondents work experience and related information

Work Experience	Frequency	Percent (%)	Significance
Total length of experience			
<3 years	22	28.2	<0.001
3-8 years	38	48.7	
9-15 years	9	11.5	
16-25 years	5	6.4	
> 25 years	4	5.1	
Where do you work?			
Academic (university based)	37	47.4	<0.001
Private practice	15	19.2	
Ministry of health	14	17.9	
University clinics	4	5.1	
National guard	5	6.4	
Armed forces	3	4.5	

Respondents' satisfaction with the prosthodontist profession: Majority of the participants, 70.3% stated that prosthodontic was their primary choice among all dental specialities. Followed by 49.3% claiming to be satisfied with working quality of auxiliary staff. Furthermore, 63.7% of the subjects were pleased with overall, quality of life as a Prosthodontist. Moreover, 65.5% of the participants were gratified with facilities and resources in the clinics for adequate delivery of quality oral health care. However, 27.3% of the respondents were not content with the income from their prosthodontic practice. In addition, nearly 49.3% of the dentists were

unhappy with medical facilities provided to them as job benefits. (Table 5)

Respondents' satisfaction of the workplace environment: Eighty four percent of the participants affirmed that they are content with working environment within the practice team because it was conducive. Likewise, 90.8% of the participating subjects agreed that their professional senior colleagues were kind and they enjoyed working as team. Moreover, 60.8% of the participants stated that their organization was supportive for professional development and quality of work (Table 6). The results presented in Table 7 show that 49.1% of the dentists were satisfied with practice management and care delivery system. patients were not punctual and did not adhere to the appointment schedule. Likewise, 85.4% of the respondents agreed that overall they were satisfied with their job as a prosthodontist. In contrast, a significant percentage (33.6%) of their patients had unrealistic expectations regarding the outcome of their prosthodontic treatment. (Table 8). The present cross-sectional survey displayed a unique assessment of prosthodontists job satisfaction in the region of KSA. To our understanding this is one of the distinctive surveys done in KSA on prosthodontists job satisfaction. The response rate in the present study was 78 (52%) which was quiet less then expected, despite reminder emails. The low response rate can be due to busy schedule of prosthodontist, inability to follow up with emails regularly, hectic clinical hours and other office obligations.

Low response rate was also observed in other studies but from different dental specialities (Al-Jewair et al., 2016; Alqahtani et al., 2018). Moreover, fewer response

Table 4: Respondents motivation for choosing prosthodontics

Reason for choosing periodontics	Frequency	Percent (%)	Significance
Motivation for opting prosthodontics			
Professional growth	16	20.5	
Prestigious specially	6	5.35	
Challenging profession	48	61.6	<0.001
Family influence	2	1.78	
Financial gains	6	5.45	

Table 5: Satisfaction factors related to the Prosthodontic profession

Satisfaction Factor	Strongly agree +agree	fair	Strongly disagree+ disagree	Significance
Prosthodontic specialty being first choice	70.3	15.5	14.2	
My job description and responsibilities	67	16	17	
where I work are well- defined and clear Satisfied with working	49.3	27.6	23.1	
quality of my auxiliary staff Satisfied with working	28.2	59.8	12	
quality of my technicians Facilities and resources	65.5	10.1	25.4	<0.001
in the clinics are adequate for delivery of quality care to patients My current practice	68	15	17	
situation is what I envisioned when I chose to be a Prosthodontics Satisfied with the	49	23.6	27.4	
salary/ wages and other financial benefits Satisfied with the medical and	31	19.7	49.3	
dental treatment services provided to me as job benefits Overall, I am satisfied with	63.7	27.2	9.1	
quality of life as Prosthodontist				

Table 6: Respondents satisfaction with the workplace environment

Satisfaction Factor	Strongly agree + agree	fair	Strongly disagree+ disagree	Significance
I am treated respectfully by the Head of my department	89.1	7.3	4.5	
Support from administrative offices, secretaries and clerical staff is adequate	63.1	22.4	14.5	
In general, I am treated respectfully by my senior colleagues	90.8	7.3	1.9	<0.001
My organization supports professional development for improvement of their efficiency and quality of work	60.8	20.1	19.1	
I am satisfied with working environment within the practice team because it is conducive and professional	84.0	12.7	4.0	

Table 7: Respondents satisfaction with staff and facilities

Satisfaction Factor	Strongly agree +agree	Fair	Strongly disagree + disagree	Significance
I have adequate time for my professional development activities	43.6	27.6	26.6	
I have adequate time for my personal and family life	50	30	20	<0.001
I am satisfied with the practice management and care delivery system	49.1	28.6	22.1	

rate were reported from females 25 (32%) which may indicate their family commitment (Shigli et al., 2012). Furthermore, 67.9% male responses show male dominancy in this field this finding was found to be in concurrent with studies by Maharjan and Mathema, (2018) and Nash and Benting, (2019). In the present study most of the respondents belonged to age group 31-40 years (53.8%) had experience between 3-8 years 38 (48.7) and were from academic universities 37 (47.4%) this may give a reflection that postgraduate institution provides better avenues to young faculty for academic development (Shigli et al., (2012). Moreover, when inquired about postgraduate qualification 38 (48.7%) subjects completed their postgraduation from Saudi Boards. This trend directs that Saudi students after graduation didn't waste time efforts to fly abroad for postgraduation and were content with postgraduate opportunities within the country. This tendency also specifies that students were motivated, eager and enthusiastic in obtaining lifelong education. This finding was found to be in harmony with a study by Al-Dlaigan et al., (2011)

The survey consisted of seven domains and thirty-eight item questions. A cross sectional type study design was used as it is easy, simple and cost-effective to perform, it may help in generating a hypothesis for a more complex investigation and possibilities of loss to follow up are minimum (Sedgwick, 2014). In addition, responses were gathered through survey monkey instrument. The survey has an advantage of usability, comprehensive feature set and security. Moreover, it gives real time results for quick and easy analysis (Buchanan and Hvizdak, 2009). The overall job satisfaction among prosthodontist in the present study was 85.4%. This high percentage of job satisfaction corelates with prosthodontic speciality being the first choice among the respondents (70.3%). Evidence suggests job satisfaction is one of the foremost attitude that predicts job performance, and it can affect customer satisfaction and health care delivery inside and outside the health care services (Lo Sasso et al., 2015b). Moreover, quality of life satisfaction among prosthodontist in the current study was 63.7%. Choosing profession and specialty of first choice helps a lot in

balancing QoL and also may reduce occupational stress levels (DiMatteo et al., 1993).

48(61.8%) respondents were of the view that they specialized in prosthodontics as they felt it's a challenging profession. This attitude reflects a mature, professional and highly motivated mind set of the respondents as challenging profession was by far advanced by other motivational factors (Alqahtani et al., 2018). This finding also relates that professional growth along with challenging profession were given priority over financial gains. This finding contradicts the findings of a study by Noble et al., (2010) where financial motives were prime indicator for choosing dental profession. Overall satisfaction of respondents with workplace environment and prosthodontics was more than 60%. Conducive work environment plays a major role in providing job satisfaction and decreasing work related stress. Work related factors contribute to job gratification and organizational commitment. Henceforth, enhancing the success and progress of organization and dental practice (Khalighi et al., 2018). Furthermore, almost 50% of the respondents were not satisfied in giving time to their family members. These findings were found to be in concurrent with work from Alqahtani et al., (2018); Soma et al., (2012) Comfortable family life and support plays a major role in job satisfaction of dentist. Prosthodontics working in KSA regret in not spending adequate time can be due to work related pressure, non-flexible clinical hours and other clinical commitments (Alqahtani et al., 2018).

Most of the respondents were not satisfied and were unhappy with patients coming late to the dental practice i.e., being not punctual. These results are parallel to a study by Rada et al., indicating that such act by the patients causes high level of stress and anxiety among dentists (Rada and Johnson-Leong, 2004). Furthermore 45.5% of the respondents were of view that load of paperwork and administrative duties affect professional capabilities. This finding is in line with a study by Jari et al., explaining office and administrative issues hampers performance of a dentists, increases stress and anxiety issues which indirectly affects job satisfaction (Hakanen et al., 2005). From the results of this study it can be inferred, that though job satisfaction among prosthodontist working in KSA was satisfying. More, studies with better study designs should be executed with comparison between different dental specialty. This is the first study among prosthodontist job satisfaction in KSA and more studies should be performed to validate the findings of the present study. The study has limitation based on its small sample size. More studies with increase sample size and prosthodontists from other parts of KSA should be taken in account to get a better representation of the population. For future studies, a comparison between Saudis and Non-Saudis job satisfaction as prosthodontists should be also performed.

Table 8: Respondents satisfaction about their relationship with patients

Satisfaction Factor	Strongly agree +agree	fair	Strongly disagree+ disagree	Significance
I have good relations with my patients	90	6.4	3.6	
My colleagues are courteous, and we enjoy working in a team	68.2	12.7	10.0	
My work is recognized and appreciated by my colleagues and seniors	74.5	10	6.3	
Patients are always on time and adhere to the appointment schedule	31.8	32.7	35.5	
I feel no problem while communicating with staff	74.5	10.9	5.4	
I feel no problem while communicating with my patients	70	10.9	10.0	
The load of paperwork and administrative duties affect my professional capabilities	45.5	17.3	28.2	
Patients' unrealistic expectations burn me out	33.6	30.9	35.5	<0.001
Amount of workload is too much	36.4	30	33.6	
I face too much pressure from my seniors	12.7	13.6	64.5	
I can freely utilize my potentials and capabilities	48.2	26.4	16.3	
I have a liberty to choose	64.5	17.3	9.1	
appropriate working methods and materials				
Overall, I am satisfied with my job as a prosthodontist	85.4	7.3	7.3	

CONCLUSION

Prosthodontists working in KSA are content and satisfied with their job. Moreover, improvements in administrative responsibilities of prosthodontists should be enhanced to further boost their professional capabilities.

Conflict of interest: The authors declare that they have no conflict of interests for the present study and all authors have read and approved the final draft.

FUNDING AND ACKNOWLEDGEMENT

The authors would like to thank the College of Dentistry Research Centre and Deanship of Scientific Research at King Saud University, Saudi Arabia, for funding this research project (#FR 0507).

REFERENCES

Al-Dlaigan, Y.H., Al-Sadhan, R., Al-Ghamdi, M., Al-Shahrani, A., Al-Shahrani, M., (2011). Postgraduate specialties interest, career choices and qualifications earned by male dentists graduated from King Saud University. Saudi Dent. J. 23, 81–86.

Al-Jewair, T.S., Hamidaddin, M.A., Alotaibi, H.M., Alqahtani, N.D., Albarakati, S.F., Alkofide, E.A., Al-Moammar, K.A., (2016). Retention practices and factors affecting retainer choice among orthodontists in Saudi Arabia. Saudi Med. J. 37, 895–901.

Al-Mudaf, B.A., Moussa, M.A.A., Al-Terky, M.A., Al-Dakhil, G.D., El-Farargy, A.E., Al-Ouzairi, S.S., (2003). Patient satisfaction with three dental speciality services: A centre-based study. Med. Princ. Pract. 12, 39–43.

Alqahtani, N.D., Alshehry, K., Alateeq, S., Alturki, H., Albarakati, S., Asiry, M.A., Ahmedani, M.S., (2018). An assessment of job satisfaction: A cross-sectional study among orthodontists of Saudi Arabia. J. Orthod. Sci. 7, 23–30.

Bergström, K., Söderfeldt, B., Berthelsen, H., Hjalmers, K., Ordell, S., (2010). Overall job satisfaction among dentists in Sweden and Denmark: A comparative study, measuring positive aspects of work. Acta Odontol. Scand. 68, 344–353.

Buchanan, E.A., Hvizdak, E.E., (2009). Online survey tools: Ethical and methodological concerns of human research ethics committees. J. Empir. Res. Hum. Res. Ethics 4, 37–48.

Cooper, C.L., Watts, J., Baglioni, A.J., Kelly, M., (1988). Occupational stress amongst general practice dentists. J. Occup. Psychol. 61, 163–174.

DiMatteo, M.R., Shugars, D.A., Hays, R.D., (1993). Occupational stress, life stress and mental health among dentists. J. Occup. Organ. Psychol. 66, 153–162.

Goetz, K., Campbell, S.M., Broge, B., Dörfer, C.E., Brodowski, M., Szecsenyi, J., (2012). The impact of intrinsic and extrinsic factors on the job satisfaction of dentists. Community Dent. Oral Epidemiol. 40, 474–480.

Hakanen, J.J., Bakker, A.B., Demerouti, E., (2005). How dentists cope with their job demands and stay engaged: The moderating role of job resources. Eur. J. Oral Sci. 113, 479–487.

Judge, T.A., Bono, J.E., Thoresen, C.J., Patton, G.K., (2001). The job satisfaction-job performance relationship: A qualitative and quantitative review. Psychol. Bull. 127, 376–402.

Kay, E.J., Scarrott, D.M., (1997). A survey of dental professionals' health and well-being. Br. Dent. J.

Khalighi, H., Mortazavi, H., Anbari, F., Dehghan, M., (2018). Job Satisfaction Level among Dental Faculty Members Based on Their Specialty. J. Med. Educ. 17.

Lo Sasso, A.T., Starkel, R.L., Warren, M.N., Guay, A.H., Vujicic, M., (2015a). Practice settings and dentists' job satisfaction. J. Am. Dent. Assoc. 146, 600–609.

Lo Sasso, A.T., Starkel, R.L., Warren, M.N., Guay, A.H., Vujicic, M., (2015b). Practice settings and dentists' job satisfaction. J. Am. Dent. Assoc. 146, 600–609.

Maharjan, S.K., Mathema, S., (2018). Survey on Status of Prosthodontists in Nepal, Service Rendered and Their Level of Satisfaction. J. Nepal. Prosthodont. Soc. 1, 26–32.

Moore, R., Brødsgaard, I., (2001). Dentists' perceived stress and its relation to perceptions about anxious patients. Community Dent. Oral Epidemiol. 29, 73–80.

Myers, H.L., Myers, L.B., (2004). "It's difficult being a dentist": Stress and health in the general dental practitioner. Br. Dent. J.

Nash, K.D., Benting, D.G., (2019). Private Practice of Prosthodontists in the United States: Results from the 2017 Survey of Prosthodontists and Trends Since 2001. J. Prosthodont. 28, 49–63.

Noble, J., Hechter, F.J., Karaiskos, N., Wiltshire, W.A., (2010). Motivational factors and future life plans of orthodontic residents in the United States. Am. J. Orthod. Dentofac. Orthop. 137, 623–630.

Puriene, A., Janulyte, V., Musteikyte, M., Bendinskaite, R., (2007). General health of dentists. Literature review. Stomatologija 9, 10–20.

Rada, R.E., Johnson-Leong, C., (2004). Stress, burnout, anxiety and depression among dentists. J. Am. Dent. Assoc. 135, 788–794.

Sedgwick, P., (2014). Cross sectional studies: Advantages and disadvantages. BMJ.

Shigli, K., Hebbal, M., Nair, K.C., (2012). Teaching, research, and job satisfaction of prosthodontic faculty members in Indian Academic Dental Institutions. J. Dent. Educ. 76, 783–791.

Soma, K.J., Thomson, W.M., Morgaine, K.C., Harding, W.J., (2012). A qualitative investigation of specialist orthodontists in New Zealand: part 2. Orthodontists' working lives and work-life balance. Aust. Orthod. J. 28, 170–180.

Stack, S., 2001. Occupation and suicide. Soc. Sci. Q. 82, 384–396.

Wells, A., Winter, P.A., (1999). Influence of practice and personal characteristics on dental job satisfaction. J. Dent. Educ. 63, 805–812.

Environmental
Communication

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp-60-67

Spatial and Temporal Changes in Land Resources of Belgorod Region Under the Influence of Anthropogenic Factors

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ABSTRACT

This article contains an assessment of the historical data on the land resources of the Belgorod Region with a breakdown by municipal districts. Some historical documents from the State Archive of the Belgorod Region and the recent reports on the land status and use of the Belgorod Region Office of the Federal Service for State Registration, Cadastral Records and Cartography (Rosreestr) were used to analyse the spatial and temporal changes in the structure of the land resources. The land area was quantitatively analysed by categories. The degree of transformation of the land resources was evaluated. The historical data on the commercial lands is presented using GIS application ArcGIS 10.2, including the use of toolkits Spatial Analyst and Spatial Statistics. Anthropogenic load was assessed with the use of multidimensional cluster analysis methods (Ward's method). For the development of information base on transformation of the land resources in the territory of municipal districts of the Belgorod Region a time interval from the formation of the region (1954) until 2017 was used. During this, period significant changes occurred in the structure of the land resources. The area of the arable land was reduced by 7.8%. The area of natural forage and forestlands increased by 17.1% and 13.2% respectively. The area of land occupied by industrial buildings and built-up territory has increased by 31.2%. The area of land under water facilities has increased by 133.2%. The dynamics of land transformation under swamps is unstable. Drainage of swamps and their transformation into hayfields and pastures has reduced the amount of wetland by 12% for the period from 1954 to 1975. Wetlands in floodplains are reported to be increased by 25.2% since 1976. The areas under perennial plantations (14.3%) and disturbed lands, which are unsuitable for agricultural use (11.6%), have slightly decreased. The municipal districts are divided into three large clusters by cluster analysis. The determinant classification criterion is spatial and temporal change in land resources over 63 years. The municipal districts of the Belgorod Region have been typified by historical data on land and resource potential of agricultural landscapes.

KEY WORDS: LAND FUND, ANTHROPOGENIC IMPACT, LAND TRANSFORMATION, LAND MONITORING.

ARTICLE INFORMATION

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Received 8th Feb 2020 Accepted after revision 25th March 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/11

INTRODUCTION

The significant rates of build-up of the gross regional product of the Belgorod region, which is mainly, composed of manufacturing, agriculture and forestry, mining, as well as increase in agricultural production in the crop and livestock sectors influences substantially on the state of land resources in the agricultural landscapes of the Belgorod region. This region is not only agricultural with the share of arable land being more than 60% of the total area of the territory, but also an industrial zone. However, disturbed lands (ferrous metallurgy quarries, construction material production facilities) make up 14.1% in the total share of industrial and other special purpose lands. It is essential to analyse the interaction between man and nature in a comprehensive way, to evaluate the ecological and resource state of agricultural landscapes, to classify and to typify those (Lisetsky et al., 2015).The data on quantitative changes in the Belgorod Region lands are constantly updated. The rates of anthropogenic degradation of vegetation and soils have already been established (Chendev et al., 2008; Chendev et al., 2016; Zelenskaya et al., 2019).

We have brought up to date the historical data on woodlands (Kuzmenko et al., 2013, Degtyar and Grigoreva, 2018), including according to satellite survey materials (Terekhin and Chendev, 2018), calculated natural grassland area variation (Kitov, 2015, Kitov and Tsapkov, 2015), evaluated the fallow area (Marinina et al., 2013), covered broadly in the paper the issues related to the development of urban agglomerations (Chugunova et al., 2019), analysed the structural transformation of the suburban areas of the Belgorod agglomeration in the paper (Tsapkov, 2016) and assessed the dynamics in the structure of the land resources of the Belgorod Region (Grigorieva, 2017 a, b). The accumulated data bank on qualitative and quantitative changes in the land cover (Shtompel' et al., 1998, Lisetskii et al., 2016, Lisetskii, 2019) is needed to manage land and property relations, to carry out monitoring functions in relation to the land resources and the entire ecological environment of the Belgorod region. Typological diversity of the river basins of the European Russia (Yermolaev et al., 2018) calls for the development of adapted systems of water and land use (Lisetskii et al., 2014 a, b; Pozachenyuk et al., 2015, 2018, Grigoreva and Buryak, 2016, Buryak and Grigoreva, 2019).

Land cover transformation is a dynamic process in the land and property system (Srivastava et al., 2010), and its cumulative effect affects the life-sustaining functions of the Earth's biomes (Chhabra et al., 2006) as well as has socio-economic consequences (Shifaw et al., 2019). One of the main lines of global socio-economic and environmental studies is the development of land monitoring system, including specific monitoring activities for the study of land cover dynamics changes (Zhou and Kang, 2011). It is essential to have available definite sets of estimated data on land cover condition and use in order to study environmental changes, to manage land resource, to forecast and to simulate sustainable development of territories (Cai et al., 2011, Manakos et al., 2017, 2019).

The limited land resources and the growing need for their use cause dissonance between stakeholders, which requires continuous correction of decisions in land management (Felsenstein and Lichter, 2014). The priority land management task is to integrate historical approach into land potential evaluation (Fischer and Black, 1995, Zia, 2012, Rinfret and Pautz, 2014, Comber et al., 2016, Bao et al., 2019). It is impossible to have long-term research, improvement and forecasting in land monitoring system without the use of geoinformation approaches (Grigoreva, 2015); in particular, remote sensing is the most important source of data for agricultural monitoring mapping (Teluguntla et al., 2018, Nduati et al., 2019). GIS-approach is largely based on the integrated use of different types (Terekhin, 2016, 2018) of a multi-time set of satellite medium-resolution data (250 m or more) and a time-limited high-resolution set (Landsat 30 m) (Nduati et al., 2019).

MATERIAL AND METHODS

Study area: The study included all land resources of the Belgorod Region with their breakdown by municipal districts. The Belgorod Region has an area of 27100 km2 and it is located in the Central Federal District of the Russian Federation. It is located on the south-western spurs of the Central Russian Upland and it is an elevated plain, which is uplifted in the northern part and has mild slopes to the west-south-west and east-south-east (Lisetskii et al., 2015). The slope type terrain prevails on the territory of the region (48% of the area) (Yudina, 2013). The share of agricultural land in the region is 78.8%, including 60.8% of arable land, 12.7% of forest area, 1.7% of land under water facilities and 4.6% of land under buildings and roads and 2.2% (data from the Rosreestr Administration for the Belgorod Region for 2017) of disturbed and other land not suitable for agricultural use. The erosion of arable soils is distributed unevenly, within the region it increases from 25 to 50% from west and north-west to east and south-east (Spesivy and Lisetskii, 2014).

Data used: Historical documents: A data set for the analysis of historical changes in the structure of the land resources is collected from the documents of the State Archive of the Belgorod Region. We have used 266 annual reports on the condition and use of the Belgorod Region lands, as well as plans and reports on land planning and reclamation works, information on the condition of the forest resources, information on the number and distribution of acreage. These materials are collected for each municipal district and have a time interval from 1954 (year of foundation of the region) to 2017.

Modern documents: We have obtained up-to-date information on land condition and use from the Rosreestr

Office for the Belgorod Region for the period from 2010 to 2017, which has also become an important source of data.

Methods: Information on quantitative indicators of the land resources has been collected in 21 districts for six temporary sections (1954, 1965, 1975, 1985, 1995, 2005, 2017). A quantitative land area analysis has been carried out by land categories, which has made it possible to assess land transformation. The extent of changes in land areas is shown on map charts with the use of GIS technologies. We have used a multi-functional GIS application ArcGIS 10.2, including tool sets Spatial Analyst and Spatial Statistics, as a software product. Anthropogenic load was assessed with the use of multidimensional cluster analysis methods (Ward's method).

RESULTS AND DISCUSSION

An analysis of historical documents and current records on the structure of the land resources of the Belgorod Region for the period 1954-2017 has shown a positive increase for natural grassland, forests, and lands under industry, roads and buildings, lands under wetlands. A negative growth has been reported for arable land, perennial plantations and disturbed lands not suitable for agricultural use (Table 1). A high share of arable land in the region has required managers and farmers to take measures to preserve land resources. As a result, since 1954 one can see ongoing land transformation with its highest peak being from 1966 to 1985. The in-house land use planning interventions by collective and communal farms (1964-1997) has resulted in reduced arable land mainly due to reclamation works (transformation of unproductive arable land for hay and pastures, the development of anti-erosion forest strips and forested areas). As a result, by 2017 the area of natural forage land increased by 17.1% as compared to 1954. Over the same period, the area of forest plantations has consistently increased and cumulatively made up 3%, 4.3%, 6.4%, 12%, and by 2017 this figure reached 13.2% due to large forest cover percent of degraded and erosion-hazardous lands. The biggest increase in forest cover was reported to be in the period from 1986 to 1995. This decade more than 16,000 hectares of forest crops were planted.

Land areas under industry, roads and buildings also increased. Their share became higher by 31.2% by 2017 as compared to 1954. The land of this type was reported to be increased between 1954 and 1995, then starting from 1996 its area was reduced by 2.8%, which was possibly related to some errors in statistical records, and it is these lands that were partially counted as disturbed lands not suitable for agricultural use.

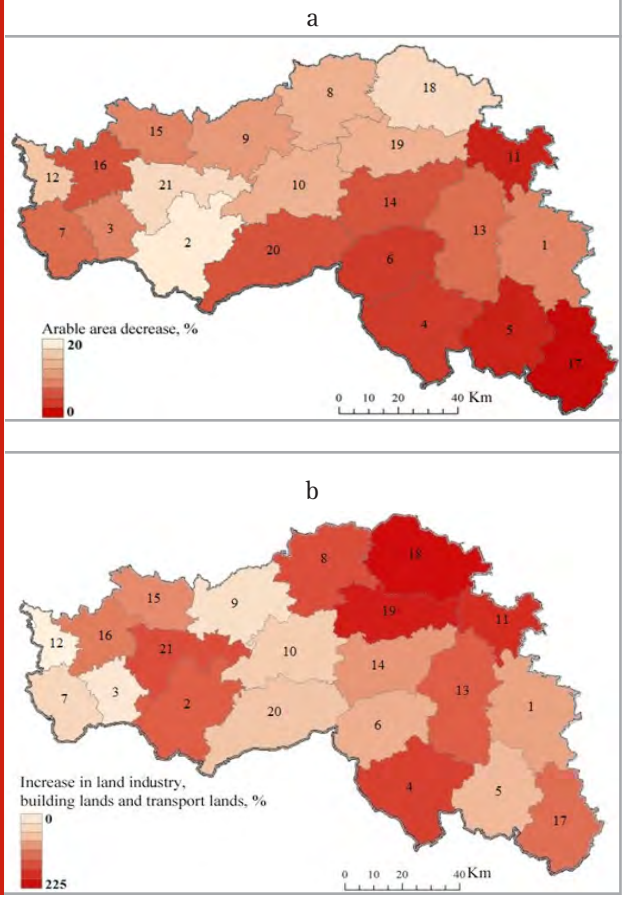
Table 1. The dynamics of the land fund of the Belgorod region

Commercial lands		1954	Time slice			1986-1995	1996-2005	2006-2017	Total* +/-
			1955-1965	1966-1975	1976-1985				
			Positive increase						
Natural forage land	ha	388194	+47720	-8764	+3605	+27222	+943	-4316	+66410
	%	-	+12.3	-2.2	+0.9	+7.0	+0.2	-1.1	+17.1
Forests and forest strips	ha	293428	+8911	+3916	+6110	+16278	+3487	0	+38702
	%	-	+3.0	+1.3	+2.1	+5.6	+1.2	0	+13.2
Land under industry, roads	ha	95941	+4228	+8353	+5177	+14962	-2331	-402	+29987
and buildings	%	-	+4.4	+8.7	+5.3	+15.6	-2.4	-0.4	+31.2
Land under water	ha	11001	+104	+4560	+5214	+4080	+298	+396	+14652
	%	-	+0.9	+41.5	+47.4	+37.1	+2.7	+3.6	+133.2
Lands under the swamps	ha	18021	-1482	-669	+2136	+4620	-116	+39	+4543
	%	-	-8.2	-3.7	+11.8	+25.7	-0.6	+0.2	+25.2
			Negative growth						
Arable land	ha	1791295	-44076	-20516	-17939	-47305	-8040	-1969	-139845
	%	-	-2.5	-1.2	-1.0	-2.6	-0.4	-0.1	-7.8
Perennial plantings	ha	39833	-560	-2967	-3700	-11285	+8913	+3911	-5688
	%	-	-1.4	-7.4	-9.2	-28.3	+22.4	+9.8	-14.3
Disturbed land	ha	75729	-11051	+15812	-1253	-11582	-2478	1758	-8794
unsuitable for									
agricultural use	%	-	-14.6	+20.9	-1.7	-15.3	-3.2	+2.3	-11.6

Note: * dynamics of the land area achieved by 2017 relative to the values of 1954.

The area of land under water facilities (by 133.2%) is reported to be increased drastically throughout the region. This is due to beams flooding and the development of ponds, which have come to be used for irrigation of agricultural fields. It is reported to have a negative land growth for arable soil, perennial plantations and disturbed land not suitable for agricultural use. The area of arable land was annually reduced, and totally decreased by 7.8% over 63 years (by 2017). At the same time, there are significant annual losses of arable land because of its transfer to medium-stabilizing lands. The author has found that in each decade (for the period from 1954 to 1995) the area of arable land decreased by 2.5%, 1.2%, 1.0%, 2.6%, then the rate of reduction of arable land was lower and over the next 20 years (1996-2017) it made up only 0.5%. This process is related with renewal of land and property relations in the Russian Federation and the transition from a controlled economy to a market one.

Figure 1. Territorial changes in the structure of the land fund for the period 1954-2017: a – reduction in arable land, %; b – land expansion under industry, transport and residential development, %; c – increase in the share of natural forage land, %; d – forest cover increase, %; e – increase in the share of land under water bodies, %; f – change in the proportion of disturbed land, %



The area of land under perennial plantations showed progressive decreasing tendency (14.3%). This process was related with reduction in mulberry plantings, vineyards and orchards, which have lost their life cycle.

The area of disturbed land not suitable for agricultural use also decreased by 11.6%. An analysis of statistical land records has made it possible to differentiate municipal districts by dynamic changes in quantitative parameters of the land structure using GIS tools. As a result, we have developed a number of map charts for spatial and temporal land distribution in the Belgorod Region (Figure 1, a-f). The over 60-year transformation of arable land is less pronounced in the southern and eastern areas of the region (Fig. 1a). The situation is different in Belgorod, Starooskolsky and Gubkin districts where the arable land was mainly reduced due to the transfer of land for individual housing construction (further – IHC). The eastern areas of the region have also undergone a slight change in the structure of the land resources. A map chart of changes in the share of hayfields and pastures (Fig. 1c) demonstrates that distribution is of mosaic type.

A map chart of dynamic changes in the land under water facilities (Fig. 1e) has made it possible for us to identify the eastern regions with the greatest positive changes in water content of the territory, which was due to the development of a large number of reclamation ponds. At the same time, one can see a reverse situation developed in the western regions where territorial water content was reported to have less significant increase (within 20-25%). An increase in the region's area covered by forests was due to political decisions to preserve land resources through the development of forest strips and percent forest cover of unproductive and degraded lands (Fig. 1d). However, despite some positive trends the forest coved percent failed to have optimal values in the Belgorod Region. A share of land occupied by industrial facilities, transport logistics and residential zone (Fig. 1b) has increased due to the rapid development of urbanization and industrialization of the region, the construction of the largest enterprise of ferrous metallurgy in the country (Starooskolsky Metallurgical Plant), machine building and sugar factories.

A growing urban population has resulted in increased share of built-up area. This trend is clearly expressed for the Belgorod district, its population has increased by 1/3 (Chugunova et al., 2019). The districts, which have had a regressive built-up share, include Krasnensky, Krasnogvardeysky and the easternmost districts of the region (Alekseevsky, Valuysky, Veydelevsky, Rovensky). The mining industry, which is represented by largest iron ore quarries of Gubkin and Starooskolsky districts, as well as by common mineral quarries (chalk, sand, clay), has contributed an increase of area of disturbed lands, which has negatively affected the ecological and resource state of agricultural landscapes (Fig. 1f). We have conducted a comprehensive assessment of the land transformation using methods of mathematical statistics, which has made it possible to identify reasonably types of municipal districts according to the dynamics of changes in their land resources. As such, we have used a multidimensional cluster analysis, which allows us to structure and break down data into homogeneous types according to aggregation level. The results of classification of 21 municipal districts of the Belgorod Region using hierarchical classification method are presented in Figure 2.

We have managed to combine all parameters of land transformation for the period from 1954 to 2017 with the use of cluster analysis and to ascertain in unbiased way three large clusters, which use dynamic land changes as determinant classification criteria. Land transformation shows that the area of destabilizing land (arable land) has decreased and the area of stabilizing land (natural forage land, forests and lands under water bodies) has increased. This distribution is generally correlated with the physical and geographical zoning and anthropogenic and social nature of the Belgorod Region. An increase in built-up area and land disturbed by industrial development and a decrease in other land not suitable for agricultural use have affected the separate allocation of districts, which include urban agglomerations (Figure 3).

Figure 2. Dendrogram of cluster analysis of 21 municipal districts of Belgorod region (A – coalescence boundary of municipal districts types, D – distance linkage, I-III – groups of the municipal districts, 1-21 – number of municipal districts)

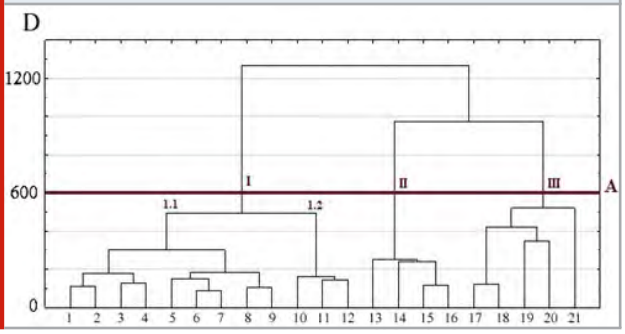
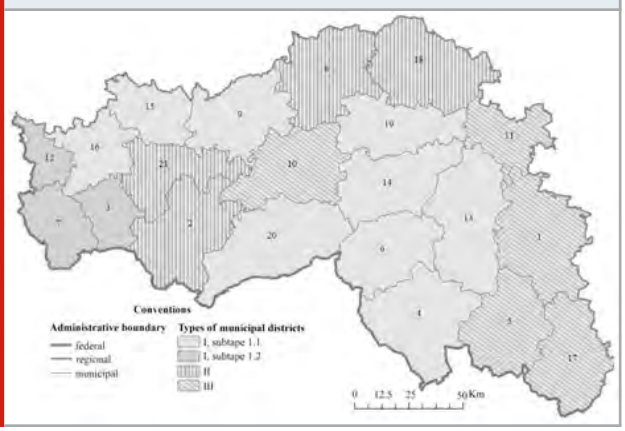


Figure 3. Typization of municipal districts of the Belgorod region on the dynamics of land and resource potential



Type I of municipal districts is confined to the forest-steppe zone and characterized by a share of arable land reduced to 12%, natural forage land down to 60% and an increase forest cover percent up to 15%. This type occupies the largest area because it includes 12 districts, which are located in the western and central parts of the region. Having in mind the community distance (D=400), the areas of type I are differentiated by anthropogenic transformation criteria in a more fractional way into two subtypes, which are geographically expressed in the western part of the region (subtype 1.1.), central and eastern parts (subtype 1.2.) respectively. These subtypes are aggregated based on differences in dynamics of land changes under the influence of anthropogenic factors. Type II of municipal districts is also confined to the forest-steppe zone, but is characterized by a share of arable land reduced to 20%, an increased share of natural forage lands up to 30%, forest cover percent up to 17% and a sharp increase in the share of built-up and industrially developed lands – up to 135%. This type is composed of four districts and includes urban agglomerations. Type II of municipal districts is confined to the steppe zone. It was reported to have a decrease in arable land down to 12%, an increase in the area under natural forage lands – up to 22%, a higher built-up area – up to 57%, but a significant role in the aggregation of this subtype had an increase in the area of water lands – 200 to 400%. This type includes four districts of the south-eastern part of the region and one district – in the central part.

CONCLUSION

It is preferable to conduct land transformation analysis by chronological sections, which can give a more objective assessment of the past land and resource reformations. It is good to add cluster analysis to statistical information processing, which allows you to structure data based on repeatability of their features, as well as to group them into types at the aggregation level and further to typify the study area by criteria, which meet the working conditions. GIS tools, in particular the ArcGis 10.2 platform, can provide ample opportunities for geospatial analysis. Using Tabulate Area and Zonal Statistics tools, we have managed to identify such municipal areas, which characterized the different degree of land transformation for individual lands, to establish the peak and stagnation of areal changes in the land resources in the Belgorod Region. Spatial and temporal transformations have most affected the arable land. It is typical for all districts of the region to have a negative balance of arable land. The areas under perennial plantations and disturbed lands, which are unsuitable for agricultural use, have slightly decreased. It is reported to have an increase in the lands occupied by industry, transport and buildings, as well as by water bodies, natural forage land and forested land. It is promising to apply the data on land tenure trends obtained with the use of GIS analysis for the implementation of regional land monitoring, as well as in the process of political decision-making on the formation of optimal ecological-economic land balance.

ACKNOWLEDGEMENTS

The study was supported within the intra-university grant of Belgorod State National Research University to support the creation and development of scientific departments – centres of excellence.

REFERENCES

Bao, Ju., Gao, Sh. and Ge, J. (2019). Dynamic land use and its policy in response to environmental and socialeconomic changes in China: A case study of the Jiangsu coast (1750–2015). Land Use Policy, 82: 169–180.

Buryak, Zh. and Grigoreva, O. (2019). A Project-Based Approach to Reduce the Risk of Soil Erosion in Agricultural Landscapes of Small Rivers Basins Using GIS Technologies. International Multidisciplinary Scientific GeoConference Surveying Geology and Mining Ecology Management, SGEM, 19(5.2): 19–26. https://doi.org/10.5593/sgem2019/5.2.

Cai, X., Zhang, X. and Wang, D. (2011). Land Availability for Biofuel Production. Environmental Science Technology, 45: 334–339.

Chendev, Yu.G., Petin, A.N., Serikova, E.V. and Kramchaninov, N.N. (2008). Degradation of geosystems of the Belgorod region as a result of economic activity. Geography and Natural Resources, 4: 69–75.

Chendev, Yu.G., Gennadiev, A.N., Zhidkin, A.P., Belevantsev, V.G. and Vagurin, I.Yu. (2016). Soils in Zone of the Tatar Road Passage. NAUCH VED BELGORO EN, 34(4): 113–123.

Chhabra, A., Geist, H., Houghton, R.A., Haberl, H., Braimoh, A.K., Vlek, P.L.G., Patz, J., Xu, J., Ramankutty, N., Coomes, O. and Lambin, E.F. (2006). Multiple impacts of land use/cover change. In: Lambin E.F., Geist H. (Eds.), Land-Use and Land-Cover Change, Global Change–The IGBP Series. Springer, Berlin Heidelberg, 71–116.

Chugunova, N., Polyakova, T., Ignatenko, S. and Buryak, Zh. (2019). Polarization of the regional settlement system as a reflection of neoliberal urbanization processes. International Multidisciplinary Scientific GeoConference Surveying Geology and Mining Ecology Management, SGEM, 19(5.2): 603–610. https://doi.org/10.5593/sgem2019/5.2.

Comber, A.J., Davies, H., Pinder, D., Whittow, J.B., Woodhall, A. and Johnson, S.C.M. (2016). Mapping coastal land use changes 1965-2014: methods for handling historical thematic data. Transactions of the Institute of British Geographers, 41: 442–459.

Degtyar, A.V. and Grigoreva, O.I. (2018). Development of Land Forests of the Belgorod Region for the 400-year period. NAUCHN VED Belgorod EN, 42(4): 574–586. https://doi.org/10.18413/2075-4671-2018-42-4-574-586.

Felsenstein, D. and Lichter, M. (2014). Land use change and management of coastal areas: retrospect and prospect. Ocean and Coastal Management, 101: 123–125.

Fischer, F. and Black, M. (1995). Greening Environmental Policy. The Politics of a Sustainable Future. Palgrave Macmillan, New York.

Grigoreva, O.I. (2015). The geoinformation analysis of soil and geomorphological communications for the rational organization of agrolandscapes on the basin principles. NAUCH VED BELGORO EN, 3(200): 157–166.

Grigorieva, O.I. (2017a). Evaluation of Distribution Dynamics of Anthropogenic Loads and Ecological State of Ecosystems of Municipal Areas in Belgorod Region. International Research Journal, 9-2(63): 75–80.

Grigorieva, O.I. (2017b). Ecological Information in Land-Cadastral Documentation. Monitoring. Science and Technologies, 3(32): 64–70.

Grigoreva, O.I. and Buryak, Z.A. (2016). Application of basin approach for soil and water protection geoplanning of territory and environmental management. RES J PHARM BIOL CHE, 7(1): 2175–2182.

Kitov, M.V. (2015). Dynamics of area abandoned grassland in the Belgorod region of the period 1990-2010. NAUCH VED BELGORO EN, 9(206): 92–102.

Kitov, M.V. and Tsapkov, A.N. (2015). Assessment of the area of fallow land in the Belgorod region and other regions of European Russia for the period 1990-2013 years. NAUCH VED BELGORO EN, 15(212): 163–171.

Kuzmenko, Ya.V., Lisetskiy, F.N., Kirilenko, J.A. and Grigorieva, O.I. (2013). Providing the Optimum Water Protection Forests Amount at Basin Organization of Nature Management. Izvestia of Samara Scientific Center of the Russian Academy of Sciences, 15(3-2): 652–657.

Lisetskii, F. (2019). Estimates of Soil Renewal Rates: Applications for Anti-Erosion Arrangement of the Agricultural Landscape. Geosciences, 9(6): 266. https://doi.org/10.3390/geosciences9060266.

Lisetskii, F.N., Buryak, J.A., Zemlyakova, A.V. and Pichura, V.I. (2014a). Basin organizations of nature use, Belgorod region. Biogeosystem Technique, 2(2): 163–173. http://ejournal19.com/journals_n/1421944356.pdf.

Lisetskii, F.N., Pavlyuk, Ya.V., Kirilenko, Zh.A. and Pichura, V.I. (2014b). Basin organization of nature management for solving hydroecological problems. Russian Meteorology and Hydrology, 39(8): 550–557.

Lisetskii, F.N., Degtyar, A.V., Buryak, Z.A., Pavlyuk, Ya.V., Naroznyaya, A.G., Zemlyakova, A.V. and Marinina, O.A. (2015). Rivers and water bodies of Belogorye. Belgorod, CONSTANTA.

Lisetskii, F.N., Smekalova, T.N. and Marinina, O.A. (2016). Biogeochemical features of fallow lands in the steppe zone. Contemporary Problems of Ecology, 9(3): 366–375. http://dx.doi.org/10.1134/S1995425516030094.

Lisetsky, F.N., Buryak, J.A., Grigoreva, O.I., Marinina, O.A. and Martsinevskaya, L.V. (2015). Implementation of the basin-administrative and ecoregional approaches to environmentally oriented arrangement inter-settlement areas of the Belgorod region. Biogeosystem technique, 1(3): 50–63.

Manakos, I., Karakizi, C., Gkinis, I. and Karantzalos, K. (2017). Validation and Inter-Comparison of Spaceborne Derived Global and Continental Land Cover Products for the Mediterranean Region: The Case of Thessaly. Land, 6(34). https://doi.org/10.3390/land6020034.

Manakos, I., Tomaszewska, M., Gkinis, I., Brovkina, O., Filchev, L., Genc, L., Gitas, I.Z., Halabuk, A., Inalpulat, M., Irimescu, A., Jelev, G., Karantzalos, K., Katagis, Th., Kupková, L., Lavreniuk, M., Mesaroš, M., Mihailescu, D., Nita, M., Rusnak, T., Stych, P., Zemek, F., Albrechtová, Ja. and Campbell, P. (2019). Comparison of Global and Continental Land Cover Products for Selected Study Areas in South Central and Eastern European Region. Remote Sensing, 10. https://doi.org/10.3390/rs10121967.

Marinina, O.A., Terekhin, E.A., Kirilenko, Z.A., Kurlovich, D.M. and Kovalchik, N.V. (2013). Characteristics Remote Detection Fallow Land Trust and Problems of Agricultural Land Use. Modern problems of science and education, 5. http://science-education.ru/ru/article/view?id=10211.

Nduati, E., Sofue, Yu., Matniyaz, A., Park, J.G., Yang, W. and Kondoh, A. (2019). Cropland Mapping Using Fusion of Multi-Sensor Data in a Complex Urban/Peri-Urban Area. Remote Sensing, 11(207). https://doi.org/10.3390/rs11020207.

Pozachenyuk, E.A., Lisetskii, F.N., Vlasova, A.N. and Kalinchuk, I.V. (2018). Justification of landscape and biotechnical solutions for designing water protection zones. RES J PHARM BIOL CHE, 9(2): 716–722.

Pozachenyuk, E.A., Lisetskii, F.N., Vlasova, A.N., Buryak, Zh.A., Marinina, O.A. and Kalinchuk, I.V. (2015). Model of position-dynamic structure of river basins. RES J PHARM BIOL CHE, 6(6): 1776–1780.

Rinfret, S.R. and Pautz, M.C. (2014). US Environmental Policy in Action-Practice and Implementation. Palgrave Macmillan, New York. https://doi.org/10.1057/9781137482082.

Shifaw, E., Sha, Ji., Xiaomei, L.E., Bao, Zh. and Zhou, Zh. (2019). An insight into land-cover changes and their impacts on ecosystem services before and after the implementation of a comprehensive experimental zoneplan in Pingtan island China. Land Use Policy,

82(2019): 631–642.
Shtompel', Yu.A., Lisetskii, F.N., Sukhanovskii, Yu.P. and Strel'nikova, A.V. (1998). Soil loss tolerance of Brown Forest Soils of Northwestern Caucasus under intensive agriculture. Eurasian Soil Sci., 31(2): 185–190.
Spesivy, O.V. and Lisetskii, F.N. (2014). Estimate of the intensity and regulation of erosion soil losses in central chernozem region based on the basin approach. Nausch Ved Belgorod En, 10(181): 125–132.
Srivastava, P.K., Mukherjee, S. and Gupta, M. (2010). Impact of urbanization on land use/land cover change using remote sensing and GIS: a case study. International Journal Ecological Econonomics and Statistics, 18: 106–117.
Teluguntla, P., Thenkabail, P., Oliphant, A., Xiong, J., Gumma, M.K., Congalton, R.G., Yadav, K. and Huete, A. (2018). A 30-m Landsat-derived cropland extent product of Australia and China using random forest machine learning algorithm on Google Earth Engine cloud computing platform. ISPRS Journal of Photogrammetry and Remote Sensing, 144: 325–340.
Terekhin, E.A. and Chendev, Yu.G. (2018). Estimation of Forest Cover Changes During modern Period in the South of the Central Russian Upland Using Multiyear Remote Sensing Data. Current Problems in Remote Sensing of the Earth from Space, 15(3): 114–126.
Terekhin, E.A. (2016). Geoinformation approach to monitoring and assessment of crop areas using remote sensing data (a case study of Belgorod region). NAUCH VED BELGORO EN, 18(239): 148–155.
Terekhin, E.A. (2018). Changes in forest cover of ravines-balks network in the south of the central russian upland in recent decades. NAUCH VED BELGORO EN, 42(2): 223–230. https://doi.org/10.18413/2075-4671-2018-42-2-223-230.
Tsapkov, A.N. (2016). Main areas of regional policy for the land plots of individual housing construction as the basis for the welfare of the population. NAUCH VED BELGORO Economics Information technologies, 9(230): 10–20.
Yermolaev, O.P., Mukharamova, S.S., Maltsev, K.A., Ivanov, M.A., Ermolaeva, P.O., Gayazov, A.I., Mozzherin, V.V., Kharchenko, S.V., Marinina, O.A. and Lisetskii, F.N. (2018). Geographic Information System and Geoportal «River basins of the European Russia». IOP Conference Series: Earth and Environmental Science, 107(1): 012108. https://doi.org/10.1088/1755-1315/107/1/012108.
Yudina, Yu. (2013). Mapping of geosystems in Belgorod region: regional aspects. Modern problems of science and education, 6: 933–941.
Zelenskaya, E.Ya., Kukharuk, S.A., Naroznyaya, A.G., Martsinevskaya, L.V. and Sazonova, N.V. (2019). Bioecological Assessment of Arable Soil Pollution: A Case Study of Belgorod Region. Biosci. Biotech. Res. Comm., 12(3): 548–555.
Zhou, X. and Kang, C. (2011). Land use/cover change analysis in Wuhan city based on RS and GIS. 19th International Conference on Geoinformatics, 1–5.
Zia, A. (2012). Land use adaptation to climate change: economic damages from landfalling hurricanes in the Atlantic and Gulf States of the USA, 1900-2005. Sustainability, 4: 917–932.

Dental
Communication

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp-68-72

Application of Low-Level Laser Therapy in Veneer Repair of Lithium Disilicate and Y-TZP Restorations

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ABSTRACT

To assess intra oral repair of Lithium Disilicate ceramics (LDC) and Yttria Stabilized Tetragonal Zirconia Polycrystal (Y-TZP) using low level laser therapy (LLLT) in comparison to conventional conditioning modalities on repair bond strength of composite resin bonded to ceramic structure. Fifty specimens of LDC and fifty samples of Y-TZP were used. Discs were prepared form each group having a diameter 6 mm and thickness of 2 mm and conditioned using different regimes. Group 1 and 6 were surface treated with bur, group 2 and 7 were conditioned with Nd-YAG (NYL), group 3 and 8 surface was treated with Er,Cr:YSGG (ECL), group 4 and 9 with hydrofluoric acid + salinization (HF+S) and group 5 and 10 were conditioned with Al2O3 air abrasion (AA). A single layer of Heliobond was scrubbed on the conditioned surface and bonded with composite resin. For shear bond strength testing the specimens were placed in a universal testing machine. A stereomicroscope at 40x magnification was used to analyse failure pattern. The mean repair bond strength was calculated using ANOVA and Tukey's post hoc test at a significance level of ($p < 0.05$). The highest repair bond strength observed in LDC was (19.57±3.58 MPa) in group 4 whereas, the lowest score was displayed in Group 3 (11.88±1.98 MPa). Similarly, in Y-TZP the highest repair SBS values were presented in group 10 (air abrasion) (20.32±3.21) and the lowest SBS values were exhibited by bur treated group 1 (12.25±2.33). Failures in group 1, group 2, group 3, group 6, group 7 and group 8 were dominantly adhesive. Whereas, failures in group 4, group 5, group 9 and group 10 were cohesive. HF acid with salinization remains gold standard for conditioning of LDC. AA with salinization is the most effective method to condition YTZP ceramics for better repair bond scores. Alternate methods of conditioning using LLLT (NYL and Er,Cr:YSGG) needs further investigation.

KEY WORDS: ER,CR:YSGG, ND-YAG, REPAIR BOND STRENGTH, LITHIUM DISILICATE CERAMICS, YTTRIA STABILIZED TETRAGONAL ZIRCONIA POLYCRYSTAL.

ARTICLE INFORMATION

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Received 14th Jan 2020 Accepted after revision 29th Feb 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/12

INTRODUCTION

Yttria Stabilized Tetragonal Zirconia Polycrystals(Y-TZP) ar used as crowns, long span fixed partial dentures and veneers because of their mechanical properties, biocompatibility and high flexural strength (Akyil et al., 2010). Zirconia Y-TZP is not translucent like natural teeth hence, to enhance its appearance it requires veneering of ceramic layer (Cristoforides et al., 2012). This ceramic layer tends to get fractured or chipped from cuspal tips or occlusal edges. This results in functional discomfort and aesthetic compromise for the patient (Galvão et al., 2018). It's not always possible to change fractured prosthesis as it is time consuming, expensive, and damaging to surrounding tissues (Kirmali et al., 2015). Therefore, use of conditioners, primers and adhesion promoters along with composite resins are used to improve the longevity of the restoration (Kirmali et al., 2015; Özcan et al., 2013). Conventionally fractures in lithium Disilicate (LDC), porcelain feldspathic and leucite, are repaired by conditioning the surface with hydrofluoric (HF) acid along with salinization (Colares et al., 2013). The method is well established to promote adhesion of resin-based material. However, HF acid is dangerous as it increases the risk of tissue necrosis and burns. Moreover, its effect on Y-TZP is still controversial as it does not allow topographical changes (Kirkpatrick et al., 1995; Özcan et al., 2013).

Repair fracture of zirconia Y-TZP is challenging. To improve adhesion, use of both chemical and mechanical conditioning regimes are considered. These regimes may range from sand blasting the repairing surface with Al2O3 to surface conditioned with bur and application of silane coupling agents. All these conditioning regimes improve wettability and surface energy of Y-TZP, but their efficacy and effectiveness are still controversial, (Ozcan et al 2013, Kirmali et al., 2015, Galvao et al 2018). Lately, use of lasers Er,Cr:YSGG (ECL) and Nd,YAG (NYL) for surface treating LDC has displayed convincing results (Alkhudhairy et al., 2019a; Vohra et al., 2019). Moreover, ECL along with NYL have demonstrated conclusive outcomes on conditioning of dentin and enamel surface (Alkhudhairy et al. 2018a, 2018b, 2019b, 2019c). In the authors knowledge from indexed literature, limited evidence is available on surface treatment of zirconia ceramic with the purpose of repair by composite resin. It is hypothesized surface treated with low level laser therapy (LLLT) will exhibit comparable repair bond strength to conventional surface treated regimes. Therefore, the aim of the present in vitro study was to assess repair of LDC and Y-TZP using LLLT in comparison to conventional conditioning modalities on repair shear bond strength of composite resin bonded to ceramic structure.

MATERIAL AND METHODS

In the current study hundred samples i.e., fifty specimens of lithium Disilicate ceramics (LDC) (IPS Emax Press; Ivoclar/Vivadent, Schaan, Liechtenstein) and fifty samples of Yttrium-Stabilized Tetragonal Zirconia Polycrystal ceramic (Y-TZPC) (Noritake Alliance, Noritake Co., Nagoya, Japan) were used. Discs were prepared form each group having diameter 6mm and thickness 2mm. Before the surface treatment all hundred discs were cleaned ultrasonically using 96% isopropanol for duration of 3 min (Vohra et al., 2019) and air dried. Based on surface conditioning method LDC and Y-TZPC discs were divided into ten groups (n=10 each) The present study followed CRIS (Checklist for reporting In vivo studies) guidelines. Specimens from group 1 were surface treated using diamond bur 30-µm-grit (Kerr Corporation, USA) 10 strokes under running water. Similarly, Cimara grinding bur (Cimara, Zircon, Voco, GmbH, Germany) for Y-TZPC was used 10 strokes for group 6. Samples from group 2 and 7 were surface conditioned using ECL (Millennium; Biolase Technology, Inc., San Clemente, CA, USA) at wavelength 2.78 micro-meter, 3.75 W power and 15 Hz frequency with air water ratio of 90-70% in a non-contact circular motion using tip MZ8 for 60 sec.

Specimens from group 3 and 8 were surface treated using NYL (Hoya ConBio Delight, Sweden & Martina, Padova, Italy) at wavelength 1064 nm. The laser was used perpendicular to the bonding surface from a distance of 2mm for a duration of 60 sec at 150 mJ, 10 Hz and 3 W for a duration of 60 sec in a non-contact circular motion using 320-µm quartz optical fibre Specimens from group 4 and 9 were conditioned using 9.5% hydrofluoric acid gel (etching gel Ivoclar, vivadent) for a duration of 1min, washed 20 sec and oil free air dried. Salinization was done using coupling agent (Monobond Plus ceramic primer Ivoclar vivadent) in a single layer for 60 sec and air dried. Specimen from group 5 and 10 were surface treated using aluminium trioxide (Al2O3) silicate particles (CoJet system; 3M ESPE, St. Paul, MN) maintaining a pressure of 2.3bar in a non-contact position from a distance of 8mm, for a duration of 1 min. After AA all specimens were washed under running water. After conditioning of surfaces samples from groups 1,4,5 and 6,9,10 received a coating of silane (Monobond Plus ceramic primer Ivoclar vivadent) a single layer for 60 sec and air dried. No silane coupling agent was applied on lased groups. Samples were cleaned with isopropanol for 180 sec and oil free air dried. A single layer of Heliobond (Ceramic Repair N) was scrubbed on the conditioned surface and light cured for 10 sec (Bluephase G2, Ivoclar,Vivadent). The repaired surface was bonded to composite resin (Multicore flow; Ivoclar/ Vivadent) incrementally using a tofelmire matrix holder at a height of 5mm and cured 20sec.

To replicate oral conditions all specimens were shifted to a thermocycler (Mini Opticon Real-Time PCR System, BioRad, USA) between 5C to 60°C for 30sec transfer time 5 sec. For shear bond strength testing the specimens were placed in a Universal testing machine (Zwick Roell Z2.5 MA 18-1-3/7 ulm, Germany) at a cross head speed of 1ml/min and 2.5KN force perpendicular to the bonded surface until repair failure. The repair bond strength was measured in MPa (Megapascals). A stereomicroscope at 40x magnification was used to analyse failure pattern. Failure type was categorized into adhesive, cohesive and admixed failure type. The data were normally distributed

according to Kolmogorov–Smirnov test (a=0.05). The mean repair bond strength was calculated using ANOVA and Tukey's post hoc test at a significance level of ($p < 0.05$). Data was charted using statistical program for social science (SPSS version 21, Inc., Chicago, US)

RESULTS AND DISCUSSION

A normal distribution of data was observed in the present study. Table 1 displays repair SBS values in Lithium Disilicate (LD) and Zr Y-TZP type ceramics. The highest repair bond strength observed in LDC was (19.57±3.58) in group 4 Hydrofluoric acid + salinization whereas, the lowest score was displayed in Group 3 laser irradiated using Nd-YAG (11.88±1.98). Similarly, in Y-TZP the highest repair SBS values were presented in group 10 air abrasion (20.32±3.21) and the lowest SBS values were exhibited by bur treated group 1 (12.25±2.33).

Table 1. Comparison of means and SD for repair bond strength values among study groups using ANOVA and Tukey multiple comparisons test

Type of Material	Surface treatment	Mean ± SD Mpa	P-value!
	Group 1: Bur treatment (Control)	12.27±2.11[a]	
	Irradiated using Er,Cr:YSGG (ECL)	13.52±2.68[a]	
Lithium Disilicate Ceramics (LDC)	Group 3: Laser Irradiated using Nd-YAG	11.88±1.98[a]	
	Group 4: Hydrofluoric acid + salinization	19.57±3.58[c]	<0.05
	Group 5: Air Abrasion	16.44±3.82[c]	
	Group 6: Bur treatment (Control)	12.25±2.33[a]	
Zirconia Y-TZP	Group 7: Laser Irradiated using Er,Cr:YSGG	14.25±2.24[a]	
	Group 8: Laser Irradiated using Nd-YAG laser	13.74±1.54[a]	<0.05
	Group 9: Hydrofluoric acid + salinization	18.66±3.44[c]	
	Group 10: Air Abrasion	20.32±3.21[c]	

Different upper script letters in individual materials indicate statistical differences ($p < 0.05$).
! Showing significant difference among study group (ANOVA)

Based on the conditioning regimes repair SBS values of group 1, group 2, group 3, group 6, group 7 and group 8 were comparable $p>0.05$. Similarly, repair SBS in group 4, group 5, group 9 and group 10 were also found to be comparable $p>0.05$. For bond strength values, analysis of variance (ANOVA) showed significant difference among all study groups ($p>0.05$). Failure modes observed among the de-bonded specimens are presented in table 2. Most of the failures in group 1, group 2, group 3, group 6, group 7 and group 8 were dominantly adhesive. Whereas, failures in group 4, group 5, group 9 and group 10 were cohesive. Overall, adhesive type failure was common amongst all groups. The present study was based on the hypothesis that LLLT in the form of ECL and NYL on conditioning of LDC and Y-TZP will exhibit comparable repair bond strength to conventional conditioning regimes. The laboratory-based study revealed that ECL and NYL conditioning of LDC and Y-TZP exhibited statistically lower shear bond strengths compared to conventional conditioning regimes HF acid+ salinization and AA. Therefore, the hypothesis was rejected. The quality and resilience of bond signifies clinical success of repaired ceramics. Mechanical and chemical roughening of LDC and YTZP is essential for obtaining a reliable bond. Recently, use of laser irradiation for surface roughening of ceramics in improving bond strength and adhesion of composite to resin has gained popularity (Alkhudhairy et al., 2019a; Vohra et al., 2019).

In the existing study, ECL application at wavelength of 2780nm, power 3.75W and 15Hz frequency was used, as ECL at these parameters are well absorbed by the dental tissues. Moreover, NYL was used at a wavelength of 1064nm as a conditioning strategy for LDC and YTZP. In the present study, YTZP and LDC surfaces treated with NYL (11.88±1.98) (13.74±1.54) and ECL (13.52±2.68) (14.25±2.24) exhibited comparable repair SBS scores. The findings of present study of low repair bond strength scores with NYL conditioning on YTZP corelates to the work done by (Akyil et al., 2010; Arami et al., 2014). A possible explanation to this outcome can be attributed to heat induction during laser irradiation causing damage to the superficial layer which gives weak attachment to underlying surface and composite resin resulting in repair bond failure (Akyil et al., 2010). Moreover, NYL exhibited low repair scores to LDC this finding was in harmony with study by Yucel et al., (2012). A potential clarification to this finding can be credited to laser parameters (frequency and power), nano crystalline structure of LDC (IPS Emax Press and IPS Empress 2), duration of laser irradiation and distance from the conditioning surface. Furthermore, low repair bond scores of ECL conditioning to LDC can be ascribed to high power output in the present study. This finding corelates to the study done by (Gökçe et al., 2007; Kirmali et al., 2015; Kursoglu et al., 2013).

The authors of the study suggest that with increase power SBS of ceramics decreases. It is expected that high power dislocates and causes irregularities of the crystalline structure of LDC compromising repair bond integrity. Moreover, ECL conditioning on YTZP showed increased

repair bond strength to bur treated and NYL group but this was statistically insignificant. A study by Kirmali et al., (2015) investigated that ECL with sandblasting improved repair bond values. Moreover, work by Tokar et al., (2019) suggested ECL at short pulse duration displayed better repair bond integrity. In the present study ECL laser with long pulse duration was used which might ascribe to low bond integrity scores. Scanning electron microscopy (SEM) of the conditioned surface may give better justification to this outcome.

The highest repair bond strength was seen in LDC conditioned with HF acid and salinization. This finding was parallel to a study by (Ataol and Ergun, 2018; Colares et al., 2013). A possible justification to this outcome is that HF acid reaction with glass matrix forms hexa-flouro-silicates i.e., exposing and roughening the crystalline structure increasing the micromechanical retention. In addition, silanization after HF acid forms a chemical bond with composite resin improving repair bond integrity. AA conditioning on YTZP displayed the maximum repair bond scores. These finding are comparable to the work done by Akyil et al., (2010); Arami et al., (2014) and Kirmali et al., (2015). A probable description to these findings is that air abraded area using Al2O3 increases the surface roughness, develops undercuts this provides retention and facilitates silane coupling agent into these grooves enhancing repair bond strength.

Based on mode of failure and fracture analysis of specimen lased irradiated surface of both LDC and YTZP showed adhesive failure type. This finding was in correlation to low repair bond strength values. Moreover, AA conditioning and HF salinization on LDC and YTZP predominantly resulted in cohesive fracture. A possible explanation is the salinization process in AA and HF acid group resulting in cohesive failure type. A possible limitation of the present study was the absence of topographical analysis of the conditioned surfaces and energy dispersive spectrometry of the deboned specimens. Use of thermocycling procedures and conditions similar to oral cavity provided better understanding of short- and long-term durability of repair bond strength outcomes. Future studies should be directed in using different laser parameter along with other conditioning regimes with simulated ageing. Moreover, clinical trials with ex-vivo designs are recommended to validate the findings of this laboratory-based study.

Table 2. Modes of failure among different experimental groups

Failure type	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8	Group 9	Group 10
Adhesive	6	8	9	2	2	7	6	9	1	-
Cohesive	2	2	0	5	5	1	2	1	7	8
Admixed	2	0	1	3	3	2	2	1	2	2

CONCLUSION

HF acid with salinization remains gold standard for conditioning of LDC. AA with salinization is the most effective method to condition YTZP ceramics for better repair bond scores. Alternate methods of conditioning using laser (NYL and Er, Cr:YSGG) needs further investigation.

REFERENCES

Akyil, M.S., Uzun, I.H., Bayindir, F., (2010). Bond strength of resin cement to yttrium-stabilized tetragonal zirconia ceramic treated with air abrasion, silica coating, and laser irradiation. Photomed. Laser Surg. 28, 801–808.

Alkhudhairy, F., Alkheraif, A., Bin-Shuwaish, M., Al-Johany, S., Naseem, M., Vohra, F., (2018a). Effect of Er,Cr:YSGG laser and ascorbic acid on the bond strength and microleakage of bleached enamel surface. Photomed. Laser Surg. 36.

Alkhudhairy, F., Naseem, M., Ahmad, Z.H., Alnooh, A.N., Vohra, F., (2019a). Efficacy of phototherapy with different conventional surface treatments on adhesive quality of lithium disilicate ceramics. Photodiagnosis Photodyn. Ther. 25, 292–295.

Alkhudhairy, F., Naseem, M., Bin-Shuwaish, M., Vohra, F., (2018b). Efficacy of Er Cr: YSGG laser therapy at different frequency and power levels on bond integrity of composite to bleached enamel. Photodiagnosis Photodyn. Ther. 22.

Alkhudhairy, F., Vohra, F., Naseem, M., (2019b). Influence of Er,Cr:YSGG Laser Dentin Conditioning on the Bond Strength of Bioactive and Conventional Bulk-Fill Dental Restorative Material. Photobiomodulation, Photomedicine, Laser Surg.

Alkhudhairy, F., Vohra, F., Naseem, M., Ahmad, Z.H., (2019c). Adhesive bond integrity of dentin conditioned by photobiomodulation and bonded to bioactive restorative material. Photodiagnosis Photodyn. Ther. 28, 110–113.

Arami, S., Tabatabaei, M.H., Namdar, F., Safavi, N., Chiniforush, N., (2014). Shear bond strength of the repair composite resin to zirconia ceramic by different surface treatments. J. Lasers Med. Sci. 5, 171–175.

Ataol, A.S., Ergun, G., (2018). Effects of surface treatments on repair bond strength of a new CAD/CAM ZLS glass ceramic and two different types of CAD/CAM ceramics. J. Oral Sci. 60, 201–211.

Colares, R.C.R., Neri, J.R., de Souza, A.M.B., Pontes, K.M. de F., Mendonça, J.S., Santiago, S.L., (2013). Effect of surface pretreatments on the microtensile bond strength of lithium-disilicate ceramic repaired with composite resin. Braz. Dent. J. 24, 349–352.

Cristoforides, P., Amaral, R., May, L.G., Bottino, M.A., Valandro, L.F., (2012). Composite resin to yttria stabilized tetragonal zirconia polycrystal bonding: Comparison of repair methods. Oper. Dent. 37, 263–271.

Galvão Ribeiro, B.R., Galvão Rabelo Caldas, M.R., Almeida, A.A., Fonseca, R.G., Adabo, G.L., (2018). Effect of surface treatments on repair with composite resin of a partially monoclinic phase transformed yttrium-stabilized tetragonal zirconia. J. Prosthet. Dent. 119, 286–291.

Gökçe, B., Özpinar, B., Dündar, M., Çömlekoglu, E., Sen, B.H., Güngor, M.A., (2007). Bond strengths of all-ceramics: Acid vs laser etching. Oper. Dent. 32, 173–178.

Kirkpatrick, J.J.R., Enion, D.S., Burd, D.A.R., 1995. Hydrofluoric acid burns: a review. Burns.

Kirmali, O., Kapdan, A., Harorli, O.T., Barutcugil, C., Ozarslan, M.M., (2015). Efficacy of ceramic repair material on the bond strength of composite resin to zirconia ceramic. Acta Odontol. Scand. 73, 28–32.

Kursoglu, P., Motro, P.F.K., Yurdaguven, H., (2013). Shear bond strength of resin cement to an acid etched and a laser irradiated ceramic surface. J. Adv. Prosthodont. 5, 98–103.

Özcan, M., Valandro, L.F., Pereira, S.M.B., Amaral, R., Bottino, M.A., Pekkan, G., (2013). Effect of surface conditioning modalities on the repair bond strength of resin composite to the zirconia core / veneering ceramic complex. J. Adhes. Dent. 15, 207–210.

Tokar, E., Polat, S., Ozturk, C., (2019). Repair bond strength of composite to Er,Cr:YSGG laser irradiated zirconia and porcelain surfaces. Biomed. J. 42, 193–199.

Vohra, F., Labban, N., Al-Hussaini, A., Al-Jarboua, M., Zawawi, R., Alrahlah, A., Naseem, M., (2019). Influence of Er;Cr:YSGG Laser on Shear Bond Strength and Color Stability of Lithium Disilicate Ceramics: An in Vitro Study. Photobiomodulation, Photomedicine, Laser Surg. 37, 483–488.

Yucel, M.T., Aykent, F., Akman, S., Yondem, I., (2012). Effect of surface treatment methods on the shear bond strength between resin cement and all-ceramic core materials. J. Non. Cryst. Solids 358, 925–930.

Icthyological Communication

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp-73-78

BBRC Bioscience Biotechnology Research Communications

Trends in Aquaculture Feed Development with Chitosan Nano Particles– A Review

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ABSTRACT

Chitosan is a natural, biocompatible, biodegradable ,nontoxic and effectively accessible polymer which can be utilized for arrangement of nanoparticles, it is chelated with chelaters, for example, sodium tripolyphosphate and barium chloride. Chitosan nanoparticles are utilized in pharmaceutical ventures as an antimicrobial compound. N-Acetylglucosamine (GlcNAc) is a monosaccharide that by and large polymerizes straightly through (1,4)-β-linkages. The goal of this assessment was to get ready chitosan (CS) nanoparticles related with N-acetyl-D-Glucosamine (GlcNAc), utilizing the wet turning technique, required to consolidate the GlcNAc pharmacological properties with the CS organic properties for utilization of feed arrangement. Chitosan nanoparticles from shells of arthropods and shellfish devastate and all around have the possibilities to upset aquaculture. One new approach called "Nanotechnology" can be utilized for altered the bolstering procedure. The fundamental idea of this strategy is that the fish nourishment supplements are covered in chitosan Nano-particles which expanding the extent of that go over the gut tissue and into the fish, other than going straight forwardly through stomach related support unused.

KEY WORDS: CHITOSAN, CHITOSAN NANOPARTICLE, AQUA FEED, FEED FORMATION.

ARTICLE INFORMATION

Corresponding Author: kumarun23@gmail.com
Received 15th Feb 2020 Accepted after revision 28th March 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)
A Society of Science and Nature Publication, Bhopal India

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/13

INTRODUCTION

Nanotechnology is that the rising science that deals with nm scale and nanoparticles are one among the building blocks in engineering. Recently, engineering and polymers along have captivated a large interest in several areas as well as pharmaceutical trade and therapeutic innovation among others. Nanoparticles are the solid mixture particles in metric linear unit vary. Chitosan may be a cationic saccharide that's usually obtained by alkalescent deacetylation of polyose poly (N-etylglucosamine). It's biocompatible, perishable, muco adhesive, and nontoxic. These nice biological properties create chitosan an honest candidate for a stage in developing drug delivery systems having improved bio distribution, multiplied specificity and sensitivity, and reduced medicine toxicity. Chitosan may be a chemical compound derived from polyose, that has been found in a very wide scope of natural sources (the exoskeletons of the crustaceans, crabs, and shrimps, and therefore the cell walls of fungi) that exhibit biodegradability, biocompatibility, and styptic capability, moreover because the ability to inhibit the expansion of microorganisms and fishes. NAG loaded with chitosan nanoparticles is used for fish feed formation. N-acetyl glucosamine may be a chemical that has been derived from the outer shells of shellfish (Vert et al., 2012). Chitosan may be an appropriate chemical compound for medical and pharmaceutical applications (Cheung et al., 2015).

Commonly, success of any cultivation venture in the main depends on "Three Pillars". They're quality seed, quality feed and quality management. Nutritionally balanced blue feed is that the most vital and essential input for the undefeated fish production because it is one among the foremost limiting factors within the enlargement of the overall fish production, (Behera et al., 2014). The aquaculture business has recently been growing quicker than some other segments of nourishment creation, so as to cover the protein interest for human utilization, (Luis et al., 2017). Chitosan particles are of high enthusiasm attributable to their physicochemical highlights, for example, biocompatibility, biodegradability, non-lethality, bioactivity, and polycationic nature by Divya and Jisha, (2018). The improvement of nanotechnological details for application in aquaculture has especially been the principle focal point of research because of worries about sanitation for customers (Jennings et al., 2016).The utilization of nanotechnology is a shelter for sustenance, as at nanometer sizes, m aterials show extraordinary highlights not at all like those of mass material and separated particles. Nanotechnology has a wide scope of uses in aquaculture furthermore, can contribute fundamentally to several emerging fields, (Luis et al., 2017, Angelica et al., 2019).

Table 1. Potential Use of Nanotechnology In Fish Feed: (Shrivastava et al., 2015)

S.NO	FUNCTION	JUSTIFICATION
1.	Antimicrobial or Antifungal agents	Preserving sacks or during storage of fish feed.
2.	Delivery of micronutrients or other ingredients	To surround or coat (Nanoencapsulation technology) nutrients that would normally degrade, such as fatty acids, or have limited absorbtion efficiency. eg. free fatty acid
3.	Increasing bioavailability	Carotenoids, Trace minerals, Vitamins and Fatty acids
4.	Nanoscale mineral supplements	Improve absorption
5.	Alternative to organic forms of feed supplements	To reduce the anti-nutritional factors
6.	Stability of the food ingredients	Alter the physical properties reduce food wastage

Nanotechnology uses in fish feed formulation: Nanotechnology includes a intensive usage potential in cultivation and food industries. significantly to beat feeding methods issues of culture organism, Nano technologies provide a promising results, attributable to it nano size particle and economical mixing property that makes the feed delivery system additional adequate and self-made. on account of studies, nanoparticles of parts like antioxidant, iron, etc. sources supplemented in diet may improve the expansion of fish scale back the value of water treatment (Albrecht et al., 2006). There are varied potential applications of NMs in blue feeds (Table.1).

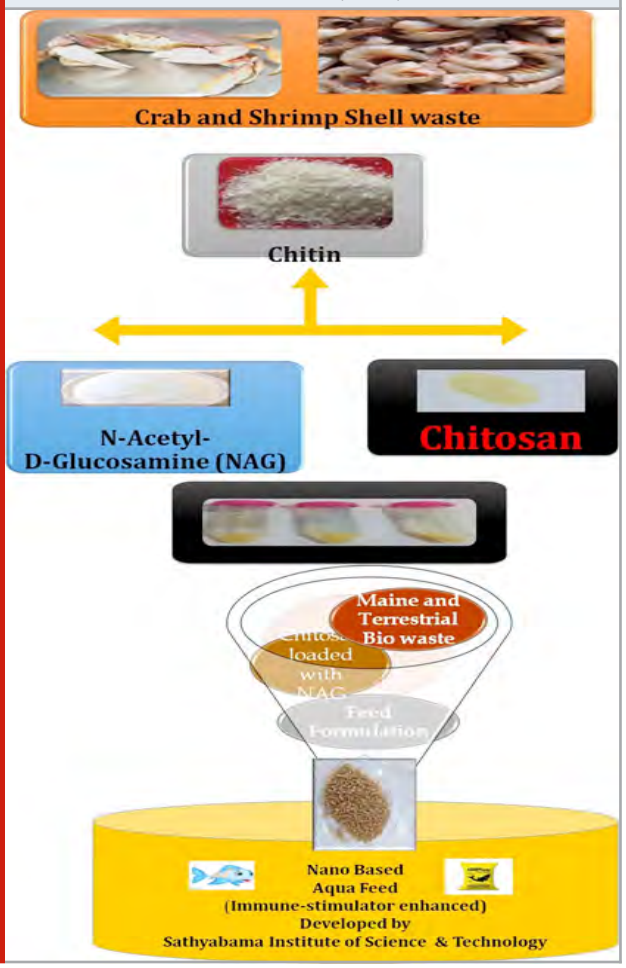
Role of Nano technology in feed formulation: Nanotechnology is associate rising science that is includes a immense potential to revolutionize agriculture and different fields as well as cultivation and fisheries (Ashraf et al., 2011). It will offer new Facilities for cultivation, fish nutrition, fish biotechnology, fish biological science, fish copy and aquatic health. Nano technology includes a wide selection of application in cultivation and food industries. for instance, production of more practical fish feed for cultivation species by application of nano technology (Choi et al., 2010).

Chitosan Nano particle preparation methods: Fathima et al., (2019) worked out the dietary supplementation with ChNP and reported that it decidedly influences the monetarily significant freshwater O. niloticus as far as development is concerned with regard to body organization, intestinal bacterial check, stomach related compounds, hematology, resistant reaction and liver status. In spite of the fact that the most elevated feed and protein usage, hematological profile, amylase and lipase exercises, body rough lipid substance, and decrease in the check of anaerobic microscopic organisms were seen in fish that got 5 g/kg diet ChNP; the lower portion of 3g/kg diet was additionally adequate to fundamentally upgrade certain parameters, for example,

development execution, and checks of high-impact and anaerobic microbes. In this manner, the organization of low be that as it may, successful degrees of ChNP can be a valuable methodology in fish cultivating, (Fathima et al., 2019).

Udo and his team (2017) formulated of experimental diets, formulations based on linear programming technology, have been developed using the feed-formulating software on windows. Dry matter was used for both diets. The protein supply was blood meal, soya and fishmeal (65 percent anchovy). In a freezer with nitrogen gas to avoid spoilage, all the products were ground up to less than 40 µm. Dry ice has been used to avoid decomposition when grinding all nutrients. Before the food planning, Vitamin Premix was prepared to ensure the ingredients are fresh. The percentage-based diets have been converted in a 5 kg bag size weight basis. Individually weighted by electronically sensitive Chitosan and chitosan nanoparticles are then added and thoroughly mixed in order to achieve a standardized mixture of 5 g kg-1. Cassava starch was used as a binder and was placed in a 50% cool (50-50 mL-water-50-g-starch) solution.

Figure 1. Schematic diagram of Nano enhanced Fish Feed Formulation designed by Subramanian Kumaran S., Wilson Aruni and Karthika M.(2020)



Method of NAG Loaded Chitosan Nano Particles: NAG-loaded nanoparticles were shaped by the addition of chitosan resolution to TPP solution containing totally different concentrations of NAG (Reddy et al., 2015). During this review paper the results of NAG concentrations (50, 75, 100, 200, three hundred and five hundred µg/mL) and chitosan concentrations (1, 2, three mg/mL) on nanoparticle's characteristics are studied (Yudinetal et al., 2014).

Nanoparticles have guarantee for building up the assurance of cultivated fish against maladies brought about by pathogens. Chitosan nanoparticles are utilized as transporters for an oral plasmid DNA antibody. For instance, oral organization with chitosan actuated a counter acting agent invulnerable reaction in fish. Fish Growth Promoter: The selenium (Se), iron (Fe) ang chitosan containing nanoparticles are likewise used to advertiser of high last body weight pick up and improve the cancer prevention agent property.

Fish Feed Formulation: However, as highlighted in this topic, the integration of or greater progressive technologies may be the manner to Gain vast advances for the aquaculture enterprise, Together with the use of nanotechnology in aggregate with that Of important oils. The manufacturing of biodegradable nanoformulations with important oils can make contributions to solving issues of efficiency in sickness manipulate, as well as, to remedy. The problems of contamination. The contamination can be decreased by way of the fact of the opportunity to discount within. The use of traditional chemicals in disorder control lies on the reality that nanoparticles can act as well managed launched structures and in this context, large amount of organic active compounds have been used to treat several diseases, (Angelica et al., 2019). The experimental trail were conducted and got a high FCR and weight of fishes. The feed formulation will be patented. The nutrient content of fish meal relies upon at the kind of uncooked materials and production approaches used in its production. In wellknown, notable fish meal produced the use of whole fish contains sixty six%–74% crude protein, eight%–eleven% crude lipids, and <12% ash.33 In evaluation, fish meal made out of byproducts incorporates fifty two%–67% crude protein, 7%–14% crude lipids, and 12%–23% ash.

As an instance, white fish meal constructed from byproducts incorporates 60%–67% crude protein, 7%–eleven% crude lipids, and 21%–23% ash,18,34 and tuna fish meal produced from byproducts contains fifty seven%–60% crude protein, eight%–14% fat, and 12%–21% ash.35, 36, 37, 38 The lower protein content material and higher ash content in byproduct fish meals are not sudden, as the nutrient composition differs among complete fish, fillets, and different parts of the frame (viscera, heads, pores and skin, bones, and blood). The extraordinary proportions of numerous byproducts which might be used to provide fish meal will therefore also contribute to the nutrient variability of the fish meal crafted from byproducts. Regardless

of this, fish meal derived from fishery and aquaculture byproducts has been effectively used in aquafeeds, and its use is not unusual exercise in some countries (FAO, 2018) research at the nutritive values of byproduct fish food has validated their right capacity as alternative raw substances. Fish meal from tuna byproducts can substitute 25%–30% of the protein from top rate-grade fish meal without affecting the increase performance of noticed rose snapper (Lutjanus guttatus) whilst blanketed at a price of 15.8%–21.4%.37 For olive flounder (Paralichthys olivaceus), 30% of fish meal may be substituted through tuna byproduct meal at a dietary inclusion price of 21%.38 For Korean rockfish (Sebastes schlegeli), 75% of fish meal may be substituted via tuna byproduct meal at a nutritional inclusion charge of fifty eight.1%, with out compromising boom and feed usage.39 The much less than best dietary profile of byproduct fish food affords challenges within the complete alternative of outstanding fish meal. Despite the fact that, byproduct fish meal remains a possible opportunity to conventional fish meal, and, greater importantly, is a greater most economical and sustainable protein supply Table 2 & Table 3 (Kim et al., 2018).

Fish feed ingredients: Wong et al., (2019) concluded the usage of meals wastes to formulate fish feed pellets

Table 2. Different levels of some conventional fish feed stuffs

S. NO	Type of fish meal	Maximum level (%)	Minimum level (%)
1.	Fish meal (tuna waste, 56% protein)	50	10
2.	Fish meal (miscellaneous, 60% protein)	50	10
3.	Poultry byproduct meal (58% protein)	10	_
4.	Fish protein concentrate (soluble, 70% protein)	5	2
5.	Blood meal (80% protein)	3	_
6.	Soybean meal (38% protein)	30	_
7.	Soybean meal (solvent extract, 48% protein)	45	_
8.	Groundnut cake	5	_
	meat (45% protein)		
9.	Brewers dried yeast (30% protein)	5	2
10.	Brewers dried grains (18% protein)	5	_
11.	Palm kernel cake meal (18% protein)	5	_
12.	Wheat middling (17% protein)	5	_
13.	Rice brans (12% protein)	3	_
14.	Maize (10% protein)	20	5
15.	Sorghum (guinea corn, 10% protein)	10	3

Table 3. General Quantities of Nutrients Incorporated Into Diets For Growing Fish

S. NO	Nutrients	Requirement (% by dry diet)
1.	**Protein:** These include 10 essential amino acids, viz., lysine, phenylalanine, arginine, valine, leucine, isoleucine, methionine, threonine, tryptophan and histidine.	32-45%
2.	**Fat:** Used as a source of energy and polyunsaturated fatty acids. Generally, freshwater fish require fatty acids of the linolenic (w-3) and linoleic (w-6) series; while saltwater and coldwater fish require EPA and DHA (w-3).	4-28%
3.	**Carbohydrates:** These are an inexpensive source of energy and are binding agents. No essential Requirements have been identified. These are poorly digested when fed raw; highest digestibility is attained when cooked. Major carbohydrates are starch, cellulose and pectin.	10-30%
4.	**Minerals:** There can be some 20 inorganic mineral elements, including, calcium, phosphorous, magnesium, iron, copper, manganese, zinc, iodine and selenium	.1.0-2.5% (fed as a multi-mineral premix)
5	**Vitamins:** These are inorganic substances required in trace amounts that can be divided into fat soluble (vitamins A, D, E and K) and water-soluble (vitamin B-complex, viz., thiamine, riboflavin, pyridoxine, pantothenic acid, cyanocobalamin, niacin, biotin, folic acid choline and myoinositol; and vitamin C).	1.0-2.5% (fed naturally as a multi-vitamin premix; because of their chemical instability vitamin choline and C are added separately from the premix)

appears to be feasible. Specific combinations of meal wastes need to be chosen for healthy feeding modes of various fish species, extensively freshwater fishes related to low-trophic stages, Herbivores (which include grass carp) and omnivores (together with gray mullet). That is because of the fact that the protein and dietary necessities of these low trophic stage fishes are lower in comparison with carnivorous fishes (consisting of freshwater bass). Food waste based pellets may be upgraded by adding enzymes and baker's yeast, leading to better Boom costs and better immunity of the cultured fish.

Figure 2. Feed Formulation Using of NAG loaded chitosan Nanoparticle

Inclusion of Chinese herbs would additionally help to replace positive antibiotics utilized in aquaculture. In preferred, fish fed with meals waste primarily based diets are safer for human consumption, whilst in comparison with the ones fed the economic diets, because of the better contaminant concentrations in Fishmeal contained within the industrial diets. In addition research must take cognizance of the feasibility of including Chinese medicinal herbs. Employments of NAG stacked chitosan nanoparticle in fish feed: In aquaculture framework the development of the fish is significant on the grounds that the interest of fish is extremely high in advertise. The feeds utilized for fish doesn't improve the better development just as it doesn't have the ability to oppose the ailment. Along these lines the NAG stacked chitosan nanoparticles is utilized in feed definition on account of its capacity to expand quick development in angles and furthermore has a decent obstruction against sicknesses (Figure 1& 2).

CONCLUSION

The best demanding situations to alternative protein assets in aquafeeds encompass variable protein content and the feasibility of growing production, which is a characteristic of to be had processing technologies, fee, and scalability. Customer popularity also varies among these uncooked materials. Given these challenges, there's huge potential for technological enhancements to constantly produce fantastic opportunity protein products with stronger nutritional profiles, at the same time as economies of scale can bring about progressed fee competitiveness. The chitosan loaded with NAG for enrichment and disesase resistant of culturing fishes. A few protein assets, which include fish by-products and bug meals, are feasible and promising options to traditional fish meal, while a few raw substances inclusive of food waste may also nonetheless need to conquer some of boundaries before becoming a staple in formulated aqua feeds and can find a recent trend in aquafeed development.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the management of Sathyabama Institute of Science and Technology, Chennai and the Professor K. R. Venakatesan, Principal, Sri Sankara Arts and Science College, Kanchipuram for their help and cooperation.

REFERENCES

Albrecht, M.A.,Evans, C.W., Raston, C.L., (2006); Green chemistry and the health implications of nanoparticles. Green Chemistry, (8),417-432.

Ashraf M. Rather, Rupam Sharma, Md Aklakur, M. S. Akhtar, Alexander Ciji, Shabir Ahmad1 and Mujahid Khan1 (2011); Nanotechnology: An emerging avenue for aquaculture and fisheries. World aquaculture, September 42:3, 9-11.

Behera, T., Swain, P., Rangacharulu, P.V., & Samanta, M., (2014). Nano-Fe as feed additive improves the haematological and immunological parameters of fish, Labeo rohita (H). Springer(4). 687-694.

Cheung, R.C.F., Ng, T.B., Wong, J.H., Chan, W.Y.,(2015); Chitosan: An update on potential biomedical and

pharmaceutical applications. Mar. Drugs, 13, 5156–5186.
Choi, M.J., Ruktanonchai, U., Min, S.G., Chun, J.Y., Soottitantawat, A.,(2010); Physical characteristics of fish oil encapsulated by β-cyclodextrin using an aggregation method or polycaprolactone using an emulsion-diffusion method. Food Chem. 119:1694–1703.
Divya, K., Jisha, M., (2018); Chitosan nanoparticles preparation and applications. Environ Chem Lett. 16, 101–112
FAO., (2018). The State of World Fisheries and Aquaculture 2018—Meeting the Sustainable Development Goals.
Fathima, S., Naby, S.A., Mohammed, A.E., Adham, A., Sagheer, A., Samar, S., Negm .S., (2019); Dietary chitosan nanoparticles enhance the growth, production performance, and immunity in Oreachromis niloticus. Aquaculture Volume 501,82-89.
Jennings, S., Stentiford, G.D., Leocadio, A.M., Jeffery, K.R., Metcalfe, J.D., Katsiadaki, I., Auchterlonie, N.A., Mangi, S.C., Pinnegar, J.K., Ellis, T., (2016); Aquatic food security: insights into challenges and solutions from an analysis of interactions between fisheries, aquaculture, food safety, human health, fish and human welfare, economy and environment. Fish Fish. 17, 893-938.
Kim, K.D., Jang, J.W., Kim, K.W., Lee B.J., Hur, S.J., Han, H.S, (2018);Tuna by-product meal as a dietary protein source replacing fishmeal in juvenile Korean rockfish Sebastes schlegeli. Fish. Aquat. Sci., 21, 29
Luis, A.I.S., Ramos Campos E.V., De Oliveira, J.L., Fraceto, L.F., (2019); Trends in aquaculture sciences: from now to use of nanotechnology for disease control. Reviews in Aquaculture, 11, 119–132
Luis, A.I.S., Campos, E.V.R., de Oliveira, J.L., Fraceto, L.F., (2017); Trends in aquaculture sciences: from now to use of nanotechnology for disease control. Rev Aquacult. 1–14. doi: 10.1111/raq.12229.
Shrivastava, V., Lende, S.R., Baraiya,K.G., Khileri, R.A., and Vikas., (2015); Nanotechnology in aquaculture feed : A Review ,National Conference on Innovative Research in Agriculture, Food Science, Forestry, Horticulture, Aquaculture, Animal Sciences, Biodiversity, Environmental Engineering and Climate Change (AFHABEC) ISBN: 978-93-85822-05-6.
Udo., Imefon, U., Etukudo., Uwana., Anwana., Udo U.I. (2017);. Effects of Chitosan and Chitosan Nanoparticles on Water Quality, Growth performance, Survival Rate and Meat Quality of the African Catfish, Clarias gariepinus. Volume 1.
Vert, M., Hellwich, K.H., Hess, M., (2012).Terminology for biorelated polymers and applications. Pure Appl. Chem , 84, 377–410.
Wong, M.H., Wing, Y.M., Choi W.M., Cheng, Z., Man, Y.B.,(2016); Recycle food wastes into high quality fish feeds for safe and quality fish production. Environmental pollution. 1-8.
Yudin, V.E., Dobrovolskaya, I.P., Neelov, I.M., Dresvyanina, E.N., Popryadukhin, P.V., Ivan Kova, E.M., Elokhovskii, V.Y., Kasatkin, I.A., Okrugin, B.M., Morganti, P., (2014); Wet spinning of fibers made of chitosan and chitin nanofibrils. Carbohydr. Polym.108, 176–182.

Entomological Communication

BBRC Bioscience Biotechnology Research Communications

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp-79-83

The Potential Use of Open Data Kit Application for the Mosquito Larvae Monitoring Program to Control Dengue Vector in Indonesia

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ABSTRACT

Dengue fever is an infectious disease which has been a major health problem in Indonesia. There are numerous approaches to reduce the incidence of dengue fever in Indonesia, including the eradication of mosquito nests, establishment of the Early Warning Alert and Response System (EWARS), as well as monitoring and analyzing the high incidence areas for easier management. Data collection is an essential component of the surveillance of mosquito spread, with both policy makers and health service providers requiring accurate and up-to-date data to implement an appropriate policy to control dengue fever. The increasing use of smartphones and rapid growth of technology must be utilized for information delivery in dengue fever surveillance to support the program of mosquito larvae monitoring by larvae monitoring officers ("*Jumantik*" in Indonesian language). The Open Data Kit (ODK) is an application which can assist the data collection process, making it more efficient as well as supporting the health program. This application can efficiently map the activity of dengue fever spread, with the interactive map displaying the location of data collection points, thereby accurately presenting the information related to disease distribution based on the Global Positioning System (GPS). Thus, the utilization of ODK in the EWARS is expected to increase the efficiency and optimize the success in controlling the incidence of dengue fever.

KEY WORDS: OPEN DATA KIT; MOSQUITO LARVAE MONITORING OFFICER; JUMANTIK; DENGUE; SURVEILLANCE.

INTRODUCTION

Disease surveillance is an activity whereby disease data is collected systematically, using the epidemiological information for planning and assessing disease control. The components of surveillance activity include data collection, compilation, analysis, and data interpretation, as well as the analysis of disease spread to determine appropriate action. The main surveillance activities include case detection, case record, case confirmation, report, data analysis, as well as giving response and feedback. The main purpose of this surveillance is using the collected data to develop policies and programs to promote health and prevent disease (McNabb et al., 2002). Furthermore, data collection must be conducted repeatedly and periodically to be consistent and relevant regarding the disease incidence

ARTICLE INFORMATION

*Corresponding Author: a.berbudi@unpad.ac.id
Received 10th Feb 2020 Accepted after revision 12th March 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)
A Society of Science and Nature Publication, Bhopal India

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/14

within a specific population. Data collection in the field is challenging, with various obstacles to overcome. For instance, the location may be difficult to reach, thereby slowing the data collection. In addition, it can be costly, requiring adequate human resources. Such difficulties can mean that the surveillance is incomplete, so stakeholders, such as primary health care units, health departments and other health institutions, cannot develop strategic and accurate policies in response to current health issues.

Industrial Revolution of 4.0: The development of Industrial Revolution 4.0 was marked by the emergence of cyber technology and automation technology used in combination in various fields including health. The implementation of this concept is centered on automation that can be conducted by technology without any human assistance in the implementation process. Such automation can increase efficiency in various aspects where time management is vital and required. Indeed, optimal time management will exponentially increase the quality of the work. The utilization of technology in surveillance means that the information can be delivered easily and is readily accessible by everyone at any time. Currently, electronic media has been the dominant information media used by people in accessing information. Indeed, with the appropriate management, electronic media could be a beneficial health information source, delivering information related to health problems, disease spread, the impact and the prevention efforts (Sujatmiko, 2018).

Open Data Kit: Data collection and sharing can be conducted easily anytime and everywhere through the internet. Moreover, data collection can be conducted digitally using applications, such as the Open Data Kit (ODK) (Macharia et al., 2013). ODK is android-based application developed by the University of Washington to accommodate community's requirements, which is freely and easily accessible. Currently, ODK has been applied globally for various needs, such as general election data collection, tropical rainforest observation, infectious disease surveillance, etc. Indeed, the scope of ODK usage is large and its use can be managed to adjust with the type of data collection required (Brunette et al., 2013; Hartung et al., 2010). ODK can be utilized for survey data processing in various fields including health, and has been used by Google, WHO, and USAID for data collection, reporting, and processing. ODK has a server that can be accessed anywhere if it is connected to internet, but has the advantage of data entry without any internet connection. A comparison of popular data collection applications, including features such as "program license","skip logic", "calculation", "query", "custom-field" etc. is presented in Table 1. Typically, the more features an application has, then the better the application (Steinberg and Schindler, 2019).

Dengue Fever: As a developing country with a tropical climate, infectious diseases are a major problem in Indonesia (Karyanti and Hadinegoro, 2016). Indeed, thousands of people are affected by infectious diseases every year, resulting in fatalities and huge economical loss for the country (Nadjib et al., 2019). Dengue fever is one such infectious disease and typical symptoms include sudden fever, headache, pain behind the eye, nausea, bleeding signs (positive result on tourniquet test or rumple lead), skin rash (petechial), nose bleeding, and bleeding gums. Massive bleeding can occur in severe cases, such as digestive tract bleeding in the stomach and intestine, resulting in bloody stools and vomit. Dengue

Table 1. Comparison of data collection applications based on their features

Feature	EC5	ODKv1	ODKv2	Kobo	Ohmage	SurveyCTO	Magpi	COBWEB
Active development	■	■	■	■	-	■	■	-
Open Source	-	■	■	■	■	-	-	■
Programing Language	-	Java Javascript Python	Java Javascript	Java Javascript Python	Java ObjectiveC	-	-	Javascript Python
License	-	Apache	Apache	Apache GNU	Apache	-	-	BSD3
Self-hosting	–	■	■	■	■	–	–	■
Form designer	■	■	–	■	■	■	■	-
Skip logic	■	■	■	■	–	■	■	-
Localization	–	■	■	■	–	■	–	-
Calculation	–	■	■	■	–	■	■	-
Queries	–	–	■	–	–	–	–	-
Link tables	–	–	■	–	–	–	–	-
Required fields	■	■	■	■	■	■	■	-
Validation	■	■	■	■	■	■	■	-
Building custom prompts		–	–	–	–	–	–	-

fever is caused by infection of dengue virus from the Arbovirus, which is spread through the mosquito bites (Aedes aegypti and Aedes albopictus). The dengue virus is mostly found in tropical areas, particularly in urban densely populated areas, and conditions in Indonesia are ideal for the spread of this disease-bearing Aedes mosquito. Consequently, dengue fever is a major health problem, with increasing incidence due to the high mobility of the urban population, climate change, density change and other epidemiological factors (Harapan et al., 2019; Utama et al., 2019).

Indonesia is ranked second state in the thirty countries with dengue fever endemic area (Haryanto, 2018). In 2017, the number of dengue fever cases was 68,407, of which, 493 resulted in death. There are three provinces with the highest incidence of dengue fever in Java Island, West Java had the largest number of dengue fever cases (10,061), followed by East Java and Central Java (7,838 and 7,400 respectively) (Indonesian Ministry of Health, 2018). The high incidence of dengue fever in Indonesia has caused both huge social and economic loss with around US$381.15 million was expensed in 2015 (Nadjib et al., 2019). Unfortunately, the effectivity of dengue vaccine is still questionable to prevent infection of dengue virus, therefore, the most effective prevention is by controlling the transmitter vector (Aedes mosquito) through the eradication of mosquito nests (EMN). It has been mandated in the Minister of Health Regulation number 581/MENKES/SK/VII/1992 on Eradicating the Dengue Fever and the Minister of Health Regulation number 92 Year 1994, which emphasizes the prevention effort by eradicating mosquito nests to control dengue fever.

Control of Dengue Fever: To optimize the prevention of dengue fever by EMN in Indonesia, the target location of EMN must be on target, effective, and efficient. The location of large populations of mosquitoes and high risk of dengue fever must be prioritized for intervention to prevent dengue fever, thereby reducing the incidence and possible epidemic as well as huge losses. However, the mapping of such locations is difficult. Indeed, there has not been any available data about mosquito spread that could be used to detect the risk of dengue fever. The rapid development of technology and application of digital data management programs, such as ODK, could be a solution for this problem. ODK could be utilized to collect data regarding mosquito spread to guide preventive action to reduce the incidence of dengue fever as well as associated losses.

ODK for Data Collection and Reporting of Surveillance Data regarding the Dengue Vector: The early benefit of the ODK application is changing the information documentation collected (reported) from a paper-based system to a digital report of information collected in real-time, as the information collected is delivered to the server allowing reporting of the survey information collected according to the time and geographical area (Hartung et al., 2010). ODK is also useful in the surveillance effort and program evaluation, significantly reducing the time needed to report the data to regional, local, state or national levels, which is beneficial for stakeholders.

Figure 1. The design process and survey report using the ODK application

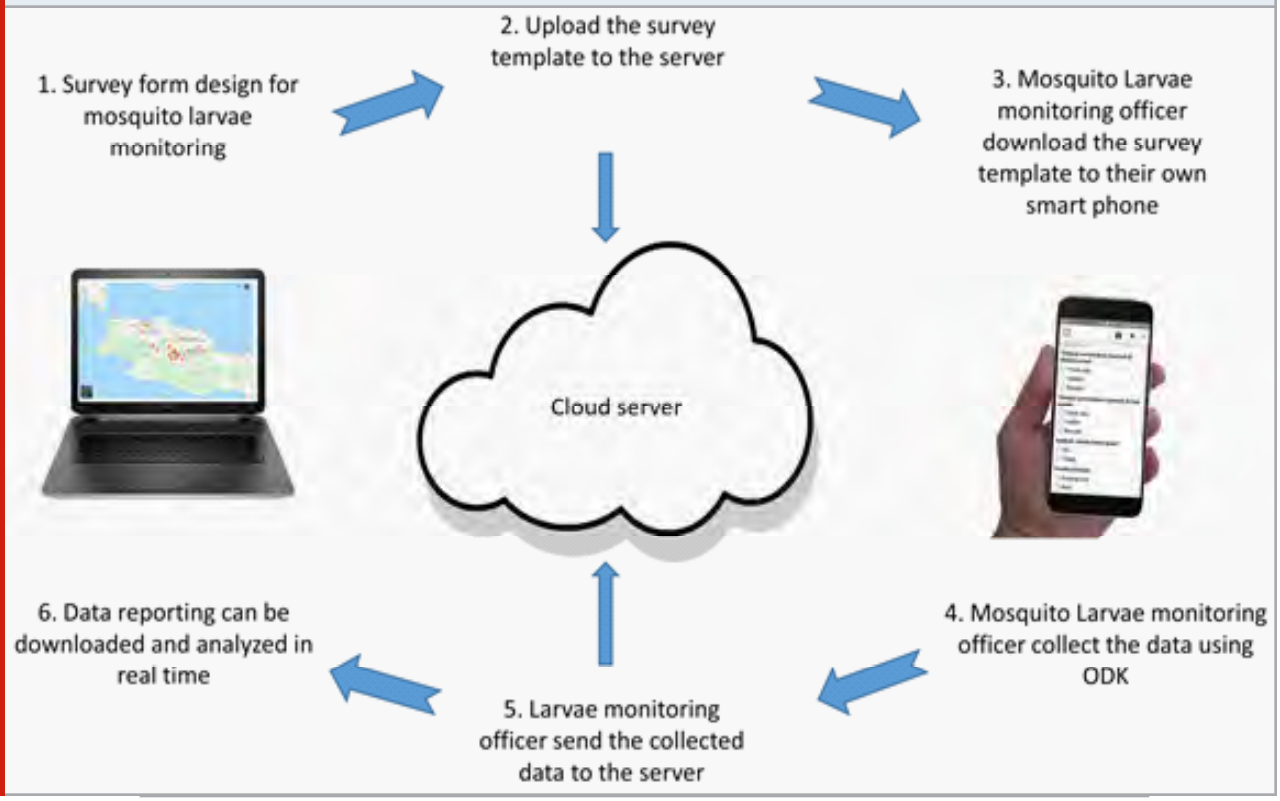
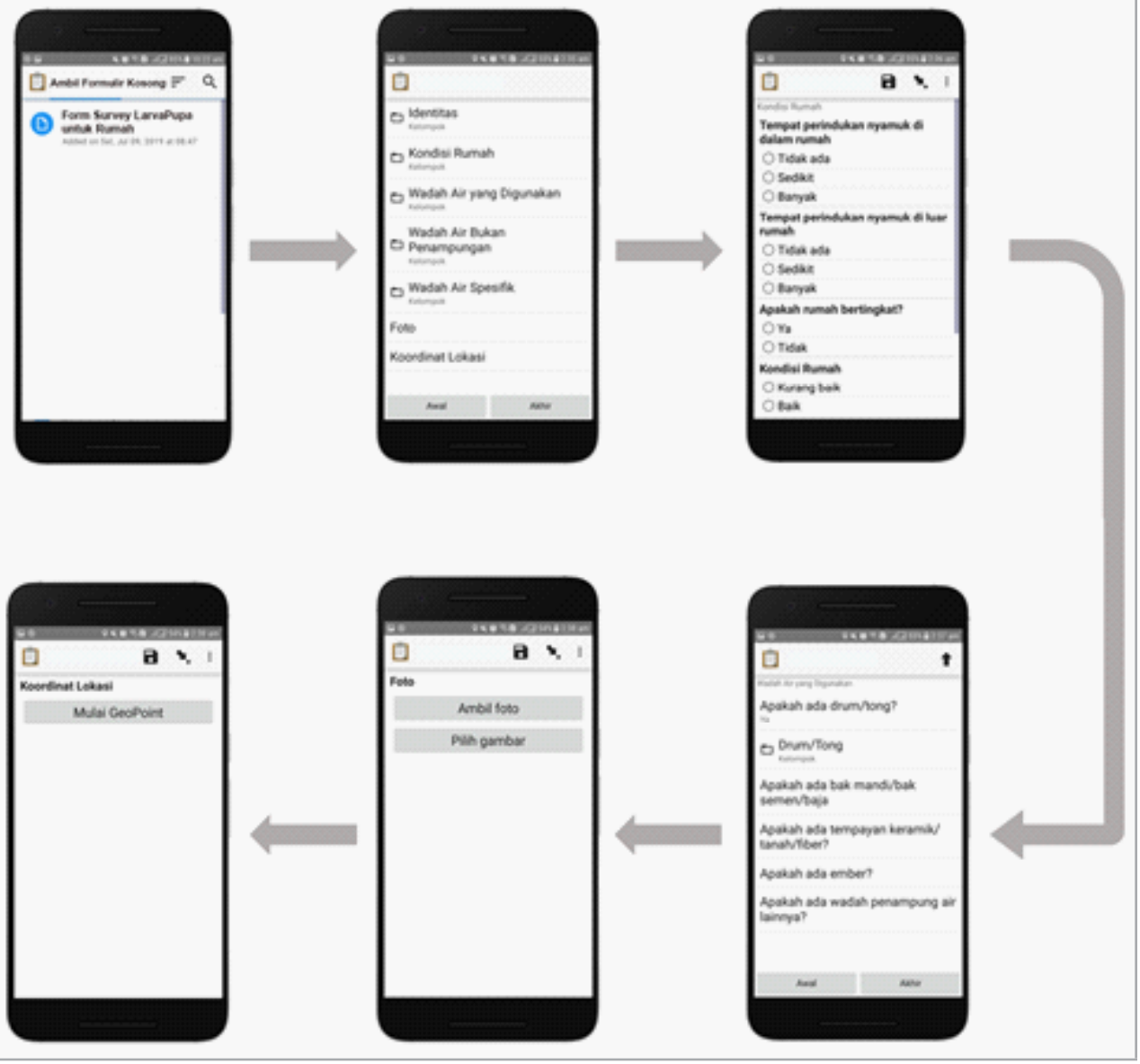


Figure 2. Display of ODK form list for Larvae monitoring Officers (*Jumantik*) in the field



Mosquito Larvae Monitoring: The government effort to prevent dengue fever involves terminating the infectious mosquito chain by spreading larvasida, fogging, and EMN, therefore, the ODK application will be useful to report helpful information to assist this effort. The survey form for dengue fever prevention can be created with the ODK application, comprising several questions to identify the risk of dengue fever spread in every house. In addition, the picture-captured and location-recorded features of ODK directly connect to the server, thus accurately identifying the area at risk of dengue fever. The ODK application can also be used for the Early Warning Alert and Response System (EWARS) and various surveillance actions periodically. Areas with large mosquito populations can be identified, so that preventive actions, such as fogging and increasing the EMN, can be conducted to prevent the incidence of dengue fever. Previous efforts to establish this system were initiated by conducting a mosquito larvae monitoring program. Mosquito larvae monitoring officers (*Juru Pemantau Jentik / Jumantik* – in Indonesian Language) check, observe, and eradicate mosquito larvae, referring to the technical guidelines for eradication of nests of Aedes aegypti mosquito. Their duties include socialize the EMN, check the mosquito breeder area (inside and outside the house), mobilize people to conduct EMN, and record mosquito larvae observations as well as record the conduct of EMN. These paper-based observations are used to determine high risk areas, thereby reducing the incidence of dengue fever. Therefore, the ODK application would be helpful in this surveillance, as it is simple, easy and free to use, allowing mosquito larvae monitoring officers to digitally record their observations, thereby improving the efficiency and effectiveness of the EWARS.

Survey Design in ODK: The process of ODK implementation for the EWARS begins by creating the form list which must be completed by every mosquito larvae monitoring officer. This form is created in Microsoft Excel or ODK form builder that can be accessed on the ODK website, then uploaded to the server, which can be made by us or provided by the cloud server. Every officer who has downloaded the application can then install the form list on their smartphone, so it can be displayed and completed on their phone screen. The data collected is then delivered to the server when the phone is connected to the internet, or automatically stored to be sent when there is an internet connection. The data will be displayed in real-time on the server for subsequent download or analysis by the authorized stakeholders. The data collection and reporting using ODK is presented in Figure 1.

Data Collection and Input Using the ODK: Tutorial videos can be created to help the mosquito larvae monitoring officers to understand the ODK procedures for data collection and input. The form list includes the identity of the house owner surveyed, the house condition, information about water canisters used in the house, as well as displaying the house condition in the form of a picture that can be taken directly using the phone camera, as shown in Figure 2. Specific data about the mosquito breeder is displayed for the next form list. Regarding location, every surveyor can easily record the GPS location that is integrated in the ODK application directly.

CONCLUSION

The utilization of technology can increase the efficiency and optimize surveillance activity to control the spread of dengue fever. The ODK application is freely available, relatively simple and easy to use, thereby allowing stakeholders to easily access up-to-date, accurate information regarding the spread of mosquitoes that cause dengue fever to establish appropriate policies to control and prevent the incidence of dengue fever in Indonesia.

Conflict of Interest: No potential conflict of interest relevant to this article was reported.

ACKNOWLEDGEMENTS

We thank the Directorate of Research and Community Service of Padjadjaran University for their supports in this scientific writing. We also thank Nabila Nurul Assyifa for her assistance in providing the proper picture in this article.

Funding: This work was funded by Internal Grant for Research and Community Service of Padjadjaran University.

REFERENCES

Brunette, W., Sundt, M., Dell, N., Chaudhri, R., Breit, N., Borriello, G., 2013. Open data kit 2.0: expanding and refining information services for developing regions, in: Proceedings of the 14th Workshop on Mobile Computing Systems and Applications. p. 10.

Harapan, H., Michie, A., Mudatsir, M., Sasmono, R.T., Imrie, A., 2019. Epidemiology of dengue hemorrhagic fever in Indonesia: analysis of five decades data from the National Disease Surveillance. BMC Res. Notes 12, 350. https://doi.org/10.1186/s13104-019-4379-9

Hartung, C., Anokwa, Y., Brunette, W., Lerer, A., Tseng, C., Borriello, G., 2010. Open data kit: Tools to build information services for developing regions, in: ACM International Conference Proceeding Series. https://doi.org/10.1145/2369220.2369236

Haryanto, B., 2018. Indonesia Dengue Fever: Status, Vulnerability, and Challenges, in: Current Topics in Tropical Emerging Diseases and Travel Medicine. IntechOpen. https://doi.org/10.5772/intechopen.82290

Karyanti, M.R., Hadinegoro, S.R., 2016. Perubahan epidemiologi demam berdarah dengue di Indonesia. Sari Pediatr. 10, 424–432.

Macharia, P., Muluve, E., Lizcano, J., Cleland, C., Cherutich, P., Kurth, A., 2013. Open Data Kit , A Solution Implementing a Mobile Health Information System To Enhance Data Management In Public Health 1–6.

McNabb, S.J.N., Chungong, S., Ryan, M., Wuhib, T., Nsubuga, P., Alemu, W., Carande-Kulis, V., Rodier, G., 2002. Conceptual framework of public health surveillance and action and itsapplication in health sector reform. BMC Public Health 2, 1–9. https://doi.org/10.1186/1471-2458-2-2

Indonesian Ministry of Health, 2018. Profil Kesehatan Indonesia 2018. Jakarta.

Nadjib, M., Setiawan, E., Putri, S., Nealon, J., Beucher, S., Hadinegoro, S.R., Permanasari, V.Y., Sari, K., Wahyono, T.Y.M., Kristin, E., Wirawan, D.N., Thabrany, H., 2019. Economic burden of dengue in Indonesia. PLoS Negl. Trop. Dis. 13, e0007038. https://doi.org/10.1371/journal.pntd.0007038

Steinberg, M.D., Schindler, S., 2019. Software solutions for form-based , mobile data collection – A comparative evaluation, in: Conference: Workshop on Big (and Small) Data in Science and Humanities. https://doi.org/10.18420/btw2019-ws-14

Sujatmiko, B., 2018. Pelatihan Pengumpulan Data KIA (Kesehatan Ibu dan Anak) DAN Gizi Berbasis Web/Internet Pada Kader POSYANDU DI Kecamatan Ujungberung Bandung. J. Pengabdi. Kpd. Masy. 2, 704–706.

Utama, I.M.S., Lukman, N., Sukmawati, D.D., Alisjahbana, B., Alam, A., Murniati, D., Utama, I.M.G.D.L., Puspitasari, D., Kosasih, H., Laksono, I., Karyana, M., Karyanti, M.R., Hapsari, M.M.D.E.A.H., Meutia, N., Liang, C.J., Wulan, W.N., Lau, C.-Y., Parwati, K.T.M., 2019. Dengue viral infection in Indonesia: Epidemiology, diagnostic challenges, and mutations from an observational cohort study. PLoS Negl. Trop. Dis. 13, e0007785. https://doi.org/10.1371/journal.pntd.0007785

Biotechnological Communication

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp-84-90

BBRC Bioscience Biotechnology Research Communications

Isolation, Purification and Characterization of Antimicrobial Metabolites from *Aspergillus ibericus*

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ABSTRACT

In the present study, isolation, purification and characterization of antimicrobial compound obtained from rhizospheric soil fungus *Aspergillus ibericus*, was carried out in order to determine the bioactive constituents present in the metabolite which are aactually responsible for the antimicrobial potential of fungus. The fungal metabolite was preliminarily screened for its antimicrobial activity against various test microorganisms. Three solvents of different polarity, ethyl acetate, chloroform and petroleum ether were tested for the extraction of antimicrobial metabolite from culture filtrate. Quantitative analysis of antimicrobial compound was carried out by using Thin Layer Chromatography (TLC). Separation of crude extract was performed by analytical HPLC followed by purification of extract through preparative HPLC. The probable structure of bioactive compound determined by NMR spectroscopy which was found to be 7-hydroxy-3-(methoxy carbonyl)-2-methylene heptanoic acid having molecular formula C10H16O5 with molecular mass 216. The Minimum Inhibitory Concentration of bioactive fraction obtained from *A. ibericus* was found to be significant as 19.5µg/ml against *Pseudomonas aeruginosa*, *Escherichia coli*, *Streptococus pyogenes*, *Candida albicans* and *Candida tropicalis*, moderate as 625µg /ml against *Staphylococcus aureus, Streptococcus mutans* and weak as 1.25mg/ml against *Bacillus subtilis*.

KEY WORDS: *ASPERGILLUS IBERICUS*, ANTIMICROBIAL METABOLITE, THIN LAYER CHROMATOGRAPHY, ANALYTICAL AND PREPARATIVE HPLC, NMR SPECTROSCOPY, MINIMUM INHIBITORY CONCENTRATION.

INTRODUCTION

Opportunistic infection is an infection caused by microbes like bacteria, viruses, fungi, or protozoa that do not cause any disease in healthy host. These opportunists can emerge from normally present in or on human body (innocuous) or from environmentally acquired microbes. These microbes take advantage of an opportunity such as a host with impaired defense system, an altered microbiota or breached integumentary barriers (Cragg and Newman, 2001, Cabrera et al., 2020). Some of the opportunistic organisms include *Candida albicans, Pseudomonas aeruginosa, Staphylococcus aureus, Salmonella, Clostridium difficile, Streptococcus pneumoniae, Streptococcus pyogenes* etc (Enoch et al., 2006; Chen et al. 2020).

Antibiotics form the most critical field of microbial biotechnology as they are found to cure various kind of bacterial and fungal infections, but one of the problem in the fight against infectious diseases is the development

ARTICLE INFORMATION

*Corresponding Author: drpranayjain@gmail.com
Received 11th Feb 2020 Accepted after revision 21th March 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)

*Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/15*

of resistance to the agents used to control them. The phenomenon of resistance in clinical isolates has been known since antimicrobial drugs entered the medicine. Drug resistance, emerging and re-emerging infectious diseases has emphasized the need of search for new strains and compounds with antimicrobial potential (Zinner, 2007; Lakoh et al. 2020).

Natural sources such as bacteria, fungi and plants can be explored for new chemical entities as natural products provide a vast source of chemically diverse biologically active leads for therapeutic agents. Medicinal plants provide enormous secondary metabolites having potential to use as natural drugs in modern medicine. These bioactive secondary metabolites synthesized by medicinal plants can also strongly affect plant-associated microbial communities and their physiological functions as microorganisms live in a world of chemical signals. Surprisingly, not only the plants themselves are able to produce substances with therapeutic properties, but research continues to show that number of natural bioactive compounds are actually produced by their associated microbes (Bull and Stach, 2007; Binyamin et al., 2019). The rhizosphere is defined as the soil zone in vicinity of plant roots, a site of high microbial activity and diversity in comparison to non-rhizosphere bulk soil. Organic compounds released by plant roots may act as basis of chemotaxis to attract some species and repel others, resulting in the existence of different communities. The microbial diversity and selection for competent microbes (for limited nutrients and space) in rhizosphere, makes it potentially an important source of natural products (Berdy, 2005; Shaikh and Mokat, 2017).

In keeping view of the above justifications, for the continuous search of new isolates from rhizosphere soil of medicinal plants, having antimicrobial activity, the present study aimed at the following objectives which included isolation of rhizosphere soil fungi *Aspergillus ibericus* from medicinal plant Ficus religiosa and screening for its antimicrobial activity against various test organisms. The fungus *A. ibericus* belongs to Order *Eurotiales*, Class *Eurotiomycetes* and Family *Trichocomaceae*. The morphological characteristics of fungus *A. ibericus* are black colony color, reverse side yellow, granular texture, biseriate sporulating structure, rough spores with maturity, conidia diameter of 5-5.5µm, conidia head diameter of 55-70 µm, colonies initiate with white hyphae and quickly form jet black conidia (Aneja, 2003). Isolated strain of *A. ibercus* seems to be broad spectrum in its mode of action as it inhibited the growth of all test microbes including gram-positive, gram-negative and yeasts. The work also included purification and characterization of antimicrobial metabolite and determination of minimum inhibitory concentration of antimicrobial metabolite.

MATERIAL AND METHODS

Soil samples were collected from rhizosphere of medicinal plant Peepal (Ficus religiosa) from Botanical garden, Kurukshetra University, Kurukshetra. by removing 1-1.5 inch of top soil with sterilized spatula. The serial dilution agar plate method was used for isolation of *Aspergillus ibericus* from soil sample (Cappucino and Sherman, 1996; Aneja, 2003). Potato dextrose agar (PDA) (CDH) for fungi was used as isolation medium. Fungal colonies appearing on their respective media were transferred to potato dextrose agar plates (one colony on each plate) at 30°C for 4-5 days. The colonies were then transferred on potato dextrose agar slants and incubated at 30°C for 4-5 days and were maintained at 4°C in a refrigerator for further studies.

The antimicrobial activity of *A. ibericus* was evaluated by using agar well diffusion assay (Nandhini and Selvam, 2011). Potato dextrose agar plates were inoculated with 100µl of standardized inoculum (0.5 McFarland Standard) of each test microbe (in triplicates) and was spread with sterile swabs. Wells were made into agar plates containing the test microbe inoculum. 200µl volume of extract was poured into a well of inoculated plates. Uninoculated potato dextrose broth (Hi-Media) was used as negative control. Antibiotics ciprofloxacin (antibacterial) and fluconazole (antifungal) were used as positive control. Then plates were left at room temperature for ten minutes allowing the diffusion of extract into agar. After incubation for 24 hours at 37°C, the plates were observed for inhibition zone surrounding the well containing extract. The zone of growth inhibition was measured and expressed in millimeters (mm). The mean and standard deviation of diameter of inhibition zones were calculated. Three solvents of different polarity viz ethyl acetate, chloroform and petroleum ether were tested for the extraction of antimicrobial metabolite from culture filtrate. The filtrate was solvent extracted with each of the solvent separately, in a separating funnel taking equal volumes of filtrate and solvent (Kekuda et al., 2013). Quantitative analysis of antimicrobial compound was carried out by using Thin Layer Chromatography (TLC). The positions of different spots and solvent (distance it covered) were marked. The relative flow (Rf) value was determined (Rajalakshmi and Mahesh, 2014 with slight modifications).

Separation of crude extract by analytical HPLC (Shimadzu) was performed at CSIR- Indian Institute of Integrative Medicine, Jammu. The analytical column RP-18e chromolith with length of 100mm and internal diameter of 4.6mm with particle size of 5µm was used for separation of crude extract.All components which showed peaks in analytical HPLC were purified by preparative HPLC at CSIR- Indian Institute of Integrative Medicine, Jammu. The preparative HPLC system (Shimadzu UFLC) consisted of pump LC20AD, autosampler SIL20A HT, column oven CTI 10AS and detector PDA SPD M20A. The column Merck Semi-prep RP-18e with length of 250mm and inner diameter of 10mm with particle size of 5µm. The analysis was carried out using a gradient of water with 0.1% formic acid and acetonitrile. Flow rate 2ml/min. and column temperature 45°C was maintained. Characterization of active component showing antimicrobial activity was

performed by Proton (1H), Carbon (13C) and 2D NMR (Heteronuclear Single Quantum Coherence (HSQC), Heteronuclear Multiple Bond Coorelation (HMBC)) at CSIR-Indian Institute of Integrative Medicine, Jammu. A 400MHz Bruker spectrophotometer was used to record the NMR of antimicrobial metabolite. Chemical shift values were given in parts per million (ppm) with tetramethylsilane as internal standard. The solvent used was deuterated methanol.

MIC of antimicrobial compound against all the test microbes was determined by two-fold dilution method. In this method, two-fold serial dilution of antimicrobial metabolite was prepared by first reconstituting the metabolite (10mg/ml) in 10% dimethyl sulphoxide (DMSO). The dilutions were made in 10% DMSO to achieve a decreasing concentration range. A 200µl volume of each dilution was introduced into wells (triplicate) in nutrient agar plates already seeded with 100µl of inoculum of the test microbes. All plates were incubated at 37°C for 24 hours and were observed for the inhibition zones to know the minimum concentration of metabolite which is sufficient to inhibit growth of test microbes (Andrews, 2001; Aneja et al., 2010 with some modifications).

Statistical analysis: The data obtained from various experiments were subjected to analysis of variance (One Way ANOVA) to evaluate the significance of each parameter by estimating p-value and f-value. The level of significance was considered as $p<0.05$ (Pan et al., 2016).

RESULTS AND DISCUSSION

Aspergillus ibericus isolated from rhizosphere soil samples of Ficus religiosa was effective against all test microbes including four gram-positive, two gram-negative and two yeasts. Antimicrobial activity of the fungus against test microbes is shown in Table 1 in terms of zone of growth inhibition. in the current study, *Aspergillus ibericus* showed maximum antimicrobial activity against most of the test microbes with very strong response against *B. subtilis, S. aureus, S. mutans, S. pyogenes, P. aeruginosa, E. coli* and *C. albicans* (24mm, 25mm, 22mm, 25mm, 21mm, 22mm and 26mm, respectively) and strong response against *C. tropicalis* (15mm). The extraction of crude bioactive metabolites from the culture filtrate by solvent extraction method is an important factor to find best solvent that have the potential to extract maximum concentration and most potent antimicrobial compounds (Haque et al., 2017). If the compounds secreted by microorganism are highly soluble in an appropriate water immiscible organic solvent, it can be easily extracted form culture broth (Haque et al., 2017; Kumar and Jadeja, 2018).

Extracellular metabolites are low molecular weight compounds that are secreted by microbial cells into a specific environment, namely the culture media (Pinu and Villas-Boas, 2017). There are some advantages of extracellular metabolite analysis over the analysis of intracellular metabolites. The separation of extracellular metabolites from microbial cells and from their intracellular metabolites can be achieved by simple techniques such as centrifugation for bacterial cells and filtration for fungi, while the extraction of intracellular metabolites from microbial cells is a complicated process (Tredwell et al., 2011; Keller, 2019). The analysis of extracellular metabolites provides invaluable information about the metabolism of different microorganisms that change in response to different environmental conditions.

The culture broth of selected strain *A. ibericus* was extracted with three solvents, ethyl acetate, chloroform and petroleum ether and metabolite was evaporated to dryness. Three solvents of different polarity were used to extract the active compound from filtrate. Antimicrobial activity of metabolites extracted with different solvents was measured in terms of diameter of zone of growth inhibition. Metabolite extracted with chloroform showed activity against only four test microbes mainly *B. subtilis, S. aureus, S. mutans* and *E. coli*. Compound extracted with petroleum ether showed antimicrobial activity against five test microbes

Table 1. Antimicrobial activity of rhizospheric soil fungus *Aspergillus ibericus* strain PIF2

Fungal isolate	Zone of growth inhibition (mm) Test microorganisms							
	Bacteria Gram-positive				Gram-negative			Yeast
	Bs	Sa	Sm	Sp	Pa	Ec	Ca	Ct
PIF2	24.66±0.57	25.00±0.00	22.66±0.57	25.00±0.00	21.00±0.00	22.33±0.57	26.66±0.57	15.00±1.00
Ciprofloxacin	24.00±0.00	NA	25.00±0.00	26.00±0.00	25.00±0.00	23.00±0.00	ND	ND
Fluconazole	ND	ND	ND	ND	ND	ND	NA	NA

Values are mean inhibition zone ± Standard deviation of three replicates
NA: No antimicrobial activity; ND: Not determined; Bs: *Bacillus subtilis*; Sa: *Staphylococcus aureus*; Pa: *Pseudomonas aeruginosa;* Ec: *Escherichia coli*; Sm: *Streptococcus mutans*; Sp: *Streptococcus pyogenes*; Ca: *Candida albicans*; Ct: *Candida tropicalis*

B. subtilis, S. aureus, S. mutans, P. aeruginosa and *C. albicans* with zone of growth inhibition between 14mm and 17mm respectively.

Metabolite extracted with ethyl acetate showed maximum activity against all test microbes with zone of growth inhibition ranging between 20 mm and 28mm. This suggests polar nature of antimicrobial compound extracted from culture filtrate of strain *A. ibericus*. One-way ANOVA analysis at 5% significance level shows calculated F value (5.87) greater than F critical value (3.46) and P value (0.000941) less than 0.05, which indicates that null hypothesis (there is no significant difference between the values) is rejected and there is significant difference between values. Table 2 and Figure 1 shows antimicrobial activity of metabolite extracted with different solvents. Figure 2 shows antimicrobial activity of metabolite extracted with different solvents against test microbes *C. albicans, E. coli* and *S. aureus*. Ethyl acetate as best solvent for extraction of antimicrobial compound from microbes was reported by many researchers (Awla et al., 2016; Ahsan et al., 2017; Haque et al., 2017; Hussaini and Gulve, 2019). The fungal crude extract was subjected to TLC analysis for the separation of the bioactive compounds. Two fractions designated as first and second were observed when developed in dichloromethane: methanol (85:15) on silica gel TLC sheets with Rf values 0.72 and 0.45 respectively (Figure 3).

Table 2. Optimization of solvent and antimicrobial activity

Solvent	Zone of growth inhibition (mm) Test microorganisms							
	Gram-positive Bacteria				Yeast Gram-negative			
	Bs	Sa	Sm	Sp	Pa	Ec	Ca	Ct
Chloroform	34.66±0.57	22.66±0.57	32.00±1.00	0.00±0.00	0.00±0.00	0.00±0.00	13.33±0.57	0.00±0.00
Ethyl acetate	28.00±0.00	26.33±0.57	26.66±0.57	26.33±0.57	26.33±0.57	22.66±0.57	31.33±0.57	20.00±0.00
Petroleum ether	15.66±0.57	13.66±0.57	15.33±0.57	0.00±0.00	17.00±0.00	0.00±0.00	15.66±0.57	0.00±0.00

Values are mean inhibition zone ± Standard deviation of three replicates
Bs: *Bacillus subtilis*; Sa: *Staphylococcus aureus*; Sm: *Streptococcus mutans*; Sp: *Streptococcus pyogenes*; Pa: *Pseudomonas aeruginosa*; Ec: *Escherichia coli*; Ca: *Candida albicans*; Ct: *Candida tropicalis*

Figure 1: Optimization of solvent and antimicrobial activity Bs: Bacillus subtilis; Sa: Staphylococcus aureus; Pa: Pseudomonas aeruginosa; Ec: *Escherichia coli*; Sm: *Streptococcus mutans*; Sp: *Streptococcus pyogenes*; Ca: *Candida albicans*; Ct: *Candida tropicalis*. When statistically analyzed at significance level 0.05 by One Way ANOVA, proved to be significantly different.

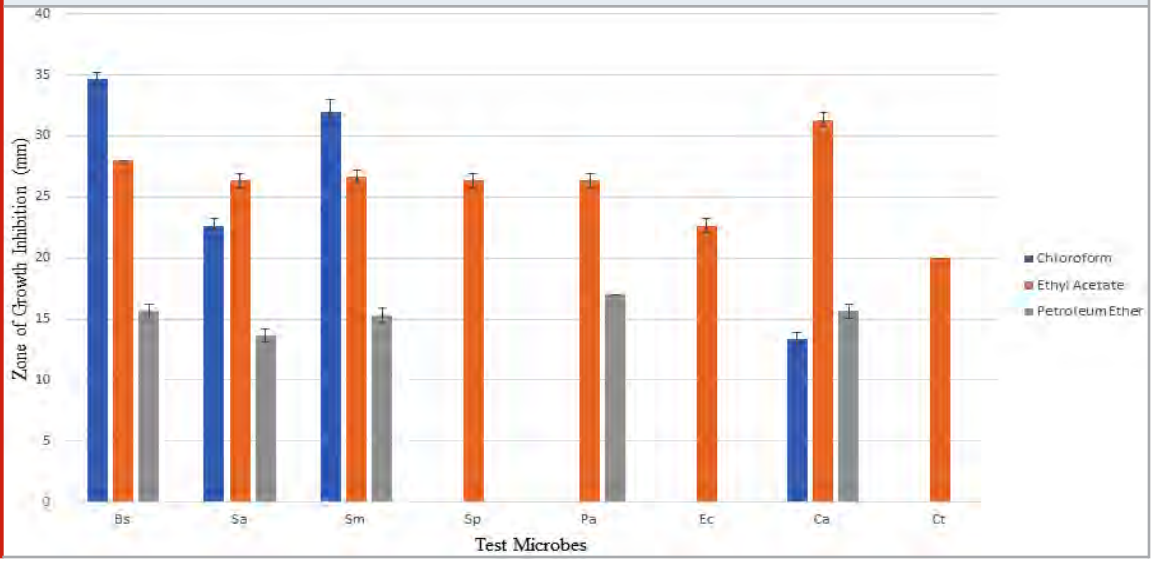
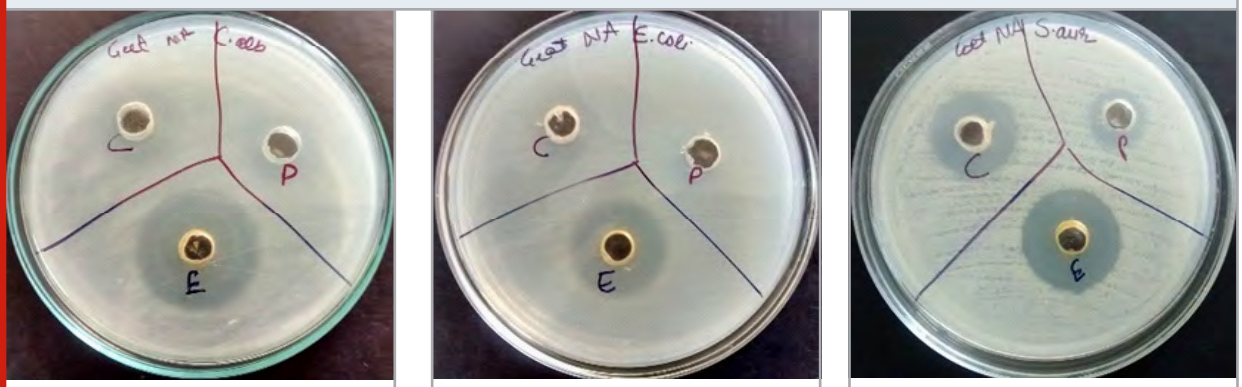
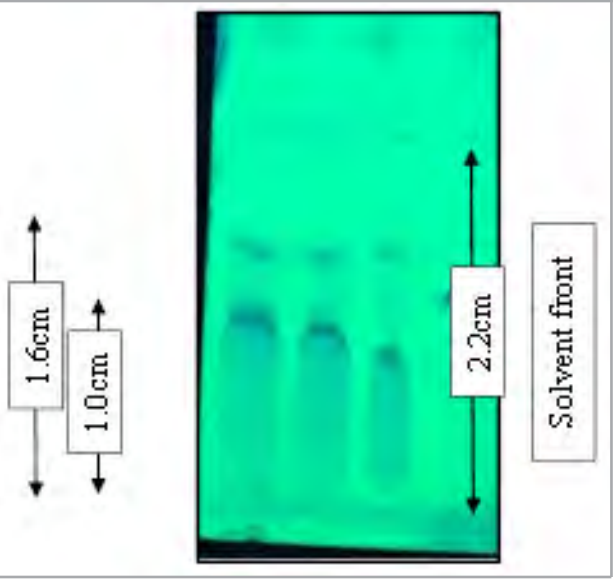


Figure 2: Optimization of solvent and antimicrobial activity against test microbes A) *C. albicans*, B) *E. coli* and C) *S. aureus*; C: Chloroform, P: Petroleum ether and E: Ethyl acetate.



A B C

Figure 3: Thin layer chromatography: Separation of crude extract with dichloromethane and methanol (85:15)



For purification by Preparative HPLC, firstly compound was subjected to analytical HPLC. In analytical HPLC, four main peaks were observed. Peak 1 at retention time 6.129, peak 2 at 6.671, peak 3 at 12.124 and peak 4 at 16.856 was observed with 4.435%, 18.554%, 24.929%, 52.081% area respectively. All four peak fractions were collected in pure form by preparative HPLC. El-Naggar et al. (2001) purified antimicrobial compound produced by Streptomyces violates using analytical and preparative high-performance liquid chromatography (HPLC). Both analytical and preparative HPLC had been performed for purification for antifungal compounds produced by *Lactobacillus plantarum* IMAU10014 by Wang et al. (2012). Analytical HPLC and Preparative HPLC had been performed for purification of ent-pimara-8(14),15-diene from engineered *Aspergillus nidulans* by Bromann et al. (2014). Alshaibani et al. (2016) performed analytical and preparative HPLC techniques for purification of active compounds from Streptomyces sp. SUK 25 with antimethicillin-resistant *Staphylococcus aureus* activity. Preparative HPLC had been performed for purification of antimicrobial substances from endophytic actinomycetes by Sunaryanto and Mahsunah (2013). Reis et al. (2018) characterized secondary metabolites from endophytic fungi *Nodulisporium sp*. isolated from the medicinal plant *Mikania laevigata* through high performance liquid chromatography coupled with mass spectrometry.

Antimicrobial activity of four fractions was analyzed against test microbes and compound showing peak 4 at retention time of 16.856 and with major fraction 52.081% showed antimicrobial activity against all test microbes. NMR spectroscopy had been performed for verification of structure of active compound obtained by purification from engineered *Aspergillus nidulans* by Bromann et al. (2014). Alshaibani et al. (2016) performed 1D and 2D NMR for characterization of active compounds from Streptomyces sp. SUK 25 with antimethicillin-resistant Staphylococcus aureus activity. Recently, Fan et al. (2020) characterized the structures of the compounds produced by seaweed derived fungus *Pyrenochaetospsis* sp. by extensive NMR. The structures of the compounds were elucidated by extensive NMR, H In the present study, purified fraction showing antimicrobial activity was analyzed by NMR spectroscopy (Pretsch et al., 2009). The probable structure of compound was found to be 7-hydroxy-3-(methoxy carbonyl)-2-methylene heptanoic acid having molecular formula C10H16O5 with molecular mass 216.MIC is defined as the lowest concentration of an antimicrobial agent which, under defined in vitro conditions, prevents the appearance of visible growth of a microorganism within a defined period of time. Extract from the isolate should be pure enough to fully characterize the activity of an antimicrobial compound (Lihan et al., 2014, Pelo et al. 2020).

The cut-off value for MIC are significant when MIC ≤ 100µg/ml, moderate in range of 100µg/ml-625µg/ml and weak when MIC ≥ 625µg/ ml. (Kuete, 2010) In the present study, MIC of bioactive fraction obtained from *A. ibericus* was found significant as 19.5µg/ml against *P. aeruginosa, E. coli, S. pyogenes, C. albicans, C. tropicalis, moderate as 625µg /ml against S. aureus, S. mutans*

and weak as 1.25mg/ml against *B. subtilis* and also compared to selected antibiotics fluconazole (showed no activity against any test microbe at any concentration) and streptomycin (120µg for P. aeruginosa, 5µg for *S. mutans, S. pyogenes, E. coli, 0.1µg for S. aureus* and *B. subtilis)*. When compared with selected antibiotics, bioactive fraction showed significant MIC value of 3.9µg against *P. aeruginosa, E. coli, S. pyogenes, C. albicans, C. tropicalis* whereas fluconazole showed no activity and streptomycin showed value of 120µg against *P. aeruginosa,* 5µg against *E. coli*, 5µg against *S. pyogenes* and no activity against two yeasts.

CONCLUSION

It may be concluded that fungus Aspergillus ibericus isolated from rhizosphere soil of medicinal plant Ficus religiosa is a promising source of antimicrobial metabolite. The research work shows rhizospheric soil of medicinal plants is a rich source of clinically important microorganisms. The antimicrobial metabolite produced by the fungal isolate *A. ibericus* was further purified and characterized. Antimicrobial metabolite obtained from A. ibericus is effective against test microbes gram-positive bacteria, *B. subtilis, S. aureus, S. mutans* and *S. pyogenes,* gram-negative bacteria, P. aeruginosa and *E. coli* and yeasts such as *C. albicans* and *C. tropicalis*. The antimicrobial metabolite is thus broad spectrum in nature. It may also be suggested that further research is needed to determine the cytotoxicity and in vivo efficacy against opportunistic pathogens before it is used for commercialization purpose.

ACKNOWLEDGEMENT

The authors are grateful to Hon'ble Vice-Chancellor, Kurukshetra University, Kurukshetra and the management and staff of CSIR-IIIM Jammu for providing necessary infrastructural facilities to carry out the research work.

Conflict of Interest: The authors declare that there is no conflict of interests.

REFERENCES

Ahsan T, J Chen, X Zhao, M Irfan and Y Wu (2017) Extraction and Identification of Bioactive Compounds (Eicosane and Dibutyl phthalate) Produced by Streptomyces Strain KX852460 for the Biological Control of Rhizoctonia solani AG-3 Strain KX852461 to Control Target Spot Disease in Tobacco Leaf. AMB Express Vol 7 Page 54.

Alshaibani MM, J Jalil, NM Sidik, R Edrada-Ebel and NM Zin (2016) Isolation and Characterization of Cyclo-(tryptophanylprolyl) and Chloramphenicol from Streptomyces sp. SUK 25 with Antimethicillin-Resistant Staphylococcus aureus Activity. Drug Design, Development and Therapy Vol 10 Pages 1817-1827.

Andrews JM (2001) Determination of Minimum Inhibitory Concentration. The Journal of Antimicrobial Chemotherapy Vol 48 Pages 5-16.

Aneja KR (2003) Experiments in Microbiology, Plant Pathology and Biotechnology. 4th ed. New Age International (P) Publishers, New Delhi.

Aneja KR, R Joshi and C Sharma (2010) In Vitro Antimicrobial Activity of Sapindus mukorossi and Emblica officinalis Against Dental Caries Pathogens. Ethnobotanical Leaflets Vol 14 Pages 402-412.

Awla HK, J Kadir, R Othman, TS Rashid and MY Wong (2016) Bioactive Compounds Produced by Streptomyces sp. Isolate UPMRS4 and Antifungal Activity Against Pyricularia oryzae. American Journal of Plant Sciences. Vol 7 Pages 1077-1085.

Berdy J (2005) Bioactive Microbial Metabolites. The Journal of Antibiotics Vol 58 No 1 Pages 1-26.

Binyamin R, Nadeem SM, Akhtar S, Khan MY and Anjum R. (2019). Beneficial and pathogenic plant-microbe interactions- A review. Soil and Environment. Vol 38(2) Pages 11-33.

Bromann K, K Viljanen, VM Moriera, J Yli-Kauhaluoma, L Ruohonen and T Nakari-Setala (2014) Isolation and Purification of Ent-pimara-8(14),15-diene from Engineered Aspergillus nidulans by Accelerated Solvent Extraction Combined with HPLC. Analytical Methods Vol 6 Pages 1227-1234.

Bull AT and JE Stach (2007) Marine Actinobacteria: New Opportunities for Natural Product Search and Discovery. Trends in Microbiology Vol 15 No 11 Pages 491-499.

Cabrera R, Fernández-Barat L, Motos A. (2020) Molecular characterization of methicillin-resistant Staphylococcus aureus clinical strains from the endotracheal tubes of patients with nosocomial pneumonia. Antimicrobial Resistance & Infection Control. Vol 9 Page 43.

Cappucino JG and N Sherman (1996) Microbiology- A Laboratory Manual. 4th ed. The Benjamin/Cummings Publishing Company, Inc. US.

Chen Y-H, Huang K-Y A, Huang Y-C, Chi H, Lu C-Y, Chang L-Y, Ho Y-H, Chi C-Y, Liu C-C, Huang L-M, Yang TYO and Huang Y-C (2020) Prevalence and molecular characterizations of Staphylococcus aureus nasal colonization among patients in pediatric intensive care units in Taiwan. Antimicrobial Resistance & Infection Control Vol 9: Page 41.

Cragg GM and DJ Newman (2001) Medicinals for the Millennia: The Historical Record. Annals of the New York Academy of Sciences Vol 953 Pages 3-25.

El-Naggar MY, MA Hassan and WY Said (2001) Isolation and Characterization of an AntimicroAVISbial Substance Produced by Streptomyces violates. Egyptian Journal of Biology Vol 3 Pages 11-21.

Enoch DA, HA Ludlam and NM Brown (2006) Invasive Fungal Infections: A Review of Epidemiology and Management Options. Journal of Medical Microbiology Vol 55 Pages 809-818.

Fan B, Dewapriya P, Li F, Blumel M and Tasdemir D (2020) Pyrenosetins A-C, new Decalinoylspirotetramic acid derivatives isolated by bioactivity-based molecular networking from the seaweed-derived fungus

Pyrenochaetopsis sp. FVE-001. Marine Drugs. Vol 18 Page 47.
Haque MA, H Imam, HK Hana, S Islam, D Ganim and Haque MR (2017) Maintenance and Optimization of Culture/Fermentation Media to Achieve Maximum Bioactive Metabolites from Marine Streptomyces sp. and their Cytotoxic Effects. Indian Journal of Geo Marine Sciences Vol 46 No 01) Pages 170-175.
Hussaini SZ and Gulve R (2019) Antimicrobial activity of ethyl acetate extract of antibiotic isolated from fresh water actinomycetes. International Journal of Research Culture Society Vol 3 No 6 Pages 56-61.
Kekuda PTR, KN Rakesh, S Junaid and N Dileep (2013) Antibacterial and Antioxidant Activities of Streptomyces species SRDP-H03 Isolated from Soil of Hosudi, Karnataka, India. Journal of Drug Delivery and Therapeutics Vol 3 No 4 Pages 47-53.
Keller NP (2019) Fungal secondary metabolism: regulation, function and drug discovery. Nature Reviews Microbiology Vol 17: Pages 167-180.
Kuete V (2010) Potential of Cameroonian Plants and Derived Products against Microbial Infections: A Review. Planta Medica Vol 76 No 14 Pages 1479-1491.
Kumar RR and Jadeja VJ (2018) Characterization and partial purification of an antibacterial agent from halophilic actinomycetes Kocuria sp. strain rsk4. Bioimpacts Vol 8 No 4 Pages 253-261.
Lakoh S, Li L, Sevalie S, Guo X, Adekanmbi O, Yang G, Adebayo O, Yi Le, Coker JM, Wang S, Wang T, Sun W, Habib AG and Klein EY (2020) Antibiotic resistance in patients with clinical features of healthcare-associated infections in an urban tertiary hospital in Sierra Leone: a cross- sectional study. Antimicrobial Resistance & Infection Control. Vol 9: Page 38.
Lihan S, CS Lin, I Ahmad, FM Sinang, NK Hua and AA Sallehin (2014) Antimicrobial Producing Microbes Isolated from Soil Samples Collected from Nanga Merit Forest in Sarawak, Malaysian Borneo. European Journal of Experimental Biology Vol 4 No 1 Pages 494-501.
Nandhini SU and MM Selvam (2011) Bioactive Compounds Produced by Streptomyces strain. International Journal of Pharmacy and Pharmaceutical Sciences Vol 5 No 1 Pages 176-178.
Pan F, Z Liu, Q Chen, YW Xu, K Hou and W Wu (2016) Endophytic Fungus Strain 28 Isolated from Houttuynia cordata Possesses Wide-Spectrum Antifungal Activity. Brazilian Journal of Microbiology Vol 47 Pages 480-488.
Pelo S, Mavumengwana V and Green E (2020) Diversity and antimicrobial activity of culturable fungal endophytes in Solanum mauritianum. International Journal of Environmental Research and Public Health. Vol 17: Page 439
Pinu FR and SG Villas-Boas (2017) Extracellular Microbial Metabolomics: The State of the Art. Metabolites Vol 7 No 3 Page 43.
Pretsch E, P Buhlmann and M Badertscher (2009) Structure Determination of Organic Compounds Tables of Spectral Data. Fourth, Revised and Enlarged Edition. Springer-Verlag Berlin Heidelberg.
Rajalakshmi S and N Mahesh (2014) Production and Characterization of Bioactive Metabolites Isolated from Aspergillus terreus in Rhizosphere Soil of Medicinal Plants. International Journal of Current Microbiology and Applied Sciences Vol 3 No 6 Pages 784-798.
Reis IMA, Ribeiro FPC, Almeida PRM and Branco A (2018) Characterization of the secondary metabolites from endophytic fungus Nodulisporium sp. isolated from the medicinal plant Mikania laevigata (Asteraceae) by reversed phase high performance liquid chromatography coupled with mass spectrometric multistage. Pharmacognosy Magazine. Vol 14 No 59 Pages 495-498.
Shaikh MN and Mokat DN (2017) Bioactive metabolites of rhizospheric fungi associated with Cimbopogon citratus (DC.) Stapf. Journal of Pharmacognosy and Phytochemistry. Vol 6 No 6 Pages 2289-2293.
Sunaryanto R and AH Mahsunah (2013) Isolation, Purification, and Characterization of Antimicrobial Substances from Endophytic Actinomycetes. Makara Journal of Sciences Vol 17 No 3 Pages 87-92.
Tredwell GD, B Edwards-Jones, DJ Leak and JG Bundy (2011) The Development of Metabolomic Sampling Procedures for Pichia pastoris, and Baseline Metabolome Data. PLoS ONE. Vol 6 No 1 Page e16286.
Wang H, Y Yan, J Wang, H Zhang and W Qi (2012) Production and Characterization of Antifungal Compounds Produced by Lactobacillus plantarum IMAU10014. PLoS ONE Vol 7 No 1 Page e29452.
Zinner SH (2007) Antibiotic Use: Present and Future. New Microbiologica Vol 30 No 3 Pages 321-325.

Sports Science Communication

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp 91-94

BBRC Bioscience Biotechnology Research Communications

On the Method of Using Variance Analytical Skills in Sport-Pedagogical and Biomedical Research

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ABSTRACT

Researchers are actively working to improve the training process of athletes, as demonstrating theory and correctly interpreting the results provide an opportunity to be advance about the effectiveness of the proposals. Important tests are based on variance analysis. The purpose of the research is to develop a method for the training of practical skills using variance analysis to analyze the results of sports, pedagogy and biomedical research for students in the field of physical education and sports. The present work suggests a method of forming students practical skills to perform variance analysis of sports-pedagogical and biomedical data is proposed. The implementation of the method involves focusing students' attention on performing variance analysis using computer programs. Automating the calculation process allows students of the department of physical education and sports to perform high-level statistical data processing.

KEY WORDS: TRAINING, ANALYSIS, FACTORS, STATISTICS, RESEARCH.

INTRODUCTION

Sport often comes with maximizing the reserve of the body's function. Therefore, at present, the evolution of scientists in order to optimize the training load is increasingly realistic in sports training practice.However, the quality of the study directly depends on the adequacy of the applied statistical analysis methods, because the use of false statistics negates the significance of the research activity. In fact, the publication of research results involves authoritative statistical processing of empirical data like the Fundamental Principles of Research Jobs for Higher Education Graduates in Culture of Sports. Only under such conditions, theoretical results can be used for reasoning in sports practice. Therefore, a highly qualified expert in physical culture and sports should be well versed in statistical research tools, (Byshevets, 2017) Byshevets and Denysova 2019, Kostiukevych et al. 2019).

An assessment of scientific, methodological and specialized materials, reflecting the issues preparing students for the field of physical education and sports, shows that today, scientists are widely used Mathematical and statistical apparatus to prove their findings (Stroganov and Sergienko 2013). Incorrect use of statistical methods and criteria can lead to deviations in test results and false conclusions. In experimental medicine and in the field of physical culture and sports, there are scientific works, the published results raise suspicion (Lang and Sesyk 2016).Evidence-based medical research of Leonov, (2017) suggests the use of mathematical estimates of the probability of its influence or absence under the variance analysis to analyze sports and education data

ARTICLE INFORMATION

Corresponding Author: thuchus@gmail.com

Received 8th Jan 2020 Accepted after revision 20th March 2020

Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)

Online Contents Available at: http//www.bbrc.in/

DOI: 10.21786/bbrc/13.1/16

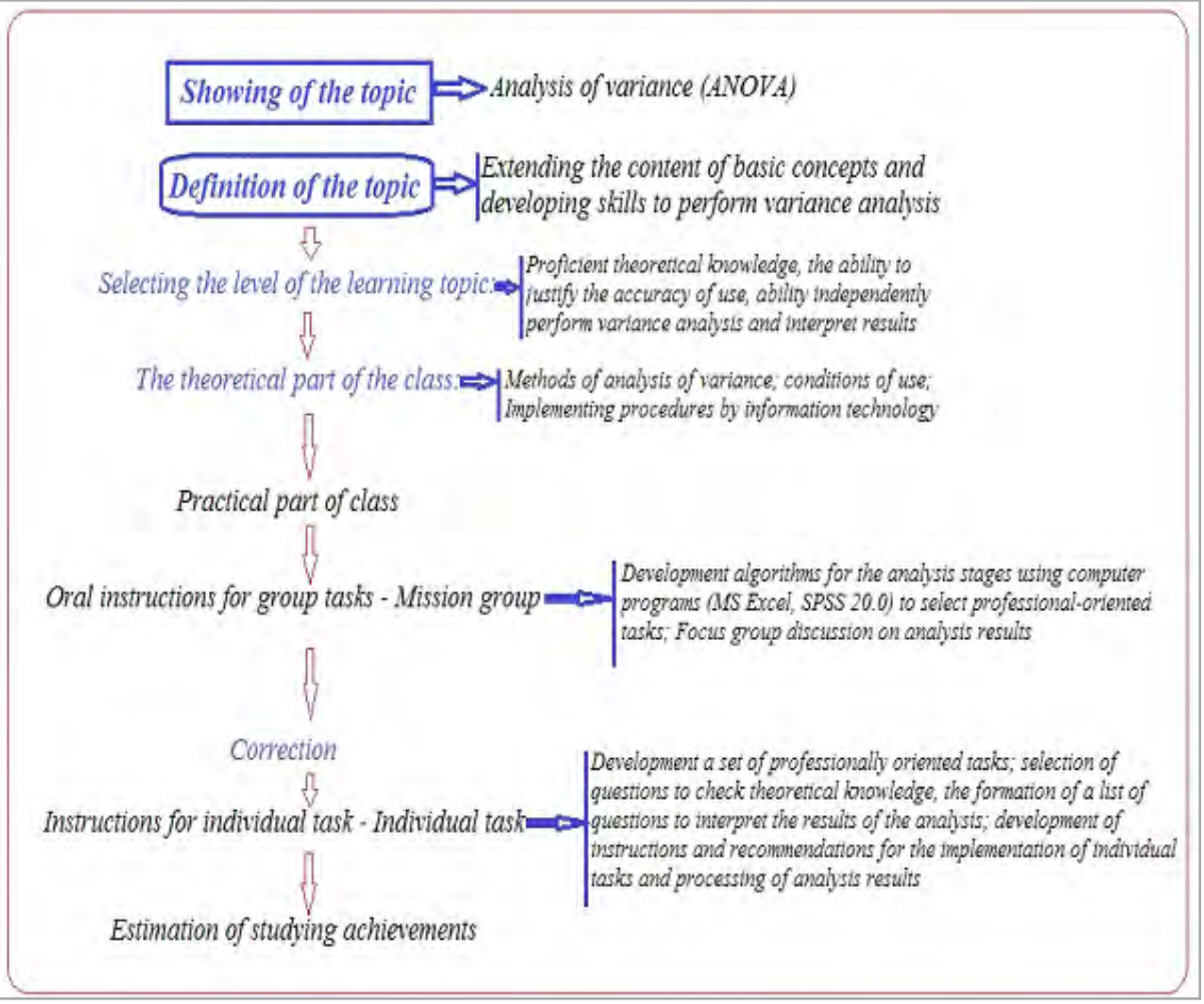
education and biomedical. An analysis of variance is influence of scientific studies the athlete models leads to decide to apply a specific training method. Therefore, future experts need to have statistical knowledge and formulate practical skills in the processing of empirical data statistics.

When analyzing literary sources, it is shown that currently experts have not presented methods for forming practical skills in using students' a well-known method for assessing the statistical importance of differences between sample populations, developed in the 1920s by English mathematician Fisher; it was the basis of a broad class of important criteria (Glantz, 1998). Looking at the practical aspect of using variance analysis to improve the system of sport choices (Drozdovska, 2015). Research purpose - to develop a method of forming practical skills using variance analysis for students in physical education and sports to analyze the results of sports and pedagogical research and biomedical. The methods of this study included analysis of scientific literature and related methods, internet sources, data and other mathematical statistical methods, including standard computer data processing.

During the study, we identified pedagogical conditions to form practical skills in using variance analysis for sports, pedagogy and biomedical data at high facilities. Among such conditions should include:- Expand the theoretical basis for methods of analysis of variance, conditions of use, characteristics of the ANOVA process;- Use of professionally oriented tasks;- A clear algorithm is available for the analysis phase using IT methods of student formation, where the practical skills for performing variance analysis are shown in Figure 1.

The study considers methodological implementation to develop students' practical skills in performing variance analysis of sports-pedagogical and biomedical data. It should be noted that: The peculiarity of the proposed method is due to the professional training content of future experts on sports and sports culture. First, they are:

Figure 1. Method of forming students' practical skills to perform variance analysis of sports-pedagogical and biomedical data



Minimize theoretical reports;– Focus on forming practical skills in students: Automate the ANOVA counting procedure. At the stage of the theoretical part, students are informed that the purpose of variance analysis is to test hypotheses about the relationship between certain characteristics and unrelated research elements in terms of quantity, as well as to establish the level of influence on the factors - circumstances affecting their results and interactions. The basis of variance analysis, considered a parameter criterion for comparing media between several sample sets, is the study of the components of variance. We emphasize that a variance analysis, depending on the number of factors, is equal to a one-way ANOVA, two-way ANOVA, or multivariate analysis. The results emphasize the need to meet the following conditions: the normal distribution of the characteristics from which the sample is taken; equality of dispersion of experimental properties (Gusev, 2000). At the stage of practical skills formation, we suggest problem-solving. For example, 34 athletes with the same fitness level were trained in four different methods. At the end of the study, they performed a pilot exercise. The input data is shown in Figure 2. The task is to assess the impact of the training method on the outcome of the test exercise.

Using the variance uniformity criterion for Leven's Test, we test the hypothesis that all distributions of the dependent variables for the compared samples have the same variance. As can be seen in Figure 2 (a), p> 0.05, so the variances of the groups were compared to be homogeneous, meaning that there was no qualitative difference between them. Therefore, it is reasonable to use one-way ANOVA variance analysis.

After performing the standard procedure for calculating Fisher criteria using MS Excel or SPSS 20.0 (b), we established the presence of statistically significant differences between the results of the test exercise in the transport teams. The motivations were compared with Fisher criterion (p <0.05). Therefore, the training method has a significant impact on the results of the test exercise performed by the athletes. Figure (2) shows that Method 4 turns out to be the most effective and Method 1 turns out to be the least effective. In addition, the use of the procedure for comparing pairs of means both by Bonferroni (c) and by Scheffe (d) proves that the difference is statistically significant (p <0.05) between results. of the test exercises performed by athletes involved in methods 1 and 4.

Figure 2: Perform an analysis of the variance factor in the SPSS 20.0 program

	MS Effect	MS Error	F	p
Result	*0.025121*	*0.211356*	*0.131724*	*0.960312*

Effect	Degr. of Freedom	Result SS	Result MS	Result F	Result p
Intercept	1	1321.721	1321.721	1426.215	0.000000
Technique	3	11.720	3.829	4.078	0.007641
Error	30	18.017	0.912		
Total	33	31.025			

a) *Estimate the variance homogeneity for Levene's Test;* b) *Fisher test calculation*

Cell No.	Technique	{1} 7.1426	{2} 8.0060	{3} 7.6000	{4} 9.2600
1	Technique1		0.620790	1.000000	0.013702
2	Technique2	0.620790		1.000000	0.307824
3	Technique3	1.000000	1.000000		0.109822
4	Technique4	0.013702	0.307824	0.109822	

Cell No.	Technique	{1} 7.1426	{2} 8.0060	{3} 7.6000	{4} 9.2600
1	Technique1		0.506371	0.904083	0.022875
2	Technique2	0.506371		0.902421	0.260019
3	Technique3	0.904083	0.902421		0.136743
4	Technique4	0.022875	0.260019	0.136743	

c) *Collating averages for Bonferroni test;* d) *Collating averages for Scheffe test*

That is, method 4 is more effective than method 1. Apparently the use of computer programs allows you to automatically simplify the calculation process and greatly simplify the process of data analysis. This allows students in the field of physical culture and sports, who often do not have a solid mathematical background, to process statistical mathematical data. Research shows that analysis of variance is quite common in psychology and pedagogical studies. Clearly, due to the increasing demand for the quality of science work in physical education, the evolution of practical skills to apply dispersed analysis among students of institutions higher education to analyze sports results - pedagogical and biomedical research. A method of forming students' practical skills to perform variance analysis of sports and biomedical data is proposed. Its characteristic feature is to focus the attention of future experts on physical culture and sports on the practical part of performing variance analysis by IT.

The prospect for further research is to illustrate methods for forming statistical knowledge and practical skills of students in the field of physical education and sports for statistical analysis of data in physical studies sports, education and health. Gusev (2010), addressed the characteristics of analysis using variance in empirical studies. Indeed, analysis of variance helps to solve a number of journalistic issues related to estimating differences between sample averages of different number of groups, surveys or differences between groups identified by factors. The factors are controlled and the influence value of each factor and their interactions determined (Lupan and Avramenko 2010), these reasons make it quite important for analyzing sports and educational data. The present study has paid attention to the importance of learning the basics of variance analysis. For the training of future statisticians: Lupan and Avramenko (2010) proposed practical tasks to analyze variance and give examples of their solutions using the most popular computer statistics packages.

In addition, it has long been tested and has a famous system algorithm for computing. Some computer programs automate the implementation of the ANOVA procedure, which makes sense. Prospects for analysis using variance for a variety of researchers. However, though, students of higher education and health institutions have no clear idea of the purpose of analyzing variance and interpreting the results of computational procedures. Theory and practice of sports training require more accurate and reasonable research results. The scientific level of empirical work depends on the proficiency of the scientific and pedagogical community studies by empirical statistical data processing methods. Misuse of the statistical system falsifies test results and leads to erroneous conclusions. One of the tasks of the student training system in the field of physical education and sports are to formulate research results handling skills, they have the necessary theoretical knowledge, practical skills and skills in using modern information technology to solve professional tasks. Analysis of variance involves establishing a relationship between qualitative (nominal) and quantitative (continuous) variables.

REFERENCES

Byshevets N., Denysova L. (2019) Using the methods of mathematical statistics in sports and educational research of masters in physical education and sport. Journal of Physical Education and Sport, V19(3), 1030 – 1034.DOI:10.7752/jpes.2019.s3148

Byshevets N.G. (2017). Experience of application of modern means of education in Practical lessons on the probability theory. Information technology in education. 2(31):95–108.

Drozdovska, S.B. (2015). Complex Molecular-Gene tic Diagnostics of Physical Performance in Sports. Bulletin of Biology and Medicine. 3(123):332-8.

Glantz, S. (1998). Biomedical statistics. M.: Praktika. 459 p.

Gusev, A. N. (2000). Analysis of variance in experimental psychology. M.: Textbook. collector Psychology.136 p.

Kostiukevych, V., Natalia Shchepotina , Oksana Shynkaruk , Iryna Kulchytska (2019). Training process construction of the qualified volleyball women players in the preparatory period of two-cycle system of the annual training on the basis of model training tasks. Journal of Physical Education and Sport, 19 (Supplement issue 2), 427-435. DOI:10.7752/jpes.2019.s2063

Lang, T. A, Sesyk, M. (2016). How to describe statistics in medicine: a guide for authors, editors and reviewers; Transl.from English. M.: Practical medicine. 480 p.

Leonov, VP. (2017). Mistakes of use of statistics in the biomedical data. International Journal of Medical Practice. 2:19-35.

Lupan I V and Avramenko AV. (2010). Computer statistical packages: Teach method guidances. Kirovograd. 218 p.

Stroganov, S., Sergienko, K. (2013). Modern approaches to the analysis of the features of the interaction between the foot and the support during the game of basketball. Theory and methods of physical education and sport.2:122-7.

Dental
Communication

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp 2020 95-100

BBRC
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Bond Strength of Resin Composite of Bioactive Restorative Materials Using Different Surface Treatments

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ABSTRACT

The current study was performed to analyse the shear bond strength of resin composite of bioactive restorative materials (Activa) using mechanical and chemical surface treatments. A total of 60 bioactive composite discs were prepared using a putty index mould and further divided into 6 groups (n= 10) each undergoing different surface treatment procedures. Study groups included, a control group (Group 1-no ageing, no surface treatments), Group 2- Aged non treated, Group 3- Acid etch/adhesive, Group 4- Acid etch-Silane/adhesive, Group 5- Grinding and Group 6 Grinding–Silane adhesive. All Activa discs were tested for shear bond strength using universal testing machine. Ten samples from each group were assessed for modes of failure. Data was assessed using analysis of variance and Tukey multiple comparisons test. In addition, among the mechanical and chemical surface treatment employed the highest mean value was of group 6 (20.86 (± 2.41) and least value of group 3 13.47 (±2.23). Among all groups the most common type of observed failure was adhesive followed by the admixed type. ANOVA displayed a significant difference between types of surface treatments on SBS of the repaired material ($p<0.01$) thus testifying the hypothesis. To increase surface energy for bonding of Bioactive (Activa) material, mechanical and chemical surface treatments are a prerequisite. A combination of mechanical grinding and silane adhesive (chemical) surface treatments produced desirable outcomes.

KEY WORDS: BIOACTIVE MATERIAL; REPAIR; RESIN COMPOSITE; SILANE; GRINDING; ADHESIVE BOND.

INTRODUCTION

Restorative repair is one of the immense challenges faced by clinicians. Many clinicians believe that total replacement of the restoration is the best way for long term integrity of the filling. However, the risk of removing tooth structure along with defaulted restoration is a commonly associated problem with total replacement. In contrast, repairing a defaulted restoration is considered a more conservative approach, which increases the serviceability of the restoration. Adhesive dentistry has created the possibility to employ a conservative approach to save the tooth from the extensive widening of the tooth cavity. The tooth is repaired based on two important factors the cavity preparation size reduction and the materials bonding ability (resin-based composite) to tooth structure, (Fernández et al., 2015 Zhang et al., 2017).

Using resin composite is a challenge in this type of approach as it undergoes degradation over time. The salivary enzymes, pH and wet environment are the factors that are responsible for early degradation process (Fernández et al., 2015). Previously the reparation of the composite was difficult because aging causes degradation of the available unsaturated double bond (Bektas et al., 2012). In addition, water sorption, chemical degradation

ARTICLE INFORMATION

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Received 15th Jan 2020 Accepted after revision 10th March 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/17

and constituent leaching out also influence the bonding between old and repair material (Neis et al., 2015, Bektas et al., 2012). Bioactive materials (Activa) are resin glass ionomer, which provides the advantage to chemically bond and create a seal against microleakage by releasing calcium, phosphate and fluoride (Garcia-Godoy and Morrow, 2015, Garcia-Godoy et al., 2014). Activa is one of the first ionic resin mixtures that bears a shock-absorbing resin component and bioactive fillers exhibiting similar chemical and physical properties as the teeth (Kaushik and Yadav, 2017, Amaireh et al., 2019).

In addition, it continuously recharges the environment with calcium, phosphate and fluoride,(Tezvergil et al., 2003). This makes the restoration durable and fracture resistant compared to the composites (Garcia-Godoy et al., 2014). Previous studies had observed the repair bond strength based on mechanical and chemical surface treatment on aged resin composite (Wendler et al., 2016, Palasuk et al., 2013). Authors claimed that increased surface roughness is an important factor for the bonding between old and new restoration (Kaushik and Yadav, 2017, Elsaka, 2016). The purpose of the surface treatment is to remove the surface layer contaminated by saliva and increase surface irregularities (Croll et al., 2015). This process exposes the high energy composite surface and increases the surface area (Wendler et al., 2016, Wiegand et al., 2012). However, there is no significant evidence to establish the relation of surface treatment and bonding strength between the composite restorations and Bioactive material (Activa). The present study aims to evaluate the shear bond strength in repaired restoration with the condition applied to different surface treatments. Therefore, the study aimed to investigate the shear bond strength of resin composite to bioactive restorative materials (Activa) using mechanical and chemical surface treatments.

MATERIALS AND METHODS

The present study conducted the shear bond strength test of resin composite of bioactive restorative material using different surface treatment after the approval from institutional research review board. A total of 60 bioactive composite discs were prepared using a putty index mould (Ø 6 mm, depth 3 mm). The materials used in this study and products details are presented in Table 1. For the preparation of the Bioactive disc (Activa) with dimension (5x3mm), the Activa material was condensed into layers in the putty index mold covered by a mylar strip. Each disc layer was light cured with a light intensity of 1100 mW/cm2, as measured by a digital radiometer (Marc Resin CalipratorTM, Blue Light Analytics Inc, Nova Scotia, Canada) for 20 secs at a distance of 1mm. A final curing was performed after removal from the mould.

The prepared disc were stored in the distilled water for 24h at 37°C. All the composite disc except 10 were subject to 100,000 thermocycles at 5 to 55 degree in the thermocycler (THE-1100, SD Mechatronik GmbH, Germany). Each cycle continues for 30 secs with 5 s interval between the baths. This process continues over a period of 7 days. Each specimen disc was embedded in an acrylic resin mould to create a base. Subsequently each disc was polished for 30 seconds to remove the flashes using a number 600 silicon carbide paper discs (CrbiMet® Abrasive Discs, BUEHLER, Lake Bluff, Illinois, USA) on a grinding machine (Automata, Jean Wirtz, Dusseldorf, Germany) driven at a speed of 300 rev/min under water spray followed by air drying.

Out of total 60 disc 10 water aged non thermocycled Activa disc were categorised as a control group for the evaluation of active shear bond strength in repaired material. The remaining disc were divided into 5 groups (n=10) to evaluate the effect of the different chemical and mechanical surface treatment procedures on the shear bond strength of repaired material. After the aging process, there were three major types of surface treatment performed on the aged composite disc: acid etching, grinding or combination of etching/grinding with silane adhesive. Each group was subjected to different treatment protocol. Group 2 consisted of thermocycled specimen disc without any surface treatment. In case of Group 3 and 5, the repair material was applied over a mechanical treated surface (etched with 37 % phosphoric and grinded with abrasive stone) in contrast to group 6 and 4 that were treated by the combination of mechanical and chemical surface treatment (silane adhesive).

Table 1: Study materials and composition

Material	Product detail
Shade A3.5 resin composite	Filtek supreme- 3M
Silane coupling agent	3M™ RelyX™ Ceramic Primer, 2721
37% phosphoric acid etch	Ivoclar Vivadent AG, FL-9494 Schaan/ Liechtenstein
Primer-adhesive system	Adper™ Scotchbond™ Multi-Purpose Adhesive

Preparation and surface treatment of the repaired disc

Group 3 and 4: The specimens were prepared with 400 – 600 grit carbide paper (Buehler) polish under a water coolant spray. Consequently, the surface is etched by 37 % phosphoric acid (Condac 37, FGM, Joinville, SC, Brazil) for 15 seconds, rinsed with water spray for 30 seconds and air dried for 10 seconds. The adhesive system in group 3 is the Scotchbond Multipurpose; however, in group 4 etching is accompanied by silane (relyx ceramic primer) and adhesive (single bond- all in one). The adhesive is applied with a micro brush and after 15 seconds it is air dried for 5 seconds and light cured for 10 seconds.

Group 5 and group 6: The specimen discs were grinded using the abrasive stone (016, Komet, CE 0197, Germany) rotated at 40,000 rpm for 5 secs, in one direction in group

5 whereas the specimen discs in group 6 were coated with silane and adhesive after grinding with abrasion stone. The detailed step by step surface treatment is explained in table 2.

The Activa discs with an acrylic resin based material (aged and non-aged) were repaired with a resin composite material (3mm x4mm), shade A3.5 with or without any surface treatment. A putty index mould (Ø 3 mm, depth 4 mm) is placed on the top of the Activa disc to support the repair material. Excess cement was removed with the micro brush. Each side was cured for 20 seconds, with the total time for curing 40 seconds using LED light cure (Elipartm S10 LED Curing Light, 3M ESPE, MN, USA) operated with a light intensity of 1100 mW/cm2. The repair material is light cured for additional 20 seconds after the mould removal followed by half of the bonded specimens from each groups were tested after the 24-hour storage in distilled water at 37 °C, whereas the other half underwent thermocycling (5000 cycles, THE-1100, SD Mechatronik GmbH, Germany) in distilled water at 5 °C to 55 °C. Each cycle runs for 30 seconds with an interval of 5 seconds between the cycles.

Table 2: Materials and surface treatment details.

Study Groups	Surface Treatments
Group 1- Control group (non thermocycled) Group 2-Aged non treated	No ageing and No treatment was applied to the repair-surface Only thermocycled and No treatment was applied to the repair-surface.
Group 3- Acid etch/adhesive	37% phosphoric acid etching was applied for 15 s, rinsed for 30 s and then air dried for 10 seconds. Subsequently, the adhesive was applied to the repaired surface of the disc for 10 s. After the application, it was air dried for 3 s to remove the excess solvent.
Group 4- Acid etch-Silane/adhesive	Acid etch was applied as described for group 3. Silane was applied for 1 min according to the manufacturer's instruction, gently air dried followed by a thin layer of the adhesive (same procedure as mention in group 3).
Group 5- Grinding	The disc surface was gently grinded using abrasive stone (016, Komet, CE 0197, Germany) rotated at 40,000 rpm with Sirona T2 Revo-R 40 hand piece (Sirona Dental Systems GmbH, Bensheim-Germany), for 5 s in one direction. Each disc cross sectional area before and after grinding was measured using a digital calliper (Mitutoyo, Mitutoyo Corporation, Japan) for standardising the quantity of composite removed. After grinding, the disc was rinsed for 15 s and dried for 3 s.
Group 6- Grinding– Silane adhesive	Repaired surface was grinded as mentioned above followed by the application of silane adhesive (Similar procedure as described above).

Shear bond strength: The specimens were transferred onto the universal testing machine (Instron 5965, Instron Corporation, Norwood, MN, USA) to evaluate the shear bond strength. The specimen was positioned in a direction where the 5-kN load cell at 90 degrees applies shear force at a cross speed of 0.5 mm/min on the Activa interface until debonding occurs. Using a digital calliper each bonded Activa-RBC cross sectional area before and after the application of the load was measured in order to calculate shear bond strength in megapascal (MPa) as per respective load applied. After the debonding, the surface was closely examined under a digital microscope (Hirox KH-8700, Europe) at 40X magnification for determination of the type of fracture at the interface. The fracture can be classified into three types mainly cohesive, adhesive and admixed. Any part of activa observed on the surface of composite resin indicates cohesive fracture whereas debonding at the interface reveals adhesive failure. Any residue of either material indicates the admixed type of fracture.

Statistical analysis: The data collected for the SBS was analysed using Statistical software for social sciences (SPSS 20.0 version), considering $p < 0.05$ statistically significant. The normality of the data was assessed through Kolmogorov-Smirnov test. A brief comparison between the groups can be appreciated through the mean and standard deviation. To determine the significant difference in shear bond strength between different surface treatments group's analysis of variance (ANOVA) and Tukey's post hoc test were employed.

RESULTS AND DISCUSSION

Kolmogorov-Smirnov test displayed even distribution of the normality data. The Analysis of variance (ANOVA) displayed a significant difference between different types of surface treatments on SBS of the repaired material ($p=0.01$). Comparing the mean and standard deviation between the groups, it can be observed that group 1 (24.61 (± 2.18)) demonstrated high value of shear strength bond closely followed by group 6, group 4, group 5, group 3 and group 2 respectively (table 3). Furthermore, under the mechanical and chemical surface treatment

employed the highest mean value was for group 6 [20.86 (± 2.41)] and least value for group 3 [13.47 (±2.23)].

Table 3: Means and SD for Shear bond strength values among study groups.

Study Groups	SBS-Mean (SD)	ANOVA-p value	Tukey HSD
Group 1	24.61 (2.18)		A
Group 2	11.38 (1.74)		B
Group 3	13.47 (2.23)	p <0.01	B
Group 4	17. 36 (2.45)		C
Group 5	16.38 (2.73)		C
Group 6	20.86 (2.41)		D

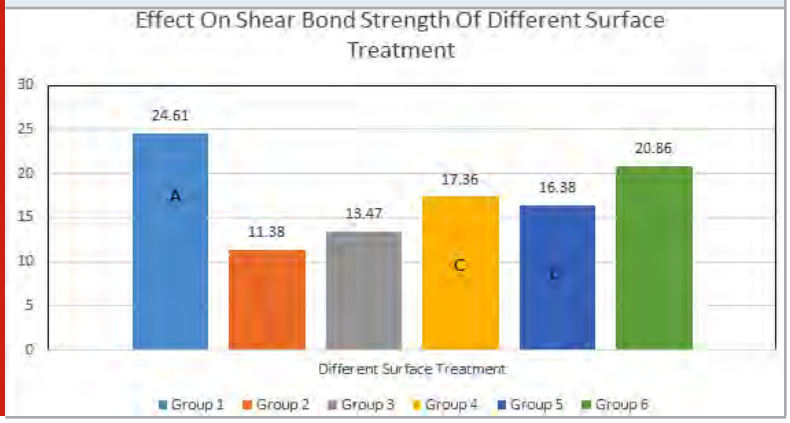
Note: Tukey HSD. Different alphabets denote a significant difference in study groups compared.

Multiple comparison tests clearly displayed that each surface treatment had an effect on the shear bond strength that includes the thermocycling, etching, silane adhesive application, grinding and a combination of etching/ grinding and silane. Nevertheless, the comparable results were appreciated between two groups using different surface treatments such as the no treatment (group 2), and etching group (group 3) (p= 0.0552) and etching with silane (group 4), and grinding group (group 5) (p= 0.7612). The no-treatment group recorded the least mean value with a similar mean value of etched specimens signifying no evident effect of etching independently compared to no treatment. On the other hand, etching with silane adhesive also did not demonstrate a significant difference in mean value in comparison to grinding if used independently. The digital microscope imaging revealed that the most common type of failure identified was an adhesive fracture, which clearly displayed the assessment of the shear bond strength. Only group 2 (no treatment) showed 100% adhesive failure indicating that surface treatment has a positive effect on the bond strength (table 4). Only 20% to 30% of the specimens demonstrated admixed fracture whereas only 1 or 2 specimens in groups 1, 4 and 6 exhibited cohesive failures.

The present study aimed to observe the effects of mechanical and chemical surface treatment on the shear bond strength between composite and bioactive material. The results revealed that the major factors affecting the bond strength between old and new restoration include surface roughness, bonding material, repair material, ageing and oral conditions (Palasuk et al., 2013, Wiegand et al., 2012). Theoretically, the bond between each fresh layer depends upon the present oxygen inhibiting layer over unsaturated monomers,(Hamano et al., 2012, Cho et al., 2013). Therefore, it is necessary to perform the surface treatment to expose high energy surface as it is difficult for aged restoration to form bonds, (Carvalho et al., 2012). The study result displayed that there was a significant effect of the mechanical and chemical surface treatment on the bond of repaired material. A multiplicity of explanation can be viewed in the light of similar studies conducted related to application of different surface treatment employed.

The curing of the Activa disc was carried out using a polyester strip matrix thereafter to standardise each specimen according to the oral conditions, to form an oxygen inhibiting layer and a smooth finish (Elsaka, 2016). To further imitate the oral conditions and initiate the aging process (except for 10 specimens) the samples were placed in the distilled water and thermocycled respectively (Bektas et al., 2012, Özcan et al., 2013). The placement of composite in the water removes the free radicals, which can react with new repair material,(Arumugam et al., 2014). In addition, the non thermocycled specimens were used as a positive control against the other groups to display the profound effect of oral condition on the repaired restoration. The purpose was to compare an ideal situation to the real possibilities in an oral cavity in order to test the materials efficacy.The result displayed the superior efficiency of the bioactive material bonding with resin composite under ideal conditions. This can be easily appreciated by comparing group 1 (non thermocycled) with other groups displaying the highest mean value.

Figure 1. Comparison between different surface treatments



Analysing the results, it can be observed that there was no significant difference between the only thermocycled and etched samples. Thermocycling ages the material by reducing the unsaturated bonds available for bonding; hence, it compromises the ability to chemically bond (Bektas et al., 2012).

Table 4: Failure mode among study groups

Study Groups	Adhesive (%)	Cohesive (%)	Mixed (%)
Group 1	50	20	30
Group 2	100	0	0
Group 3	70	0	30
Group 4	60	10	30
Group 5	80	0	20
Group 6	70	10	20

Furthermore, the results revealed that etching with the phosphoric acid presented with the lowest mean value among surface treatments employed. Previous studies explained that the phenomena of etching creates entrance voids upto the fillers that come in contact with water (Takamizawa et al., 2015, Torres-Gallegos et al., 2012). This deranges the silane layer and stabilises the filler matrix; hence, causing weakening of the bond strength (Rengo et al., 2012). Rengo et al., claimed that change in microstructure of the composite depends upon the variation in the intensity of etching (Rengo et al., 2012). However, an evident difference is observed in the application of silane and adhesive after etching. This indicates that applying silane adhesive creates a silane bridge (Si–O–Si) with the remaining filler particles of the composite and allows easy penetration of the adhesive into etched retentive cavities (Wiegand et al., 2012, Eslamian et al., 2012).

Thus, this indicates the microstructure and composition of the composite play an important role in developing bond strength in a repaired material. Divergent results were appreciated when grinding was used on the samples and compared with other surface treatment. Grinding with an abrasive stone (group 5) demonstrated comparable results to the etching and silane adhesive (group 4); however, when silane adhesive was applied after grinding a significant difference was appreciated. Authors had suggested that grinding the surface with the abrasive grit bur causes the removal of the fillers from the surface leading to voids formation (Özcan and Koc-Dundar, 2014). This reduces the amount of silica to react and form bonds. Therefore, adding the silane and adhesive after grinding causes an increase in the surface activation of bond formation (Lee et al., 2017, Dickens, 2015).

According to Lee et al, they failed to present improve bond strength following above-mentioned procedure (Lee et al., 2017). The authors stated the bond failure was appreciated due to the presence of smear debris (Lee et al., 2017). Whereas, the present study indicates silane and adhesive efficiently bonding with the repaired material. Therefore, it can be concluded that the difference in results might be due to different study materials and types of methodology testing. Hence, it can be appreciated that by comparing the two mechanical surface treatments grinding (singly used) showed better outcomes in contrast to etching and silane adhesive.

The author suggests that the ideal weight to test the shear bond strength is about 15 MPa to 25 MPa, typical resin-dentine bond strength values. In the present study, the samples were tested for shear bond strength under a load of 15 MPa. The adhesive failure was not completely 100% (except group 2) which demonstrated that mechanical and chemical surface treatment has a positive impact on the shear bond strength of the repaired material. Limited failure in the cohesive failure reflected the validity of the test based on bond strength in contrast to material strength (Hickel et al., 2013). Therefore, it can be apposite that surface treatment has an evident effect on the bonding strength of the repaired material. Analysing the limitation of the study, multitude of barriers can be viewed. The present study was able to efficiently evaluate the shear bond strength of the repaired material; however, the repaired bond strength was not compared with the previous strength of the material.

In addition, two particular different substrates were used to evaluate the bond strength. One of the most common problems associated with this material is the aesthetic shade. It has a narrow range of shade available, which limits its use. Therefore, the bioactive material can be used under a sandwich technique to overcome the esthetic compromise. In addition, the literature showed limited data availability on repair bond strength of Activa with a composite resin that intrigued to conduct such study. Thus, the study outcome provides some clinical perspective in using different surface modifications for repair of bioactive material with resins for durable clinical performance. Moreover, the study recommends further in-vitro and in-vivo studies to assess the cohesive strength of non-repaired materials to compare with repaired shear bond strength for validation of current findings.

CONCLUSION

Within the limitations, it was observed that thermocycling has a negative impact on the shear bond strength; therefore, to increase surface energy for bonding of Bioactive (Activa) Material, mechanical and chemical surface treatments are necessary. A combination of mechanical grinding and silane adhesive (chemical) surface treatment produced a desirable outcome.

REFERENCES

Amaireh, A., Al-Jundi, S. and Alshraideh, H. (2019). In Vitro Evaluation Of Microleakage In Primary Teeth Restored With Three Adhesive Materials: Activa™, Composite Resin, And Resin-Modified Glass Ionomer. European Archives Of Paediatric Dentistry, 20(4):359-67.

Arumugam, M. T., Nesamani, R., Kittappa, K., Sanjeev, K. and Sekar, M. (2014). Effect Of Various Antioxidants On The Shear Bond Strength Of Composite Resin To Bleached Enamel: An In Vitro Study. Journal Of Conservative Dentistry: Jcd, 17(1):22.

Bektas, Ö. Ö., Eren, D., Siso, S. H. and Akin, G. E. (2012). Effect Of Thermocycling On The Bond Strength Of Composite Resin To Bur And Laser Treated Composite Resin. Lasers In Medical Science, 27(4):723-8.

Carvalho, R. M., Manso, A. P., Geraldeli, S., Tay, F. R. and Pashley, D. H. (2012). Durability Of Bonds And Clinical Success Of Adhesive Restorations. Dental Materials, 28(1):72-86.

Cho, S., Rajitrangson, P., Matis, B. and Platt, J. (2013). Effect Of Er, Cr: Ysgg Laser, Air Abrasion, And Silane Application On Repaired Shear Bond Strength Of Composites. Operative Dentistry, 2013;38(3):E58-E66.

Croll, T. P., Berg, J. H. and Donly, K. J. (2015). Dental Repair Material: A Resin-Modified Glass-Ionomer Bioactive Ionic Resin-Based Composite. Compend Contin Educ Dent, 36(1):60-5.

Dickens, C. B. (2015). Waterproof Silane-Endcapped Adhesive Mixture. Google Patents.

Elsaka, S. E. (2016). Influence Of Surface Treatments On The Bond Strength Of Resin Cements To Monolithic Zirconia. J Adhes Dent, 18(5):387-95.

Eslamian, L., Borzabadi-Farahani, A., Mousavi, N. and Ghasemi, A. (2012). A Comparative Study Of Shear Bond Strength Between Metal And Ceramic Brackets And Artificially Aged Composite Restorations Using Different Surface Treatments. The European Journal Of Orthodontics, 34(5):610-7.

Fernández, E., Martín, J., Vildósola, P., Junior, O. O., Gordan, V., Mjor, I., Bersezio, C., Estay, J., De Andrade, M. and Moncada, G. (2015). Can Repair Increase The Longevity Of Composite Resins? Results Of A 10-Year Clinical Trial. Journal Of Dentistry, 43(2):279-86.

Garcia-Godoy, F. and Morrow, B. (2015). Wear Resistance Of New Activa Compared To Other Restorative Materials. J Dent Res, 2015;94:3522.

Garcia-Godoy, F., Morrow, B. and Pameijer, C. (2014). Flexural Strength And Fatigue Of New Activa Rmgics. J Dent Res, 93:254.

Hamano, N., Chiang, Y.-C., Nyamaa, I., Yamaguchi, H., Ino, S., Hickel, R. and Kunzelmann, K.-H. (2012). Repair Of Silorane-Based Dental Composites: Influence Of Surface Treatments. Dental Materials, 28(8):894-902.

Hickel, R., Brüshaver, K. and Ilie, N. (2013). Repair Of Restorations–Criteria For Decision Making And Clinical Recommendations. Dental Materials, 29(1):28-50.

Kaushik, M. and Yadav, M. (2017). Marginal Microleakage Properties Of Activa Bioactive Restorative And Nanohybrid Composite Resin Using Two Different Adhesives In Non Carious Cervical Lesions-An In Vitro Study. Journal Of The West African College Of Surgeons, 7(2):1.

Lee, H.-Y., Han, G.-J., Chang, J. and Son, H.-H. (2017). Bonding Of The Silane Containing Multi-Mode Universal Adhesive For Lithium Disilicate Ceramics. Restorative Dentistry and Endodontics, 42(2):95-104.

Neis, C. A., Albuquerque, N. L. G., Albuquerque, I. D. S., Gomes, E. A., Souza-Filho, C. B. D., Feitosa, V. P., Spazzin, A. O. and Bacchi, A. (2015). Surface Treatments For Repair Of Feldspathic, Leucite-And Lithium Disilicate-Reinforced Glass Ceramics Using Composite Resin. Brazilian Dental Journal, 26(2):152-5.

Özcan, M., Corazza, P. H., Marocho, S. M. S., Barbosa, S. H. and Bottino, M. A. (2013). Repair Bond Strength Of Microhybrid, Nanohybrid And Nanofilled Resin Composites: Effect Of Substrate Resin Type, Surface Conditioning And Ageing. Clinical Oral Investigations, 17(7):1751-8.

Özcan, M. and Koc-Dundar, B. (2014). Composite–Composite Adhesion In Dentistry: A Systematic Review And Meta-Analysis. Journal Of Adhesion Science And Technology, 28(21):2209-29.

Palasuk, J., Platt, J., Cho, S., Levon, J., Brown, D. and Hovijitra, S. (2013). Effect Of Surface Treatments On Microtensile Bond Strength Of Repaired Aged Silorane Resin Composite. Operative Dentistry, 38(1):91-9.

Rengo, C., Goracci, C., Juloski, J., Chieffi, N., Giovannetti, A., Vichi, A. and Ferrari, M. (2012). Influence Of Phosphoric Acid Etching On Microleakage Of A Self-Etch Adhesive And A Self-Adhering Composite. Australian Dental Journal, 57(2):220-6.

Takamizawa, T., Barkmeier, W. W., Tsujimoto, A., Scheidel, D., Erickson, R., Latta, M. A. and Miyazaki, M. (2015). Effect Of Phosphoric Acid Pre-Etching On Fatigue Limits Of Self-Etching Adhesives. Operative Dentistry, 40(4):379-95..

Tezvergil, A., Lassila, L. and Vallittu, P. (2003). Composite–Composite Repair Bond Strength: Effect Of Different Adhesion Primers. Journal Of Dentistry, 31(8):521-5

Torres-Gallegos, I., Zavala-Alonso, V., Patiño-Marín, N., Martinez-Castañon, G., Anusavice, K. and Loyola-Rodríguez, J. (2012). Enamel Roughness And Depth Profile After Phosphoric Acid Etching Of Healthy And Fluorotic Enamel. Australian Dental Journal, 57(2):151-6.

Wendler, M., Belli, R., Panzer, R., Skibbe, D., Petschelt, A. and Lohbauer, U. (2016). Repair Bond Strength Of Aged Resin Composite After Different Surface And Bonding Treatments. Materials, 9(7):547.

Wiegand, A., Stawarczyk, B., Buchalla, W., Tauböck, T. T., Özcan, M. and Attin, T. (2012). Repair Of Silorane Composite—Using The Same Substrate Or A Methacrylate-Based Composite? Dental Materials, 28(3):e19-e25.

Zhang, K., Zhang, N., Weir, M. D., Reynolds, M. A., Bai, Y. and Xu, H. H. (2017). Bioactive Dental Composites And Bonding Agents Having Remineralizing And Antibacterial Characteristics. Dental Clinics, 61(4):669-87.

Psychological Communication

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp 101-105

The Impact of Physical Education on Self-Esteem and Confidence of Orphaned Children

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ABSTRACT

The confidence scale and self-esteem scale have been passed to 68 mental health checkups for children in Grades 3 and 4, at Ho Chi Minh City Primary School, Vietnam. For better resolution, the problem, physical education intervention has been used for comparative research. Previous research indicates that Self-esteem and self-confidence of children in general are poor; Physical education interventions play an important role in improving their self-esteem and confidence. It suggests that the intervention of physical education should be applied more to the study of mental health of children with orphans. From a physical perspective. Educational institutions should focus more on motivating students. The ability to teach as well as encourage them to work in chartered schools. From another perspective, the need for professional training for physical education teachers in orphan schools The school popularizes science teaching methods so that the mental health of children can be improved effectively.

KEY WORDS: CHILDREN ORPHAN, PHYSICAL EDUCATION INTERVENTION, SELF-ESTEEM, SELF-CONFIDENCE.

INTRODUCTION

Self-esteem (SE) is considered integral to the self-concept, and can be defined in terms of positive feelings about the self. SE has become a household word. Teachers, parents, therapists, and others have focused efforts on boosting self-esteem, on the assumption that high self-esteem will cause many positive outcomes and benefits (Baumeister et al., 2003). It is integral to an individual's sense of their own value (Fox & Corbin, 1989), a principal component of mental health (Jambor & Elliott, 2005) a strong indicator of a healthy lifestyle (Smolenáková & Bendíková, 2017), and an important indicator of well-being (Shek & McEwen, 2012, Lyu et al, 2019). The Rosenberg Self-Esteem Scale (RSES) (Rosenberg, 1989) is the most widely used self-report measure of self-esteem and was designed to measure self-esteem as a one-dimensional construct (Dhingra, 2013). The factor structure of this scale, however, has been the subject of considerable debate (Corwyn, 2000). While numerous studies support this one-dimensional model (Shevlin, Bunting & Lewis, 1995), other research has found evidence of a range of multi-factorial solutions (Huang & Dong, 2012). A considerable number of researchers contend that the RSES is more appropriately conceptualized as a two-factor solution, comprised of positive and negative aspects of self-esteem (Kaufmann et al., 1991 Lyu et al 2019). Self-confidence is a person's belief in own ability to carry out life tasks, and relates to someone's competences and self-esteem (Vealey & Chase, 2008) defined self-efficacy as "beliefs in one's capabilities to organize and execute courses of action required to produce given attainments".

According to the theory of self-efficacy, sources of confidence include enactive mastery experiences, vicarious experience, verbal persuasion and physiological and affective states experiences (Bandura, 1997;

ARTICLE INFORMATION

*Corresponding Author: thuchus@gmail.com
Received 1st Jan 2020 Accepted after revision 25th Feb 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)
A Society of Science and Nature Publication, Bhopal India

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/18

Hays, Maynard, Thomas & Bandura, 2007). Feltz and Reissinger (1990) applied the self-efficacy theory and found that performance accomplishments was the most significant source of self-efficacy among athletes, followed by physiological states, verbal persuasion and vicarious experiences. The number of orphan children has increased greatly in Ho Chi Minh City - Vietnam, along with the dissemination of agricultural population policies, the complete expansion of the second child policy and the gradual improvement of the orphan policy school-age population. According to the latest statistics of the Ho Chi Minh City Education Commission, today, more than 8900 students attend an orphaned school run by the private sector.

In terms of related surveys, because the quality of private school education is worse than that of public schools, orphans, it is easy to encounter psychological problems due to poor quality teachers and terrible teaching environment as well as photographs potential effects of personality itself. Concerning crimes of orphan children, especially so social groups have sparked widespread public concern. Therefore, more and more scholars are transforming their research into children who are considered orphans, but only at the theoretical level instead of performing physical education interventions for pragmatic discovery. This study aims to find a way to intervene in physical education that affects the confidence and confidence of children who are considered to be able to develop a sound assessment and intervention program. Scientific side for reference.

MATERIAL AND METHODS

Based on the previous interview with head teachers and questionnaire accomplished by students of Grade 5, 30 students with less self-esteem and self-confidence in Class 3, Grade 5 were chosen as an experimental group imposed with physical education intervention while 38 students in Class 4, Grade 5 was taken as a control group taught by their former physical education teacher. In this study, the author used a research tool to request self-esteem and confidence assessment. Using SPSS 20.0 software to statistics research results were analyzed, (Dao Chanh Thuc, 2018).

RESULTS AND DISCUSSION

Self-esteem study: The self-esteem scale (SES) designed by Rosenberg and applied in this article has been widely used at home and abroad to measure the overall feeling of youth about village values body and self-acceptance, high reliability, validity and operability. And a total of 136 self-esteem scales with 68 for previous and 68 for the following test is distributed, while each test also distributes 30 for the experiment group and 38 for the corresponding control group, so it is collected with an effective rate of over 80%. According to Earl Babble, the questionnaire with a response rate of over 70% is very effective so that the results of this survey are valid.

Finally, the paper conducted analytical data with SPSS20.0 and obtained the following analysis results: According to Table 1, there were 19 people with extremely low self-esteem and 23 people with low self-esteem in the test group, accounting for 61.76% of the total before experiment. After the experiment, the number of people with extremely low self-esteem decreased and 12 people with low self-esteem dropped to15, accounting for 39.71% of the total in the group. Data analysis showed significant differences (P <0.05) that physical education intervention plays an important role in improving the self-esteem of these students. According to Table 2, there are 20 people with extremely low self-esteem and 22 people with low self-esteem in the control group, accounting for 61.76% of the total before intervention. After the intervention, the number of people with extremely low self-esteem still did not change much. Data analysis showed that there was no significant difference (P> 0.05), moreover, students in the control group had less improvement in self-esteem, compared with those in the test group.

According to Table 3, before the intervention, the experimental group had 19 people with extremely low confidence levels and 23 people with low confidence in the test group, accounting for 61.76% of the total. After the intervention, the number of people with extremely low confidence fell to 9, while those with low confidence fell to 11, accounting for 29.41% of the total in the group. Data analysis shows that significant differences (P <0.01) that intervention plays an important role in improving the confidence of these students. With the control group, before the intervention, there are 20 people with extremely low confidence and 24 people with low confidence in the control group, accounting for 64.71% of the total.

Table 1. Number of people n = 68 having a change in self-esteem in the experiment group after three months of physical education intervention

	10-15 points (very low)	16-25 points (low)	26-35 points (normal)	35-40 points (high)	P
Before experiment	19	23	14	12	
After experiment	12	15	24	17	<0.05*
*indicates the significant difference (P<0.05)					

After the intervention, the number of people with extremely low confidence levels dropped to 18, while those with low confidence levels dropped to 26, accounting for 64.71% of the total. Data analysis showed that there was no significant difference (P> 0.05) (Dao Chanh Thuc, 2018), moreover, that the students in the control group had poor performance in terms of confidence level compared to those in the control group. Teaching experiment: Program Design: Because teaching in the classroom is the main part of the physical education intervention program, the author first consulted with many experts and professors in designing the education program, substance of the Institute of Physical Education at the University of Sports in Ho Chi Minh City, Vietnam, then refered to the documents related to the psychological health of teenagers and children. Based on full theoretical support of self-esteem and confidence, the program not only focuses on basketball and karate, but also includes many interesting and challenging sub-projects, considering both benefits, physically and mentally for children to see. Details are shown in the following table (Table 4):

Experimental methods: Students with lower self-esteem and confidence are grouped together during practice or group games. Meanwhile, based on the principle of discrimination, challenging goals are set in Teaching at different levels of students Self-esteem and confidence. For example, when it comes to the chest of basketball, pay more attention to standards than accuracy as students have low self-esteem and confidence and then give a corresponding assessment of passing, allowing them to accurately identify their strengths and weaknesses (Lyu, et al 2019). Combined with the rule of principle teachers, students should be encouraged to actively participate in activities so that teachers can provide timely and full recognition.

Also, it is necessary to control their negative emotions caused by positive failures. Playing basketball, as an illustration, teachers will find its results to inspire students to create a record and compare distances before and after they learn about standard movement. At the end of this class, the discussion was introduced to class recollections and shared comments, especially for students with lower confidence and confidence. Therefore, what they conclude from reflection can be done to solve the problem of practical problems.

Result of experiment: Through distributing some challenging assignments which are modifiable in practice, teaching activities are not only suitable for students with lower self-esteem and self-confidence to increase self-awareness in a positive way, but also applicable for students with better self-recognition to achieve sense of joy and sense of accomplishment, from which both groups are able to obtain impetus for future improvement. With regard to students with less progress, it is necessary to help them draw lessons from the past experience to strengthen self-confidence. Once they have a conception of self-evaluation will it be possible to further stimulate their potential? In the review section, discussion amongst students and teachers contributes to a broader horizon and intensified capacity of expression. Testing extracurricular activities: Program design: Extracurricular activities are complementary to the entire teaching system, with autonomy, flexibility and reality. Details are presented as follows (Table 5):

Table 2. Number of people n = 68 having self-esteem changes in the control group after three months of normal intervention.

	10-15 points (very low)	16-25 points (low)	26-35 points (normal)	35-40 points (high)	P
Before experiment	20	22	16	10	
After experiment	19	23	17	9	>0.05*

Table 3. Number of people n = 68 with a change in confidence in the intervention experimental group and control group after a three-month intervention

	0-50 points	50-70 points	70-80 points	80-100 points	P
Experimental group					
Before experiment	19	23	15	11	
After experiment	09	11	31	17	<0.01*
Control group					
Before experiment	20	24	14	10	
After experiment	18	26	15	9	>0.05*

Table 4. Physical education intervention program

Time	Intervention project	Times per week	Time required	Intensity
1-3 week	Track and field	3	45-50 min	Medium
4-8 week	Basketball	3	45-50 min	Medium
9-12 week	Karatedo	3	45-50 min	Medium

Table 5. Extracurricular activities intervention program

Intervention time	Intervention project	Time required
The beginning of the first month	Quality development	45-50 min
The beginning of the second month	Inspirational storytelling	45-50 min
The beginning of the third month	See microfilms inspirational sports	45-50 min

Experimental process: The first intervention of extracurricular activity took place before the class officially started; Quality development activities are carried out on the topic of ice breaking. After a brief introduction, the teachers and students were grouped to play the "Meo duoi chuot" game, asking students to tear up the name tag stuck on each other's back in the order of the teacher. The three rounds of the game have led to a good relationship between teachers and students as well as better mutual understanding among students, creating a solid foundation for the intervention of the following physical education system. The second intervention of extracurricular activities was put into practice after a month of physical education intervention in class, to share inspirational stories of sports celebrities.

To begin with, students were asked about self-esteem and self-confidence, then shared stories about how sports celebrities win over difficulties and create an achievement. another great (Yu, 2019). They were not only asked to discuss stories, but were also given homework to read more relevant documents. Such activities are conducive to exemplary and influential examples for students with lower self-esteem and confidence to understand the relationship between success and self-assertion and enhance self-awareness, (Jun, 2017). The third extra-curricular activity is the appreciation of the movie "Hai Phuong".

The main character regained self-esteem and confidence underpinned great achievements after a long struggle in hard times, providing students with a motivation to improve themselves. Special instructions have been applied for students with extremely low self-esteem and confidence, and the results are remarkable. For example, opportunities have been provided to them as a stage for self-implementation. Some of them will be encouraged to play a team building role so they can exploit their potential and appreciate what they have. On the other hand, under its frustration caused by many failed experiences, mainly in-class instruction focused on positive instruction while reducing the task's difficulty to get the feeling of accomplishmen through successful experience and laying the foundation for positive self-assessment. After the intervention, the number of students with extremely low self-esteem and serious guilt declined by half, confirming the positive effect of physical intervention.

CONCLUSIONS

Children with orphans often have poor mental health. More than half of the students have shown abnormal levels of self-esteem and self-esteem, and even some of them have extremely low self-esteem and low self-esteem tends to deviate from average development. Therefore, it is necessary to have timely and reasonable interventions to improve. Physical education interventions have the benefit of improving self-esteem and confidence. After performing physical education interventions for three months, data show that some students increase confidence and significantly increase confidence in group experiments while there are no major differences occurring before and after the experiment in the control group. Moreover, after the intervention, students in the experimental group became more active and active in class, especially when there was a significant improvement in students in harsh conditions, thus improving the whole atmosphere, manifesting that physical education intervention has a positive impact on improving the effectiveness of self-esteem and confidence of students in the experimental group.

The effect of physical education applied in this study has been shown to be effective in improving the confidence and self-esteem of children who are considered, so it is hoped that the system will beat The price of confidence and confidence and the sports intervention program set up in this study can be applied by many children who consider schools in society to help them improve their mental health better. In addition, physical education interventions need to be strengthened among students from vulnerable groups, including children with visual impairments, HIV-infected children and children with hearing loss. And should also make corresponding changes based on the physical education intervention program to better explore the impact of their mental health intervention. According to the shortage of physical education teachers at orphanage schools, as well as the lack of long-term curriculum and the orientation of the students in these schools, it is important that colleges and the University of physical education must strengthen the combination of physical education of psychological theory of adolescents.

Through establishing cooperative relationships with migrant children's schools, universities play an active role to encourage students to practice, which not only improves student practice but also contributes part to strengthen psychological health conditions

and complete the construction of physical education systems. Furthermore, colleges and universities of physical education can also provide appropriate training for teachers at orphanage schools to improve teaching capacity.

REFERENCES

Bandura, A. (1997). Self-efficacy: The Exercise of Control. New York: W.H. Freeman.

Baumeister, R.F., Campbell, J.D., Krueger, J.I. & Vohs, K.D. (2003). Does high selfesteem cause better performance, interpersonal success, happiness, or healthier lifestyles? Psychological Science in the Public Interest, 4(1), 1-44.

Corwyn, R. F. (2000). The factor structure of global self-esteem among adolescents and adults. Journal of Research in Personality, 34(4), 357–379.

Dao Chanh Thuc, (2018). Measurement in sports. LAP LAMBERT Academic Publishing, ISBN 978-613-9-90198-2.

Dhingra K. (2013). Validity and Composite Reliability of the Rosenberg Self-Esteem Scale among U.K. University Students. Journal of Humanistics & Social Sciences, 2(1), 24-30.

Feltz, D. L., & Riessinger, C. A. (1990). Effects of in vivo emotive imagery and performance feedback on selfefficacy and muscular endurance. Journal of Sport and Exercise Psychology, 12, 132 – 143.

Fox, K.R. & Corbin, C.B. (1989). The physical self-perception profile: Development and preliminary validation. Journal of Sport and Exercise Psychology, 11(4), 408–430.

Hays, K., Maynard, I., Thomas, O., & Bandura, M. (2007). Sources and types of confidence identified by world class sport performers. Journal of Applied Sport Psychology, 19 (4), 434-456.

Huang, C. & Dong, N. (2012). Factor structures of the Rosenberg Self-Esteem Scale: A meta- analysis of pattern matrices. European Journal of Psychological Assessment, 28(2), 132-138.

Jambor, E. & Elliott, M. (2005). Self-esteem and coping strategies among deaf students. Journal of Deaf Studies and Deaf Education, 10(1), 63–81.

Jun, J., (2017). Research on the present situation of college students' sense of inferiority and its intervention easures in physical exercise. J Anyang Teach College, 5, pp. 120-124.

Kaufman, P., Rasinski, K.A., Lee, R. & West, J. (1991). National Education Longitudinal Study of 1988. Quality of the responses of eighth-grade students in NELS88. Washington, DC: U.S. Department of Education.

Lyu H, Gang Du, and Kimberly Rios (2019) The Relationship Between Future Time Perspective and Self-Esteem: A Cross-Cultural Study of Chinese and American College Students Front Psychol. 2019; 10: 1518.

Rosenberg, M. (1989). Society and the adolescent self-image. (Rev. Ed.). Middeltown, CT: Wesleyan University Press.

Shek, Y.L. & McEwen, M.K. (2012). The relationships of racial identity and gender role conflict to self-esteem of Asian American undergraduate men. Journal of College Student Development, 53(5), 703– 718.

Shevlin, M., Bunting, B.P. & Lewis, C.A. (1995). Confirmatory Factor Analysis of The Rosenberg Self-Esteem Scale. Psychological Reports, 76(3), 707-710.

Smoleáková, N. & Bendíková E. (2017). Effect of the Content Standard for Changing the Level of Knowledge of Secondary School Students. Journal of Physical Education and Sport, 17(1), 182 – 187.

Vealey, R. S., & Chase, M. A. (2008). Self-confidence in sport: Conceptual and research advances. In Horn T. S. (Ed), Advances in Sport Psychology (3rd ed.) (pp. 65 – 97). Champaign, IL: Human Kinetics.

BIOMEDICAL
Communication

BBRC
Bioscience Biotechnology
Research Communications

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp 106-112

Incorporating Etoposide into PUFA-rich Oils Nanoemulsion Potentiates its Inhibitory Effect on the Cellular Growth of A549 Non-Small Cell Lung Cancer Cells

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ABSTRACT

Incorporation of a chemotherapeutic drug into natural oils based-nanoemulsion may facilitate its delivery to the cancer cells and improve its cytotoxic effect. The current study is aimed to formulate etoposide (EP) in a nanoemulsion formula (BLO-EVO-N) produced by blending black currant seed (BLO) and evening primrose (EVO) oils with an emulsifying agent and to in vitro assess its anti-cancer activity on the A549. The size and charge of the produced formulas, BLO-EVO-N and EP/BLO-EVO-N, were characterized using the dynamic light scattering techniques. The viability and invasion of the treated A549 cells were assessed using a cell counting kit- 8 and collagen-based cell invasion assay. Apoptosis of A549 cells was evaluated with mitochondrial staining, annexin V- fluorescein isothiocyanate (FITC), cell death assays. The z-average diameter of EP/BLO-EVO-N (75.19 ± 9.0 nm) was increased when loaded with EP (236.9 ± 6.8 nm). Most of the A549 cells remained viable following treatment with a single EP, while a remarkable reduction in the percentage of the viable cells was noticed at the EP/BLO-EVO-N treatment. The signs of apoptosis, such as the change in the mitochondrial permeability, increased amount of apoptotic cells and enhancement in the mono- and oligonucleosomes ratios were larger when cells were treated with EP/BLO-EVO-N formula. Regarding the invasion assay, the EP/BLO-EVO-N has a potent anti-invasion effect since it can suppress the invasion of 66% of the A549 cells. In conclusion, combining EP in BLO-EVO-N formula had improved the anti-tumor effect of EP in A549 cells.

KEY WORDS: ANTI-CANCER ACTIVITY; APOPTOSIS; CELL VIABILITY; CHEMOTHERAPEUTICS; MITOCHONDRIAL PERMEABILITY; POLYUNSATURATED FATTY ACIDS.

INTRODUCTION

Despite the extensive developments in cancer therapy, lung cancer remains the foremost cause of cancer-related deaths in both males and females (Yin et al., 2017; Bray et al., 2018). Moreover, most of the lung cancer patients ~80% are diagnosed with non-small cell lung cancer (NSCLC). The low survival rate after treatments of NSCLC patients could be attributable to the late diagnosis and the cancer metastasis. Therefore, handling of NSCLC metastasis is one of the most crucial ways for effective lung cancer therapies (Townsend et al., 2017; Altorki et al., 2019). Despite the extensive uses of chemotherapeutics agents, the undesirable side effects, drug resistance, short biological half-life, low permeability, and poor solubility are the main causes leading to failure delivery of the drug to the cancer cell Therefore, colloidal delivery systems were proposed to overcome the main hurdle related to the conventional chemotherapeutic agent and to enhance its efficacy (Reddy et al., 2006; Pandey, 2020).

ARTICLE INFORMATION

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Received 17th Jan 2020 Accepted after revision 25th March 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)

*Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/19*

The natural oils of BLO and EVO are a rich source of polyunsaturated fatty acids (PUFAs) that have a potent anti-cancer activity (Sayegh et al., 2016; Timoszuk et al., 2018). The possible mechanisms for PUFAs apoptotic effect in cancer cells are owing to the disruption of the cell cycle progression, stimulation of the cellular oxidative stress, and alteration of the membrane fluidity which might facilitate the permeation of the anti-cancer drugs to the target cell (Menendez et al., 2001; Xu and Qian, 2014; Zajdel et al., 2015). Nanoemulsions (NEs) are one of the most effective dispersed nanosystems that are developed to improve the delivery of therapeutic agents. The NEs offer several advantages in the therapeutic field, such as enhanced bioavailability, physical stability, and solubility of the lipophilic drug (Jaiswal et al., 2015; Ting et al., 2015; Khan et al., 2018; Halnor et al., 2018). In the present study, a novel EP-loaded NE formula based on mixing BLO and EVO oils was produced to potentiate the efficacy of EP. The antitumor activity of the produced formula was in vitro evaluated in the A549 cells.

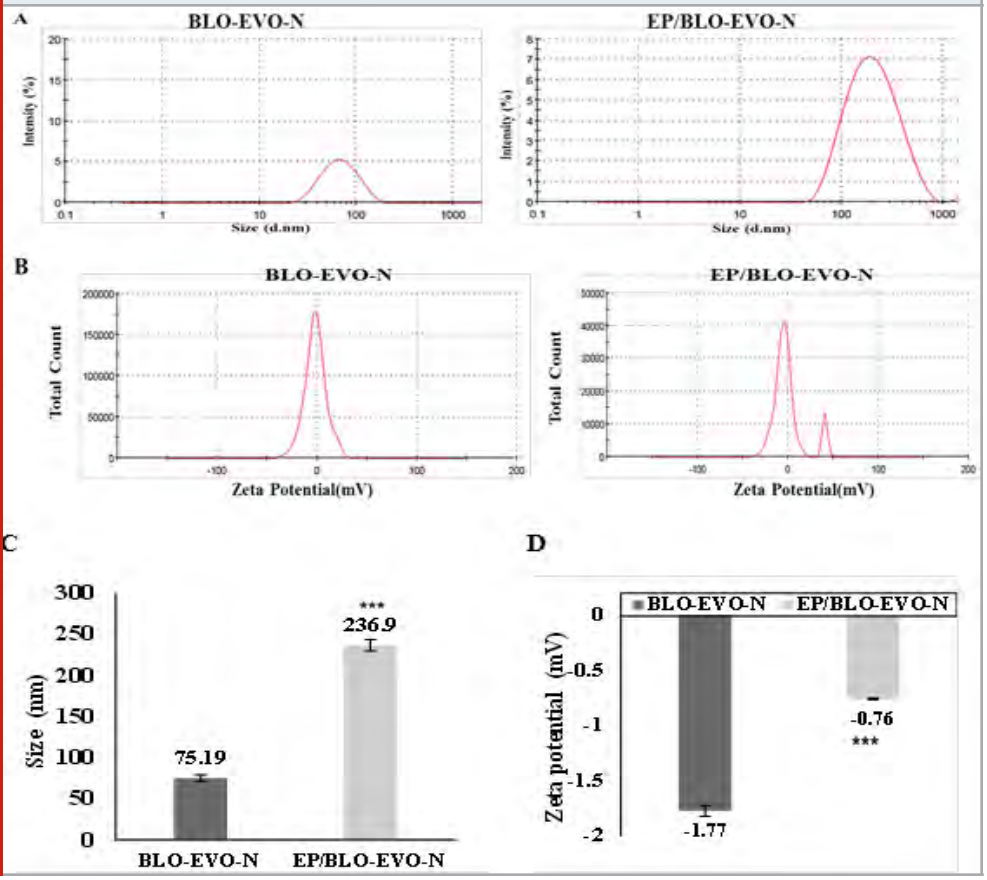
MATERIALS AND METHODS

Materials: EP was obtained from Ebewe Pharma GmbH (Unterach, Austria). Tween 80 and span 20 were purchased from Al-Rowad Modern Est. for the Medical Supply. The natural oils (EVO and BLO) were acquired from Chateau Cosmetics Botanical Beauty (Miami, Florida US). Cell Counting Kit- 8 (CCK-8) (Lot. No. LE612) was acquired from Dojindo Molecular Technologies, Inc. (Japan). Mitochondria Staining Kit (Cat. No. CS0390) was purchased from Sigma Aldrich.

Preparation of BLO–EVO–N formulas: The formula (BLO-EVO-N) was produced by the ultrasonication method. A volume fraction of 2 % of each BLO and EVO were combined with 86 % of buffer (50 mM, pH7) and then stabilized by the addition of 3 % Span 20 and 7 % Tween 80. The mixture was sonicated for 30 min using the Omni Sonic Ruptor 4000 Ultrasonic Homogenizer (New York, US) until it becomes clear. The EP-loaded formulations (EP/BLO-EVO-N) were prepared by directly solubilizing the EP in the BLO-EVO-N formula.

Physical measurements of formulation: The zeta potentials, z-average diameters, and polydispersity index (PDI) for the produced formulas were measured using zetasizer (Malvern Instruments Ltd., UK).

Figure 1. A Particle size distributions of BLO-EVO-N and EP/BLO-EVO-N, measured using zetasizer. *B Zeta* potential spectrum of BLO-EVP-N and EP/BLO-EVO-N. C & D The graphs displayed the amount of z-average diameters and zeta potential for the produced formula, respectively. Data were expressed as mean ± SD (n =3). *Error bars* represent the standard deviation. The P-values were assessed using the independent t-tests. The (***, P < 0.001) represents the very highly significant difference between the variables.



In vitro drug release: The in vitro release of EP and EP/BLO-EVO-N was assessed using the dialysis technique. 1 mL of the EP and EP/BLO-EVO-N were relocated into the dialysis bag separately (Cut-off 3.5 Da, Spectra Lab, California) submerged into 200 mL buffer (50 mM, pH 7.4) and stirred at 100 rpm. Almost 1.0 mL of the buffer was taken at a predetermined interval and replaced by fresh buffer (1.0 mL). The optical density of the removed buffer at a specific time (A1) was measured by using a UV-Vis spectrophotometer (Thermo Scientific™, US). The % of EP released was estimated through dividing A1 by A0 (absorbance of the initial sample added to the dialysis bag) and multiplying by 100.

Cell culture: Non-small cell lung cancer cell line (A549) was generously provided by King Abdulaziz University Hospital (Jeddah, KSA). Approximately 2×106 A549 cells were plated in a culture flask (25 cm2) containing 5 mL of Dulbecco's modified eagle's medium (DMEM), complemented with 10 % (v/v) fetal bovine serum (FBS), 1 % (v/v) penicillin/streptomycin and incubated at 37oC in a 5 % CO2 humidified atmosphere.

Cell counting kit-8 assay: A549 cells (1×104/well) were cultured into 96-well plates at which each well-contained 100 µL of DMEM followed by incubation at 37oC for 24 h in a CO2 incubator. Then, 200 µL of tested formula, EP, BLO-EVO-N, and EP/BLO-EVO-N were added. The cellular viability was evaluated by adding 5 µL of CCK-8 reagent to each well, mixed gently, and incubated for 3 h at culture conditions. After that, the absorbance (A) of treated cells, control cells, and media (blank) were measured in a BioTek Microplate Reader (λ = 450 nm). The cellular viability (% CV) was calculated by subsequent calculation: % CV = ((A of treated cells –A of blank) / (A of control)) *100

Cell morphology characterization: Cells (1×104/well) were cultured and treated into 96-well plates at the same procedure done in the CCK-8 experiment. Following treatments, the DMEM was removed, cells were rinsed with 100µL phosphate buffer saline (0.5mM, PBS), fixed by the addition of formaldehyde (200 µL, 4 %), and stained with100 µL of 5 % Coomassie Brilliant Blue dye for 10 min. Finally, cells were cleaned with tap water followed by drying overnight at 25oC. The morphological alteration of treated cells was detected using a phase-contrast inverted microscope (Olympus, Japan).

DAPI Staining: In a 24 well-plate, A549 cells (5×104/well) were seeded in each well-containing 0.5 mL of growth media. Then, 0.5 mL of the IC50 for each BLO-EVO-N and EP/BLO-EVO-N was added to each well. After that, cells were rinsed with 300 µL PBS (50 mM, pH7.4), fixed via 4% formaldehyde, stained for 2 min with DAPI stain (300 µL, 300 nM) and visualized using fluorescent microscope at λ = 437nm (Leica CRT6000, Germany).

Mitochondrial permeability assay: The 1×104 of A549 cells were grown in 100 µL of DMEM in each well of 96 well-plates. Then, cells were incubated with 200 µL of IC50 for BLO-EVO-N and EP/BLO-EVO-N for 24 h at 37oC. After that, cells were stained with a cytofluorimetric, cationic dye, JC-1 stain solution (JC-1 stain, JC-1buffer solution, and DMEM) and incubated at 37 oC. Finally, the fluorescence was examined at excitation (Ex): 525 nm / emission (Em): 590 for red fluorescence (depolarization) and at Ex: 490nm / Em: 530 for green fluorescence (hyperpolarization) using fluorescence microplate reader (Synergy™ HTX, BioTek, US).

Annexin V-FITC/PI assay: A549 cells apoptosis was assessed using Annexin V-FITC Apoptosis Detection Kit (Cat. No. MBS668896, MyBioSource, San Diego, USA). Briefly, cells (3×10^5/well) were planted into 6 well- plates contained 2 mL of DMEM and incubated at 37°C. Then, 2 mL of IC50 for the various formulation was added. After that, cells were harvested, rinsed twice with pre-cold PBS, spun down (300 g), re-suspended in 1X binding buffer (100 µL). Then, 5 µL of each FITC and PI added, incubated for 20 min, and completed with 400 µL of binding buffer. Finally, cells estimated within one hour using flow cytometer (FACS AriaTM III, BD Biosciences, CA, US).

Nuclear fragmentation assay: The DNA fragmentation ratio was examined by applying a Cell Death Detection ELISAplus kit (Lot. No. 19315700, Mannheim, Germany). Briefly, cells (1×104/well) were cultured into 96 well-plates contained 100 µL of growth medium. Then, 0.2 mL of IC50 of tested formulas were added. After that, 20 µL of cell lysates were relocated into the streptavidin-coated microplate, supplemented with 80µL of immunoreagent and incubated on a shaker (300 rpm, 2 h) at the dark. Then, the ABTS stain was add and incubated on a plate shaker (10 min). The optical density (A) was determined (405 nm) and the DNA fragmentation ratio was estimated according to the following equation:

DNA fragmentation ratio = (A of treated (dead cells)/ A of control (viable cells)).

Cell invasion assay: The ability of A549 cells to invade the collagen membrane was evaluated by utilizing Chemicon collagen-based cell invasion assay kit (Cat. No. ECM551, Merck KGaA, Darmstadt, Germany). Briefly, Cells (3×103/well) were cultured and treated into six-well plates similar to the procedure done in the Annexin V-FITC/PI assay. After treatments, the insert was incubated with serum-free medium (300 µL, 30 min), and then 250 µL of cell suspension was added. The insert was incubated at 500 µL of complete medium for 24 h in a CO2 incubator. After that, the insert was stained, gently cleaned, and then incubated with the extraction buffer (200 µL, 15 min). The extraction solution (100 µL) was shifted to a 96 well-plate for colorimetric measurements (560 nm). The % of A549 cell invasion was measured by the subsequent equation:

% Relative Invasion = (A of treatment / A of control) *100

Statistical Analysis: The variations between groups were assessed through one-way ANOVA. The significant

differences between the two groups were estimated by the independent sample t-test ($P < 0.05$). Values were presented as mean ± SD and done in triplicate. All statistical calculations were carried out using MegaStat Excel Software (Butler University, Indianapolis).

RESULTS AND DISCUSSION

Physical characterization of formulations: As exhibited in Figure 1 (A and C), the dispersed nanodroplets of EP-loaded BLO-EVO-N had noticeably increased when compared to the ones of free BLO-EVO-N ($P < 0.001$). The greater size of EP/BLO-EVO-N might indicate that EP was located inside the core of the BLO-EVO-N micelles. It has been recently demonstrated that the droplet size of sorafenib merging in a carrot-NE was larger than free carrot-NE (Alkhatib et al., 2018). Additionally, the polydispersity indexes (PDI) for both formulations, BLO-EVO-N and EP/BLO-EVO-N, which were calculated by dividing the SD over the average size, were smaller than 0.08, indicating a monodisperse size distribution. According to Figure 1 (B and D), the negative charge magnitudes of BLO-EVO-N was considerably larger than EP/BLO-EVO-N ($P < 0.001$).

In vitro drug release: As demonstrated in Figure 2, the EP-loaded BLO-EVO-N showed a faster release than the native EP solution. Approximately, half of the EP/BLO-EVO-N was released at the third hour compared to the 38% of the released EP solution. The rapid release of EP/BLO-EVO-N than free EP could be ascribed to the negative charge and monodisperse size distribution of the EP/BLO-EVO-N formula. After 24 h, nearly 85 % of EP/BLO-EVO-N was released, while 50% of the EP solution was released.

Cell viability assay: The % of viable A549 cells following incubation with formulations at different concentrations (0.7, 0.5, 0.3, and 0.1 µM) for 24 h was displayed in Figure 3A. Most of the A549 cells remain viable after treatment with the free drug formula (EP). In contrast, a remarkable decrease in the % of viable A549 cells was detected following treatment with BLO-EVO-N and EP/BLO-EVO-N. Furthermore, the graph showed a steady decline in the % of viable A549 cells once the concentration of drug formulas increased. Regarding half-maximal inhibitory concentration (IC50) of the produced formula, the EP/BLO-EVO-N (0.2 ± 0.03 µM) has a considerably less IC50 than that of BC/EP-NE (0.4 ± 0.01 µM) ($P < 0.001$). Interestingly, the incorporating of EP in the NE based on EVO and BLO might facilitate its delivery and enhanced its antineoplastic effects on A549 cells. In agreement with our findings, several previous studies have pointed out that using an essential oil or synthetic drug in a nanoemulsion form exhibited a strong cytotoxic effect on various cancer cells (Farshi, et al., 2017; Milhomem-Paixão et al., 2017; Pereira et al., 2017; Alkhatib et al., 2018; Alkhatib et al., 2019). According to the light microscopy images presented in Figure 3B, the treated A549 cells showed a reduction in cell count, loss of cell membrane, irregular cell shape and membrane blubbing compared to the untreated cells (control).

Apoptosis detection: The apoptosis of A549 cells treated with BLO-EVO-N and EP/BLO-EVO-N was evaluated using different assays, mitochondrial permeability, Annexin V-FITC flow cytometry, cell death, light, and fluorescent microscopy. The failure in the electrochemical gradient of the mitochondrial membrane (lowering red/green fluorescence intensity ratio) was detected when A549 cells were treated with various formulations

Figure 2: In vitro release profiles of free EP and EP/BLO-EVO-N in phosphate buffer (50 mM, pH7.4) using dialysis technique.

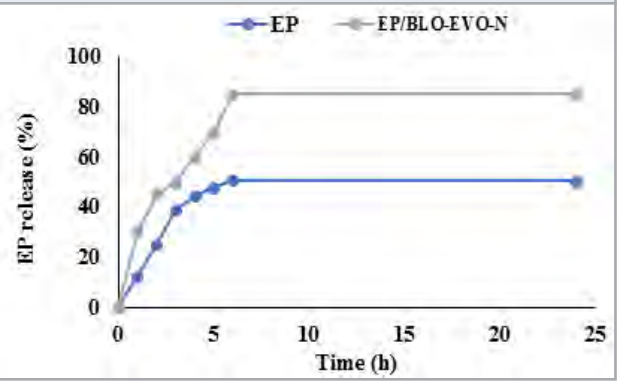
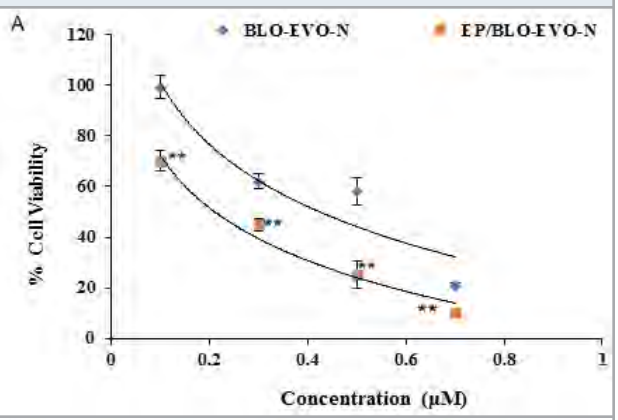
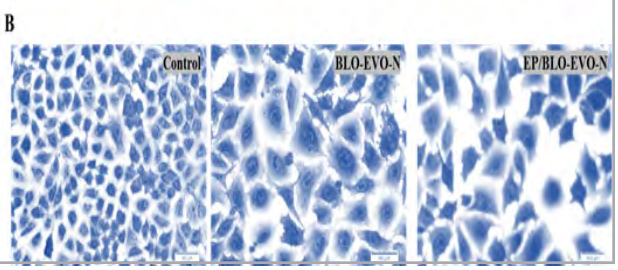


Figure 3: A Cytotoxicity effect of various concentrations of the tested formulas on the A549 cells incubated for 24h at 37°C determined by the CCK-8 assay. Data were presented as mean ± SD (n = 3). *Error bars* display the ± SD. The P-values were assessed using the independent t-tests. The highly significant differences between formulas were represented as (**, $0.001 \le P < 0.01$). B Light microscopy images of the control (untreated) and treated A549 cells stained with Coomassie blue. Images were magnified at 40 X.





relative to the untreated cells (P < 0.001). Moreover, the loss of the A549 cells mitochondrial gradient (lowering red/green fluorescence intensity ratio) was higher with the EP/BLO-EVO-N treatment (Figure 4) (P < 0.001). Additionally, the flow cytometry plots (Figure 5A), displayed that cells treated with the formulations, BLO-EVO-N and EP/BLO-EVO-N, were distinguished to the different stages of apoptosis. The highest amount of early apoptotic cells was noticed when cells were treated with BLO-EVO-N (Figure 5B). In contrast, the amount of late apoptotic cells was considerably increased upon the EP/BLO-EVO-N treatment (P < 0.001).

The results of the flow cytometry analysis were confirmed with the cell death experiment that indicates the increased ratio of the mono- and oligonucleosomes of the apoptotic A549 cells upon the EP/BLO-EVO-N treatment (Figure 6A). The nuclear fragmentation ratios were increased to 2.2 ± 0.05 when A549 cells were treated with EP/BLO-EVO-N (P < 0.001). According to the fluorescence microscopy images exhibited in Figure 6B, the changes of the A549 nuclear morphology was more considerable in the treated A549 cells. The active component of PUFAs such as arachidonic acid (AA, ω-6), gamma-linolenic acid (GLA, ω-6), eicosapentaenoic acid (EPA, ω-3), and docosahexaenoic acid (DHA, ω-3) have a selectively tumoricidal effect on cancer cells (Dai et al., 2013). Zajdel et al. (2015) have found that ω-3 PUFAs specifically, EPA and DHA possess many cytotoxic effects on the A549 cells such as inhibition of cellular growth, enhanced cell death, stimulated activation of caspase-3/7, and potentiated intracellular oxidative DNA.

Likewise, a previous study of Dai et al. (2013) emphasized that PUFAs can induce apoptosis in the gastric carcinoma cells by lipid peroxidation process. The valuable effects of EVO have been confirmed in the treatments of many diseases such as atopic dermatitis, psoriasis, asthma, and anti-cancer therapy (Timoszuk et al., 2018). Therefore, combining EP in the NE containing PUFAs could enhance

Figure 4: Mitochondrial permeability change of A549 cells detected by measuring the red/green fluorescence intensity ratio using JC-1stain. Data were expressed as mean ± SD (n = 3). *Error bars* display the ± SD. The independent t-tests evaluated the P-values. The (***) indicates the very highly significant differences between formulas (P < 0.001).

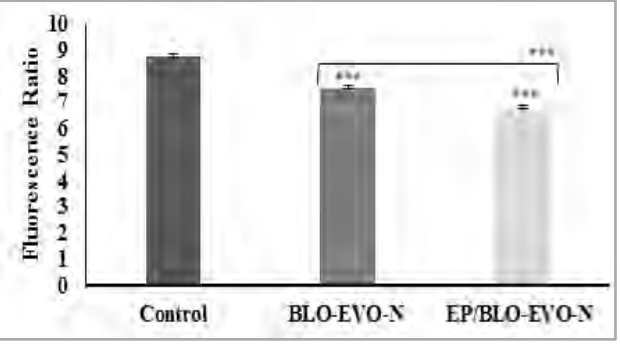


Figure 5: A FITC/PI flow cytometry plots of A549 cells subjected to the formulation. Cells were treated with the IC50 of BLO-EVO-N and EP/BLO-EVO-N for 24 h. Cells were categorized as necrotic (Q1), late apoptotic (Q2), viable (Q3), and early apoptotic cells (Q4). B Bar chart reveals the percentages of A549 cells undergoing apoptosis. Data were expressed as mean ± SD (n = 3). Error bars display the ± SD. The independent t-tests assessed the P-values. The (***) referred to the very highly significant differences (P < 0.001).

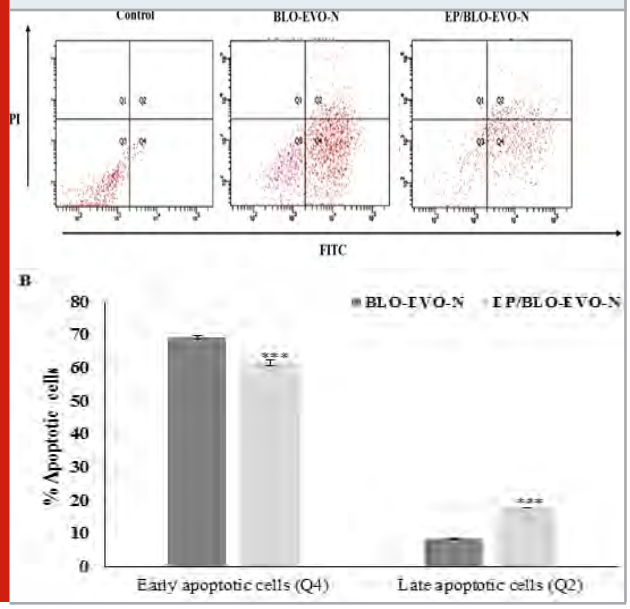
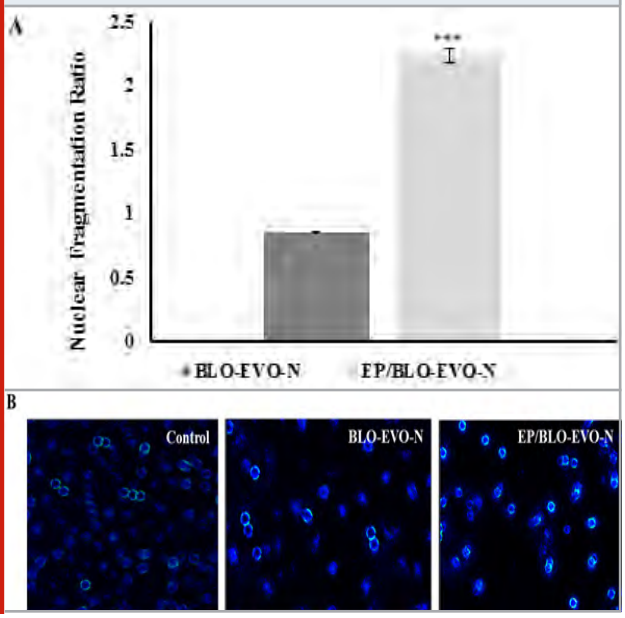
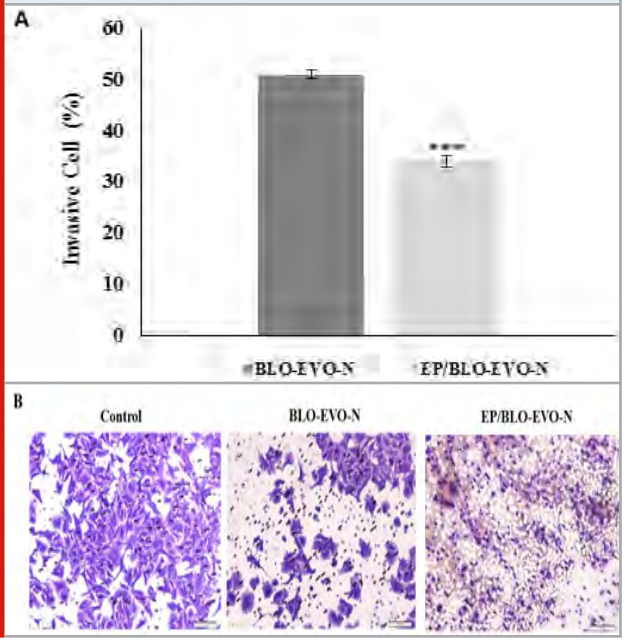


Figure 6: A Nuclear fragmentation ratio in the cytoplasm of the apoptotic A549 cells following treatments with the IC50 of BLO-EVO-N and EP/BLO-EVO-N for 24 h. *Error bars* display the ± SD. The P-values were calculated using the independent t-tests. The (***) described the very highly significant differences between BLO-EVO-N and EP/BLO-EVO-N (P < 0.001). B Nuclear changes of the treated A549 cells stained with DAPI and observed using fluorescence microscopy. Images were magnified at 20X.



the delivery of EP to the target site and improve the induction of A549 cells apoptosis.

Figure 7: A The percentages of invasive A549 cells after treatments with the IC50 of BLO-EVO-N and EP/BLO-EVO-N for 24 h. Data were expressed as mean ± SD. *Error bars* represent the ± SD. The P-values were measured by the independent t-tests. The (***) referred to very highly significant differences (P < 0.001). B Images of the invasive A549 cells following treatments with the formulation using light microscopy (Magnification 20X).



A549 cell invasion: The effect of BLO-EVO-N and EP/BLO-EVO-N in repressing the invasion of A549 cells was displayed in Figure 7 (A and B). The invasion of 49 % of A549 cells was repressed when cells were treated with free BLO-EVO-N formula relative to the untreated cell (control). Upon treatments with EP/BLO-EVO-N, the A549 cells showed a noticeable decrease in the invasive rate than BLO-EVO-N (P < 0.001). Increased susceptibility of the tumor cells to apoptosis, diminished angiogenesis and suppressed metastasis are some of the various signaling pathways of n-3 PUFA (Gorjao et al., 2019).

The DHA and EPA, as a ω-3 PUFAs, are vital for the treatments and prevention of metastatic lung cancer (Yin et al., 2017). In a previous study, Zhang et al. (2012) have reported that PUFAs, DHA and EPA, have an impact on breast cancer proliferation, differentiation, and prognosis. In the present study, the BLO-EVO-N and EP/BLO-EVO-N could inhibit the invasion of A549 cells by 49% and 66%, respectively. The obvious decrease in the percentage of invaded A549 cells at the EP/BLO-EVO-N treatments compared to the BLO-EVO-N might be attributed to the combined effect of EP and the active metabolites of the natural oils, BLO and EVO.

CONCLUSION

The free-EP did not induce any noticeable inhibitory effect on the A549 cellular growth. However, incorporating EP in a mixed natural oils based-nanoemulsion (EP/BLO-EVO-N) had significantly reduced the A549 cellular growth and invasion. The new formula EP/BLO-EVO-N is a promising antitumor agent that has beneficial properties owing to the inclusion of oils that are rich with the PUFAs. Given that, EP/BLO-EVO-N needs to be further investigated in animal models.

REFERENCES

Alkhatib, M. H., Al-Otaibi, W. A., & Wali, A. N. (2018). Antineoplastic activity of mitomycin C formulated in nanoemulsions-based essential oils on HeLa cervical cancer cells. Chemico-Biological Interactions, 291, 72-80.

Alkhatib, M. H., AlMotwaa, S. M., & Alkreathy, H. M. (2019). Incorporation of ifosfamide into various essential oils-based nanoemulsions ameliorates its apoptotic effect in the cancers cells. Scientific Reports, 9(1), 695-703.

Alkhatib, M. H., Alnahdi, N. S., & Backer, W. S. (2018). Antitumor activity, hematoxicity and hepatotoxicity of sorafenib in a nanoemulsion based on the carrot seed oil. International Journal of Life Science and Pharma Research, 8, 50-57.

Altorki, N. K., Geoffrey, J. M., Dingcheng, G., Jeffrey, L. P., Ashish, S., Brendon, S., Timothy, M., and Vivek, M. (2019). 'The lung microenvironment: an important regulator of tumour growth and metastasis', Nature Reviews Cancer, 19, 9-31.

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., & Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: A Cancer Journal for Clinicians, 68(6), 394-424.

Dai, J., Shen, J., Pan, W., Shen, S., & Das, U. N. (2013). Effects of polyunsaturated fatty acids on the growth of gastric cancer cells in vitro. Lipids in Health and Disease, 12(1), 71-85.

Farshi, P., Tabibiazar, M., Ghorbani, M., & Hamishehkar, H. (2017). Evaluation of antioxidant activity and cytotoxicity of cumin seed oil nanoemulsion stabilized by sodium caseinate-guar gum. Pharmaceutical Sciences, 23(4), 293-300.

Gorjao, R., dos Santos, C. M. M., Serdan, T. D. A., Diniz, V. L. S., Alba-Loureiro, T. C., Cury-Boaventura, M. F., Hatanaka, E., Levada-Pires, A.C., Sato, F.T., Pithon-Curi, T.C., Fernandes, L.C., Curi, R., Hirabara, S.M. (2019). New insights on the regulation of cancer cachexia by N-3 polyunsaturated fatty acids. Pharmacology & Therapeutics, 196, 117-134.

Halnor, V.V., Pande, V.V., Borawake, D.D., and Nagare, H.S. (2018). 'Nanoemulsion: A novel platform for

drug delivery system', Journal of Materials Science & Nanotechnology, 6, 104-114.

Jaiswal, M., Dudhe, R., & Sharma, P. (2015). Nanoemulsion: an advanced mode of drug delivery system. 3 Biotech, 5(2), 123-127.

Khan, I., Bahuguna, A., Kumar, P., Bajpai, V. K., & Kang, S. C. (2018). In vitro and in vivo antitumor potential of carvacrol nanoemulsion against human lung adenocarcinoma A549 cells via mitochondrial mediated apoptosis. Scientific Reports, 8(1), 144-158.

Menendez, J., del Mar Barbacid, M., Montero, S., Sevilla, E., Escrich, E., Solanas, M., Colomer, R. (2001). Effects of gamma-linolenic acid and oleic acid on paclitaxel cytotoxicity in human breast cancer cells. European Journal of Cancer, 37(3), 402-413.

Milhomem-Paixão, S. S. R., Fascineli, M. L., Muehlmann, L. A., Melo, K. M., Salgado, H. L. C., Joanitti, G. A., Grisolia, C. K. (2017). Andiroba oil (Carapa guianensis Aublet) nanoemulsions: development and assessment of cytotoxicity, genotoxicity, and hematotoxicity. Journal of Nanomaterials, 2017,e362046.

Pandey and Abhishek. (2020). Solid Lipid Nanoparticles: A Multidimensional Drug Delivery System.' in, Nanoscience in Medicine Vol. 1 (Springer).

Pereira, F. G., Marquete, R., Oliveira-Cruz, L., Quintanilha-Falcão, D., Mansur, E., & de Lima Moreira, D. (2017). Cytotoxic effects of the essential oil from leaves of Casearia sylvestris Sw. (Salicaceae) and its nanoemulsion on A549 tumor cell line. Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas, 16(5), 506-512.

Reddy, L. H., Adhikari, J. S., Dwarakanath, B. S. R., Sharma, R. K., & Murthy, R. R. (2006). Tumoricidal effects of etoposide incorporated into solid lipid nanoparticles after intraperitoneal administration in Dalton's lymphoma bearing mice. The AAPS Journal, 8(2), E254-E262.

Sayegh, F., Elazzazy, A., Bellou, S., Moustogianni, A., Elkady, A. I., Baeshen, M. N., & Aggelis, G. (2016). Production of polyunsaturated single cell oils possessing antimicrobial and anticancer properties. Annals of Microbiology, 66(3), 937-948.

Timoszuk, M., Bielawska, K., & Skrzydlewska, E. (2018). Evening Primrose (Oenothera biennis) Biological Activity Dependent on Chemical Composition. Antioxidants, 7(8), 108-118.

Ting, Y., Chiou, Y.-S., Pan, M.-H., Ho, C.-T., & Huang, Q. (2015). In vitro and in vivo anti-cancer activity of tangeretin against colorectal cancer was enhanced by emulsion-based delivery system. Journal of Functional Foods, 15, 264-273.

Townsend, M. H., Anderson, M. D., Weagel, E. G., Velazquez, E. J., Weber, K. S., Robison, R. A., & O'Neill, K. L. (2017). Non-small-cell lung cancer cell lines A549 and NCI-H460 express hypoxanthine guanine phosphoribosyltransferase on the plasma membrane. OncoTargets and Therapy, 10, 1921-1932.

Xu, Y., & Qian, S., Y. (2014). Anti-cancer activities of ω6 polyunsaturated fatty acids. Biomedical Journal, 37(3), 112-119.

Yin, Y., Sui, C., Meng, F., Ma, P., & Jiang, Y. (2017). The omega-3 polyunsaturated fatty acid docosahexaenoic acid inhibits proliferation and progression of non-small cell lung cancer cells through the reactive oxygen species-mediated inactivation of the PI3K/Akt pathway. Lipids in Health and Disease, 16(1), 87-95.

Zajdel, A., Wilczok, A., & Tarkowski, M. (2015). Toxic effects of n-3 polyunsaturated fatty acids in human lung A549 cells. Toxicology in Vitro, 30(1), 486-491.

Zhang, H., Zhou, L., Shi, W., Song, N., Yu, K., & Gu, Y. (2012). A mechanism underlying the effects of polyunsaturated fatty acids on breast cancer. International Journal of Molecular Medicine, 30(3), 487-494.

Biotechnological Communication

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp-113-118

In Vitro Activity of Selected Medicinal Plant Extracts Against *Mycobacterium tuberculosis* and other Non Mycobacterial Pathogens

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ABSTRACT

Use of medicinal plants for the treatment of infectious and life style diseases is since time immemorial. This study reports the in vitro antibacterial activity of selected medicinal plant extracts against *Mycobacterium tuberculosis* H37Rv and other non-mycobacterial pathogens. Fruits of *S. torvum, Z. mauritiana* and leaves of *V.negundo* were sequentially extracted using n-hexane, ethyl acetate and methanol. All n-hexane, ethyl acetate and methanol extracts were screened against clinical pathogens viz., *S. aureus* ATCC 29213, *P. aeruginosa* ATCC 27853, Carbapenem resistant *K. pneumoniae* ATCC 700603 and *V. parahaemolyticus* by agar well diffusion method at 10 µg/µL concentration. The methanol extract of S. torvum and ethyl acetate extract of *V.negundo* and *Z.mauritiana* demonstrated 17 and 23 mm inhibition against the non-mycobacterial pathogens, respectively. The three extracts were also screened for anti-tubercular activity against *M. tuberculosis* H37Rv using Luciferase Reporter Phage (LRP) assay. All the three extracts were exhibited anti TB activity at 500 µg/ml concentration. In particular, the *S. torvum* extract was showed 98.46% inhibition. GC-MS analysis of the afore mentioned extracts yielded peaks of compounds of ethnomedicinal value/significance. Findings of this study depicted that the medicinal plant *S. torvum* deserves the potential for isolation of anti TB molecules.

KEY WORDS: ANTI-TB ACTIVITY, MEDICINAL PLANTS, MYCOBACTERIUM TUBERCULOSIS, TUBERCULOSIS.

INTRODUCTION

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, remains among the top ten mortality causing diseases in the world. According to WHO, 10 million new cases of TB and 1.3 million deaths were estimated globally in 2017. This puts the disease burden of TB to be equivalent to 133 deaths per 1,00,000 people. Around 27% of the global TB cases were contributed by India in 2017 which highlights the burden of the disease on the Indian population. The severity of multi-drug resistance TB still persists and the success rates of treatment for MDR/RR-TB (Multi-drug resistant/Rifampicin resistant TB) and XDR-TB (Extensively drug resistant TB) are strikingly low, being 55% and 34% respectively. The problem of drug resistance does not appear to have an easy solution in the near future. Synthetic drugs used in the TB treatment programme pose a heavy burden on the already weakened body of TB patients, (WHO Global TB report 2019).

The drugs are most commonly nephrotoxic (Hussein et al., 2015) and hepatotoxic (Ramappa et al., 2012), thereby leading to harmful side effects of the treatment. This shifts

ARTICLE INFORMATION

*Corresponding Author: jerrine.jj@gmail.com
Received 8th Jan 2020 Accepted after revision 7th March 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)

*Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/20*

the focus of the treatment regime to natural products and medicinal herbs for treatment of infectious diseases. Use of ethno-medically significant plants for treatment can be initiated by validating the traditional methods that were reported to be used since ancient times. These methods were used to treat multiple ailments including bacterial and viral diseases. With several reports on the activity of herbal medicines against pathogens, there is a need to focus on the potential of herbal medicines in the treatment of infectious diseases,(Ladda and Magdum 2018).

Solanum torvum is a shrub of the Solanaceae family widely available and extensively used in India for the treatment of bacterial diseases and for relief from cough and cold (Yousaf et al., 2013). It is native to other tropical countries and has been traditionally used for treatment of various ailments such as a sedative and diuretic, TB, skin infections, fever and tooth decay (Naimon et al., 2015; Silva et al., 2011). The plant extracts have been reported to be possess cardioprotective, nephroprotective, anti-viral, anti-microbial, anti-oxidant, anti-ulcerogenic and haemostatic properties (Jaiswal et al., 2012). Such properties could be attributed to the presence of phytoconstituents such as flavonoids, steroids, saponins, tannins, alkaloids, vitamin B, vitamin C and phenols, (Jaiswal et al., 2012; Yousaf et al. 2013 Ladda and Magdum (2018). *Ziziphus mauritiana* is a member of the Rhamnaceae family and is native to the Indian subcontinent, Africa, Iran and parts of Southern Asia. It is known to be traditionally used for the treatment of pain, vomiting and diarrhoea (Mahesh et al., 2008). It has also been reported to possess anti-plasmodial effect (Sameera et al., 2015); its seeds are good sedatives while its leaves are used for treatment of sores, cuts and ulcers. It has also been demonstrated to resolve liver troubles, asthma and experimentally induced liver damage (Abalaka et al., 2010). The juice obtained from the bark of its root is known to alleviate gout and rheumatism according to traditional medicinal practises (Priyanka et al., 2015).

It is a pharmacologically important plant since it is known to possess anti-typhoid, anti-cancer, antioxidant and anti-inflammatory properties (Abdallah et al., 2016). *Vitex negundo* is a member of the *Verbenaceae* family and is native to South Asia, East Africa, South America, Indonesia and Japan. It has been demonstrated to possess analgesic, anti-oxidant, anti-inflammatory, hypoglycemic, anti-tumour, anti-rheumatism and insecticidal activities (Zheng et al., 2015, Gupta et al., 2010). The leaves of the plant have been proven to possess anti-convulsant and anti-parasitic activities (Ladda and Magdum 2018) which is an addition to the traditional medicinal properties that the plant has been reported to possess. In this study, we have investigated the antibacterial and anti-mycobacterial properties of different solvent extracts of *S. torvum* fruits, *Z.mauritiana* fruits and leaves of *V.negundo*. GC-MS analysis was performed to identify the potential compounds present in the extracts. With the help of the present in vitro studies, it may be possible to maximize the traditional use of the plants for treatment of various infections.

MATERIALS AND METHODS

The fruits of Solanum torvum, fruits of *Ziziphus mauritiana* and leaves of *Vitex negundo* were collected in 2018, from Chennai, India, and duly authenticated by a botanist. The fruits and leaves were processed by washing thrice with distilled water and surface sterilization by rinsing with 70% acetone. Then the samples were shade dried and powdered using mixer grinder. Sequential extraction was performed using the solvents n-hexane, ethyl acetate and methanol to extract the compounds from the powdered plant material. Ten gram of powdered plant material was added to 100 mL n-hexane and incubated at room temperature in an orbital shaker at 80 rpm for 24 hrs. The extract was filtered using Whatman filter paper and the powdered plant material was reused upon drying. To the dried powder, 100 mL of ethyl acetate and subsequently methanol were added and the extracts were filtered and collected in a similar manner. The n-hexane, ethyl acetate and methanol extracts were concentrated using rotary evaporator and dried by incubating the extracts at 50 ^{o}C for 24-36 hrs. The weight of the extracts was determined and the extracts were dissolved in 10% DMSO for evaluating their antibacterial and anti-mycobacterial activities. Anti-bacterial activity of the extracts was evaluated by agar well diffusion method. Overnight grown cultures of bacterial pathogens viz. *Staphylococcus aureus* ATCC 29213, Pseudomonas aeruginosa ATCC 27853, Carbapenem resistant *Klebsiella pneumoniae* ATCC 700603 and *Vibrio parahemolyticus* were swabbed on individual Muller-Hinton Agar plates. Wells of 5 mm diameter were cut on the plate using well cutter and 10 µg/µL of n-hexane (HF), ethyl acetate (EAF) and methanol (MF) extracts were added to each well respectively.

The plates were then incubated at 37 ^{o}C for 18 hours and zones of inhibition were measured. The assay was performed in triplicates and mean values were calculated and tabulated. The antitubercular activity of HF, EAF and MF was screened against M. tuberculosis H37Rv by Luciferase Reporter Phage assay (Radhakrishnan et al., 2016). Briefly, 400 µL of middlebrook 7H9 broth was added to two cryovials (Control) and another 400 µL of rifampicin containing Middlebrook 7H9 broth at concentration of 2 µg/mL was added to a cryovial (drug control). About 350 µL of Middlebrook 7H9 was added to two cryovials (solvent control and test). Fifty µL of extract (HF) was added to test vial to achieve a final concentration of 500 µg/mL and 50 µL of 10% DMSO was added to solvent control vial. All the vials were added with 100 µL of M. tuberculosis H37Rv (McFarland unit 2) and incubated at 37 ^{o}C for 72 hrs. After incubation, 50 µL of phAE202 and 40 µL of 0.1 M CaCl2 were added to all the vials (Cell-phage mixture) and incubated at 37 ^{o}C for 4 hrs. About 100 µL of cell-phage mixture from each vial was added into a luminometer cuvette, to which 100 µL of D- Luciferin was added. The relative light unit

(RLU) was measured immediately at 10 sec integration time using the luminometer (Lumat 9508, Berthold, Germany). The above mentioned procedure were followed for the other extracts MF and EAF as well. The percentage reduction was calculated using the formula:% RLU reduction = Control RLU- Test RLU/ Control RLU x 100 On comparison with control, if the sample showed 50% or more reduction of RLU, the extract was deemed to have anti tubercular activity. The potential MF extract was analysed by GC-MS (Shimadzu QP2010 Ultra).

RESULTS AND DISCUSSION

Sequential extraction procedures are commonly used to isolate a number of compounds from plant extracts. The process offers improved phase-specificity due to combined use of multiple solvents of varying polarity. In sequential extraction, the plant residue from the first extraction is used as the material for the second extraction and the process may be continued as required (Kaplan et al., 2009). In this study, sequential extraction was carried out using solvents of increasing polarity. Due to the difference in chemical nature of the solvents, the process is very selective in extraction of compounds from plants. Sequential extraction from 10 g of plant extracts using 100 mL of solvents n-hexane, ethyl acetate and methanol yielded fractions whose stock concentrations were maintained at 10 mg/mL. The crude extracts were diluted and a final concentration of 10 µg/µL was used for evaluating anti-microbial activity against the selected non-mycobacterial pathogens. The inhibitory activity of n-hexane, ethyl acetate and methanol extracts of the fruits of *S. torvum,* fruits of *Z.mauritiana* and leaves of *V.negundo* were screened against clinical pathogens and results were summarized in Table 1.

The methanol extract (MF) of *S.toruvum* fruits demonstrated inhibitory activity against *S. aureus* ATCC 29213 (17mm), *P. aeruginosa* ATCC 27853 (17mm) and *V. parahaemolyticus* (25mm) at a concentration of 10 µg/µL whereas the ethyl acetate extract (EAF) showed inhibition against *P. aeruginosa* ATCC 27853 (12mm) and V. parahaemolyticus (12mm) at the same concentration. None of the pathogens tested were inhibited by hexane extract (HF). Chah et al. (2000) demonstrated the antimicrobial activity of methanolic and ethanolic extracts of S. torvum against Actinomyces pyogenes, B. subtilis, S.pyogenes, A.niger and *C.albicans*. In other reports, plant extracts of *S. torvum* showed inhibition against *B.cereus, Staphylococcus epidermidis*, *E. coli, V. cholerae, Salmonella cibrum* and *Salmonella typhimurium* (Naimon et al., 2015; Sivapriya et al., 2011).

The ethyl acetate extract (EAF) of *Z.mauritiana* fruits inhibited the growth of *S. aureus* ATCC 29213 (23mm), *P.aeruginosa* ATCC 27853 (11mm) and Carbapenem resistant *K.pneumoniae* ATCC 700603 (11mm) at a concentration of 10 µg/µL. However, 10 µg/µL of MF inhibited the growth of only *S.aureus* ATCC 29213 (12mm) and *V.parahaemolyticus* (12mm), while the HF did not inhibit the growth of any of the pathogens tested. The antibacterial activity of the extracts demonstrated against *S.aureus* is in accordance with previous reports of the same being exhibited by extracts of *Z.mauritiana* as reported by Mahesh et al., 2008, Abdallah et al., 2016 and Priyanka et al., 2015. Their reports also illustrated the anti-bacterial activity of *Z.mauritiana* extracts against bacterial pathogens such as *B.subtilis, E.coli*, *Pseudomonas fluorescens* and *K.pneumoniae.*

EAF of *V.negundo* leaves was inhibited the growth of *S.aureus* ATCC 29213 (15mm) and *P.aeruginosa* ATCC

Table 1. Activity of extracts tested against non-mycobacterial pathogens

S. No.	Plant	Extract	Zone of inhibition against pathogen (mm in diameter)			
			S. aureus ATCC 29213	*P. aeruginosa* ATCC 27853	Carbapenem resistant *K. pneumoniae* ATCC 700603	*Vibrio parahem-olyticus*
1	*Solanum torvum*					
	HF	-	-	-	-	
		EAF	-	12	-	12
		MF	17	17	-	25
2	*Ziziphus mauritiana*					
	HF	-	-	-	-	
		EAF	23	11	11	-
		MF	12	-	-	12
3	*Vitex negundo*					
	HF	-	-	-	25	
		EAF	15	15	-	-
		MF	-	-	-	-

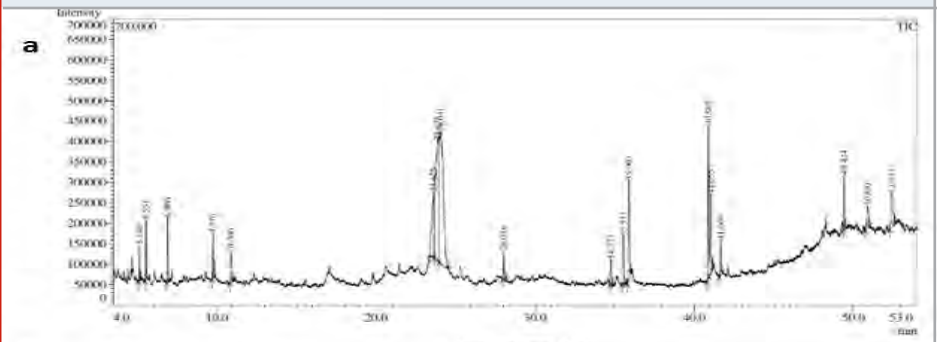
27853 (15mm) at a concentration of 10 µg/µL. The HF only inhibited the growth of *V. parahaemolyticus* (25 mm), while the MF did not inhibit the growth of any of the pathogens tested. This was similar to the reports of antibacterial activity of *V.negundo* extracts against *S.aureus*, *V.parahaemolyticus* and *K.pneumoniae* (Zheng et al., 2015). This helps to substantiate the ethnomedicinal use of *V.negundo* for the treatment of cold, cough and bacterial dysentery (Gupta et al., 2010).

Table 2. Anti-tubercular activity of extracts against *M. tuberculosis* H37Rv by LRP

Extracts	% inhibition against *M. tuberculosis* H37Rv
S.torvum-MF	98.46
Z.mauritiana-EAF	84.41
V.negundo-EAF	59.2

Upon screening of the extracts against *M. tuberculosis* H37Rv by Luciferase Reporter Phage (LRP) assay, MF of *S.torvum* showed promising inhibitory activity against *M. tuberculosis* H37Rv at 500 µg/mL concentration and the RLU reduction in terms of inhibition was found to be 98.46% (Table 2). Our results are in correlation with that of Mohamad et al., (2011), in which hydromethanolic fruit extracts from *S.torvum* displayed moderate antimycobacterial activity against *M. tuberculosis*

Figure 1: (a) GC-MS chromatogram (b) GC-MS Peak Report of Methanol fraction of *S. torvum*

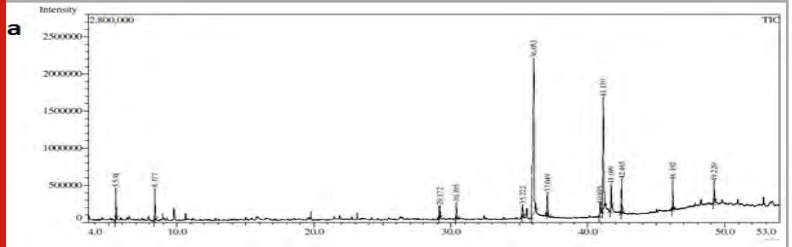


b

Peak Report TIC

Peak#	R.Time	Area	Area%	Name
1	5.140	259030	1.30	4-Methylpiperidine-1-carboxylic acid, pheny
2	5.551	392052	1.96	1,2,3-Propanetriol, 1-acetate
3	6.906	447519	2.24	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-
4	9.770	424886	2.13	1,2,3-Propanetriol, 1-acetate
5	10.900	321485	1.61	.beta.-Alanine, N-acryloyl-, isobutyl ester
6	23.625	1520222	7.61	1,2-CYCLOPROPANEDIACETIC ACID
7	23.875	4950139	24.79	1,3,4,5-TETRAHYDROXYCYCLOHEXAN
8	24.041	5546944	27.78	Quinic acid
9	28.036	335246	1.68	4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyp
10	34.773	288487	1.44	Benzenepropanoic acid, 3,5-bis(1,1-dimethyl
11	35.553	457019	2.29	Dibutyl phthalate
12	35.903	946639	4.74	n-Hexadecanoic acid
13	40.885	1379261	6.91	9,12-Octadecadienoic acid (Z,Z)-
14	41.055	885544	4.44	Oleic Acid
15	41.669	312407	1.56	Octadecanoic acid
16	49.424	374181	1.87	Pyrazolo[3,4-b]thiopyrano[4,3-d]pyridin-1-a
17	50.897	379027	1.90	STIGMAST-5-EN-3-OL, (3.BETA.)-
18	52.442	746291	3.74	Hexacontane
		19966379	100.00	

Figure 2: (a) GC-MS chromatogram (b) GC-MS Peak Report of Ethyl acetate fraction of *Z.mauritiana*.



b

Peak Report TIC

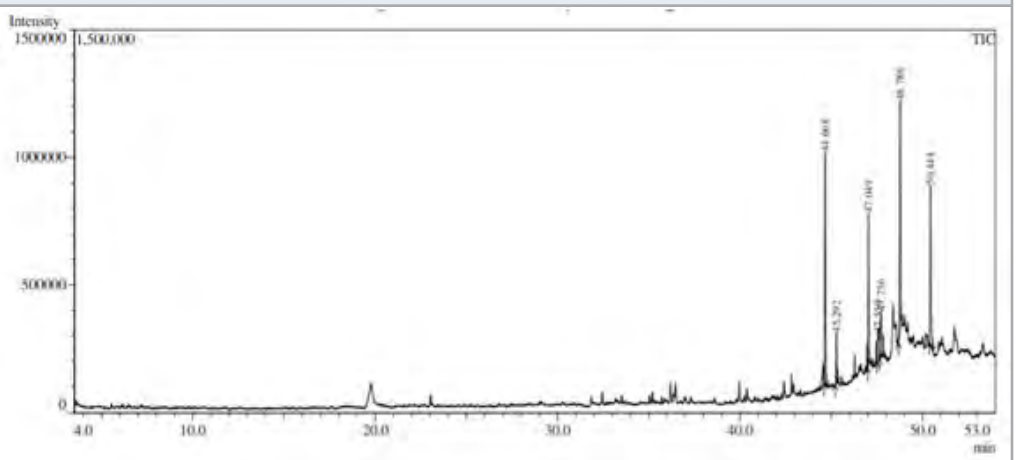
Peak#	R.Time	Area	Area%	Name
1	5.538	1006609	3.31	1,2,3-Propanetriol, 1-acetate
2	8.377	1196836	3.93	4-(1-HYDROXY-ETHYL) .GAMMA. BUT
3	29.172	507078	1.67	TETRADECANOIC ACID
4	30.395	681681	2.24	1-Nonadecene
5	35.222	584657	1.92	Palmitoleic acid
6	36.053	14232713	46.79	l-(+)-Ascorbic acid 2,6-dihexadecanoate
7	37.049	1051697	3.46	1-Nonadecene
8	40.895	489123	1.61	9,12-Octadecadienoic acid (Z,Z)-
9	41.130	6818319	22.42	Oleic Acid
10	41.699	942149	3.10	Octadecanoic acid
11	42.465	1235942	4.06	1-Heptacosanol
12	46.192	983341	3.23	Heneicosyl trifluoroacetate
13	49.220	687762	2.26	1-Triacontanol
		30417907	100.00	

H37Rv.

Previously other parts of plants are also evaluated for antimycobacterial activity includes the ethanol extract of leaves from *S. torvum,* which displayed activity against *M. smegmatis* and *M. tuberculosis* H37Rv (Nguta et al., 2016). In this study, *Z.mauritiana* EAF was showed 84.41% inhibition against *M.tuberculosis* H37Rv at a concentration of 500 µg/mL. Using the alamar plate assay, *Z.mauritiana* was reported to have anti-mycobacterial activity against *M.tuberculosis* H37Ra as reported by Panseeta et al., (2011). *V.negundo* EAF demonstrated an anti-mycobacterial activity of 59.2% at a concentration of 500 µg/mL. This is along the lines of the published reports of Ladda et al., (2018) and Gupta et al., (2010) where the plant extracts were tested against *M. tuberculosis* H37Rv. GC-MS analysis of MF of *S.torvum* (Fig. 1) showed several compounds among which the prominent peaks denotes the presence of Quinic acid, n-hexadecanoic acid, oleic acid and 9,12-octadecadienoic acid. The GC-MS peaks of EAF of *Z. mauritiana* (Fig. 2) yielded significant peaks corresponding to ascorbic acid-2,6-dihexadecanoate and oleic acid, while those of *V.negundo* yielded peaks (Fig. 3) which indicated the presence of 2-heptanone, 4,8,13-cyclodecatriene and 2-pentanoic acid. The antimicrobial and anti-mycobacterial activity of the extracts could be attributed to the presence of oleic acid as reported by Ojo et al., (2018; Kalita et al., 2018 and Santhosh et al., 2013).

Fatty acids such as n-hexadecanoic acid, otherwise known as palmitic acid has also been reported to possess anti-mycobacterial activity (Ojo et al., 2018). Ascorbic acid, 2,6-dihexadecanoate is an antioxidant which could play a significant role in combatting deficiency or imbalance of essential nutrients which is a common problem that occurs in patients infected by TB. The ascorbic acid from the extracts could compensate for the decreased anti-oxidant levels and further elevate and complement the effects of the treatment (Turchenko et al., 2008).

Figure 3: (a) GC-MS chromatogram (b) GC-MS Peak Report of Ethyl acetate fraction of *V.negundo*.



b

Peak Report TIC

Peak#	R.Time	Area	Area%	Name
1	44.668	2733406	22.73	2-HEPTANONE, 6-(3-ACETYL-1-CYCLO
2	45.292	564349	4.69	Sclareoloxide(Cis-A/B)
3	47.049	1717107	14.28	TETRAHYDROEDULAN D
4	47.550	842980	7.01	Octadecane, 3-ethyl-5-(2-ethylbutyl)-
5	47.736	1083374	9.01	Tetrapentacontane
6	48.786	2821424	23.46	4,8,13-Cyclotetradecatriene-1,3-diol, 1,5,9-tr
7	50.444	2261534	18.81	2-PENTENOIC ACID, 5-(DECAHYDRO-5
		12024174	100.00	

CONCLUSION

In conclusion, *S. torvum* fruits, fruits of *Z.mauritiana* and leaves of *V.negundo* have potential anti-mycobacterial properties which can be taken up for further in vitro and in vivo studies. Phyto constituents of the plant extracts can be purified and investigated further to identify lead compounds responsible for anti-bacterial and anti-mycobacterial activity.

ACKNOWLEDGEMENT

Authors thank the authorities of Sathyabama Institute of Science and Technology for the research facilities provided.

Conflict of Int7erest: There are no conflict of Interest

REFERENCES

Abalaka ME Daniyan SY Mann A (2010) Evaluation of the antimicrobial activities of two *Ziziphus species* (*Ziziphus mauritiana* L. and *Ziziphus spinachristi L.*) on

some microbial pathogens African Journal of Pharmacy and Pharmacology Vol 4 No 4 Pages 135-139.
Abdallah EM Elsharkawy ER Ed-Dra A (2016) Biological activities of methanolic leaf extract of *Ziziphus mauritiana*. Bioscience Biotechnology Research Communications Vol 9 No 4 Pages 605-614
Abhishek RU Thippeswamy S Manjunath K Mohana DC (2015) Antifungal and antimycotoxigenic potency of *Solanum torvum* Swartz. Leaf extract: Isolation and identification of compound active against mycotoxigenic strains of *Aspergillus flavus* and *Fusarium verticillioides* Journal of Applied Microbiology Vol 119 Pages 1624-1636.
Balachandran C Duraipandiyan V Dhabi NAA Balakrishna K et al (2012) Antimicrobial and antimycobacterial activities of methyl caffeate isolated from *Solanum torvu*, Swartz. fruit Indian Journal of Microbiology Vol 52 No 4 Pages 676-681
Chah KF Muko KN Oboegbulem SI (2000) Antimicrobial activity of methanolic extract of *Solanum torvum* fruit Fitote Vol 71 Pages 187-189.
Gupta VK Shukla C Bisht GRS Saikia D et al (2010) Detection of anti-tuberculosis activity in some folklore plants by radiometric BACTEC assay Letters in Applied Microbiology Vol 52 Pages 33-40.
Hussein OE Germoush O Mahmoud AM (2016) Ruta graveolens protects against Isoniazid/Rifampicin-induced nephrotoxicity through modulation of oxidative stress and inflammation Global Journal of Biotechnology and Biomaterial Sciences Vol 1 No 1 Pages 017-022
Jaiswal BS (2012) *Solanum torvum:* A review of its traditional uses, phytochemistry and pharmacology International Journal of Pharmacy and Biological Sciences Vol 3 No 4 Pages 104-111
Kalita RD Hussain I Deka RC Buragohain AK (2018) Antimycobacterial activity of linoleic acid and oleic acid obtained from the hexane extract of the seeds of *Mesua ferrea L.* and their in silico investigation Indian Journal of Natural Products and Resources Vol 9 No 2 Pages 132-142.
Kaplan O Yaman M (2009) Selective and sequential extraction of lead in soil samples and plant parts taken from a serpentine and copper mining area Atomic Spectrometry Vol 30 No 1
Ladda PL Magdum CS (2018) Antitubercular activity and isolation of chemical constituents from plant Vitex negundo Linn Iranian Journal of Pharmaceutical Research Vol 17 No 4 Pages 1353-1360
Mahesh B Satish S (2008) Antimicrobial activity of some important medicinal plant against plant and human pathogens World Journal of Agricultural Sciences Vol 4 Pages 839-843
Mohamad S Zin NM Wahab HA Ibrahim P Sulaiman SF Zahariluddin ASM (2011) Antituberculosis potential of some ethnobotanically selected Malaysian plants Journal of Ethnopharmacology Vol 133 Pages 1021-1026.
Naimon N Pongchairerk U Suebkhampet A (2015) Phytochemical analysis and antibacterial activity of ethanolic leaf extract of *Solanum torvum Sw.* against pathogenic bacteria. Kasetsart Journal Natural Science Vol 49 No 4 Pages 516-523
Nguta JM Opong RA Nyarko AK Manu DY Addo PGA Otchere I Twum AK (2016) Antimycobacterial and cytotoxic activity of selected medicinal plant extracts Journal of Ethnopharmacology Vol 182 Pages 10-15.
Ojo PO Babayi H Olayemi IK et al (2018) Anti-tubercular activities and molecular characterization of salivary extract of Leech (*Hirudo medicinalis*) against Mycobacterium tuberculosis Journal of Tuberculosis Research Vol 6 Pages 1-9.
Panseeta P Lomchoey K Prabpai S Kongsaeree P et al (2011) Anti-plasmodial and antimycobacterial cyclopeptide alkaloids from the root of *Ziziphus mauritiana*. Phytochemistry Journal Vol 72 Pages 909-915.
Priyanka C Kumar P Bankar SP Karthik L (2015) In vitro antibacterial activity and gas chromatography-mass spectroscopy analysis of Acacia karoo and *Ziziphus mauritiana* extracts. Journal of Tal Uni Sciences Pages 13-19.
Radhakrishnan M Sekar P Jerrine J Vanaja K (2016) Anti-tubercular activity of pigment from forest soil *Streptomyces sp* SFA5 Bangladesh Journal of Pharmacology Vol 11 Pages 138-140.
Ramappa V Aithal GP (2013) Hepatotoxicity related to anti-tuberculosis drugs: mechanisms and management Journal of Clinical and Experimental Hepatology Vol 3 Pages 37-49
Sameera NS Mandakini BP (2015) Investigations into the antibacterial activity of *Ziziphus mauritiana Lam.* and *Ziziphus xylopyra* (Retz.) Willd. International Food Research Journal Vol 22 No 2 Pages 849-853.
Santhosh RS Suriyanarayanan B (2014) A source for new antimycobacterial drugs Planta Medica Vol 80 Pages 9-21.
Silva KN Silva RC Coelho VPM Agra MF (2011) A pharmacobotanical study of vegetative organs of *Solanum torvum* Brazilian Journal of Pharmacognosy Vol 21 No 4 Pages 568-574
Sivapriya M Dinesha R Harsha R Gowda SST Srinivas L (2011) Antibacterial activity of different extracts of sundakkai (*Solanum torvum*) fruit coat International Journal of Biological and Chemical Sciences
Turchenko LV Voloshchuk EO Ivanov V et al (2008) Clinical improvement of active tuberculosis patients with complex treatment and nutritional supplementation Open Natural Products Journal Vol 1 Pages 20-26.
WHO, Global tuberculosis report (2018) https://www.who.int/tb/publications/ global_report/en/
Yousaf Z Wang Y Baydoun E (2012) Phytochemistry and pharmacological studies on *Solanum torvum* Swartz Journal of Applied Pharmaceutical Sciences Vol 3 No 4 Pages 152-160
Zheng CJ Li HQ Ren SC Xu CL et al (2015) Phytochemical and pharmacological profile of *Vitex negundo*. Phytotherapy Research

Biomedical
Communication

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp-119-122

BBRC
Bioscience Biotechnology
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On the Knowledge and Practices of Family Planning Methods in Hail Region: A Cross-Sectional Study

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ABSTRACT

Present study focuses on knowledge and practices of family planning methods in Hail Region. This study is a cross-sectional study conducted in Hail province and its villages. Married men and women were the study subjects. A detailed questionnaire is prepared containing demographic profiles and questions related to knowledge about family planning (FP) methods. A multistage sampling is used for the selection of subjects. Firstly, a list of villages were made and selected randomly. After the selection of villages, subjects were selected starting from a pin-point made in the village till the final subject selected from the village. More than one third of the subjects were ≤25 and 26-35 years each constituted 36.3%. Most of the subjects were females (55.6%). Overall, the knowledge about family planning methods was among 95.2% (95%CI=89.8-97.7%) of the subjects. The knowledge about family planning methods was higher among female subjects (97.1%) than males (92.7%), however, the association was statistically insignificant (p>0.05). Drug use was in majority of subjects as the method of family planning (70.3%). About half of the subjects got knowledge about FP methods from doctors (51.7%) and one third got from family & friends (30.5%). The study showed almost universal knowledge about family planning methods with higher knowledge among women. The family planning and birth spacing interventions need to focus on alleviating fears about side-effects among men and women through effective counseling and providing adequate information to both men and women about method-related side-effects and how to manage them. In addition, involving community leaders, religious clerics, and health workers in awareness raising campaigns can help address sociocultural and religious concerns.

KEY WORDS: KNOWLEDGE, PRACTICES, FAMILY PLANNING, AWARENESS.

ARTICLE INFORMATION

Corresponding Author: jahooralam@gmail.com
Received 10th Feb 2020 Accepted after revision 28th March 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/21

INTRODUCTION

The dynamics of decision-making between a husband and wife also create barriers to access. Several studies have examined the influence of social and cultural factors on contraceptive use. These studies have emphasized the influence of the mother-in-law and the husband on family planning decision-making and have highlighted the importance of communication between spouses regarding the use of contraception (Pasha et al., 2001; Kadir et. al., 2003 Al-Mousa et al., 2019). Despite the huge benefits, family planning is one of the most difficult and least discussed topics, particularly amongst males in a conservative and patriarchal society where men have the final decision-making power regarding most issues, including reproductive health. Nevertheless, there have been some efforts to target men through either advocacy or behavioral change interventions, but very little have been achieved (Omolase et. al., 2009). Healthy timing and spacing of pregnancy (HTSP) is a family planning intervention to help women and couples delay, time, space, or limit their pregnancies to achieve the healthiest outcomes for women, newborns, infants, and children regardless of the total number of children (Marston, 2005). It has been documented that perinatal outcomes and child survival can be improved mainly by lengthening inter-pregnancy intervals. Over one million maternal deaths were averted between 1990 and 2005 because the fertility rate in developing countries has declined and by reducing high parity births family planning contributed to reducing the maternal mortality ratio.

On the contrary, birth to pregnancy intervals of less than 18 months are associated with risk of low birth weight, preterm birth, small size for gestational age, and stillbirth (Celand et. al., 2012; Stover and Ross, 2010 Alhusain, 2018). Use of family planning (FP) methods can contribute to a substantial reduction in fertility and reduce the proportion of unwanted pregnancies as well as maternal deaths that would otherwise occur in the absence of contraception. In 2008, contraceptive use averted over 250,000 maternal deaths worldwide by reducing unintended pregnancies, which is equivalent to 40% of the 355,000 maternal deaths that occurred in that year (Alege, et.al., 2016).Fear of contraceptive side effects and associated treatment costs, cultural barriers and low male involvement continue to hamper effective use of FP services in most countries. Lack of knowledge of where to obtain FP methods and lack of information on what women consider to be trusted sources of FP information and services, are key barriers that affect access to and utilization of FP methods (Celand et. al., 2012, Abdulreshid and Dadi, 2020). The presented study was designed to study the knowledge and practices of family planning methods adopted in Hail Region of Kingdom of Saudi Arabia.

MATERIAL AND METHODS

This study was a cross-sectional study conducted in Hail province and its villages. The consent was taken from each subject before the interview. Married men and women were the study subjects. A detailed questionnaire was prepared containing demographic profiles and questions related to knowledge about family planning (FP) methods. A multistage sampling was used for the selection of subjects. Firstly, a list of villages were made and selected randomly. After the selection of villages, subjects were selected starting from a pin-point made in the village till the final subject selected from the village.

Statistical Analysis: The results are presented in frequencies and percentages. Chi-square test was used to assess the associations. The p-value<0.05 was considered significant. All the analysis was carried out on SPSS 16.0 version.

RESULTS AND DISCUSSION

More than one third of the subjects were ≤25 and 26-35 years each constituted 36.3%. Most of the subjects were females (55.6%). More than one third of the subjects had college level of education (44.4%) and majority of the subjects were employed (73.4%). Marriage duration was 16-20 years among more than one third of subjects (37.1%). More than one third of subjects had 1-3 kids (45.2%) (Table-1). Overall, the knowledge about family planning methods was among 95.2% (95%CI=89.8-97.7%) of the subjects (Fig. 1). The knowledge about family planning methods was higher among female subjects (97.1%) than males (92.7%), however, the association was statistically insignificant (p>0.05) (Table-2)More than one third of subjects had good knowledge about FP methods (42.4%) and 32.2% had excellent knowledge. The percentage of good and excellent knowledge was found to be higher among females than males (Table-3). Drug use was in majority of subjects as the method of family planning (70.3%). However, 26.3% used condom as the method of family planning. Majority of males used drugs (70.6%) and condom (60.8%) as the method of family planning. However, 70.1% females used drugs for family planning (Table-4).About half of the subjects got knowledge about FP methods from doctors (51.7%) and one third got from family & friends (30.5%). Males (54.9%) and females (49.3%) also got knowledge from doctors (Table-5).

DISCUSSION

In the present study, more than one third of the subjects were ≤25 and 26-35 years each constituted 36.3%. Most of the subjects were females (55.6%). (Gupta et. al., 2012) showed that 32 (32% of the married women belonged to 20-24 years of age group.In this study, the knowledge about family planning methods was among 95.2% (95%CI=89.8-97.7%) of the subjects. This finding is in agreement with the study by (Gupta et. al., 2012) in which 94 (94%) of participants have knowledge about contraception. In the present study, drug use was in majority of subjects as the method of family planning (70.3%). However, 26.3% used condom as the method of family planning. However, in a study (Gupta et.

al., 2012), 64 (68%) were using modern contraception methods. They also showed that injectables hormonal contraceptives were most commonly practiced by 34 (54%) women. However, in the present study, Majority of males used drugs (70.6%) and condom (60.8%) as the method of family planning. However, 70.1% females used drugs for family planning. This study showed that about half of the subjects got knowledge about FP methods from doctors (51.7%) and one third got from family & friends (30.5%). It is reported that clinic providers, friends and the media were the most trusted sources of contraceptive information while government and private health facilities were the main sources of FP methods (Celand et. al., 2012; Al-Turki, 2010, Abdulreshid and Dadi, 2020, Kantorova et. al., 2020). There are some of the limitations of this study. One them is fewer sample size. Studies on larger sample size are recommended to have robust findings.

Table 1. Demographic profile of study subjects

Demographic profile	No. (n=124)	%
Age in years		
≤25	45	36.3
26-35	45	36.3
36-45	13	10.5
46-55	10	8.1
>55	11	8.9
Gender		
Male	55	44.4
Female	69	55.6
Level of education		
Collage	55	44.4
High school	12	9.7
Elementary school	21	16.9
Pre-primary school	14	11.3
None	22	17.7
Occupation		
Employed	91	73.4
Farmer	1	0.8
Business	11	8.9
Housewife	10	8.1
Retired	11	8.9
Marriage duration in years		
1-5	14	11.3
6-10	19	15.3
11-15	33	26.6
16-20	46	37.1
>20	12	9.7
No. of kids		
1-3	56	45.2
4-7	46	37.1
>7	22	17.7

Table 2. Knowledge about family planning methods among study subjects

Gender	No. of subjects	Knowledge about family planning methods				p-value[1]
		Yes		No		
		No.	%	No.	%	
Male	55	51	92.7	4	7.3	0.25
Female	69	67	97.1	2	2.9	

1Chi-square test

Table 3. Level of knowledge about family planning methods among study subjects

Level of knowledge about family planning methods	Male (n=51)		Female (n=67)		Total (n=118)	
	No.	%	No.	%	No.	%
Little	14	27.5	4	6.0	18	15.3
Moderate	4	7.8	8	11.9	12	10.2
Good	18	35.3	32	47.8	50	42.4
Excellent	15	29.4	23	34.3	38	32.2

Table 4. Attitudes towards use of family planning methods among study subjects

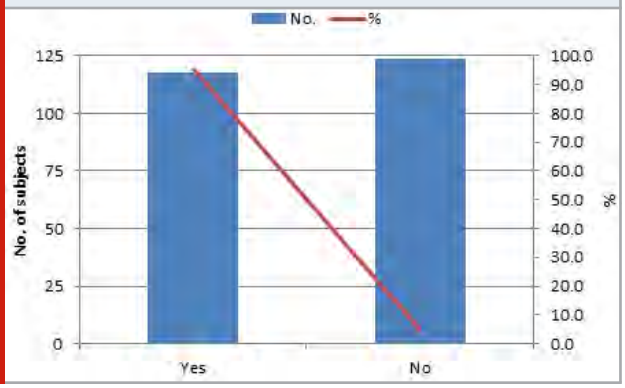
Contraceptive methods used*	Gender Male (n=51)		Female (n=67)		Total (n=118)	
	No.	%	No.	%	No.	%
Drugs	36	70.6	47	70.1	83	70.3
IUD	0	0.0	22	32.8	22	18.6
Condom	31	60.8	0	0	31	26.3
Uterine barrier	0	0.0	3	4.5	3	2.5
Withdrawal	6	11.8	9	13.4	15	12.7
Injections	0	0.0	6	9.0	6	5.1

*Multiple response

Table 5. Source of knowledge about family planning methods among study subjects

Source of knowledge*	Gender Male (n=51)	Female (n=67)		Total (n=118)		
	No.	%	No.	%	No.	%
Family and friends	8	15.7	28	41.8	36	30.5
TV	5	9.8	9	13.4	14	11.9
Internet	5	9.8	6	9.0	11	9.3
Doctors	28	54.9	33	49.3	61	51.7
*Multiple response						

Figure 1: Knowledge about family planning methods



CONCLUSION

The study showed almost universal knowledge about family planning methods with higher knowledge among women. The family planning and birth spacing interventions need to focus on alleviating fears about side-effects among men and women through effective counseling and providing adequate information to both men and women about method-related side-effects and how to manage them. In addition, involving community leaders, religious clerics, and health workers in awareness raising campaigns can help address sociocultural and religious concerns.

REFERENCES

Al-musa H.M, Alsaleem M. A., Alfaifi W. H., Alshumrani Z., et al. (2019). Knowledge, attitude, and practice among Saudi primary health care attendees about family planning in Abha, Kingdom of Saudi Arabia. J Family Med Prim Care; 8(2): 576–582.

Alhusain F., Alkaabba F., Alhassan N., Alotaibi S., Breakeit S., Musaudi E., Alhasan A. (2018). Patterns and knowledge of contraceptive methods use among women living in Jeddah, Saudi Arabia. Saudi Journal of Health Science; 7(2): 121-126.

Alege S. G., Matovu J. K., Ssensalire S., and Nabiwemba E. (2016). Knowledge, sources and use of family planning methods among women aged 15-49 years in Uganda: a cross-sectional study. Pan Afr Med J.; 24(39): 1-12.

Al-Turki H. A., (2010). Contraception: Attitudes and Experiences of Saudi Arabian Women. Health Care for Women International; 32(2): 134-139.

Abdulreshid M. and Dadi H.B. (2020) Assessment of Family Planning Counseling Provided for Postpartum Women and Associated Factors. International Journal of Reproductive Medicine; 2020 (2649340): 1-5.

Cleland J., Conde-Agudelo A., Peterson H., Ross J., and Tsui A., (2012). Contraception and health. The Lancet; 380(9837):149–156, 2012.

Gupta S, Singh A, Gupta N, Shrestha VL. (2012). Family planning knowledge and practices among women in a district hospital. JNMA J Nepal Med Assoc.; 52(188): 159-61.

Kadir M. M., Fikree F., Khan A., and Sajan F. (2003). Do mothers-in-law matter? Family dynamics and fertility decision-making in urban squatter settlements of Karachi, Pakistan. Journal of Biosocial Science; 35(4): 545–55.

Kantorová V., Wheldon M.C., Ueffing P., Dasgupta A.N.Z, (2020). Estimating progress towards meeting women's contraceptive needs in 185 countries: A Bayesian hierarchical modelling study. PLoS Med; 17(2): e1003026.

Marston C. (2005). Report of WHO Technical Consultation on Birth Spacing", World Health Organization, Geneva, Switzerland.

Omolase C.O., Faturoti S.O., and Omolase B.O. (2009). Awareness of family planning amongst antenatal patients in a nigerian community: an exploratory study. Ann Ib Postgrad Med; 7(1): 36–39.

Pasha O., Fikree F. F., and Vermund S. (2001). Determinants of unmet need for family planning in squatter settlements in Karachi, Pakistan. Asia-Pacific Population Journal; 16(2): 93–108.

Stover J. and Ross J., (2010). How increased contraceptive use has reduced maternal mortality. Maternal and Child Health Journal; 14(5), 687–695.

Dental
Communication

Biosc.Biotech.Res.Comm. Vol 13 (1) Jan- March 2020 123-128

Adhesive Bond Strength of Bioactive Cement to Er Cr YSGG Laser Treated Lithium Disilicate Ceramics

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ABSTRACT

The aim of the study was to evaluate the adhesive bond strength of bioactive cements to lithium disilicate ceramics in comparison to the resin based luting cements with two surface treatments, hydrofluoric acid (HF) and Er Cr YSGG laser (ECL). Ninety ceramic disks were fabricated and divided into three groups (n=30) based on the surface treatment employed; HF-S, HF-S (with Silane as control), ECL-2, for 2 minute duration, and ECL-4 for 2 minute duration and ECL4 for 4 minute duration. The laser treated groups were prepared with the use of a gold handpiece using an MZ10 tip size (Er Cr: YSGG, water lase I plus). Surface treated disks in each group were further divided into three sub groups (n= 10) based on the type of cement applied; Bioactive cements, Rely X unicem and Rely X ARC. Subsequent to the application of the cements, Multicore Flow composite was build up with the help of the putty mould; specimens were placed in a thermocycler for 5000 cycles at 5^{0}C and 55^{0}C. Each specimen disk was tested under the universal testing machine for shear bond strength (SBS). Data was analysed using analysis of variance and Tukey's – Kramer multiple tests ($p<0.05$). The maximum mean value for shear bond strength achieved was 23.55 (±2.26) for Rely-x unicem¬-HF-S and the minimum mean value was 14.30 (±2.08) for the group bioactive and ECL-2. Therefore, it was concluded that the adhesive bond strength of the bioactive cements on lithum disilicate ceramics was lower than the resin based luting cements. Difference in surface treatments did not influence the adhesive bond strength of bioactive cements to the lithium disilicate ceramic.

KEY WORDS: LITHIUM DISILICATE, ER CR YSGG LASERS, SELF ETCH RESIN LUTING AGENTS, RELY X AND BIOACTIVE CEMENTS.

INTRODUCTION

The most popular technology that has become the centre of choice in dental restoration recently is all ceramic restorations (Zarone et al., 2016). These restorations offer a series of advantage over the metal porcelain used, such as biocompatibility, low thermal conductivity, high optical properties, better chemical stability and comparable coefficients of thermal expansion to the teeth (Niu et al., 2014, Niu et al., 2013, Aboushelib and Sleem, 2014). Lithium disilicate (LD) is the most common type of contemporary adhesive glass ceramics employed in modern dentistry (Elsayed et al., 2017). In Comparison to zirconia, lithium disilicate ceramics are processed through the hot press technique exhibiting greater potential for restoration displaying translucency and aesthetics (Palla et al., 2018). The lithium silicate ceramics comprise of silica glass matrix and lithium oxide (Li2O) that presents greater flexural strength compared to the leucite-reinforced glass ceramics (Klosa et al., 2013). The crystals in the lithium disilicate ceramic are the unit of foundation that offers better mechanical strength to the

ARTICLE INFORMATION

*Corresponding Author: Malshowaish@ksu.edu.sa
Received 5th Jan 2020 Accepted after revision 10th March 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/21

ceramics such as fracture toughness, chemical durability and abrasion resistance (Lee et al., 2017).

Efforts have been made in formulating a resin based luting agent with improved mechanical strength, toughness and fracture resistance (Koizumi et al., 2012). These bioactive cements have demonstrated improved and better functional properties critical for clinical function and survival. The volumetric shrinkage of these materials is <1.7% compared to the resin based composites (Al-Sowygh, 2017). These materials have successfully presented with properties equivalent to glass ionomer including intimate contact with the dentin walls and display hydrophilicity (Pekkan and Oezcan, 2012). Studies have pointed out that the adhesive bond between the ceramic restoration and the cement is the main factor for fracture resistance and marginal adaptation rather than the strength of the ceramic (Özcan and Mese, 2012, Koizumi et al., 2012).

Few authors have employed different surface treatments for stronger adhesive bond strength between LD ceramics and bioactive cements through increasing surface roughness (Bagis et al., 2011, Al-Sowygh, 2017). According to Koizumi et al. (2012), sandblasting reported serious damage to the ceramic surface resulting in low flexural strength; however, hydrofluoric surface treatment chemically modified the surface creating irregular interlocking surface. This technique improved interfacial bonding through an increase in the bonding area. In many instances, clinicians have applied silane coupling agent to decrease the wettability contact angle and increase the free energy of the ceramic surface (Koizumi et al., 2012, Al-Sowygh, 2017). Recently, the use of lasers in modern dentistry has led to increased beneficial effects in terms of painless removal of infected dentine and long term retention of the restoration. Er Cr YSGG (ECL) laser working is based on micro explosion creating surface irregularities for micro retention, which is considered favourable surface treatment compared to hydrofluoric acid that has the tendency to ablate the tissue in process of etching surface. Hence, using hydrofluoric acid on the chairside is risky for the patient, (Kursoglu et al., 2013, Vohra et al., 2019).

In the current view, the self-etched resin had replaced the conventional bonding technique of the glass ceramics using the resin based luting agents (Yilmaz-Savas et al., 2016). Self etch agents such as Rely X unicem are commonly employed as bonding cement in ceramic restoration. The recent introduction of Bioactive cements with inherent beneficial properties such as an efficient dentinal bond, resin tag supported adhesion, caries resistance, continuous fluoride release, dentin formation, and pulp protection compels to opt as a luting agent in the ceramic restoration (Al-Sowygh, 2017, Chaharom et al., 2018). Therefore, it is hypothesized Bioactive cements would show higher adhesive bond strength in Er Cr YSGG laser treated disilicate glass ceramics. Literature provides series of studies comparing the self etch and conventional luting agents bond strength in ceramics; however, there is no plausible data for displaying the adhesive bond strength in regards to bioactive cements in ceramic under different surface treatment (Yaman et al., 2014, Aguiar et al., 2014). Thus, the present study intends to evaluate the adhesive bond strength of bioactive cements in Lithium disilicate ceramics under two surface treatment; hydrofluoric acid and Er Cr YSGG treatment in comparison to the resin based luting agents.

MATERIALS AND METHODS

The present in vitro study evaluated the adhesive bond strength of Bioactive cement to Er-Cr-YSGG laser treated Lithium disilicate ceramics. Under the heat pressed technique, a total of 90 lithium disilicate ceramic disks were fabricated (EMax Press, Ivoclar Vivadent, AG, Schaan/Liechtenstein MO1 lot no.10721) with height (Ø) and diameter of 3 mm x 3 mm, respectively. The materials details are provided in table 1. To achieve a flat base, the ceramic disc was embedded into the acrylic resins with PVC (polyvinyl chloride) particles. The disks were finished through grinding under running water using silicon carbide paper on a polishing machine (Buehler Polishing Machine type: 49-5100-230, No 620-PXB-22061, Germany). The 90 specimens were initially divided into three main groups (n= 30) followed by distribution into 3 groups in each category (n= 10) depending upon the surface treatment and the type of luting agent used, correspondingly.The group distribution according to the surface treatment is described as follows:

Group 1 HF -S (silane) (Control): The specimen's surface was treated with the 9.6% concentration hydrofluoric acid (Pulpdent Corporation, USA). The etchant is applied for 1 min and washed along with air dried for 2 minutes. Subsequently, after cleaning, silane adhesive (Silane bond enhancer, Pulpdent- Watertown, MA, USA) was smeared over the etched surface using a micro brush and allowed to dry for 5 mins.

Group 2 ECL-2: The specimen's surface was treated with Er Cr:YSGG laser (water lase I plus, Biolase, USA) at a power of 3.75W and frequency of 15 Hz (L1), air-water 90-70% for 2 mins. Followed by the application of silane adhesive with micro brush and allowing it to dry for 5 mins.

Group 3 ECL-4: A similar procedure as group 2 proceeded; however, the laser surface treatment prolonged for 4 Minutes. Moreover, the specimens in both laser treated groups were prepared with the use of a gold handpiece using an MZ10 tip size (Er Cr:YSGG , water lase I plus, Biolase, USA). The focus of the handpiece was in the centre for 30 seconds followed by a standard clockwise rotational movement for the remaining time at 2 mm distance. Subsequently, each of the three groups was further divided according to the type of luting agent used.

Group HF-S-Bioactive: Bonding with ACTIVA- Bioactive cement (Pulpdent- Watertown, MA, USA).
Group HF-S-Rely-X: Bonding with RelyX Unicem (3M, St. Paul, MN, USA)

Group HF-S-Rely-ARC: Bonding with RelyX-ARC (3M, St. Paul, MN, USA)
Group ECL-2-Bioactive: Bonding with ACTIVA- Bioactive cement
Group ECL-2-Rely-X: Bonding with RelyX Unicem,
Group ECL-2:Rely-ARC: Bonding with RelyX-ARC
Group ECL-4-Bioactive: Bonding with ACTIVA- Bioactive cement
Group ECL-4-Rely-X: Bonding with RelyX Unicem
Group ECL-4:Rely-ARC: Bonding with RelyX-ARC

Following the preparation, each of the specimen disks was coated with either of three different types of the luting agent. The luting agents were polymerised using curing light (Bluephase®, Ivoclar Vivadent, Schaan, Liechtenstein) at a light intensity of 1,000 mW/cm2 for 10 seconds. Consequently, after complete preparation of the specimen, using a putty mould (Polyvinyl siloxane-Express, 3M Center St. Paul, MN, USA) the resin composite (Multicore flow, Ivoclar Vivadent Schaan, Liechtenstein) (Ø 2 mm, depth 2 mm) was build upon the specimen disk and polymerised from each side for 40 seconds in total. Each specimen was placed in thermocycler afterward (Thermocycler SD Mechatronik, GmbH Dental Research Equipment, Germany) for 5000 cycles at 5^0C and 55^0C (dwelling time: Cold bath, 30 sec; Hot bath, 30 sec).

Subsequent to thermocycling, the specimen from each group was placed under load in the universal testing machine (Instron 8500 Plus, 100 Royal St. Canton USA). The chisel of the universal machine is placed on the specimen at a perpendicular direction at a control force rate of 1 mm/min until the build-up materials were detached from the ceramic surface. To examine the failure mode of the detached surface, the digital microscope (Hirox- KH7700) was used. The failures are observed at three levels: adhesive failure at the luting agent and ceramic interface, cohesive: failure within the cement or the ceramic or composite materials, and admixed: Failure at luting/ceramic interface, progressing into luting cement.

All the data collected for the adhesive bond strength was processed and tabulated using a statistical program for social science (SPSS). The analysis of normally distributed data was performed using Kolmogorov-Smirnov test. The shear bond strength was analysed using analysis of variance and Tukey's – Kramer multiple tests ($p<0.05$). Furthermore, all data recorded was subjected to Levene's test of homogeneity of variance ($\alpha= 0.05$) following the assumption of equal variances.

RESULTS AND DISCUSSION

Kolmogorov-Smirnov test presented with normal distribution of data. The analysis of variance (ANOVA) displayed a significant difference in the adhesive bond strength of bioactive cement to Er Cr YSGG laser treated Lithium disilicate ceramics in comparison to the resin based luting cements ($p=0.01$). The outcome of the present study displayed significant difference ($p<0.05$) for the two different surface treatments employed in the bioactive cement group. Resin luting cements also presented significant difference with the two surface treatment; HF and Er Cr YSGG laser treatment employed ($p<0.05$).

The present study demonstrated that the maximum mean value for adhesive shear bond strength was 23.55 (±2.26)

Table 1: Details of the materials used in the study

Material	Composition Details	Filler
RelyX™ Unicem 3M, ESPE, St Paul, MN,USA	Methacrylated phosphoric ester Dimethacrylate (TEGDMA, Bis-GMA), Stabiliser, Peroxy compound, Substituted pyrimidine Pigment, Calcium hydroxide	Barium glass, ytterbium trifluoride, and mixed oxide
RelyX™ ARC 3M, ESPE, St Paul,MN. USA	TEGDMA, Bis-GMA, Benzoic peroxide, amine, photo-initiator, pigment.	Barium-alumino-fluoro-borosilicate glass, Strontium alumino -fluoro-silicate glass , Zirconia powder
ACTIVA TM, Bioactive dental cement Pulpdent, Watertown, MA, USA	Blend of diurethane and other methacrylates with modified polyacrylic acid (44.6%) contain no Bisphenol A, No Bis-GMA, No BPA derivatives	Amorphous silica (6.7%) Sodium fluoride (0.75%)

for the group Rely-x unicem ¬- HF-S and the minimum mean value was 14.30 (±2.08) for the group bioactive cement-ECL-2. The means and standard deviations of bond strength obtained are summarized in table 2. Each luting agent used in the study presented with a different set of bond strength mean value varying according to the employed surface treatments, respectively. Comparing the luting agent outcomes indicated an evident difference in shear bond strength of Activa and Relyx cements (unicem and ARC) ($p < 0.01$). The surface prepared with the silanised process (HF-S) specifically produced better shear bond strength results. Multiple comparisons test demonstrated a significant difference between the bioactive and Relyx cements (unicem and ARC) only under the hydrofluoric – silane surface treatment. In contrast, the laser treated surface (ECL-2 and ECL-4) presented with an evident difference between bioactive and Rely – ARC cement whereas exhibiting comparable results between the Activa and RelyX Unicem. Despite the fact there was a significant difference in the result; nevertheless, Bioactive cements presented with lower bond strength compared to the resin luting agents.

Analysing the effect of the surface treatment on each material, the result of bioactive cements presented a significant difference between the two laser groups [ECL-2 (14.30 (2.08), ECL-4 (17.37 (2.23)] and HF-S group (17. 45 (2.40)) with ECL-2 (14.30 (2.08)) respectively. Whereas comparable outcome was observed between the HF-S and ECL -4 groups ($p > 0.05$). Rely-X cements (unicem and ARC) showed an evident difference between the HF-S and laser treated group (ECL-2); however, Rely X unicem presented similar results between the two types of laser treatment. In addition, a significant difference was observed between the HF-S and laser treated group (ECL-4) in Rely X unicem while no significant difference in Rely X ARC. Thus, bioactive cements showed an insignificant difference in adhesive bond strength in Er-Cr-YSGG treated lithium disilicate compared to the HF-S group. Failure mode outcomes exhibited adhesive failure in the majority of the specimens compared to cohesive and admixed failures. The result indicated that only specimens in ECL-2 group presented in Bioactive material showed 100% adhesive failure. Overall, only 20 – 30% of observed failures were admixed type. The results directed that the ECL-2 had a profound effect on the shear bond strength of bioactive cement compared to Rely X cements. These Rely X cements in between them demonstrated a comparable number of failures in each surface treatment. However, ECL- 4 treatment did not show an apparent difference in the type of failure among either cement.

The present study evaluates the adhesive bond strength of bioactive cement to Er-Cr-YSGG laser treated lithium disilicate ceramics based on the hypothesis that bioactive cements exhibit better adhesive bond strength compared to self etch resin based luting agents. The study compared bonding strength of bioactive cements under hydrofluoric acid and Er Cr YSGG surface treatment for 2 and 4 minute duration. Self etched resin cements (Rely X unicem) demonstrated higher bond strength compared to the bioactive cements. Furthermore, under different surface treatments bioactive cements showed significant change in shear bond strength to LD ceramics. Therefore the hypothesis was rejected. A multitude of explanations can be provided for the outcomes in the present study.

To maintain the homogeneity and standardisation the adhesive shear bond strength was assessed using a universal testing machine. All the specimens were thermocycled to ensure the shear bond strength tested was in a standard environment. Studies have reported that thermocycling determines a positive change in surface bonding, which has beneficial long term effects on the ceramic restoration (Brum et al., 2011, Lee et al., 2017). Thermocycling aids in the water resorption property that causes the ageing of the bond resulting in weak bonds (Brum et al., 2011). The bioactive cements in previous studies have demonstrated an insignificant decrease in adhesive bond strength to ceramics compared to resin based luting cement despite the fact some detrimental effects are associated with water absorptiondue to the methacrylate in resin cements (Brum et al., 2011, Ahrari et al., 2017). In the present study, Rely X cements showed higher bond strength compared to the bioactive cements. Rely X unicem (self etched) displayed higher mean value in HF surface treatment while Rely X ARC (resin based) displayed high mean value in laser treated group. Nevertheless, comparable bond strength was observed between the self etch and resin cements. The results of the present study partially supported the outcomes noted by the previous studies regarding higher bond strength in bioactive cements (Al-Sowegh, 2017).

The plausible explanation identified was that self etch cements uses dual cure method to polymerise; therefore, presented with better bond strength (Chaharom et al.,

Table 2: Means and SD for shear bond strength among study groups

Luting agent	HF-S	ECL-2	ECL-4	P value
Bioactive (Activa)	17. 45 (2.40)^{aA}	14.30 (2.08)^{bB}	17.37 (2.23)$^{a\ A}$	< 0.01
RelyX- Unicem	23.55 (2.26)^{bA}	16.37(3.11)^{abB}	18.40 (2.61)^{abB}	
Rely-X ARC	21.37 (2.38)^{bA}	18.33 (2.83)^{aB}	20.65 (2.76)^{bAB}	

Dissimilar superscript small alphabets in same column show significant difference ($p< 0.05$)
Dissimilar superscript capital alphabets in same row show significant difference ($p< 0.05$)

2018). The lower bond strength of the bioactive cements suggested that the durability of the bond depends upon the nature of the mechanical bond between the ceramics and cements (Gurney et al., 2016). As ceramics are inert in nature they do not display chemical change that would contribute to the bonding strength (Kursoglu et al., 2013). In addition, theoretically, the silane bond on the ceramic surface binds effectively to the micro retentive etched surface. However, the bioactive cements offer limited methacrylate group for bonding to the silane groups compared to resin based cements; thus, resulting in low bond strength (Ahrari et al., 2017, Al-Sowygh, 2017).

Several studies documented the evident effect of hydrofluoric acid on the interfacial bonding surface (Lee et al., 2017, Kalavacharla et al., 2015). Whereas few previous studies used different intensity laser application to identify the effect on the ceramic surface (Passia et al., 2015, Kalavacharla et al., 2015). Therefore, to observe the evident effect of the varying surface treatment on the ceramic surface, two types of surface treatments were employed HF-S and Er Cr YSGG laser treatment. In the present study, the duration of the laser was varied in order to observe the effect on the adhesive bond strength to lithium disilicate ceramics. It is reported that laser treated surface often displays greater surface roughness irregularities and patterns, unlike the HF that dissolves the ceramics glassy matrix to form interlocking surface (Neis et al., 2015). The interlock mechanism implicates better micromechanical retention compared to an irregular surface. Intriguingly, a prolonged period of laser application increase surface roughness; nevertheless, the laser produces excessive heat that results in the weakening and over destruction of the surface (Gurney et al., 2016). Hence, laser treated ceramics with prolonged duration demonstrated higher bond strengths but comparatively less than the HF study groups.

Table 3: Failure type percentage among study groups

Study Groups	Adhesive	Cohesive	Mixed
Group HF-S-Bioactive	70	0	30
Group HF-S-RelyX	80	0	20
Group HF-S-Rely-ARC	80	0	20
Group ECL-2-Bioactive	100	0	0
Group ECL-2-RelyX	70	0	30
Group ECL-2-Rely-ARC	80	0	20
Group ECL-4-Bioactive	70	0	30
Group ECL-4-RelyX	70	0	30
Group ECL-4-Rely-ARC	60	0	40

The mode of failure assessment presented a higher number of adhesive failures followed by admixed. No specimen demonstrated cohesive failure in the present study attesting no measurement of material strength. The bioactive cements displayed an increased number of adhesive failure only in ECL 2 group while comparable results were appreciated in other groups. This indicates employing bioactive cements at laser treated surface with 2 minutes duration results in the formation of weak adhesive bonds comparatively to laser treated surface at 4 minutes duration. Nevertheless, failure mode assessment displayed comparable adhesive bond strength of bioactive cements and resin luting cements.

Certain limitations were identified in the present study despite the fact it provided a clear comparison between the bioactive cements and resin based luting agents. The results of the study are applicable only in reference to the particular surface treatments employed, particularly the type of laser (Er Cr YSGG) used. For better understanding of the bonding strength of bioactive cements to ceramics, studies comparing different types of ceramics are recommended. Although the study presented lower bond strength of bioactive cements to the ceramics; however, the status of fracture resistance of bioactive cements is questionable and needs to be evaluated. Lithium disilicate ceramics are well established restoration used in dentistry exhibiting inherent properties necessary for efficient adhesive bonding and long term retentive restoration. The mechanical and biological properties of bioactive cements shows great potential in bonding with the tooth; however, literature provides limited data with respect to its bonding with the ceramics. Therefore it is recommended to evaluate the fracture resistance of these Biocements prior to the application of different laser with different types of ceramic restorations.

CONCLUSION

The adhesive bond strength of the bioactive cements to lithum disilicate ceramics was lower than the resin based luting cements. Use of 4 minutes of Er Cr YSGG laser treatment displayed an increase in the adhesive bond strength of bioactive cement to Lithium disilicate ceramics. Despite the low bond strength, Bioactive-luting agents exhibited durable bond strength to the ceramics.

REFERENCES

Aboushelib, M. N. and Sleem, D. (2014). Microtensile Bond Strength Of Lithium Disilicate Ceramics To Resin Adhesives. J Adhes Dent, 16(6):547-52.

Aguiar, T. R., André, C. B., Correr-Sobrinho, L., Arrais, C. A., Ambrosano, G. M. and Giannini, M. (2014). Effect Of Storage Times And Mechanical Load Cycling On Dentin Bond Strength Of Conventional And Self-Adhesive Resin Luting Cements. The Journal Of Prosthetic Dentistry, 111(5):404-10.

Ahrari, F., Boruziniat, A., Mohammadipour, H. S. and Alirezaei, M. (2017). The Effect Of Surface Treatment With A Fractional Carbon Dioxide Laser On Shear Bond Strength Of Resin Cement To A Lithium Disilicate-Based Ceramic. Dental Research Journal, 14(3):195.

Al-Sowygh, Z. H. (2017). Bond Strength Of Novel Bioactive Resin Modified Luting Agent To Lithium

Disilicate Dental Ceramic. Journal Of Biomaterials And Tissue Engineering, 7(12):1349-54.

Bagis, B., Bagis, Y. H. and Hasanreisoglu, U. (2011). Bonding Effectiveness Of A Self-Adhesive Resin-Based Luting Cement To Dentin After Provisional Cement Contamination. Journal Of Adhesive Dentistry, 13(6).

Brum, R., Mazur, R., Almeida, J., Borges, G. and Caldas, D. (2011). The Influence Of Surface Standardization Of Lithium Disilicate Glass Ceramic On Bond Strength To A Dual Resin Cement. Operative Dentistry, 36(5):478-85.

Chaharom, M. E. E., Azar, F. P., Mohammadi, N. and Nasiri, R. (2018). Effect Of Surface Preparation With Nd: Yag And Er, Cr: Ysgg Lasers On The Repair Bond Strength Of Lithium Disilicate Glass Ceramic To A Silorane-Based Composite Resin. Journal Of Dental Research, Dental Clinics, Dental Prospects, 12(1):12.

Elsayed, A., Younes, F., Lehmann, F. and Kern, M.(2017). Tensile Bond Strength Of So-Called Universal Primers And Universal Multimode Adhesives To Zirconia And Lithium Disilicate Ceramics. J Adhes Dent, 19(3):221-8.

Gurney, M. L., Sharples, S. D., Phillips, W. B. and Lee, D. J. (2016). Using An Er, Cr: Ysgg Laser To Remove Lithium Disilicate Restorations: A Pilot Study. The Journal Of Prosthetic Dentistry, 115(1):90-4.

Kalavacharla, V. K., Lawson, N., Ramp, L. and Burgess, J. (2015). Influence Of Etching Protocol And Silane Treatment With A Universal Adhesive On Lithium Disilicate Bond Strength. Operative Dentistry, 40(4):372-8.

Klosa, K., Boesch, I. and Kem, M. (2013) Long-Term Bond Of Glass Ceramic And Resin Cement: Evaluation Of Titanium Tetrafluoride As An Alternative Etching Agent For Lithium Disilicate Ceramics. Journal Of Adhesive Dentistry, 15(4).

Koizumi, H., Nakayama, D., Komine, F., Blatz, M. B. and Matsumura, H. (2012). Bonding Of Resin-Based Luting Cements To Zirconia With And Without The Use Of Ceramic Priming Agents. Journal Of Adhesive Dentistry, 14(4).

Kursoglu, P., Motro, P. F. K. and Yurdaguven, H. (2013). Shear Bond Strength Of Resin Cement To An Acid Etched And A Laser Irradiated Ceramic Surface. The Journal Of Advanced Prosthodontics, 5(2):98-103.

Lee, H.-Y., Han, G.-J., Chang, J. and Son, H.-H. (2017). Bonding Of The Silane Containing Multi-Mode Universal Adhesive For Lithium Disilicate Ceramics. Restorative Dentistry and Endodontics, 42(2):95-104.

Neis, C. A., Albuquerque, N. L. G., Albuquerque, I. D. S., Gomes, E. A., Souza-Filho, C. B. D., Feitosa, V. P., Spazzin, A. O. and Bacchi, A. (2015). Surface Treatments For Repair Of Feldspathic, Leucite-And Lithium Disilicate-Reinforced Glass Ceramics Using Composite Resin. Brazilian Dental Journal, 26(2):152-5.

Niu, E., Agustin, M. and Douglas, R. D. (2013). Color Match Of Machinable Lithium Disilicate Ceramics: Effects Of Foundation Restoration. The Journal Of Prosthetic Dentistry, 110(6):501-9.

Niu, E., Agustin, M. and Douglas, R. D. (2014). Color Match Of Machinable Lithium Disilicate Ceramics: Effects Of Cement Color And Thickness. The Journal Of Prosthetic Dentistry, 111(1):42-50.

Özcan, M. and Mese, A. (2012). Adhesion Of Conventional And Simplified Resin-Based Luting Cements To Superficial And Deep Dentin. Clinical Oral Investigations, 16(4):1081-8.

Palla, E.-S., Kontonasaki, E., Kantiranis, N., Papadopoulou, L., Zorba, T., Paraskevopoulos, K. M. and Koidis, P. (2018). Color Stability Of Lithium Disilicate Ceramics After Aging And Immersion In Common Beverages. The Journal Of Prosthetic Dentistry, 119(4):632-42.

Passia, N., Lehmann, F., Freitag-Wolf, S. and Kern, M. (2015). Tensile Bond Strength Of Different Universal Adhesive Systems To Lithium Disilicate Ceramic. The Journal Of The American Dental Association, 146(10):729-34.

Pekkan, G. and Oezcan, M. (2012). Radiopacity Of Different Resin-Based And Conventional Luting Cements Compared To Human And Bovine Teeth. Dental Materials Journal, 1201190225.

Vohra, F., Labban, N., Al-Hussaini, A., Al-Jarboua, M., Zawawi, R., Alrahlah, A. and Naseem, M. (2019). Influence Of Er; Cr: Ysgg Laser On Shear Bond Strength And Color Stability Of Lithium Disilicate Ceramics: An In Vitro Study. Photobiomodulation, Photomedicine, And Laser Surgery, 37(8):483-8.

Yaman, B. C., Ozer, F., Takeichi, T., Karabucak, B., Koray, F. and Blatz, M. B. (2014). Effect Of Thermomechanical Aging On Bond Strength And Interface Morphology Of Glass Fiber And Zirconia Posts Bonded With A Self-Etch Adhesive And A Self-Adhesive Resin Cement To Natural Teeth. The Journal Of Prosthetic Dentistry, 112(3):455-64..

Yilmaz-Savas, T., Demir, N., Ozturk, A. N. and Kilic, H. S. (2016). Effect Of Different Surface Treatments On The Bond Strength Of Lithium Disilicate Ceramic To The Zirconia Core. Photomedicine And Laser Surgery, 34(6):236-43.

Zarone, F., Ferrari, M., Mangano, F. G., Leone, R. and Sorrentino, R. (2016). Digitally Oriented Materials: Focus On Lithium Disilicate Ceramics. International Journal Of Dentistry, 2016.

Biotechnological Communication

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp-129-133

Ascorbic Acid and Total Phenolic Contents of Dried Roasted Chestnut (*Castanea sativa*) Affected by Drying, Roasting and Preservation

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ABSTRACT

This Chestnut (*Castanea sativa*) is a gluten-free seasonal nut. Chestnut has become important composition for human diet owing to their demonstrated nutritional qualities and healthy benefits. Its seed has radical scavenging activity with a rich source of natural antioxidants for different applications. It contains various alkaloids, flavonoids, volatile oils and terpenoids essential for variety of antimicrobial, antitumor, antioxidant activities. Owing to the healthy constituents in chestnut seed, there is a potential supplement for human meal. Drying and roasting are very important in enhancing color, flavor, appearance and taste in seeds. There was not many research related to the change of antioxidant during hot air drying as well as storage. So purpose of this study was to examine the effectiveness of thermal treatment in hot air drying, roasting, packaging and storage to vitamin C and total phenolic in the dried chestnut (*Castanea sativa*) seeds. Results revealed that drying temperature (45 ^{o}C), roasting (135 ^{o}C in 10 min), packing in polyethylene bag and keeping in dry cool place were suitable to preserve the vitamin C, total phenolic contents in the samples for 12 months. The drying, roasting temperature and duration were thoroughly identified to manufacture the dried roasted chestnut seeds.

KEY WORDS: *CHESTNUT, DRYING*, ROASTING, PRESERVATION, VITAMIN C, PHENOLIC.

INTRODUCTION

Chestnut (Castanea sativa Mill) belongs to the family Fagaceae. This nut has a smooth and coriaceous epicarp, which can be light brown or deep brown in colour with more or less evident stripes, (Kosnovska,2013). It's nutritionally rich with high content of sugar, starch, dietary fibre, high quality protein, low lipids, rich in vitamins and minerals, good source of fatty acids and antioxidants (Chenlo F. et al., 2007; Borges et al., 2007; Kunsch et al., 1999; Vasconcelos et al., 2007). There were several studies mentioned to processing of chestnut. Effects of roasting on chemical composition and quality of different chestnut (*Castanea sativa Mill*) varieties were examined (Kunsch et al., 2001). The total vitamin C content and antioxidant activity of raw and cooked chestnuts was evaluated. The cooking process significantly changed the antioxidant activity of the chestnuts. A significant decrease in the vitamin C content of the chestnuts was observed (Barros et al., 2011). The effects of pan and microwave roasting on physicochemical, functional, rheological and antioxidant properties of sweet chestnut were compared.

Roasting increased the water absorption capacity and oil absorption capacity of chestnut flours making them potentially useful in flavor retention and improvement

ARTICLE INFORMATION

*Corresponding Author: minh.np@ou.edu.vn
Received 9th Feb 2020 Accepted after revision 25th March 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/23

of palatability. Roasted flours had higher TPC and antioxidant activity. The gelatinization temperatures were higher in roasted flours while as their viscoelastic behavior was lower as compared to the native flour. Roasting also improved the flavor of chestnuts with microwave roasted chestnuts expressing better aroma as compared to the pan roasted (Wani et al., 2017). The effect of hot air convective drying on the organic acid profile of chestnut was evaluated (Delgado et al., 2018). Most studies conducted on hot air drying of chestnuts have examined various technological varibale such as chemical parameters (Correia, et al., 2009, 2012; Moreira et al., 2013; Zhang et al., 2011), temperature effect on morphological and rheological attributes of chestnut flours, rehydration effect (Attanasio et al., 2004; Moreira et al., 2008; Moreira et al., 2011), energetic requirements (Koyuncu et al., 2004), drying kinetics (Cletus et al., 2008; Delgado et al., 2014; Guiné et al., 2006; Moreira et al., 2005). Purpose of the this study was to identify the efficacy of thermal treatment in hot air drying, roasting, packaging and preservation to vitamin C, total phenolic in the dried Chestnut (*Castanea sativa*) seed.

MATERIAL AND METHODS

Materials: Chestnut, Castanea sativa was collected from Tien Giang province, Vietnam. They were cultivated following VietGAP without using pesticide or insecticide to ensure food safety. After collecting, harvested seeds were preserved at dry cool place and conveyed to laboratory as soon as possible for experiments. These seeds were cleaned thoroughly by air blowing to remove foreign matters. The seeds were sorted to get the uniformity and defect-free ones. Before roasting treatment, chestnut kernels were submersed in 15% (w/w) salt solution for 30 min. Then, the draining water of sieved seeds was removed. Apart from chestnut, Castanea sativa, we also used other materials during the research such as NaCl, HCl, Na2CO3, Folin-Ciocalteau. Lab utensils and equipment included weight balance, hot air dryer and spectrophotometer.

Phytochemical constituents inside fresh Chestnut (*Castanea sativa*) seed: The phytochemical constituents such as protein (%), fat (%), moisture (%), vitamin C (mg/g), total phenolic (mg/g) in fresh Chestnut (*Castanea sativa*) were measured as follows: Protein (by Kjeldahll), fat (by Soxhlet) and moisture (drying to constant weight) were used. Vitamin C (mg/g) were performed by a validated hydrophilic interaction chromatography method (Barros et al., 2010). Total phenolic (mg/g) was determined colorimetrically using Folin- Ciocalteau reagent by spectrophotometer. Efficacy of hot air drying temperature to vitamin C, total phenolic in dried Castanea sativa seed: In order to examine the efficacy of hot air drying temperature to the dried Chestnut (*Castanea sativa*) seed, the vitamin C, total phenolic were measured before and after drying in different hot air drying temperature (35 °C, 40 °C, 45 °C, 50°C). Efficacy of roasting conditions on vitamin C, total phenolic in the dried Chestnut (Castanea sativa) seed: At the end of drying treatment, the dried seeds were roasted at different conditions (130 °C for 15 min, 135 °C for 10 min, and 140 °C for 5 minutes). The vitamin C (mg/g), total phenolic (mg/g) were analyzed to determine the optimal roasting condition. Efficacy of storage temperature to vitamin C, total phenolic in dried Chestnut (Castanea sativa) seed. The dried Chestnut (Castanea sativa) seeds were preserved in PA bag in 4°C, 30°C. The vitamin C (mg/g), total phenolic (mg/g) were monitored in 3 month-interval for 12 months. Statistical analysis: All experiments were run in triplicate with three different lots of samples. Statistical analysis was performed by the Statgraphics Centurion XVI.

RESULTS AND DISCUSSION

Phytochemical constituents in raw Chestnut (*Castanea sativa*). The phytochemical constituents in raw Chestnut (*Castanea sativa*) seed were analyzed. The present results revealed that raw chestnut seed has high content of vitamin C and total phenolic (Table 1).

Table 1. The phytochemical constituents in raw Chestnut (*Castanea sativa*) seed

Variables	Protein (%)	Fat (%)	Moisture (%)	Vitamin C (mg/g)	Total phenolic (mg/g)
Value	24.76± 0.01	6.25± 0.00	57.38± 0.03	12.63± 0.01	49.28± 0.00

Note: the values were expressed as the mean of three repetitions; the same characters (denoted above), the difference between them was not significant (α = 5%)

Efficacy of hot air drying temperature to vitamin C, total phenolic contents in dried Chestnut seeds: Convective drying depended on different variables such as temperature, relative humidity and velocity of the drying air; size, shape and loading density of the samples (Moreira et al., 2015). In order to examine the efficacy of hot air drying temperature to the dried Chestnut (*Castanea sativa*) seed, the vitamin C, total phenolic was measured before and after drying in different hot air drying temperature (35 °C, 40 °C, 45 °C, 50°C). From table 2, the Chestnut (*Castanea sativa*) was dried at 45oC to preserve the highest amount of vitamin C (mg/g), total phenolic (mg/g). In another report, the thermal process at 50°C caused equivalent losses of ascorbic acid (Delgado et al., 2018). Roasting, boiling and frying procedures lead to significant reduction of total organic acids contents (Barbara Ribeiro et al., 2007). Fresh chopped chestnuts were dried using a hot air convective tray dryer at different temperatures (45, 65 and 85°C) and loading densities (2.5 and 6.3 kg/m2). Total and damaged starch varied significantly with drying temperature and loading density (Moreira et al., 2015).

Efficacy of roasting conditions on vitamin C, total phenolic in the dried Chestnut (Castanea sativa) seed:

Chestnut has the high proportion of tannins in the inner shell. Tannins are known for their astringent bitter taste that reduce the palatability. Roasting before their utilization is a normal handling to enhance the color and flavor. The thermal treatment received during roasting changes the nutritional compositions of chestnuts by increasing the antioxidant activity and reducing the anti-nutritional factors such as tannins (Chang et al., 2016). In this process, the nuts are heated applying the conventional thermal treatment, such as air convection and pan or sand roasting at 250–300°C for a short time (Demir et al., 2002; 2005;Schlörmann et al., 2015; Sharma et al., 2011).

In this research, at the end of drying treatment, the dried seeds were roasted at different conditions (130 °C for 15 min, 135 °C for 10 min, and 140 °C for 5 minutes). The vitamin C, total phenolic were analyzed to determine the optimal roasting condition. Results were elaborated in table 3. Chestnut (Castanea sativa) seed must be roasted at 135 °C for 10 min to preserve vitamin C (mg/g), total phenolic (mg/g) at the highest level. In another research, the effects of roasting on starch, sugars and fatty acid composition and on chestnut quality were verified. Weight loss of chestnut by roasting from 23-30% while causing little change in composition (Kunsch et al., 2001).

Roasting reduced the anti-nutritional factors in chestnut. Protein, fat, and ash contents displayed insignificant variation upon roasting. Significant increase in water absorption capacity, oil absorption capacity, and antioxidant properties was observed following roasting. Flour obtained from roasted chestnuts exhibited a significant decrease in light transmittance, foaming, and pasting properties. Higher gelatinization temperatures and lower enthalpies were reported in microwave and pan roasted chestnut flours. Roasting also reduced the viscoelastic behavior of native sweet chestnut and

Table 2. Vitamin C (mg/g), total phenolic (mg/g) in dried Chestnut (*Castanea sativa*) seed by the effect of hot air drying temperature (°C)

Parameter	Raw Chestnut (*Castanea sativa*) before drying	Dried Chestnut (*Castanea sativa*) seed by the effect of hot air at drying temperature (°C)			
		35	40	45	50
Vitamin C (mg/g)	12.63±0.01^{a}	9.37±0.00^{b}	9.13±0.02^{bc}	9.04±0.00^{bc}	8.35±0.00^{c}
Total phenolic (mg/g)	49.28±0.00^{a}	31.53±0.01^{b}	31.05±0.02^{bc}	30.48±0.01^{bc}	29.74±0.02^{c}

Note: the values were expressed as the mean of three repetitions; the same characters (denoted above), the difference between them was not significant (α = 5%).

Table 3. Efficacy of roasting conditions on vitamin C (mg/g), total phenolic contents (mg/g) in the roasted dried Chestnut (*Castanea sativa*) seed

Roasting conditions	130 °C for 15 min	135 °C for 10 min	140 °C for 5 min
Vitamin C (mg/g)	7.15±0.03^{ab}	7.94±0.00^{a}	6.84±0.03^{b}
Total phenolic (mg/g)	26.49±0.00^{ab}	27.81±0.02^{a}	25.70±0.00^{b}

Note: the values were expressed as the mean of three repetitions; the same characters (denoted above), the difference between them was not significant (α = 5%).

Table 4. Vitamin C (mg/g), total phenolic (mg/g) in dried Chestnut (*Castanea sativa*) seed by the effect of storage temperature

Storage duration (months)	Dried Chestnut (*Castanea sativa*) seed stored in PA bag at 4°C		Dried Chestnut (*Castanea sativa*) seed stored in PA bag at 30°C	
	Vitamin C (mg/g)	Total phenolic (mg/g)	Vitamin C (mg/g)	Total phenolic (mg/g)
0	7.94±0.00^{a}	27.81±0.02^{a}	7.94±0.00^{a}	27.81±0.02^{a}
3	7.83±0.02^{ab}	27.24±0.03^{ab}	7.74±0.03^{ab}	27.19±0.00^{ab}
6	7.59±0.03^{b}	27.03±0.00^{b}	7.50±0.03^{b}	26.95±0.01^{b}
9	7.48±0.00^{bc}	26.86±0.02^{bc}	7.39±0.01^{bc}	26.77±0.01^{bc}
12	7.39±0.01^{c}	26.50±0.01^{c}	7.31±0.02^{c}	26.39±0.03^{c}

Note: the values were expressed as the mean of three repetitions; the same characters (denoted above), the difference between them was not significant (α = 5%).

changed the transmittance of identical functional groups (Wani et al., 2017).

Efficacy of storage temperature to vitamin C, total phenolic in dried Chestnut (*Castanea sativa*) seed: Roasting also improved the digestibility and stability of chestnut (Wani et al., 2017). The dried Chestnut (Castanea sativa) seeds were preserved in PA bag in 4°C, 30°C. The vitamin C, total phenolic were monitored in 3 month-interval for 12 months. From table 4, the roasted dried Chestnut (Castanea sativa) seed must be stored in PA (vaccum) bag in dry cool place so that the vitamin C (mg/g), total phenolic (mg/g) could be preserved for 12 months.

CONCLUSION

Antioxidants are valuable phytochemical elements contributing to our health benefits. Owing to the high antioxidant capacity as well as a rich source of vitamins, Castanea sativa seeds were demonstrated to prevent degenerative diseases associated with free radical damage. Drying and roasting are very important steps showing significant alterations in the manufacturing of value-added nuts with better taste, aroma, texture, crispiness. Especially, roasting can improve color and aroma through caramelization on the surface of the dried chestnut

REFERENCES

Attanasio, G.; Cinquanta, L.; Albanese, D.; Matteo, M. D. (2004). Effects of drying temperatures on physico-chemical properties of dried and rehydrated chestnuts (Castanea sativa). Food Chemistry 88: 583–590.

Barbara Ribeiro, Joana Rangel, Patrıcia Valentao, Paula B. Andrade, Jose Alberto Pereira, Hanna Bolke, Rosa M. Seabra (2007). Organic acids in two Portuguese chestnut (Castanea sativa Miller) varieties. Food Chemistry 100: 504–508.

Barros, A I Fernando M. Nunes, Berta Gonçalves, Richard N. Bennett, Ana Paula Silva (2011). Effect of cooking on total vitamin C contents and antioxidant activity of sweet chestnuts (Castanea sativa Mill.). Food Chemistry 128: 165–172.

Barros, A. I., Silva, A. P., Gonçalves, B., & Nunes, F. M. (2010). A fast, simple, and reliable hydrophilic interaction liquid chromatography method for the determination of ascorbic and isoascorbic acids. Analytical and Bioanalytical Chemistry 396: 1863–1875.

Borges O.B., Carvalho J.S., Corriera P.R., Silva A.P. (2007). Lipid and fatty acid profiles of Castanea sativa Mill. Chestnuts of 17 native Portuguese cultivars. J Food Compos Anal. 20: 80–89.

Chang S.K., Alasalvar C., Bolling B.W., Shahidi F. (2016). Nuts and their co-products: the impact of processing (roasting) on phenolics, bioavailability, and health benefits – a comprehensive review. J Funct Foods. 26: 88–122.

Chenlo F., Moreira R., Pereira G., Silva C.C. (2007). Evaluation of the rheological behaviour of chestnut (Castanea sativa mill) flour pastes as function of water content and temperature. Electron J Environ Agric Food Chem. 6: 1794–1802.

Cletus, A. B.; Carson, J. K. (2008). Drying curves and apparent diffusivity of New Zealand chestnut variety 6666666gy1015. Journal of Food Engineering 85: 381–386.

Correia, P.; Beirão-da-Costa, M. L. (2012). Effect of drying temperatures on starch-related functional and thermal properties of chestnut flours. Food and Bioproducts Processing 90: 284–294.

Correia, P.; Leitão, A.; Beirão-da-Costa, M. L. (2009). The effect of drying temperatures on morphological and chemical properties of dried chestnuts flours. Journal of Food Engineering 90: 325–332.

Delgado T, Elsa Ramalhosa, José Alberto Pereira, and Susana Casal (2018). Organic acid profile of chestnut (Castanea sativa Mill.) as affected by hot air convective drying. International Journal of Food Properties 21: 557–565.

Delgado, T.; Pereira, J. A.; Baptista, P.; Casal, S.; Ramalhosa, E. (2014). Shell's influence on drying kinetics, color and volumetric shrinkage of Castanea sativa Mill. fruits. Food Research International 55: 426–435.

Demir AD and K. Cronin (2005) Modelling the kinetics of textural changes in hazelnuts during roasting. Simulation Modelling Practice and Theory 13(2): 97–107.

Demir AD, J. M. Fraas Celayeta, K. Cronin, and K. Abodayeh (2002) Modelling of the kinetics of colour change in hazelnuts during air roasting. Journal of Food Engineering 55 (4): 283–292.

Guiné, R. P. F.; Fernandes, R. M. C. (2006). Analysis of the drying kinetics of chestnuts. Journal of Food Engineering 76: 460–467.

Kosnovska J (2013). The origin, archaeobotany and ethnobotany of sweet chestnut (Castanea sativa Miller) in the Czech Republic. Natural Sciences in Archaeology 4: 163–176.

Koyuncu, T.; Serdar, U.; Tosun, I. (2004). Drying characteristics and energy requirement for dehydration of chestnuts (Castanea sativa Mill.). Journal of Food Engineering 62: 165–168.

Kunsch U., Scharer H., Conedera M., Sassella A., Jermini M., Jelmini G. (1999). Quality assessment of chestnut fruits. Acta Hort. 494: 119–127.

Kunsch U, Hans Scharer, Bruno Patrian, Ernst Hohn, Marco Conedera, Alberto Sassella, Mauro Jermini and Giorgio Jelmini (2001). Effects of roasting on chemical composition and quality of different chestnut (Castanea sativa Mill) varieties. Journal of the Science of Food and Agriculture 81: 1106-1112.

Moreira, R., Chenlo, F., Torres, M.D., Rama, B. and Arufe, S. (2015). Air drying of chopped chestnuts at several conditions: effect on colour and chemical characteristics

of chestnut flour. International Food Research Journal 22: 407-413.
Moreira, R.; Chenlo, F.; Chaguri, L.; Fernandes, C. (2008). Water absorption, texture, and color kinetics of air-dried chestnuts during rehydration. Journal of Food Engineering 86: 584–594.
Moreira, R.; Chenlo, F.; Chaguri, L.; Mayor, L. (2011). Analysis of chestnut cellular tissue during osmotic dehydration, air drying, and rehydration processes. Drying Technology 2: 10–18.
Moreira, R.; Chenlo, F.; Chaguri, L.; Vázquez, G. (2005). Mathematical modelling of the drying kinetics of chestnut (Castanea sativa Mill.) influence of the natural shells. Food and Bioproducts Processing 83: 306–314.
Moreira, R.; Chenlo, F.; Torres, M. D.; Rama, B. (2013). Influence of the chestnuts drying temperature on the rheological properties of their doughs. Food and Bioproducts Processing 91: 7–13.
Schlörmann W, M. Birringer, V. Böhm (2015) Influence of roasting conditions on health-related compounds in different nuts. Food Chemistry 180: 77–85.
Sharma P and H. S. Gujral. (2011) Effect of sand roasting and microwave cooking on antioxidant activity of barley. Food Research International 2011; 44(1): 235–240.
Vasconcelos M.C.B.M., Bennett R.N., Rosa E.A.S., Ferreira-Cardoso J.V. (2007). Primary and secondary metabolite composition of kernels from three cultivars of Portuguese chestnut (Castanea sativa) at different stages of industrial transformation. J Agric Food Chem. 55: 3508–3516.
Wani IA, Humaira Hamid, Afshan Mumtaz Hamdani, Adil Gani, and Bilal Ahmad Ashwar (2017). Physico-chemical, rheological and antioxidant properties of sweet chestnut (Castanea sativa Mill.) as affected by pan and microwave roasting. J Adv Res 8: 399–405.
Zhang, M.; Chen, H.; Zhang, Y. (2011). Physicochemical, thermal, and pasting properties of Chinese chestnut (Castanea mollissima) starches as affected by different drying methods. Starch 63: 260– 267.

Hydrological Communication

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp-134-138

Evaluation of Traditional Wells in Hiznah Village, Albaha Region, Saudi Arabia

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ABSTRACT

This study aimed at evaluating 73 traditional wells in Hiznah village, Albaha region (Saudi Arabia). All information about the availability of water, well status, irrigation and drinking uses and associated plant species were recorded. The statistical method in terms of the determination of relationships between wells characteristics was analyzed using Spearman's correlation coefficient. Forty-seven (65.75%) of wells were concentrated in the north of study area, 14 wells (17.81%) in the middle and 12 wells (16.4%) in the south of the study area. The majority of wells (79.5%) showed water availability and almost (46.6%) of such wells were used for both irrigation and drinking purposes. Approximately, 58.9% of wells were open followed by 34.2% and 6.8% of covered and fenced, respectively. Plant species were associated with 35.6% of open and fenced wells. The plant species were; Ficus palmata (31.5%), Celtus africana (2.7%) and Ficus vasta (1.4%). The availability of water in most wells may reflect the energy of groundwater to feed them, which they are concentrated in the north of the study site and this may due to increased recharged rates in these areas. Groundwater wells need further investigations regarding their quality to avoid any health hazards to crops, animals, and humans if water is to be used for irrigation and drinking.

KEY WORDS: ALBAHA, HIZNAH, VILLAGE, HYDROLOGY, WELLS, IRRIGATION, DRINKING, TOPOGRAPHY.

INTRODUCTION

Freshwater is considered to be an important requirement for life, however, it represents only less than 3% of the total water volume in the world (Hahn, 2006). Groundwater is the one form of freshwater on earth and considered to be a renewable resource of water (APHA, 1998, 2012). Saudi Arabia is known as arid country and most of Saudi lands are deserts. The average of rainfall in Saudi Arabia is < 100 mm per year with no permanent lakes or rivers. The main sources of water are surface water, groundwater and desalination. The surface water consists of water that mainly captured in approximately 260 dam reservoirs. On the contrary, the amount of groundwater depends on the recharge rate which is higher in shallow wells than deep wells (Ouda, 2013). Groundwater in Saudi Arabia has long been used as the main source for the accomplishment of different purposes, such as domestic and agriculture supply, especially during 1970s and 1980s (Alsuhaimi et al., 2019).

The depth and water volume can be varied between traditional wells (hand-dug systems) (Yakubu, 2013). The agricultural sector consumes about 70% to 80% of freshwater in Saudi Arabia. However, up to five thousand litres of water are consumed for the production of 3 meals per person per day (Alsalah et al., 2015).In Saudi Arabia, quality assessments of groundwater have been

ARTICLE INFORMATION

*Corresponding Author: alibaggar@hotmail.com
Received 19th Feb 2020 Accepted after revision 25th March 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)
A Society of Science and Nature Publication, Bhopal India

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/24

studied previously (Al-Hasawi et al., 2018; Alsalah et al., 2015; Alsuhaimi et al., 2019; Bob et al., 2015; Ouda, 2013). To the best of author's knowledge, evaluating large number of traditional wells in one village in terms of scientific explanations for the distribution of wells, availability of water, well status, associated plant species and the possibility of water utilization for drinking and irrigation have not been addressed yet. Thus, the main objectives of this study are to evaluate all traditional wells in Hiznah village, Saudi Arabia during 2019, and to determine the relationships between the characteristics of these wells.

MATERIAL AND METHODS

The study sites: The study area Hiznah is located west of Baljurashi, between lat. 19.85387 and lat. 19.827907 and between long. 41.53561 and 41.55814, with altitude ranges between 1900 and 2167 (Fig. 1). Hiznah is a 3 km² village located in the south of Albaha region, Saudi Arabia (Fig. 1). Almost 1.25 thousand people currently live in Hiznah village. All traditional wells of Hiznah village (73 wells) were selected during 2019, (Table 1). All information about each well in terms of the availability of water, well status, irrigation and drinking uses and associated plant species were recorded (Table 1). Statistical analysis: The statistical package of SPSS, version 20 (IBM) was used to analyze the data of wells characteristics (availability of water, well status, irrigation and drinking use and also associated plant species). Spearman's rank correlations coefficient (rs) was applied to examine the relationships between wells characteristics (Field, 2009).

RESULTS AND DISCUSSION

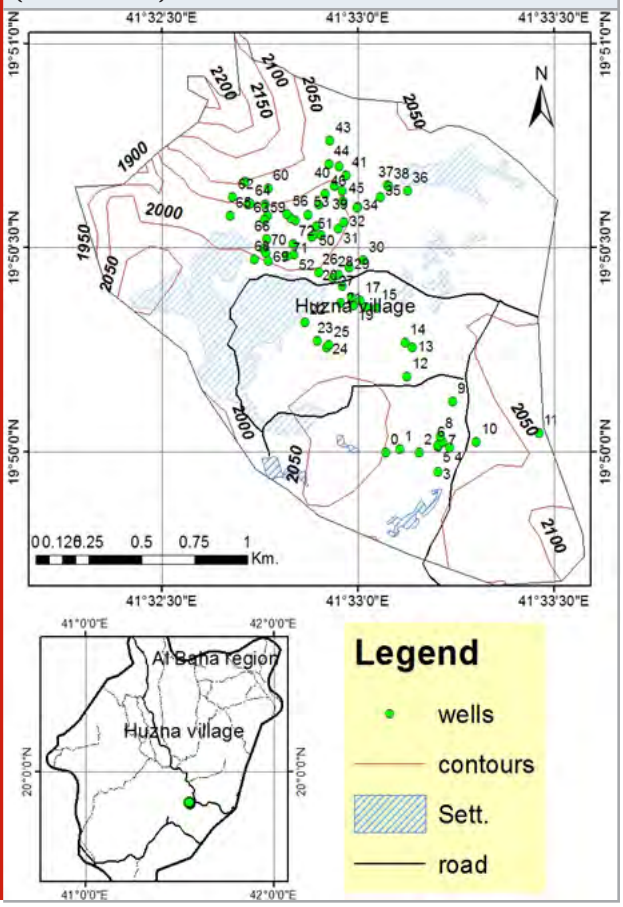
In Hiznah village, numerous traditional wells (total of 73 wells) are distributed in its valley and were used for irrigation and drinking. Geographically, the wells were distributed as follow; 47 (64.4%) of wells were concentrated in the north of the village, 14 wells (19.2%) in the middle, and 12 wells (16.4%) in the south (Figure 3). Each well was owned by different families and each one of them had a day of watering. Each family also was responsible for maintaining and protecting the well.

Area topography and recharge of wells: The age of Hiznah wells is unknown for exact. Structurally, most of the wells seem to be similar to each other reflecting the same period of time of the construction. The depth of the wells almost ranged from 15-25 m and the width range from 3 to 4 m. After precipitation of rain water, storing or moving of the water stream depends on geological locations (Plummer et al., 2013), topography, and climate (Condon & Maxwell, 2015; Grinevskii, 2014; Shahbazi et al., 1967). Groundwater moves from higher altitude areas to lower zones culminating in recharging of wells and increase water levels in this zone (Plummer et al., 2013).

In Hiznah village, wells are concentrated near agricultural fields located on the bottoms of the valleys and near the settlements, especially in the north of the study area at the southern side of Hiznah mountain where most wells (64.4%) were seen below Hiznah mountain (Figure 1). Indeed, the topography features of this region resembling a water basin that contributes to the conservation and recharge of groundwater in large quantities. Condon & Maxwell (2015) found a strong relationship between topographic gradients and the fluxes of groundwater. Additionally, the study conducted by Rukundo & Dogan (2019) highlighted the role of spatial variations in determining groundwater recharge rates in the Ergene River catchment in Turkey. The middle part of Hiznah village contains a water basin that receives the water descends from the northern side of Madhan mountain and recharge the nearby wells (19.2%) (Figure 2). On the contrary, the wells in the south of Hiznah village represent (16.4%) of total wells (Figure 2). This area is rocky and surrounded by plateaus with agricultural terraces that absorb water and the wells are poorly fed as a result. Some of the water flows southward and not westward towards the boreholes. Also, the concentration of the population is low in this location compared to the north of the village. However, wells in this area are concentrated below the south of Madhan mountain.

Availability of groundwater: The availability of groundwater depends upon the recharge rates and not

Figure 1: The location of the study area with targeted wells (n=73) in Hiznah village in Albaha region (Saudi Arabia).



just the amount of residence water (Healy et al., 2007). These rates can be physically and directly measured by estimating water fluxes. Age of groundwater can be also estimated by tracing isotopes (Plummer et al., 2013). Availability of water in Hiznah village is presented in (Table 1) and (Figure 3A). Approximately 79.5% of the studied wells have water available, while just 20.5% of them showed no water available and this may be due to the low amount of rainfall that recharge groundwater. Geologically, weathering, recharge rate, and human activities can affect the physicochemical and microbial quality of groundwater (Sethy et al., 2016; Yakubu, 2013). This water can be unsafe for drinking and irrigation due to numerous health hazards that result from polluted water (WHO, 2017). The open system represents the majority of wells (58.9%), while covered and fenced wells represent 34.2% and 6.8%, respectively (Figure 3B). This open system in the village may affect the quality of water in terms of the ease of pollutants to access the water from the surrounding area.

The uses of water for drinking and irrigation in Hiznah village are presented in (Table 1) and (Figure 3C). Approximately, 46.6% of wells that have water available are used for drinking and irrigation, while other wells (53.4%) are not used for the same purposes. The reason for the low use in the village is that some people are no longer interested in agriculture because they are tenured governmental employees with a higher income than the agricultural sector. In addition, drinking water is now available via public municipal networks (desalination).

Associated plants species: Plant species associated with open wells in Hiznah village are presented in (Table 2) and (Figure 3D). Approximately, 35.6% of plant species associated with open and fence system of the top of wells, while the remaining covered wells (64.4%) showed no association with any pant species. The natural plant species were *Ficus palmata* (31.5%), *Celtis africana* (2.7%) and *Ficus vasta* (1.4%) (locally called Hamat, Shopareq, and Roqqah, respectively). These three species are recorded from Albaha region, *Ficus palmate* and Fecus vasta are recorded between 1800 and 2800 m, and *Celtis Africana* between 1500 and 2500 m. The three species are not harmful and have economic uses (Al-Khulaidi, et al., 2016). A study on rare and endangered plant species in Albaha region conducted by Al-Khulaidi, et al., (2018) considered *Celtis africana* and Ficus vasta are rare species with frequency percentage of 1.56 and 1.25, respectively.

Green and blue-green algae are common and can be found in well water. The distinct green color in water can result from green algae with no potential health hazards. However, certain cyanobacteria (blue green algae) can produce toxins in the water that can be harmful to humans and animals (Gerba & Pepper, 2019; Sheath & Wehr, 2003). Up to 5.5% of wells showed a growth of green algae floated on the surface of water. All are open wells located in the north of the village and are not used for drinking and irrigation. The growth of green Algae in these wells may as a result of agriculture activities (in the form of fertilizers) and wastes of humans and animals, reaching into wells through runoff.

Figure 2: Figure 2. The distribution of targeted wells (n=73). Forty-seven (65.75%) of wells are in the north of the village, 14 wells (17.81%) in the middle, and 12 wells (16.44%) are located in the south.

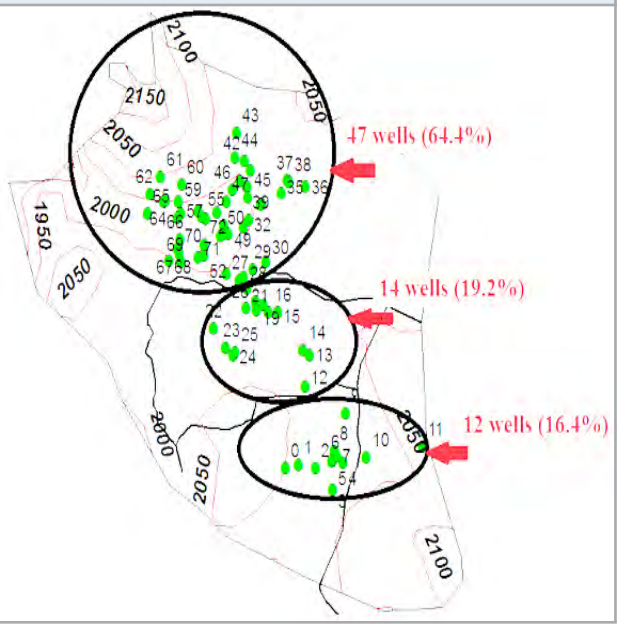


Table 1. Characters of targeted wells in Hiznah village, Albaha region, KSA (n=73).

Character of wells	Location North	Middle	South	Total
Wells with available water n (%)	41 (70.69)	11 (18.96)	6 (10.34)	58 (79.45)
Wells used for drinking & irrigation n (%)	26 (78.79)	5 (15.15)	3 (9.09)	33 (45.20)
Well status				
Covered	15 (65.22)	4 (17.39)	4 (17.39)	23(31.51)
Fenced	4 (57.14)	2 (28.57)	1 (14.28)	7 (9.59)
Open	29 (67.44)	7 (16.28)	7 (16.28)	43 (58.90)

Table 2. Number of wells associated with different plant species in different locations of Hiznah village, Albaha region, KSA

Plant	Well's location		
	North	Middle	South
Celtis africana	1 (2.08)	0 (0)	0 (0)
Ficus palmata	17 (35.42)	3 (23.08)	3 (25)
Ficus vasta	1 (2.08)	0 (0)	0 (0)

Table 3. Relationships between different characteristics of wells in Hiznah village, Albaha region Saudi Arabia

characteristics	Availability of water	Well status	Irrigation and drinking use	Associated plants
Availability	–	N.S.	rs = .475**,	rs = -.330**,
of water			p < 0.001	p < 0.001
Well status	N.S.	–	N.S.	rs = .375**,
				p < 0.001.
Irrigation	rs = .475**,			
and drinking use	p < 0.001	N.S.	–	N.S.
Associated plants	rs = -.330**,	rs = .375**,		
	p < 0.001	p < 0.001.	N.S.	–

Key symbols: SU: standard unit, N.S.: not significant, rs: Superman's rank correlation.

Figure 3. Overall characteristics of wells and their utilization purposes in Hiznah village, KSA

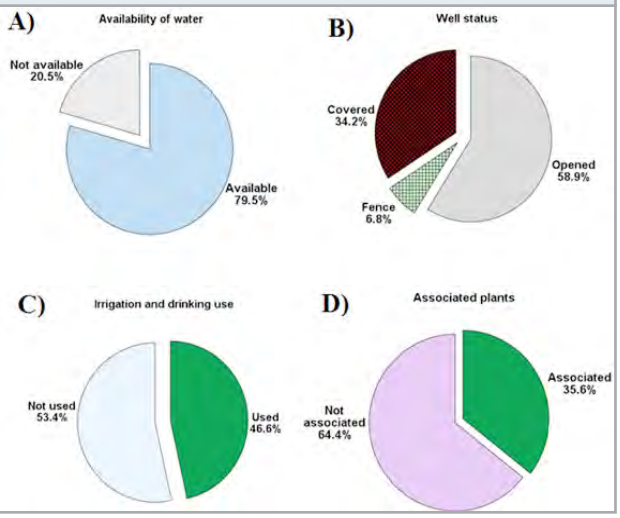
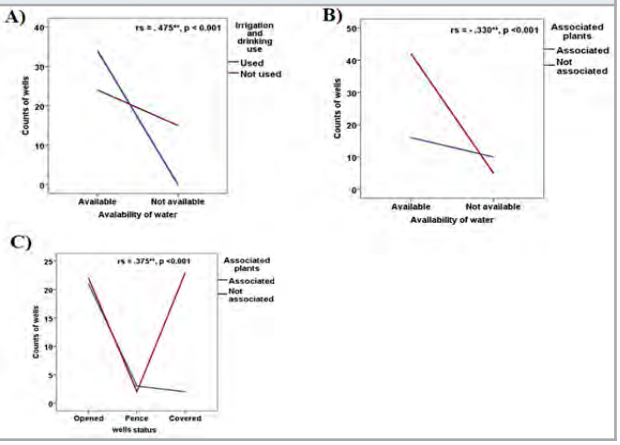


Figure 4. Relationship between wells' characters in Hiznah village, Albaha region, Saudi Arabia. Association of availability of water with utilization purposes (A), water availability and presence of associated plants (B), and well status association with plant presence (C).



Mohamed & Al-Shehri (2008) identified two cyanobacterial species (*Chroococcus minutus* and *Pannus spumosus*) in all samples of groundwater wells in Asir in Saudi Arabia. These species are known to produce toxins and can be harmful to human (Karan et al., 2017; John et al., 2015). In the investigation of twenty wells in Arar city in Saudi Arabia, Al-Shaikh (2017) detected six types of algae in four water wells. These types are *Amphora, Navicula, Nitzschia, Euglena, Cyclotella* and *Opephora*. This study recommended that the proper treatment of some wells water should be done before drinking.

Significant relationships between characteristics of wells: Correlations analysis between all characteristics of wells in Hiznah Village is presented in table 3 and figures 4. The availability of water was positively correlated with irrigation and drinking use (rs =0 .475**, p < 0.001) (Figure 4A). However, the availability of water was negatively correlated with associated plant species (rs = - 0.330**, p < 0.001) (Figure 4B). Well statuses were positively correlated with associated plant species (rs = .375**, p < 0.001) (Figure 4C). Open and fenced systems of wells in the village showed the growth of plant species.

ACKNOWLEDGEMENT

The author would like to thank Ahmed Tohman Alghamdi for his help in naming all traditional 73 wells in Hiznah village. The author would like also to thank other inhabitants in Hiznah village for their help and support. Especial thanks go to Dr. Abdul Wali Al-Khulaidi for his support in GIS mapping.

REFRENCES

Al-Hasawi, Z. Al-Hasawi, R. and S. Al-Zahrani, S.: (2018).
The study of some physicochemical and microbiological properties in water wells at Rabigh governate, Saudi Arabia. J. Biosci. Appl. Res., 4, 169-183
Al-Khulaidi, A. Al-Sagheer, N. Al-Turki, T. and F.

Filimban: (2018) Inventory of most rare and endangered plant species in Albaha region, Saudi Arabia. International Journal of Biology, Pharmacy and Allied Sciences (IJBAS), 7, 443-460.

Al-Khulaidi, A. Al-Sagheer, N. Dharfawi, M. and S. Al-Qhamidi: (2016) Tress of Albaha region and surrounding areas. FAO (http://www.fao.org/3/a-i6725a.pdf).

Alsalah, D. Al-Jassim, N. Timraz, K. and P.Y. Hong: (2015) Assessing the groundwater quality at a Saudi Arabian agricultural site and the occurrence of opportunistic pathogens on irrigated food produce. Int. J. Environ. Res. Public Health, 12, 12391-12411.

Al-Shaikh, T.M.: (2017) Study of microbial characteristics of well groundwater in the city of Arar, Kingdom of Saudi Arabia. J. North Basic Appl. Sci., 2, 41-50.

Alsuhaimi, A.O., Almohaimidi, K.M., and K.A. Momani: (2019) Preliminary assessment for physicochemical quality parameters of groundwater in Oqdus Area, Saudi Arabia. J. Saudi Soc. Agric. Sci., 18, 22-31.

APHA: (1998) Standard Methods for Examination of Water and Wastewater. 20th Edn., APHA, AWWA, WPCF, Washington DC, USA.

APHA: Standard Methods for Examination of Water and Wastewater. 22nd Edn., APHA, AWWA, WPCF, Washington DC, USA (2012).

Bob, M. Abd Rahman, N. Taher, S. and A. Elamin: (2015) Multi-objective assessment of groundwater quality in Madinah city, Saudi Arabia. Water Qual. Expo. Health, 7, 53-66.

Chao, S. Fan, J. and L. Wang: (2016) Association between the levels of calcium in drinking water and coronary heart disease mortality risk: evidence from a meta-analysis. J. Clin. Exp. Med., 9, 17912-17918.

Condon, L.E. and R.M. Maxwell: (2015) Evaluating the relationship between topography and groundwater using outputs from a continental-scale integrated hydrology model. Water Resour. Res., doi: 10.1002/2014WR016774

Fan, A.M. and V.E. Steinberg: (1996) Health implications of nitrate and nitrite in drinking water: an update on methemoglobinemia occurrence and reproductive and developmental toxicity. Regul. Toxicol. Pharmacol., 23, 35-43.

Field, A.P: (2009) Discovering statistics using SPSS: and sex and drugs and rock 'n' roll. 3rd Edn., SAGE publications Ltd, London, UK.

Gerba, C.P. and I.L. Pepper: (2019) Microbial Contaminants. In: Environmental and Pollution Science. 3rd Edn., (Eds.: M.L. Brusseau, I.L. Pepper and C.P. Gerba). Academic Press, Boston, p. 191-217.

Grinevskii, S.O.: (2014) The effect of topography on the formation of groundwater recharge. Moscow Univ. Geol. Bull., 69, 47-52.

Hahn, M.W.: (2006) The microbial diversity of inland waters. Curr. Opin. Biotechnol., 17, 256-261.

Healy, R.W. Winter, T.C. Labaugh, J.W. and O.L. Franke: (2007) Water budgets: Foundations for effective water-resources and environmental managment. U.S. Virginia: Geological Survey 1308.

John, D. W., Robert, G. S., and J. P. Kociolek: (2015) Freshwater algae of North America: Ecology and Classification. 2nd Edn., USA.

Karan, T., Altuner, Z., and R. Erenler: (2017) Growth and metabolite production of Chroococcus minutus under different temperature and light conditions. Journal of New Results in Science (JNRS), 6, 47-52.

Mohamed, Z.A. and A.M. Al-Shehri: (2008) Cyanobacteria of surface and ground waters in Asir region with new records to Kingdom of Saudi Arabi. J. King AbdulAziz Univ., 20, 113-129.

Omer, E.O.M. Algamidi, A.A. Algamidi, I. M. Fedlelmula, A. and A.S.R. Alsubaie: (2014) The hygienic-related microbiological quality of drinking water sources Al-Baha Province, Kingdom of Saudi Arabia. J. Health Spec., 2,.

Otu, R.A. Agyenim, J.B. and G.B.N. Bumah: (2014) Correlation analysis of groundwater colouration from mountainous Areas, Ghana. Environ. Res. Eng. Manage., 1, 16-24.

Ouda, O.K.M.: (2013) Towards assessment of Saudi Arabia public awareness of water shortage problem. Resour. Environ., 3, 10-13.

Plummer, L.N. Sanford, W.E. and P.D. Glynn:(2013) Characterization and conceptualization of groundwater flow systems. In: Isotope methods for dating old groundwater. (Eds.: A. Suckow, P. K. Aggarwal, and L. A. Araguás). International Atomic Energy Agency, Vienna, p. 5-20

Rukundo, E. and A. Dogan: (2019) Dominant influencing factors of groundwater recharge spatial patterns in Ergene River catchment, Turkey. J. Water, 11, 653 (2019). doi: 10.3390/w11040653

Sethy, S.N. Syed, T.H. Kumar, A. and D. Sinha: (2016) Hydrogeochemical characterization and quality assessment of groundwater in parts of Southern Gangetic Plain. Environ. Earth Sci., 75, 232. doi: 10.1007/s12665-015-5049-4

Shahbazi, M. Zand, S. and D.K. Todd: (1967) Effect of topography on ground water flow. paper for presentation to Inter. soc. of Sci. Hydrology ,14th General Assembly, IUGG. September 1967, Berne, Switzerland, 314-319.

Sheath, R. and J. Wehr: (2003) Introduction to Freshwater Algae. Freshwater Algae of North America: Ecol. Classif., 1-9. doi: 10.1016/B978-012741550-5/50002-7

WHO: (2017) Guidelines for drinking water quality. 4th Edition incorporating the first addendum. Geneva: World Health Organization.

Yakubu, S.: (2013) Assessment of water quality of hand-dug wells in Zaria LGA of Kaduna State, Nigeria. Int. J. Eng. Sci., 2, 01-04.

Microbiological Communication

BBRC Bioscience Biotechnology Research Communications

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp-139-145

Cytotoxic and Antibacterial Activity Evaluation of MDR Bacteria Mediated Synthesized Silver Nanoparticles

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ABSTRACT

In the current study cytotoxic and antibacterial activities of MDR bacteria *S.aureus* and *E.coli* mediated synthesized AgNPs (Silver Nanoparticles) were determined. The Antibacterial activities of silver nanoparticles were determined against *L. monocytogenes* and *S. abony*. The cytotoxicity of silver nanoparticles was determined against SH-SY5Y cell lines. MTT assay and DAPI staining were used to determine the cytotoxic potential. DCFH-DA was used to determine ability of ROS generation of synthesized silver nanoparticles. The MIC values of MDR S.aureus mediated synthesized AgNPs against *L. monocytogenes* and *S. abony* were found to be 36.22 µM and 35.65 µM respectively. Whereas MIC values of MDR E.coli mediated synthesized AgNPs against *L. monocytogenes* and *S. abony* were calculated to be 41.44 µM and 31.62 µM, respectively. Similarly, IC50 values of MDR S.aureus and MDR E.coli mediated synthesized AgNPs against SH-SY5Y cell line were reported to be 148.25 µM & 152.1 µM. DAPI results suggest that synthesized silver nanoparticles were able to cause nuclear condensation in the treated cell. Qualitative ROS generation via DCFH-DA assay suggest that MDR bacteria *S.aureus* & *E.coli* mediated synthesized Silver Nanoparticles have the capacity to generate significant amount of Reactive Oxygen Species(ROS), the higher amount of ROS generation was observed at a concentration of 300 µM AgNPs.

KEY WORDS: ANTIBACTERIAL, CYTOTOXIC; MDR; SH-SY5Y; ROS.

INTRODUCTION

Increase in microbial and cancer resistance is on a continuous rise and has now become a global problem. The primary cause of this increased resistance is due to rapid misuse of antimicrobial and chemotherapeutic agents (Skladanowski et al., 2016). In order to address this fast rising problem, scientific fraternity is in continuous search of alternative treatment. Researchers are now concentrating more on unconventional and alternative approaches such as use of antimicrobial peptides, nanoparticles and quorum quenching nanomaterials (Ma et al., 2018).The rapid emergence of nanotechnology and specifically nanoparticles has led to the discovery and recognition of nanoparticles as a potent antimicrobial and anticancer agent. Silver ions in particular have shown their extraordinary potential as antimicrobial and anti-inflammatory agent, and are being used widely since ancient times. Silver NPs (AgNPs), due to their unique characteristic properties and high therapeutic potential have found great application. The AgNPs are

ARTICLE INFORMATION

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Received 17th Jan 2020 Accepted after revision 29th Feb 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)
A Society of Science and Nature Publication, Bhopal India

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/25

now used in diagnosis and probing of cancer (Alshaye et al., 2017), biomolecular detection (Karim et.al., 2018), catalysis (Burdusel et al., 2018), wound healing (Orlowski et al., 2018). The antimicrobial potential of these silver nanoparticles (AgNPs) against MDR strains of a variety of pathogenic bacteria and cytotoxic potential against cancer cells has been well established (Abalkhil et al., 2017; Huang et al., 2017).

The bactericidal potential of the AgNPs can be attributed to large surface area to volume ratio, a consequence of smaller size, which permits the AgNPs to interact closely with the bacterial membrane (Helmlinger et al., 2016). Several studies propose that silver nanoparticles disturb the function of their target cells by getting attached to the cell membrane surface (Yan et al., 2018). Other studies have suggested that silver nanoparticles could induce cell death in gram negative bacteria *E. coli*, by causing the formation of small ''pores'' in the bacterial cell wall, which increases the permeability and ultimately inactivates the respiratory chain and electron transport system. In the present work the cytotoxic and antibacterial potential of previously synthesized silver nanoparticles from MDR *S.aureus* and *E.coli* was determined (Mohd Haseeb et al., 2019). Through this study we have aimed to address the challenges associated with cancerous cells and bacterial infections. The synthesized silver nanoparticles were characterized by various techniques such as UV-Vis Spectroscopy, Zeta potential, Dynamic Light Scattering (DLS), Transmission Electron Microscopy (TEM), and Fourier Transformed Infra Red (FTIR) Spectroscopy (Mohd Haseeb et al; 2019). The synthesized silver nanoparticles were subsequently tested for their antibacterial activity against *L. monocytogenes* and *S. abony*. The cytotoxic activity and the ability of these silver nanoparticles to generate ROS was determined against SH-SY5Y.

MATERIAL AND METHODS

All the Media and chemicals used in the experiment were purchased from Sigma Aldrich (St. Louis, USA) and HiMedia, India.

Antibacterial Activity: The antibacterial potential of MDR *S.aureus* and *E.coli* synthesized Silver nanoparticles was determined against *L. monocytogenes and S. abony*. These bacteria (OD600-0.60) were allowed to grow in Nutrient broth (NB) for 24 hours then incubated for 24 hrs at 37^{0}C kept at 180 rpm in a rotary shaker incubator. 100 ml of cultured broth was used to prepare bacterial lawns. The antibacterial potential of MDR *S. aureus and E.coli* mediated synthesized silver nanoparticles was determined by method discussed elsewhere (Mohd. Haseeb et al., 2019). 25 milliliters of sterile Mueller Hinton Agar (MHA) medium was poured in sterilized petriplates to prepare culture plates. Sterilized cotton swab was used to swab the bacterial strains from culture plates. Three wells of 5 mm diameter each were made in every plate by using Sterilized gel puncture. One of the well from each plate was filled with sterilized dH2O (for control) and the other two were added with MDR *S.aureus* and MDR *E.coli* mediated synthesized AgNPs. The inoculated plates were incubated 24 hours at 37^{0}C temperature. Clear zone of inhibition around the wells were examined in order to determine the antibacterial potential of as synthesized AgNPs.

Minimum Inhibitory Concentration (Mic) of AgNPs: MIC50 is the amount of drug needed to inhibit the bacterial growth to 50%. A flat-bottom, 96 well plates was employed to determine the MIC50 by broth dilution method. This enabled the inoculum to be prepared in distilled water allowing the determination of absorbance by UV- spectrophotometer and thereby eliminating the need of interference from colored media. Double diffusion method, was used to determine the MICs of the E.coli synthesized silver nanoparticles. The bacterial strains were grown till mid-log phase, culture harvesting was achieved by centrifugation, 1mM sodium Phosphate buffer (SPB) was used to wash the harvest at pH 7.4, and finally diluted to 2 × 105 CFU/ml in same buffer. AgNPs were serially diluted in desired concentrations by taking 90 microtitre (µL) of Mueller-Hinton broth in 96 well- microtitre plates. Bacterial suspension of 95×10^4 CFU/ well was taken as inoculum. After inoculation, wells were incubated for overnight at 37o C. The lowest concentration of silver nanoparticles inhibiting the bacterial growth was used to determine MIC50 of biosynthesized AgNPs.

Evaluation of Cytotoxic Potential Against SH-SY5Y Cell Line: The synthesized silver nanoparticles were analyzed for their anticancer activities against SH-SY5Y Cell line. MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay was used to determine the Cytotoxic activity of synthesized AgNPs whereas DAPI (4', 6-diamino-2-phenylindiole) toxicology assays was used to assess the nuclear degradation. In order to assess the same, the cells were treated with varying doses of silver nanoparticles. SH-SY5Y cells were grown in 96 well plates with each well having 1 X 104 cells, followed by 24 hours incubation in CO2 incubator. After a successful incubation period, silver nanoparticles of varying concentrations were used to treat the cells. The experiment was repeated in triplicate with untreated cells as the positive control and incubated for 48 hours. Spent medium was removed, followed by loading of wells with 50µl of MTT dye (5mg/ml in PBS) and freshly prepared culture media. Then the plate was incubated for 4 hours. Formazon crystals formed during the process were dissolved by adding 100µl of DMSO (Dimethyl Sulfoxide) and incubated for one hour. The reduced MTT was quantified by measuring optical density at 570 nm in ELISA reader. The Percentage inhibition of the cells was calculated using the formula

$$X = 100 - (A_{test} - A_{blank}) / (A_{control} - A_{blank}) \times 100$$

Where,
X- Percentage inhibition of cells Atest - absorbance of the test sample at 570 nm.

Ablank - absorbance of blank at 570 nm Acontrol - absorbance of the control sample at 570 nm The IC50 value was calculated by the obtained data. Nuclear condensation in silver nanoparticles treated SH-SY5Y cells was determined by using nuclear fluorescent stain 4', 6-diamino-2-phenylindiole (DAPI) dye. SH-SY5Y Cells were seeded in 96 well plates, treated with varying concentration silver nanoparticles were maintained in CO2 humidification incubator at 37 °C for 4 hrs. It was followed by careful removal of medium at appropriate times and washing the cells twice with saline phosphate buffer (SPB). The treated cells were washed again, stained with DAPI for 20 minutes at 37 °C, along with a permeabilizing buffer that allows the fixing of the stain into treated cells. Methanol and phosphate buffer saline was used to wash the stain respectively. Fluorescence microscope was used to visualize and capture the images of the stained cells and cells with fragmented and condensed nuclei were considered to be the dead cells.

Assessment of Intracellular ROS (Reactive Oxygen Species) Generation: The intracellular reactive oxygen species (ROS) level was measured by DCFH-DA method as per standard protocol. It is based on ROS-induced formation of the highly fluorescent dye 2'7'- dicholorofluorescin diacetate (DCFH-DA). Shortly, Human neuroblastoma cell lines SH-SY5Y were seeded in a 12 well plate at a density of 5 x 104 per well and incubated for 24 hr at 37 ^{0}C. After treatment with AgNPs (0, 50, 100, 200, and 300 µM) for 12 hrs, the cells were incubated with 10 µM DCFH-DA for 30 minutes at 37 ^{0}C. Cells were washed to remove the excessive amount of DCFH-DA. Images were captured by using an inverted fluorescence microscope) Nikon ECLIPDE Ti-S, Japan).

RESULTS AND DISCUSSION

The antimicrobial potential of silver nanoparticles from MDR *S.aureus* and *E.coli* was determined against *L. monocytogenes and Salmonella abony.* The antibacterial potential of as synthesized silver nanoparticles were confirmed on the basis of the clear zone of inhibition observed around the area inoculated with NPs. Different concentration of silver NPs synthesized MDR *S.aureus* and MDR *E.coli* was used to calculate and determine the minimum inhibitory concentrations (MIC).

The zone of inhibition of MDR *S.aureus* silver nanoparticles on *L. monocytogenes* and *Salmonella abony* was found to be 18 mm and 19 mm respectively. The zone of inhibition of MDR *E.coli* synthesized silver nanoparticles on *L. monocytogenes and Salmonella abony* was found to be 17 mm and 18 mm respectively. The MIC values of synthesized silver nanoparticles against treated bacteria are given in table 1. The exact mechanism of action of Ag+ ions on microorganisms is not fully known. It is believed that the DNA of Ag+ treated microbial cells loses the ability of replication and protein inactivation occurs (Divya et.al. 2016). It has also been shown that silver ions cause protein denaturation by binding to functional groups on the protein. In a study involving *E.coli* and Metal oxide nanoparticles, treated bacterial cells were observed to have a significant increase in membrane permeability, making cells incapable of regulating the proper transport across the cell membrane and ultimately leading to cell death (Gomaa, 2017).

It has earlier been reported that antimicrobial potential is dependent upon dose and size, which makes gram-negative bacteria an obvious target as compared with gram positives bacteria. AgNPs may interact directly with microbial cells, may inhibit the enzymes of respiratory chain and affect the permeability of protons and phosphates for example; by halting the transmembrane transfer of electrons, disrupting the cell envelope thereby making it susceptible to the ROS (Reactive Oxygen Species) (Rajeshkumar and Malarkodi, 2014). Additionally, Silver nanoparticles can cause damage to bacterial cells by permeating the cell and interacting with proteins, DNA and some other cell constituents containing sulfur and phosphorus (Nayak and Chitra, 2015). In a similar research experiment where *E.coli* and *K. pneumonia* were treated with AgNPs the highest antibacterial effect was recorded on E.coli with a zone of inhibition of 13 mm and the lowest was examined on *K. pneumonia* with a zone of inhibition of 7mm (Feng et.al., 2000).

Figure 1: Plates showing the zone of inhibition of MDR *S.aureus* and *E.coli* mediated synthesized Silver NPs against- *L. monocytogenes*

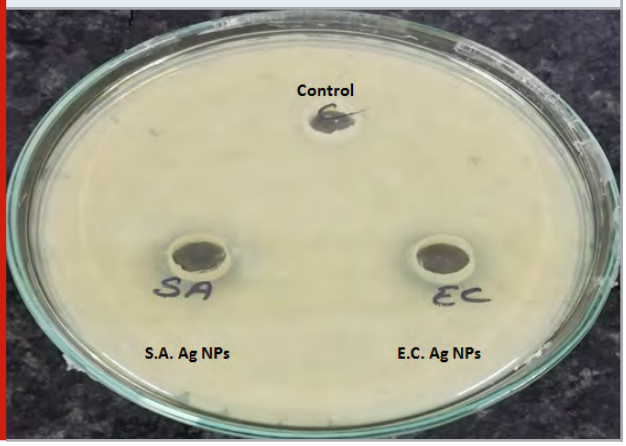
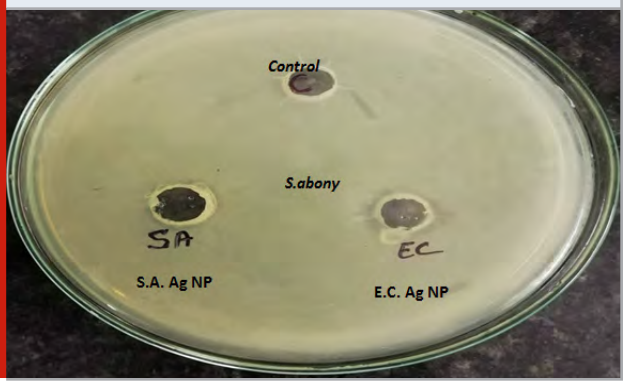


Figure 2: Plates showing the zone of inhibition of MDR *S.aureus* and *E.coli* mediated synthesized Silver NPs against- *S.abony*



Cytotoxic Potential Against SH-SY5Y: The synthesized silver nanoparticles froms MDR *S.aureus* and *E.coli* were assessed for their cytotoxic potential on SH-SY5Y neuroblastoma cancer cell line. The cell viability or cytotoxic activity was assessed via MTT assay and nuclear fragmentation was studied via DAPI staining.

Cytotoxicity of Mdr MDR S.aureus and MDR E.coli Synthesized Silver Nanoparticles: The vey aim of cell viability assays is to determine the cellular response of any toxic material that has the potential to influence the metabolic activity of the target cells. MTT analysis is used to analyse the mitochondrial succinate dehydrogenase activity as metabolically active viable cells have the capacity to convert the MTT to purple color formazon crystal which gives maximum absorbance near 570 nm. The cellular mechanism behind this reduction of MTT probably involves NADH or a similar reducing agent from which electrons are transferred to tetrazolium, which is then reduced to formazon. The dead cells lose the ability

Table 1. Representing MIC values of synthesized silver nanoparticles

Name of sample	MIC (µM) L. monocytogenes	*S. abony*
MDR *S.aureus* AgNPs	36.22 µM	35.65 µM
MDR *E.coli* AgNPs	41.44 µM	31.62 µM

Figure 3: Curve showing the MIC of silver NPs synthesized from MDR *S. aureus* and MDR *E.coli* against L. monocytogenes

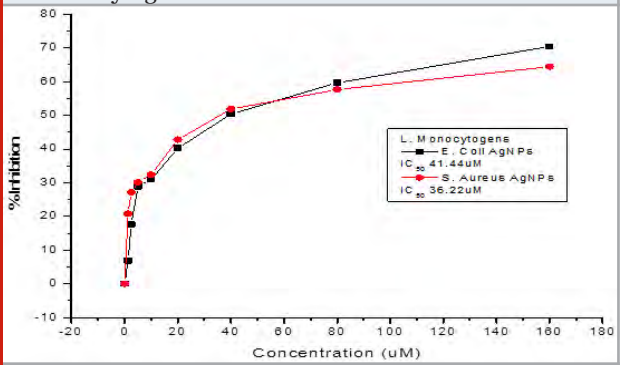
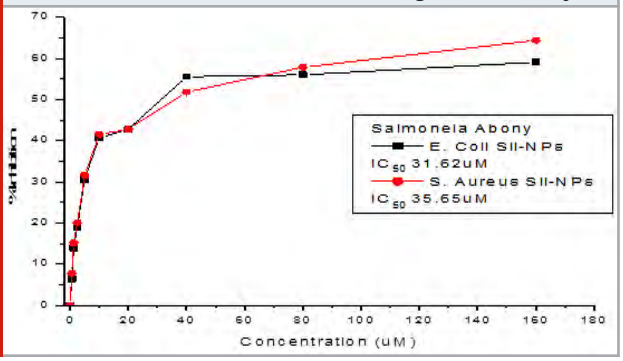


Figure 4: Curve showing the MIC of silver NPs synthesized from MDR S. aureus and MDR E.coli against S. abony



colored product decreases with decrease in cell viability hence the intensity of colored product which is directly proportional to number of viable cells in culture, is measured to determine the level of apoptosis (Präbst et al., 2017).

Here in our study, the cytotoxicity of silver nanoparticles in human neuroblastoma cancer cells SH-SY5Y was determined by treating the cells with varying dose of (10 µM to 300 µM) for 24 hr and 48 hr respectively, followed by MTT reduction assay. After a successful incubation period of 24 hr, 50% reduction in cell viability was noticed. The IC50 values were found to be 148.25 µM & 152.1 µM for MDR S.aureus and MDR E.coli mediated synthesized Silver nanoparticles. Almost same patterns of results with strong cytotoxic effects were observed after 48 hrs of treatment period, proving silver nanoparticles to be more cytotoxic and of anti-proliferative nature. Earlier studies have predicted similar findings in which the cytotoxic effects of silver nanoparticles have been reported (Singh et al., 2018). The cytotoxic efficacy of silver nanoparticles in affecting the survival of various cell types by disturbing the mitochondrial activity, structure and metabolism have been also predicted by several types of research (Suliman et al., 2015). In several previous studies, smaller sized Ag-nanoparticles have been shown to have more toxicity than the NPs with a larger size (Gurunathan et al., 2015). It has also been demonstrated that the type of capping agent dictates the potency of silver nanoparticles (Patra et.al., 2018). In order to determine the apoptotic cell death induced by as synthesized silver NPs, DAPI nuclear staining method was used. The apoptotic cells are characterized by cell shrinkage, extensive blabbing in the plasma membrane, and condensation of chromatin and formation of apoptotic bodies from separated cellular fragments (Sheikh et al., 2018).

The cells treated with AgNPs, were stained with DAPI, a DNA staining dye. DAPI is a fluorescent staining dye which binds preferentially to the AT-rich regions of double stranded DNA. When DAPI binds DNA its fluorescence intensity increases around 20 folds as compared with unbound DAPI (Baharara et.al., 2018). The intensity of stain is less in the viable cells as the membrane permeability is intact, DAPI fails to pass through but when cell permeability is compromised due to apoptosis; DAPI enters inside the cell with ease and a strong blue fluorescence is produced as it binds to DNA (Baharara et.al., 2018). After treatment with AgNPs for 24 h, significant nuclear changes in SH-SY5Y neuroblastoma cancer cells were observed (Fig. 7 & 8). As apparent from pictures of DAPI staining, AgNPs induced nuclear condensation and fragmentation in treated cancer cells in a dose dependent manner whereas the control cells exhibited normal cell morphology (Fig. 7 & 8). The apoptotic effect of silver nanoparticles can be attributed to excessive ROS generation which leads to DNA and protein damage (Foldbjerg et al., 2009; Gurunathan et al., 2018). The findings of our current research are in agreement with previously published reports that exposure of cells to NPs induces apoptotic

cell death (Kishore et al., 2018; Mohammed et al., 2018). The results were evident that AgNPs induced apoptosis in neuroblastoma cells in a time dependent and dose-dependent manner.

The ability to generate ROS by AgNPs from MDR strains of *E.coli* and *S.aureus* was analyzed in human neuroblastoma cell lines SH-SY5Y. It was measured by the H2DCF-DA assay. After 24 h of exposure, increasing concentrations of AgNPs significantly increased ROS levels at various concentrations used in this study. In general, nanoparticles are known to kill cancer cells by producing ROS. Ag ions play an important role in catalyzing ROS production in the presence of oxygen species and AgNPs can induce oxidative stress in a variety of cellular systems by generating ROS, including in human cancer cells. To examine the effect of MDR S. aureus and E.coli mediated synthesized AgNPs on ROS generation, human neuroblastoma cell lines SH-SY5Y were treated with various concentrations (0, 50, 100, 200, and 300 µM) of AgNPs for 24 h, and then, ROS generation was measured in an H2DCF-DA assay. After 24 h of exposure, increasing concentrations of AgNPs significantly increased ROS levels at various concentrations used in this study. ROS levels generated in response to AgNPs treatment were significantly higher than those in untreated cells.

Marked differences were evident in the production of ROS between treated and untreated control cells. Treated cells emitted bright fluorescence and displayed deformed morphologies because of the loss of integrity of plasma membrane due to ROS generation. The cytotoxicity effects may have been due to the induction of oxidative stress and apoptosis upon ROS overproduction. Cancer cells generate high levels of ROS that leads to a state of increased basal oxidative stress. Since this state of oxidative stress makes cancer cells vulnerable to agents that further augment ROS levels, the use of pro-oxidant agents is emerging as an exciting strategy to selectively target tumor cells (Cordero et al., 2012). However, the induction of ROS formation plays an important role in the chemotherapeutic activity of several anticancer drugs and a large number of anticancer compounds (Fruehauf et al., 2007; Trachootham et al., 2009). Our data for the qualitative analysis of ROS generation suggested that AgNPs induced ROS-mediated induction of apoptosis in SH-SY5Y cells in a concentration- dependent manner.

Figure 5: Showing the IC50 of MDR *E.coli* mediated synthesized AgNPs against human neuroblastoma cell lines SH-SY5Y

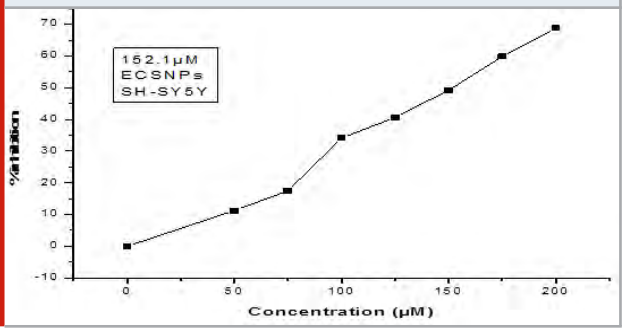


Figure 6: Graph showing the IC50 of S.aureus mediated synthesized AgNPs against Human neuroblastoma cell lines SH-SY5Y.

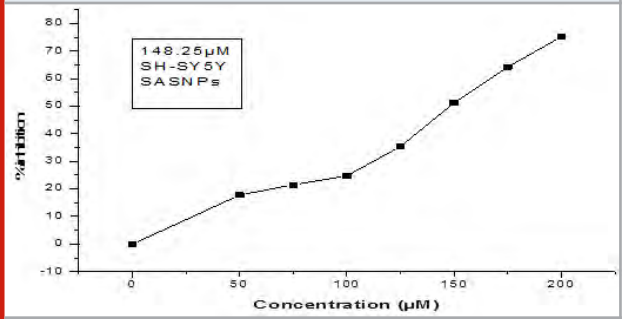


Figure 7. DAPI Images of SH-SY5Y (taken at a magnification of 40x): a) Control cells, on top left and b), c) & d) Cells treated with S.aureus mediated synthesized AgNPs, on right

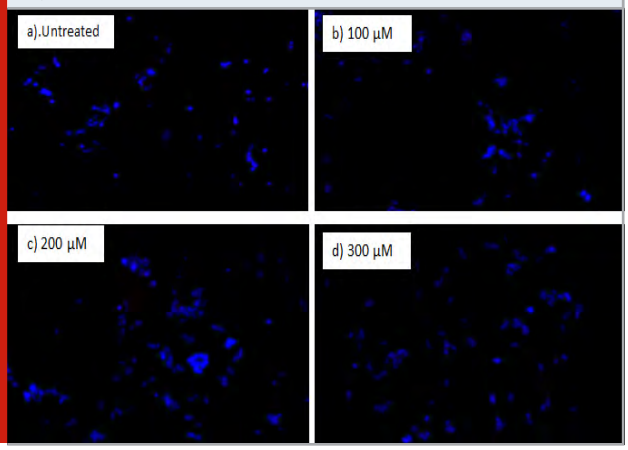


Figure 8: DAPI Images of SH-SY5Y (taken at a magnification of 40x): a) Control cells, on left and b), c) & d) Cells treated with *E.coli* mediated synthesized AgNPs, on right n clockwise.

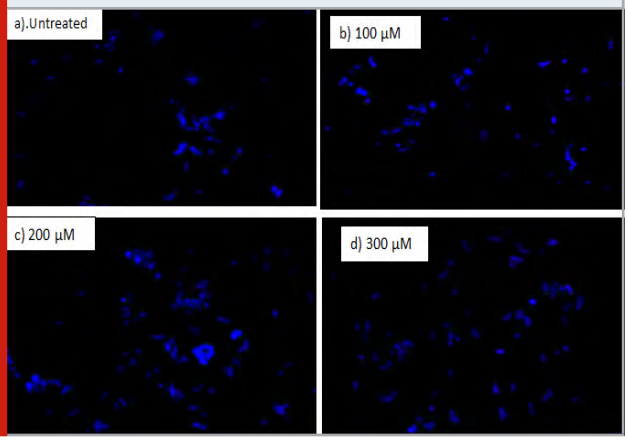


Figure 9: Effect of MDR *E.coli* mediated synthesized AgNPs on reactive oxygen species (ROS) generation. SH-SY5Y cells were treated with or without AgNPs for 24 h, and ROS generation was measured using DCFH-DA.

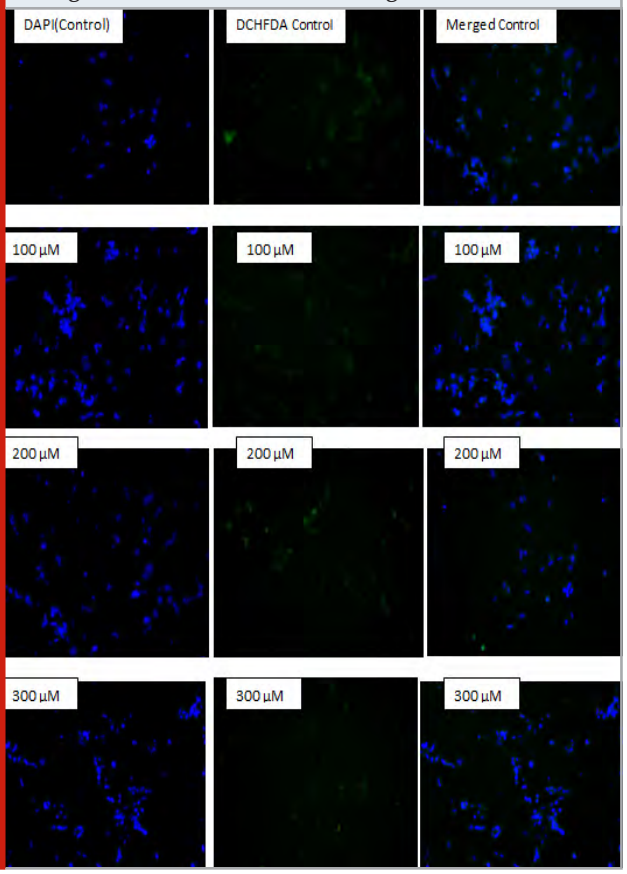
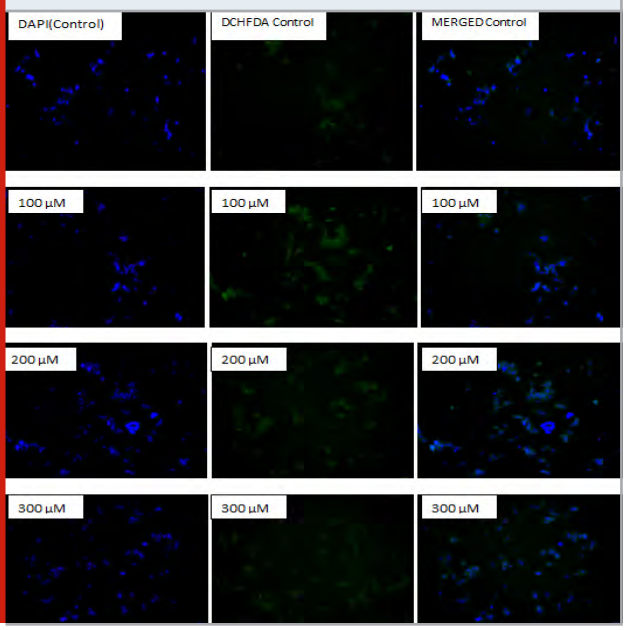


Figure 10: Effect of MDR S.aureus mediated synthesized AgNPs on reactive oxygen species (ROS) generation. SH-SY5Y cells were treated with or without AgNPs for 24 h, and ROS generation was measured using DCFH-DA.At all tested concentrations, the most pronounced effects were observed at higher concentrations, showing up to 2–3-fold higher ROS levels (Fig- 9 & 10).



CONCLUSION

In the present work, the cytotoxic and antibacterial activities of MDR *S.aureus* and *E.coli* mediated synthesized silver nanoparticles (AgNPs) were determined. The MIC values of MDR *S.aureus* mediated synthesized silver nanoparticles against *L. monocytogenes* and *S. abony* were found to be 36.22 µM and 35.65 µM respectively. Whereas MIC values of MDR *E.coli* mediated synthesized AgNPs against *L. monocytogenes* and *S. abony* were calculated to be 41.44 µM and 31.62 µM, respectively. Similarly, IC50 values of MDR S.aureus and MDR *E.coli* mediated synthesized AgNPs against SH-SY5Y cell line was reported to 148.25 µM & 152.1 µM. DCFH-DA results show that silver nanoparticles are capable of generating large amount of ROS in their target cells; the ROS generation was recorded to be increasing on the increase of silver nanoparticle concentration. It was for the first time that MDR bacteria mediated synthesized were tested for their cytotoxicity against SH-SY5Y cell lines. Further research on various other cell lines is inevitable to establish these nanoparticles as a potent anticancer agent, also, being of MDR bacteria origin; these nanoparticles must also be assessed for any kind of associated antigenic challenge in animal models in order to determine the safety of these synthesized Silver nanoparticles.

REFERENCES

Abalkhil, Tarad Abdulaziz, Sulaiman Ali Alharbi, Saleh Hussein Salmen, and Milton Wainwright: (2017). Bactericidal activity of biosynthesized silver nanoparticles against human pathogenic bacteria. Biotecnology & Biotechnological Equipment 31, no. 2: 411-417.

Alshaye, Najla A., Mudawi M. Elobeid, Dalal HM Alkhalifah, and Afrah E: (2017). Mohammed. Characterization of Biogenic Silver Nanoparticles by *Salvadora persica* leaves extract and Its Application Against Some MDR Pathogens *E. coli* and *S. aureus*. Res. J. Microbiol 12: 74-81.

Baharara, Javad, Tayebe Ramezani, Nasrein Hosseini, and Marzieh Mousavi: (2018). Silver Nanoparticles Synthesized Coating with *Zataria multiflora* Leaves Extract Induced Apoptosis in HeLa Cells Through p53 Activation." Iranian journal of pharmaceutical research: IJPR 17, no. 2: 627.

Burdusel, Alexandra-Cristina, Oana Gherasim, Alexandru Mihai Grumezescu, Laurentiu Mogoanta, Anton Ficai, and Ecaterina Andronescu: (2018). Biomedical applications of silver nanoparticles: An up-to-date overview." Nanomaterials 8, no. 9: 681.

Divya, Koilparambil, Liya C. Kurian, Smitha Vijayan, and Jisha Manakulam Shaikmoideen: (2016). Green synthesis of silver nanoparticles by *Escherichia coli*: Analysis of antibacterial activity. Journal of Water and Environmental Nanotechnology1, no. 1: 63-74.

Feng, Qing Ling, Jian Wu, G. Q. Chen, F. Z. Cui, T. N. Kim, and J. O. Kim: (2000). A mechanistic study of the antibacterial effect of silver ions on *Escherichia coli* and *Staphylococcus aureus*. Journal of biomedical materials research 52, no. 4: 662-668.

Foldbjerg, Rasmus, Ping Olesen, Mads Hougaard, Duy

Anh Dang, Hans Jürgen Hoffmann, and Herman Autrup: (2009). PVP-coated silver nanoparticles and silver ions induce reactive oxygen species, apoptosis and necrosis in THP-1 monocytes. Toxicology letters 190, no. 2: 156-162.

Fruehauf, John P., and Frank L. Meyskens: (2007). Reactive oxygen species: a breath of life or death?. Clinical Cancer Research 13, no. 3: 789-794.

Gomaa, Eman Zakaria: (2017). Silver nanoparticles as an antimicrobial agent: A case study on *Staphylococcus aureus* and *Escherichia coli* as models for Gram-positive and Gram-negative bacteria. The Journal of general and applied microbiology 63, no. 1: 36-43.

Gurunathan, Sangiliyandi, Jae-Kyo Jing, Jae Woong Han, Xi-Feng Zhang, Jung Hyun Park, and Jin-Hoi Kim: (2015). Multidimensional effects of biologically synthesized silver nanoparticles in *Helicobacter pylori*, *Helicobacter felis*, and human lung (L132) and lung carcinoma A549 cells." Nanoscale research letters 10, no. 1: 35.

Gurunathan, Sangiliyandi, Muhammad Qasim, Chanhyeok Park, Hyunjin Yoo, Jin-Hoi Kim, and Kwonho Hong: (2018). Cytotoxic Potential and Molecular Pathway Analysis of Silver Nanoparticles in Human Colon Cancer Cells HCT116. International journal of molecular sciences 19, no. 8: 2269.

Haseeb, Mohd, Mohd Sajid Khan, Abu Baker, Imran Khan, Iram Wahid, and MS Mohamed Jaabir. "Anticancer and antibacterial potential of MDR *Staphylococcus aureus* mediated synthesized silver nanoparticles." An International Peer Reviewed Open Access Journal For Rapid Publication: 26.

Helmlinger, J., C. Sengstock, C. Groß-Heitfeld, C. Mayer, T. A. Schildhauer, M. Köller, and M. Epple: (2016). Silver nanoparticles with different size and shape: equal cytotoxicity, but different antibacterial effects. RSC advances 6, no. 22: 18490-18501.

Huang, Yu, Chao-Qiang Fan, Hui Dong, Su-Min Wang, Xiao-Chao Yang, and Shi-Ming Yang: (2017). Current applications and future prospects of nanomaterials in tumor therapy. International journal of nanomedicine 12: 1815.

Karim, Md N., Samuel R. Anderson, Sanjay Singh, Rajesh Ramanathan, and Vipul Bansal: (2018). Nanostructured silver fabric as a free-standing NanoZyme for colorimetric detection of glucose in urine. Biosensors and Bioelectronics 110: 8-15.

Kishore, Medikondu, Abeer Talib Abdulqader, Hanan Shihab Ahmad, and Y. Hanumantharao: (2018). Anticancer and antibacterial potential of green silver nanoparticles synthesized from Maytenus senegalensis (L.) leaf extract and their characterization. Drug Invention Today 10, no. 4.

Ma, Zhi-Ping, Yu Song, Zhong-Hua Cai, Zhi-Jun Lin, Guang-Hui Lin, Yan Wang, and Jin Zhou: (2018). Anti-quorum sensing activities of selected coral symbiotic bacterial extracts from the South China Sea." Frontiers in cellular and infection microbiology 8: 144.

Martin-Cordero, Carmen, Antonio Jose Leon-Gonzalez, Jose Manuel Calderon-Montano, Estefania Burgos-Moron, and Miguel Lopez-Lazaro: (2012). Pro-oxidant natural products as anticancer agents. Current drug targets 13, no. 8: 1006-1028.

Mohammed, Afrah, Alaa Al-Qahtani, Amal Al-Mutairi, Bashayir Al-Shamri, and Kawther Aabed: (2018) Antibacterial and cytotoxic potential of biosynthesized silver nanoparticles by some plant extracts. Nanomaterials 8, no. 6: 382.

Nayak, B. K., and N. Chitra. Anima Nanda: (2015). Comparative antibiogram analysis of AgNPs synthesized from two *Alternaria spp*. with amoxicillin antibiotics. J. Chem. Pharm. Res 7: 727-731.

Orlowski, P., Zmigrodzka, M., Tomaszewska, E., Ranoszek-Soliwoda, K., Czupryn, M., Antos-Bielska, M., Szemraj, J., Celichowski, G., Grobelny, J. and Krzyzowska, M.: 2018. Tannic acid-modified silver nanoparticles for wound healing: the importance of size. International journal of nanomedicine, 13, p.991.

Orlowski, P., Zmigrodzka, M., Tomaszewska, E., Ranoszek-Soliwoda, K., Czupryn, M., Antos-Bielska, M., Szemraj, J., Celichowski, G., Grobelny, J. and Krzyzowska, M: 2018. Tannic acid-modified silver nanoparticles for wound healing: the importance of size. International journal of nanomedicine, 13, p.991.

Präbst, K., Engelhardt, H., Ringgeler, S. and Hübner, H., (2017). Basic colorimetric proliferation assays: MTT, WST, and resazurin. Cell Viability Assays: Methods and Protocols, pp.1-17.

Rajeshkumar, S., and C. Malarkodi: (2014). In vitro antibacterial activity and mechanism of silver nanoparticles against foodborne pathogens. Bioinorganic chemistry and applications 2014.

Sheikh, E., Bhatt, M.B. and Tripathi, M., 2018. Bio-based synthesised and characterized monodispersed *Curcuma longa* silver nanoparticles induces targeted anticancer activity in breast cancer cells. Pharmacognosy Magazine, 14(57), p.340.

Singh, H., Du, J., Singh, P. and Yi, T.H., (2018). Ecofriendly synthesis of silver and gold nanoparticles by *Euphrasia officinalis* leaf extract and its biomedical applications. Artificial cells, nanomedicine, and biotechnology, 46(6), pp.1163-1170.

Składanowski, M., M. Wypij, D. Laskowski, P. Golinska, H. Dahm, and M. Rai: (2017). Silver and gold nanoparticles synthesized from Streptomyces sp. isolated from acid forest soil with special reference to its antibacterial activity against pathogens." Journal of Cluster Science 28, no. 1: 59-79.

Suliman Y, Al Omar, Daoud Ali, Saud Alarifi, Abdul Halim Harrath, Lamjed Mansour, and Saleh Hamad Alwasel: (2015). Evaluation of cytotoxic, oxidative stress, proinflammatory and genotoxic effect of silver nanoparticles in human lung epithelial cells. Environmental toxicology 30, no. 2: 149-160

Trachootham, Dunyaporn, Jerome Alexandre, and Peng Huang. Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach Cell Tech 4 21 32-38

Yan, Xueting, Bin He, Lihong Liu, Guangbo Qu, Jianbo Shi, Ligang Hu, and Guibin Jiang: (2018). Antibacterial mechanism of silver nanoparticles in *Pseudomonas aeruginosa*: proteomics approach. Metallomics 10, no. 4: 557-564.

Biotechnological Communication

BBRC Bioscience Biotechnology Research Communications

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp-146-150

Recombinant Sericin-Cecropin B Fusion Protein Aids in the Proliferation and Cryopreservation of Human Dermal Fibroblast Cells

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ABSTRACT

Silk sericin is a natural polymer with wide utility in biomedical applications. The use of native sericin protein has been well documented in cell culture and cryopreservation of various types of cells. However, the use of recombinant sericin is limited and currently there is no report on the effect of recombinant sericin fusion protein on cell culture. In this work, we report the use of recombinant sericin-cecropin B fusion in the proliferation and cryopreservation of primary human dermal fibroblast cells. Compared with cells cultured in the control with FBS, those cultured in sericin/sericin-cecropin B supplemented media (along with FBS) showed enhanced proliferation of cells. The culture medium containing sericin or its fusion also proved effective in cryopreservation of human dermal fibroblasts with reduced amount of FBS. However, low DMSO concentration did affect the viability of cells post-freezing. Both proliferation and cryoprotection properties were found enhanced in the recombinant sericin-cecropin B treated group than in recombinant sericin alone. Our results clearly show that the use of sericin-cecropin B could act as potential media supplement for the enhanced proliferation and improved cryopreservation of human dermal fibroblasts.

KEY WORDS: SILK SERICIN, FUSION PROTEIN, CELL PROLIFERATION, CRYOPRESERVATION..

INTRODUCTION

Sericin and fibroin are the two major proteins in the silk fibre produced by the domesticated silkworm, *Bombyx mori*. Sericin proteins are produced by the middle silk gland (MSG) and Ser1 is the most abundant sericin protein. It is characterized by a serine-rich peptide consisting of 38-amino acids repeats reported to play important roles in hydrophilicity, cryoprotection, and cell proliferation (Tsujimoto et al., 2001; Terada et al., 2005). Fibroin has been used in textile manufacturing and for several biomaterial applications, whereas sericin is treated as a waste material in the textile industry. However, sericin has shown interesting applications in cosmetics and pharmaceuticals (Kundu et al., 2008; Cao and Zhang, 2017; Zhang et al., 2019). The use of cell and tissue culture is essential for various research and medical applications. Hence, the culture and storage of cells is of critical importance in a standard cell culture laboratory. Fetal bovine serum (FBS) is widely used in cell culture as it aids in cell proliferation, growth and storage. However, the serum is expensive and also causes concern regarding bovine spongiform encephalopathy (BSE). Sericin has been presented as a potential alternative to FBS, can promote proliferation of several types of cells and protect cells from freezing stress. Dermal fibroblasts are responsible for producing the extracellular matrix

ARTICLE INFORMATION

*Corresponding Author: ravikumarpillai@gmail.com
Received 10th Feb 2020 Accepted after revision 27th March 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)
A Society of Science and Nature Publication, Bhopal India

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/26

forming the connective tissue of the skin and play a crucial role in wound healing, tissue repair, and remodelling (Cao and Zhang, 2017).

Proteins derived from genetic engineering are characterized by control over composition, sequence, molecular mass, and stereochemical purity. Most importantly, unlike native sericin, recombinant sericin is free from contamination. Recently we have recombinantly expressed partial repeat sequences of sericin 1 protein and its fusion with cecropin B in bacteria (Thomas et al., 2020 - paper communicated). The recombinant sericin/sericin-cecropin B has shown strong antibacterial activity against gram-negative and positive bacteria. In this paper, we describe the effect of recombinant sericin-cecropin B fusion protein in the proliferation and cryoprotection of primary human dermal fibroblasts. This is the first report on the use of recombinant sericin fusion protein in cell culture applications.

MATERIALS AND METHODS

Recombinant sericin and sericin-cecropin B proteins: B oth sericin and its fusion with cecropin were expressed in *Escherichia coli* as described by us (Thomas et al., 2020-paper communicated). Briefly, 282 bp *Ser*1 sequence of *Bombyx mori* (Accession No. AB112019) coding for 94 amino acids containing one copy of 38-amino acid motif and two copies of decapeptide repeats alone or fused with an open reading frame of *B. mori* cecropin B (Accession No.NM_001102561) without its signal sequence were cloned in to pET-47b(+) and expressed in *E. coli* Rosetta (DE3) strain. Recombinant proteins were purified by immobilized metal affinity chromatography (IMAC). The following recombinant proteins were used for the assays: 1. sericin, 2. sericin-cecropin B fusion, and 3. proteins purified from bacteria harbouring empty vector as control.

Cells and culture conditions: Human primary adult dermal fibroblast cells (EZXpand Dermal Fibroblast, HiMedia) were cultured in HiFibroXL Fibroblast Expansion Medium, supplements and containing gentamycin-amphoterecin B (HiMedia). This modified culture medium contained reduced amount of FBS (2%, unlike the conventional 10%) as indicated by the manufacturer. Cells were cultured in an incubator at 37 °C under 5% CO_2 till they attained confluency. Each well (6-well culture plates) was seeded with $1x10^5$ cells and each treatment had three replicates. Recombinant protein concentration was 25 or 150 µg/ml and added in to medium in the respective groups. Cells were allowed to grow and observations on adherent cells under microscope followed by digestion with trypsin and counting of cells. The following groups were employed for the assays: 1. CM (Culture medium) alone, 2. CM + PB (Protein purified from bacteria harbouring empty vector), 3. CM + RS (Recombinant sericin), and 4. CM + RSC (Recombinant sericin-cecropin B).

Cryopreservation of cells: Cells were grown to confluency and resuspended at 1 x 10^5 cells/ml in the freezing medium containing culture medium as above, 23% FBS (MP Biomedicals) and 10% DMSO (Sigma). Different concentrations of FBS (11.5%) and DMSO (5%) were also tested. Recombinant protein concentration was 500 µg/ml or 2 mg/ml and added in to medium in the respective groups. Cells after adding the freezing media were chilled on ice for 5 min and then kept at -80°C. After 24 h, cells were thawed, fresh culture medium was added and centrifugation done to remove the DMSO. Cells were then cultured on 6-well plates with 1 ml fresh culture media for a day and observations taken. Cells without the addition of recombinant proteins were also used as control.

Cell morphology, number and viability: Morphology of the cells was studied by visualizing the cells under inverted microscope (Leitz, Germany). The viable and non-viable cell densities were determined by the Trypan Blue (HiMedia) exclusion method using a Neubauer haemocytometer.

RESULTS AND DISCUSSION

Cell proliferation: Results are presented in Figure 1 a and b. The cells grown in complete culture media (CM) were used as control. The attachment, growth and proliferation of cells were found in all groups and as same as that of control. The morphology of cells was typically spindle shaped and comparable to the cells grown in normal culture media. The CM + PB exhibited normal growth as it contained the complete culture media. Comparatively, the cells grown in recombinant sericin (CM + RS) and sericin-cecropin B fusion (CM + RSC) supplemented media showed marked increase in density of viable cells. Subsequently, the cell density gradually increased and attained confluence at 48-72 h. The highest proliferation of cells was observed in media supplemented with recombinant sericin-cecropin fusion protein CM + RSC. Compare to CM, the number of cells in the CM + RSC and CM + RS increased by 1.22 and 1.16 times, respectively, on 72 h. However, higher concentration of sericin and sericin-cecropin fusion protein drastically inhibited the cell growth and proliferation, resulting in round shaped and reduced number of cells (Figure 1a and b-D). We could not alter the concentration of FBS as the culture media supplied by the manufacturer already contained FBS with other ingredients. However, reduced amount of FBS was used in cryopreservation studies (below).

Cryopreservation: As shown in Figure 2, addition of recombinant sericin or its fusion with cecropin in the standard freezing media (CM containing 10% DMSO and 23% FBS) resulted in an increased number of viable cells recovered after freezing- thawing. Compared to all groups, the maximum cell viability after cryopreservation was achieved with CM + RSC followed by CM + RS. Reduced amount of FBS to 11.50% did not alter the viability of cells in the presence of CM + RS or CM + RSC when compared to cells treated with CM alone. However, FBS below 11.50% was found harmful (data not shown). The concentration of DMSO at 5% had deleterious effect on the survivability of cells although the effect was less

pronounced in CM + RS and CM + RSC groups. Further, when the concentration of recombinant sericin and its fusion protein was increased to 2 mg/ml, significant decline in viability of cells was observed (Figure 2-D).

In our study, addition of recombinant sericin or sericin-cecropin fusion proteins along with FBS supplemented media can be used for enhanced growth of human dermal fibroblast cells. Effect of sericin on serum-free or serum-reduced media on cell culture is reported (review by Cao and Zhang, 2017). However, previous studies are limited by the use of native sericin except one report which shows recombinant sericin helps in the proliferation of cells (Terada et al., 2002). In addition, no report on the recombinant sericin fusion protein on cell culture studies yet. The recombinant sericin in our constructs has repeat regions of Ser1 protein composed of 94 amino acid containing one copy of 38-amino acid motif and two copies of decapeptide repeats. The molecular mass of the recombinant sericin and sericin-cecropin is approximately 12 and 16 kDa, respectively. Low molecular weight sericin is most suitable for cell cultures (Terada et al., 2005; Cao and Zhang, 2017). The repeats in Ser1 protein is reported to have mitogenic and cryoprotective properties (Cao and Zhang, 2017). Sericin can promote proliferation of various types of cells including human fibroblast cells (Terada et al. 2002; 2005; Tsubouchi et al., 2005; Toyosawa et al., 2006; Aramwit et al., 2010; Liu et al., 2016).

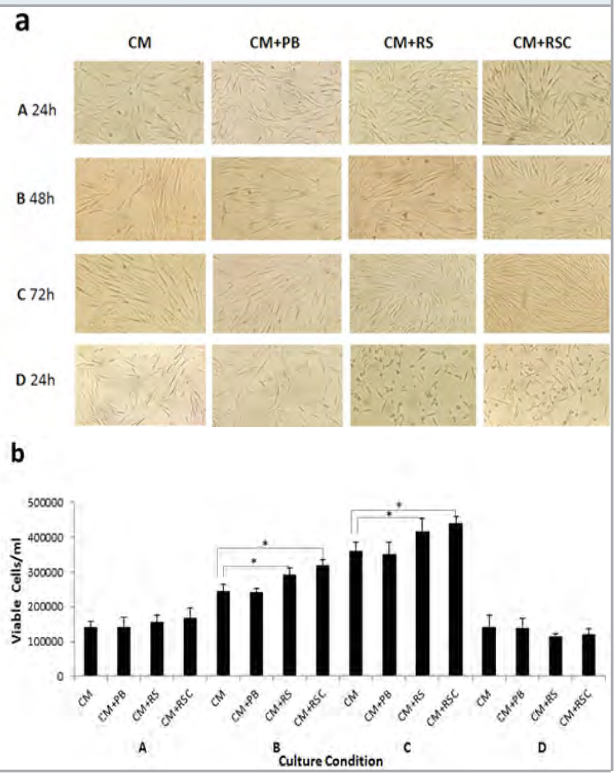
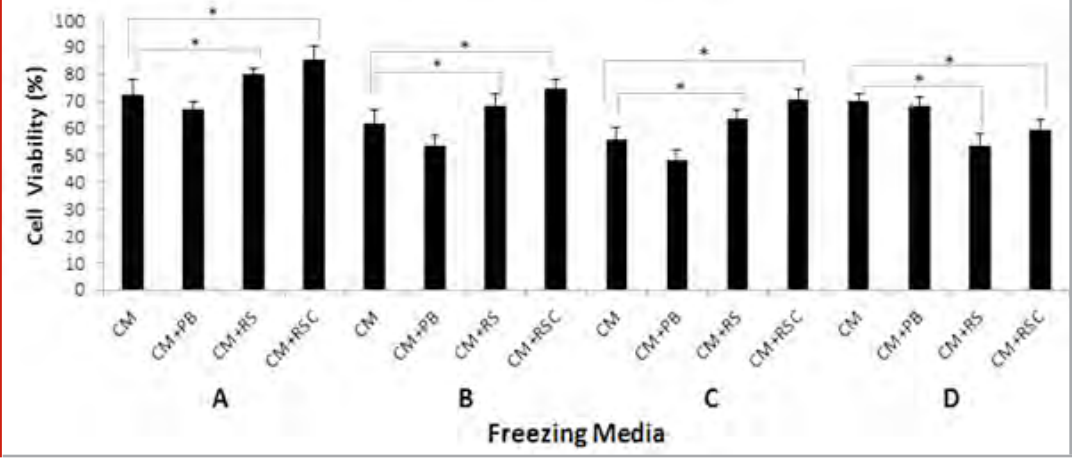


Figure 1. Effect of recombinant sericin and sericin-cecropin fusion proteins on human dermal fibroblast cell proliferation. CM: culture medium, CM + PB: culture medium containing proteins purified from bacteria harbouring empty vector, CM + RS: culture medium containing recombinant sericin, CM + RSC: culture medium containing recombinant sericin-cecropin fusion protein. Recombinant protein concentration was 25 µg/ml in the respective groups except in D where it was 150 µg/ml. Observations were taken at 24, 48, and 72 h (A-C) and 24 h in D. a: Microscope observations showing cell attachment, morphology and proliferation, b: Estimation of viable cells. *Significant difference from control $p < 0.05$ by Student's t-test

At the same time, sericin also suppresses carcinogenesis and tumour promotion by reducing oxidative stress (Zhaorigetu et al., 2003). CHO and Hela cells cultured in the medium with 15 µg/ml sericin hydrolysate instead of FBS show survivability of both cells is similar or higher than that of FBS group (Zhang et al., 2019). The addition of recombinant sericin-cecropin further increased the proliferation of cells indicating that a combination of both sericin and cecropin yielded superior cultures than sericin alone. Similar to other polycationic peptides, cecropin B possesses broad antimicrobial spectrum, antioxidant and anti-endotoxin activity (Moore et al.,1996). Further, cecropin B and analogues have shown cytotoxic activity against several cancer cell lines but do not affect normal cells and shown proliferation of fibroblast cells (Reed et al., 1992; Chen et al., 1997). The concerted action of both sericin and cecropin resulted in the enhanced proliferation of cells in our study. The proliferation of cells promoted by sericin is shown to be concentration-dependent. Sericin concentration from 0.01 to 0.1% was simulative while 1% is harmful to the culture (Terada et al., 2002) and our results are comparable with this observation. Using synthetic gene constructs, varying number of 38-amino acid repeats of *B. mori* sericin 1 protein are expressed in bacteria (Tsujimoto et al., 2001; Huang et al., 2003). In the presence of 0.01% of a recombinant sericin consists of two to four repeats of the consensus sequence, stimulated the hybridoma proliferation as that of native sericin (Terada et al., 2002). To conclude, addition of sericin-cecropin fusion protein into FBS culture medium yields superior cell viability to FBS culture medium alone.

The cryopreservation of cells is crucial for the continuous source of functional cell lines. The conventional freezing media contains 10% DMSO and 25% FBS. There is significant difference in the viability of cells in the freezing media when supplemented with either recombinant sericin or sericin-cecropin B fusion protein. However, reduced FBS concentration to half results in lower cell viability, but the cell viability is still at par with control in sericin and sericin-cecropin treated groups. Reduction in DMSO affects cell viability with a slight change in fibroblast survivability in the presence of recombinant sericin-cecropin. Our results show that replacement of FBS may not be advisable but freezing media with reduced amount of FBS, 10% DMSO along with sericin-cecropin is better for the cryopreservation of human primary dermal fibroblast cells. A 10% DMSO concentration is optimal both for the freezing of primary

Figure 2. Human dermal fibroblast cell viability after 24 h of cryopreservation using varying concentrations of freezing media. Number of frozen cells was set as 100%. A: culture medium (CM), 10% DMSO and 23% FBS; B: CM, 10% DMSO and 11.50% FBS; C: CM, 5% DMSO and 25% FBS; D: CM, 10% DMSO and 25% FBS; Recombinant protein concentration was 500 µg/ml in A-C and 2 mg/ml in D. *Significant difference from control p< 0.05 by Student's t-test.



hMSCs and SAOS-2 cell line, and that sericin cannot compensate for a 5% decrease of DMSO (Verdanova et al., 2014). Most studies to date have not shown sericin superior to FBS as a cryopreservative agent (Toyosawa et al., 2009; Miyamoto et al., 2012; Ohnishi et al., 2012).

However, freezing medium containing 1% sericin, 0.5% maltose, 0.3% proline, 0.3% glutamine and 10% DMSO in PBS is found better than medium with FBS using various cell lines (Sasaki et al., 2005). The serine-rich repetitive motif of 38 amino acid residues in the silk protein ser1 is found to act as a cryoprotectant of cells under freezing conditions and it functions as an antioxidant barrier against oxidative stress (Tsujimoto et al., 2001). The combined action of both sericin and cecropin B, which is also an effective antioxidant, gives extra protection to cells under freezing stress conditions. Our results clearly show that recombinant sericin or sericin-cecropin fusion proteins have significant impact on proliferation and cryopreservation of primary human dermal fibroblast cells and can be used as cell culture supplements. Another potential application of the recombinant sericin-cecropin fusion protein is that it can be an attractive alternative to conventional antibiotics used in cell culture media.

ACKNOWLEDGEMENTS

DST, CM, and SR are thankful to Central Silk Board (CSB), Bengaluru, India for financial assistance in the form of Junior and Senior Research Fellowships. This work was supported by a grant from CSB to RG.

REFERENCES

Aramwit P, Kanokpanont S, Nakpheng T Srichana T (2010) The effect of sericin from various extraction methods on cell viability and collagen production. Int J Mol Sci, 11:2200–2211

Cao T and Zhang y (2017) The potential of silk sericin protein as a serum substitute or an additive in cell culture and cryopreservation. Amino acids, DOI 10.1007/s00726-017-2396-3

Chen H, Wang W, Smith D and Chan SC (1997) Effects of the anti-bacterial peptide cecropin B and its analogs, cecropins B-1 and B-2, on liposomes, bacteria, and cancer cells. Biochimica et Biophysica Acta, 1336:171-179

Huang, J, Valluzzi R, Bini E, Vernaglia B, and Kaplan, D. L (2003) Cloning, expression, and assembly of sericin-like protein. J Biol Chem, 278: 46117–46123

Kundu SC, Dash BC, Dash R and Kaplan DL. (2008) Natural protective glue protein, sericin bioengineered by silkworms: Potential for biomedical and biotechnological applications. Prog Poly Sci, 33: 998–1012

Liu LY, Wang JH, Duan SH, Chen L, Xiang H, Yang Dong, Wang W (2016) Systematic evaluation of sericin protein as a substitute for fetal bovine serum in cell culture. Sci Rep,6:31516. doi:10.1038/srep31516.

Miyamoto Y, Oishi K, Yukawa H, Noguchi H, Sasaki M, Iwata H and Hayashi S. (2012) Cryopreservation of human adipose tissue-derived stem/progenitor cells using the silk protein sericin. Cell Transplant, 21: 617–622

Moore AJ, Beazley WD, Bibby MC, Devine DA.(1996) Antimicrobial activity of cecropins. J Antimicrob Chemother,37:1077–1089

Ohnishi K, Murakami M, Morikawa M, and Yamaguchi A. (2012) Effect of the silk protein sericin on cryopreserved rat islets. J Hepato Bil-Pan Sci, 19:354–360

Reed WA, White KL, Enright FM, Holck J, Jaynes JM, and Jeffers, GW. (1992) Enhanced in vitro growth of murine fibroblast cells and preimplantation embryos cultured in medium supplemented with an amphipathic peptide. Mol Rep Dev, 31:106–113

Sasaki M, Kato Y, Yamada H, Terada S. (2005)

Development of a novel serum-free freezing medium for mammalian cells using the silk protein sericin. Biotechnol Appl Biochem, 42:183–188

Terada S, Nishimura T., Sasaki M., Yamada H and Miki M. (2002) Sericin, a protein derived from silkworms, accelerates the proliferation of several mammalian cell lines including a hybridoma. Cytotechnology, 40: 3–12

Terada, S., Sasaki, M., Yanagihara, K., and Yamada, H. (2005) Preparation of silk protein sericin as mitogenic factor for better mammalian cell culture, J Biosci Bioeng,100: 667–671

Thomas DS, Manoharan C, Rasalkar, S, Mishra, RK and Gopalapillai, R. (2020) Recombinant expression of sericin-cecropin fusion protein and its functional activity (Communicated).

Toyosawa T, Terada S, Sasaki M, Yamada H, Kino-oka M. (2006) Observation of individual cell behaviors to analyze mitogenic effects of sericin. Animal Cell Technol: Basic & Appl Aspects, 3:155–161

Toyosawa T, Oumi Y, Ogawa A. (2009) Novel serum-free cryopreservation of mammalian cells using sericin. Animal Cell Technology, 15:41–45

Tsubouchi K, Igarashi Y, Takasu Y, Yamada H.(2005) Sericin enhances attachment of cultured human skin fibroblasts. Biosci Biotechnol Biochem, 69:403–405

Tsujimoto, K., Takagi, H., Takahashi, M, Yamada, H., and Nakamori, S. (2001) Cryoprotective effect of the serine-rich repetitive sequence in silk protein sericin, J Biochem,129: 979–986

Verdanova M, Pytlik R, and Kalbacova MH. (2014) Evaluation of sericin as a fetal bovine serum-replacing cryoprotectant during freezing of human mesenchymal stromal cells and human osteoblast-L like Cells. Biopreserv Biobank, 12: 99-105

Zhang M, Cao T, Wei Z, and Zhang Y. (2019) Silk sericin hydrolysate is a potential candidate as a serum-substitute in the culture of Chinese hamster ovary and henrietta lacks cells. J Insect Sci, 19: 10. doi: 10.1093/jisesa/iey137.

Zhaorigetu S, Yanaka N, Sasaki M, Watanabe H, and Kato N. (2003) Sericin consumption suppresses development and progression of colon tumorigenesis in 1,2-dimethylhydrazine-treated rats. Oncol Rep, 10: 537–543

Dental
Communication

BBRC
Bioscience Biotechnology
Research Communications

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp-151-155

Influence of Nd-YAG And Er-Cr-YSGG Laser Conditioning on the Adhesive Bond Strength of Resin Modified Glass Ionomer Cement with Dentin

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ABSTRACT

To assess and compare different conditioning regimes (laser and conventional) on shear bond strength (SBS) of resin modified glass ionomer cement (RMGIC) bonded to dentinal tissue. Sixty permanent intact human third molars were prepared and allocated into six groups (n=10 each). Group 1, dentin was conditioned with Nd-Yag laser (NYL), Group 2: Er, Cr:YSGG laser (ECL), group 3: total etch adhesive, group 4: self-etch adhesive resin, group 5: 25% polyacrylic acid (PAA) and group 6: no treatment group. After surface conditioning of all fifty samples resin modified glass ionomer cement (RMGIC) was mixed and build up was performed. For SBS testing, specimens (n=10) in each group were placed in a custom jig of a Universal testing machine. For the de-bonded surface fracture analysis was completed using stereomicroscope at 40x magnification. Descriptive statistics i.e., means and standard for SBS were compared using analysis of variance (ANOVA) and Tukey's post hoc test at a significance level of ($p<0.05$). The maximum SBS values were observed in PAA (20.44±3.92) and the minimum values were noted (12.54±2.25). RMGIC conditioned with NYL (13.45±2.44) and control group (12.45±2.25) were comparable $p>0.05$. In laser, irradiated groups admixed failure was common. Dentin conditioned with PAA exhibited cohesive failure. The use of ECL and not NYL has a protentional to be used as conditioning regime in clinical setting prior to application of RMGIC.

KEY WORDS: RMGIC, DENTIN CONDITIONING, ER,CR:YSGG, ND-YAG LASER, SHEAR BOND STRENGTH.

INTRODUCTION

In clinical practice premature restorative failure is a major concern. Most of the failures results at the interface between tooth structure and dental tissue due to poor bond strength resulting in secondary caries, microleakage, discoloration and fracture (Mjör and Gordan, 2002). Available evidence suggests glass ionomer cements (GIC) unveils improved bond integrity due to physio-chemical bonding with hydroxy appetite crystals in dentin, compared to adhesive resins (De Munck et al., 2005; Yoshida et al., 2000). In recent years resin modified glass ionomer cement (RMGIC) has become popular due to fluoride release and biocompatibility (Swartz et al., 1984). But, debate still exits on tooth conditioning prior to RMGIC application. Recent reports have shown RMGIC to improve bond integrity on application of various conditioning regimes(Cardoso et al., 2010; Hotz et al., 1977; Powis et al., 1982). Whereas, other studies have stated no improvement in bond strength after surface treatment (Hamama et al., 2014; Imbery et al., 2013). The prime purpose for conditioning dentin is to remove the smear layer formed during cavity preparation which acts as a barrier, in forming a durable bond. Currently, there are numerous conditioners available in the market, but their efficacy and effectiveness are still dubious, (Alkhudhairy, et al., 2019a).

ARTICLE INFORMATION

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Received 4th Feb 2020 Accepted after revision 20th March 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)
A Society of Science and Nature Publication, Bhopal India

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/27

Alternative methods in the form of lasers Er,Cr:YSGG (ECL) and Nd-Yag (NYL) to condition tooth surface has displayed convincing results, (Alkhudhairy, AlKheraif, et al., 2018; Alkhudhairy, Naseem, et al., 2018; Vohra et al., 2018, Alkhudhairy, Naseem, Ahmad, et al., 2019; Alkhudhairy, Vohra and Naseem, 2019a; Alkhudhairy, Vohra, Naseem, et al., 2019). Interestingly, existing data suggests that lased dentinal surface exhibit open tubules, increase roughness and caters resembling to acid etched surface, (Ekworapoj et al., 2007, Alkhudhairy, Vohra and Naseem, 2019a). Moreover, it provides a clean surface free of smear layer and enhances tooth adhesion (Kobayashi et al., 2003). To our knowledge from indexed literature limited evidence on the use of lasers (ECL, NYL) as dentin conditioner prior to GIC application exists. Moreover, efficacy of different conditioning regimes and their comparison with laser conditioning is scarce and limited. It is hypothesized that dentin conditioned with ECL and NYL will exhibit bond strength similar to dentin treated with other surface regimens. Therefore, the aim of the present study was to assess and compare different conditioning regimes (lased and conventional) on shear bond strength (SBS) of RMGIC bonded to dentinal tissue.

MATERIAL AND METHODS

Sixty permanent intact human third molars free of caries, cracks were used as a bonding substrate. Teeth were isolated and stored in 0.5% chloramine T solution at 4 °C for duration of one month to disinfect, following storage in distilled water until use. With a low speed diamond saw (Isomet, Buehler, USA) the enamel surface was removed, and dentin surface was made flat. To attain a homogenize surface roughness, dentin polishing using silicon carbide grinding discs 800 and 1200 grits (Buehler, Great Britain UK) under water irrigation on a polishing machine (Stone Liu Shanghai Smedent Medical Instrument Co., Ltd) was used. Teeth were mounted vertically in acrylic resin within the sections of polyvinyl pipes (6mm diameter) up to cement-o-enamel junction (CEJ). Now based on the conditioning protocol the samples were allocated into six groups (n=10 each).

Group 1: Dentin surface was conditioned with Nd-Yag laser (NYL) (Hoya ConBio Delight, Sweden & Martina, Padova, Italy) at 150 mJ, 10 Hz and 1W for a duration of 60 sec in a non-contact circular motion using a quartz optical fibre having diameter of 320-µm.

Group 2: The surface of bonded dentin samples were treated with Er,Cr:YSGG laser (ECL) (Biolase- Waterlase I-Plus) at 0.5W and power 30Hz in a circular, non-contact position using tip MZ6 from a distance of 1mm for duration of 60 sec.

Group 3: 37% phosphoric acid was applied on dentin for 5 sec and washed with water spray thoroughly for 10sec. Bonding agent was applied and air dried for 10sec without the surface being desiccated. The bonding agent was reapplied and photo cured (Bluephase G2, Ivoclar,Vivadent) for 10 sec.

Group 4: On bonded dentinal surface self-etching primer was implemented and left undisturbed for 20 sec, gently air dried. Bonding agent was applied, spread uniformly with a light air stream, and light cured (Bluephase G2, Ivoclar,Vivadent) for 10 s.

Group 5: Polyacrylic acid (PAA) was applied on bonded dentinal surface and was left undisturbed for 10 sec. The surface was rinsed for 10 sec and air dried 5 sec without desiccation.

Group 6: Dentinal surface of this group did not receive any surface treatment.

After surface conditioning regimes of all fifty samples resin modified glass ionomer cement (RMGIC) Fuji II LC GC, Tokyo, Japan was mixed according to manufacturer instructions and build up was done incrementally using a tofelmire matrix holder at a height of 5mm and cured 20sec (Bluephase G2, Ivoclar,Vivadent).The specimens were covered with GC varnish (GC, America, Inc) and stored in distilled water at 24C until use. Shear bond Strength (SBS) :For SBS testing specimen (n=10) each group were placed in a custom jig of a Universal testing machine (Lloyds, LF, plus, Ametek Inc, Great Britain, UK) under a cross head speed of 1mm/ min until failure occurred. The force was applied parallel to bonded samples. The maximum force required to generate fracture was recorded in MPa.

Failure mode Analysis: Fracture analysis was completed by two examiners using stereomicroscope at 40x magnification (Preconfigured Olympus Stereo Microscope Systems, SZX7, Edmund Optics UK). Type of failure was categorized into adhesive cohesive and admixed. **Statistical Analysis:** SBS data was tabulated using statistical program for social science (SPSS version 19, Inc., Chicago, US). Normality of data obtained was assessed using Kolmogorov-Smirnov test. Descriptive statistics i.e., means and standard for SBS were compared using analysis of variance (ANOVA) and Tukey's post hoc test at a significance level of ($p < 0.05$)

RESULTS AND DISCUSSION

The data observed in the existing study was normally distributed. Table 1 displays SBS of RMGIC bonded to different conditioning regimes to dentin. The maximum SBS values were observed in PAA (20.44±3.92) and the minimum values were noted (12.54±2.25). RMGIC conditioned with NYL (13.45±2.44) and control group (12.45±2.25) were comparable $p>0.05$. Furthermore, conditioning of RMGIC with ECL (18.54±3.24), TE (19.44±3.74), SE (18.22±3.55) and PAA (20.44±3.92) were comparable $p>0.05$. Mode of failure amongst all experimental groups, adhesive failure type was pertinent (Figure 1). In laser, irradiated groups admixed failure was common. Moreover, In TE and SE groups adhesive failure was found in majority. Dentin conditioned with PAA exhibited cohesive failure. The existing study was based on the hypothesis that dentin conditioned with ECL and NYL and bonded to RMGIC will exhibit comparable bond

integrity to different conditioning regimes. The present in-vitro experiment revealed that bond strength of dentin conditioned with ECL was comparable to PAA, SE and TE. Whereas, dentin conditioned with NYL displayed low bond strength scores compared to different conditioning regimes but was comparable to non-treatment group. Therefore, the hypothesis was partially accepted. The durability of a restoration is contingent on the adhesive capability, which can be assessed by SBS testing. In the present study SBS of the restoration was determined using universal testing machine. The method is simple to use, widely acceptable, provides comparative analysis and samples does not require further processing after bonding procedures (Sirisha, Rambabu, Ravishankar, et al., 2014; Sirisha, Rambabu, Shankar, et al., 2014).

Conditioners are used to promote adhesion, remove smear layer, improve surface energy and promote surface wettability (Tanumiharja et al., 2000). In the present study ECL at 0.5 W and 30 Hz was used to condition dentin prior to RMGIC. ECL works at a wavelength of 2780 micrometres displays strong affinity to hydroxyapatite crystals in dentin and water. In the existing study ECL at low level exhibited SBS (18.54±3.24) similar to dentin conditioned with PAA (20.44±3.92). This finding was in line with a study by (Alkhudhairy, Al-Johany, Naseem, et al., 2019; Ekworapoj et al., 2007; Garbui et al., 2013). A probable explanation to these conclusions is ECL at low level causes loss of water and collapse of the dentinal collagen resulting in reduction of hydrophilicity of dentin, which indirectly improves the suitability of hydrophobic polymeric materials like RMGIC to adhere well with the dentin structure (Alkhudhairy, Al-Johany, Naseem, et al., 2019). Furthermore, lased dentin results in smear free layer following easy penetration of RMGIC exhibiting strong bond affinity (Ekworapoj et al., 2007). Moreover, due to improved surface energy of lased dentin ionic exchange between the intermediary layer of RMGIC and dentin structure might influence better bod integrity (Alkhudhairy, Vohra and Naseem, 2019b).

The other laser used in the present study NYL (13.45±2.44) exhibited statistically significant difference compared to PAA (20.44±3.92) TE (19.44±3.74) and SE (18.22±3.55) experimental groups. These findings were not in harmony with the outcome of work carried out by Kobayashi et al., (2003) and Aljdaimi et al., (2018). In authors opinion diversity in results can be attributed to power and frequency of laser, duration of laser, type of dentin (human or bovine) and kind of RMGIC and conditioning regimes. Moreover, since the power of NYL used in the current study was 1W it is conceivable that NYL at low level did not significantly removed the smear layer, altered the hybrid layer and produced morphological changes in dentin compromising bond integrity (Kobayashi et al., 2003). Dentin conditioned with TE (19.44±3.74), SE (18.22±3.55) and PAA (20.44±3.92) displayed comparable bond strength. In the current study, 37% phosphoric acid was used for 5 sec to condition dentin. Reducing the conditioning time to 5 sec enhances micro-mechanical retention by forming resin tags, enhances resin infiltration of RMGIC and results in less loss of calcium ions improving ionic bonding of RMGIC with dentin (Tay et al., 2001).Moreover, SE adhesives (18.22±3.55) displayed SBS values similar to dentin conditioned with PAA (20.44±3.92) and TE (19.44±3.74).

Our findings, of the present study were in concurrent with the results of (Besnault et al., 2004; Coutinho et al., 2006). Multiple explanations can be given for such outcomes. Primary, reason is SE adhesives and RMGIC both have carbon-carbon bonds which on polymerization changes to covalent bonds improving adhesion. Secondly, presence of water in both RMGIC and SE adhesives improves their wettability on dentinal surface which indirectly improve RMGIC spread over dentin conditioned surfaces with SE adhesives. Thirdly, low modulus of elasticity of RMGIC renders low polymerization shrinkage compared to resin composites (Besnault et al., 2004; Coutinho et al., 2006). Based on the result of our finding the author recommends use of SE adhesives to condition dentin as the conditioning method is less technique sensitive and more user friendly and provide desirable results similar to TE.

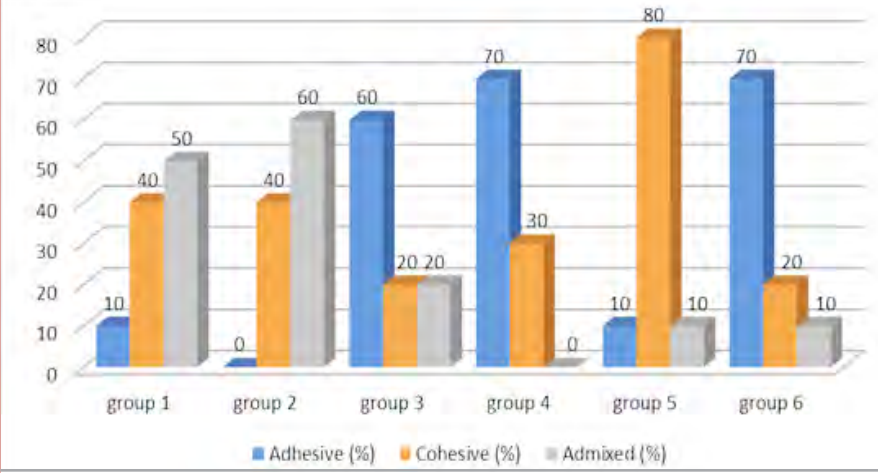
The highest bond strength scores were observed in group conditioned with PAA (20.44±3.92). Possible explanation reported for this outcome is that dentinal surface conditioned with acid creates irregularities and pores on the substrate surface boosting adhesion (Hamama et al., 2014). This finding was in synchronization with work of Tanumiharja et al., (2000). Moreover, in the same study by Tanumiharja et al.,(2000) reported that

Table 1. Comparison of means and SD for bond strength values among study groups using ANOVA and Tukey multiple comparisons test

Type of RMGIC	Group 1: Nd-Yag laser (NYL) irradiation	Group 2: Er, Cr:YSGG laser (ECL) irradiation	Group 3: Total etch (Optibond solo Plus)	Group 4: Self-Etch (Clearfill SE)	Group 5: Dentin Conditioner 10% Polyacrylic acid) PAA	Group 6: Control No Treatment	P-value!
Fiji II LC	13.45±2.44a	18.54±3.24b	19.44±3.74b	18.22±3.55b	20.44±3.92b	12.45±2.25a	<0.05

Different upper script letters indicate statistical differences ($p < 0.05$).
! Showing significant difference among study group (ANOVA

Figure 1. Modes of failure among different experimental groups



wettability of surface enhances infiltration of HEMA into collagen network of dentin increasing bond scores. Recently, work by Sauro et al., (2018) reported dentin conditioned with PAA increases the risk of collagen collapse and degradation at dentin/ material interface under prolong stress, mechanical cycling and saliva immersion ultimately leading to bond failure. Therefore, though the SBS values of PAA conditioned group is the highest in the existing study, PAA as conditioner should be used with caution. Based on fracture analysis, admixed and cohesive failure was found to be pertinent in lased dentin and surface conditioned with PAA. Such failure type can be attributed to porosity within the material which act as a centre of stress resulting in breakage within the material, implying better bond integrity (Saraç et al., 2009).

The biggest limitation of the study was not carrying out the micro morphological analysis of the bonded surface. Conditioning of dentin surface using laser is a unique concept and needs further clinical and non-clinical assessment in the form of durability testing of conditioned surface, surface energy measurements, surface profiling and scanning electron microscopic evaluation. Promising invitro results not always can assure clinical success. These laboratory-based studies act as a screening tool for selection of materials used in in-vivo study design. Therefore, more studies to extrapolate the findings and recommendations of present study should be performed.

CONCLUSION

The use of ECL and not NYL has a protentional to be used as conditioning regime in clinical setting prior to application of RMGIC.

REFERENCES

Aljdaimi, A., Devlin, H. and Dickinson, M. (2018), Effect of the Er: YAG laser on the shear bond strength of conventional glass ionomer and BiodentineTM to dentine European Journal of Dentistry, Wolters Kluwer Medknow Publications, Vol. 12 No. 3, pp. 380–385.

Alkhudhairy, F., Al-Johany, S.S., Naseem, M., Bin-Shuwaish, M. and Vohra, F. (2019), Dentin bond strength of bioactive cement in comparison to conventional resin cement when photosensitized with Er,Cr:YSGG Laser Pakistan Journal of Medical Sciences, Vol. 36 No. 2, available at:https://doi.org/10.12669/pjms.36.2.1284.

Alkhudhairy, F., AlKheraif, A., Bin-Shuwaish, M., Al-Johany, S., Naseem, M. and Fahim Vohra, Mc. (2018), Effect of Er,Cr:YSGG Laser and Ascorbic Acid on the Bond Strength and Microleakage of Bleached Enamel Surface Photomedicine and Laser Surgery, Vol. XX No. Xx, pp. 1–8.

Alkhudhairy, F., Naseem, M., Ahmad, Z.H., Alnooh, A.N. and Vohra, F. (2019), Efficacy of phototherapy with different conventional surface treatments on adhesive quality of lithium disilicate ceramics Photodiagnosis and Photodynamic Therapy, Elsevier B.V., Vol. 25, pp. 292–295.

Alkhudhairy, F., Naseem, M., Bin-Shuwaish, M. and Vohra, F. (2018) Efficacy of Er Cr: YSGG laser therapy at different frequency and power levels on bond integrity of composite to bleached enamel, Photodiagnosis and Photodynamic Therapy, Vol. 22, available at:https://doi.org/10.1016/j.pdpdt.2018.02.019.

Alkhudhairy, F., Vohra, F. and Naseem, M. (2019a), Influence of Er,Cr:YSGG Laser Dentin Conditioning on the Bond Strength of Bioactive and Conventional Bulk-Fill Dental Restorative Material Photobiomodulation, Photomedicine, and Laser Surgery, available at:https://doi.org/10.1089/photob.2019.4661.

Alkhudhairy, F., Vohra, F. and Naseem, M. (2019b) Influence of Er,Cr:YSGG Laser Dentin Conditioning on the Bond Strength of Bioactive and Conventional Bulk-Fill Dental Restorative Material Photobiomodulation, Photomedicine, and Laser Surgery, available at:https://doi.org/10.1089/photob.2019.4661.

Alkhudhairy, F., Vohra, F., Naseem, M. and Ahmad, Z.H.

(2019), Adhesive bond integrity of dentin conditioned by photobiomodulation and bonded to bioactive restorative material", Photodiagnosis and Photodynamic Therapy, Elsevier B.V., Vol. 28, pp. 110–113.

Besnault, C., Attal, J.P., Degrange, M. and Ruse, D. (2004), Self-etching adhesives improve the shear bond strength of a resin-modified glass-ionomer cement to dentin", Journal of Adhesive Dentistry, Vol. 6 No. 1, pp. 55–59.

Cardoso, M.V., Delmé, K.I.M., Mine, A., Neves, A. de A., Coutinho, E., De Moor, R.J.G. and Van Meerbeek, B. (2010), Towards a better understanding of the adhesion mechanism of resin-modified glass-ionomers by bonding to differently prepared dentin, Journal of Dentistry, Vol. 38 No. 11, pp. 921–929.

Coutinho, E., Van Landuyt, K., De Munck, J., Poitevin, A., Yoshida, Y., Inoue, S., Peumans, M., et al. (2006), Development of a self-etch adhesive for resin-modified glass lonomer Journal of Dental Research, Vol. 85 No. 4, pp. 349–353.

Ekworapoj, P., Sidhu, S.K. and McCabe, J.F. (2007), Effect of surface conditioning on adhesion of glass ionomer cement to Er,Cr:YSGG laser-irradiated human dentin", Photomedicine and Laser Surgery, Vol. 25 No. 2, pp. 118–123.

Garbui, B.U., De Azevedo, C.S., Zezell, D.M., Aranha, A.C.C. and Matos, A.B. (2013), Er,Cr:YSGG Laser dentine conditioning improves adhesion of a glass ionomer cement", Photomedicine and Laser Surgery, Vol. 31 No. 9, pp. 453–460.

Hamama, H.H., Burrow, M.F. and Yiu, C. (2014), Effect of dentine conditioning on adhesion of resin-modified glass ionomer adhesives Australian Dental Journal, Vol. 59 No. 2, pp. 193–200.

Hotz, P., McLean, J.W., Sced, I. and Wilson, A.D. (1977), The bonding of glass ionomer cements to metal and tooth substrates, British Dental Journal, Vol. 142 No. 2, pp. 41–47.

Imbery, T.A., Namboodiri, A., Duncan, A., Amos, R., Best, A.M. and Moon, P.C. (2013), "Evaluating dentin surface treatments for resin-modified glass ionomer restorative materials", Operative Dentistry, Vol. 38 No. 4, pp. 429–438.

Kobayashi, C.A., Osada, T., Fukunaga, H., Kawawa, T., Fujishima, A., Miyazaki, T., Kimura, Y., et al. (2003) Effect of Nd:YAG laser irradiation on shear bond strength of glass-ionomer luting cement to dentin surface, International Journal of Prosthodontics, Vol. 16 No. 5, pp. 493–498.

Mjör, I.A. and Gordan, V. V. (2002), Failure, repair, refurbishing and longevity of restorations Operative Dentistry.

De Munck, J., Van Landuyt, K., Peumans, M., Poitevin, A., Lambrechts, P., Braem, M. and Van Meerbeek, B. (2005), A critical review of the durability of adhesion to tooth tissue: Methods and results Journal of Dental Research, February.

Powis, D.R., Folleråas, T., Merson, S.A. and Wilson, A.D. (1982), Improved adhesion of a glass ionomer cement to dentin and enamel. Journal of Dental Research, Vol. 61 No. 12, pp. 1416–1422.

Saraç, D., Külünk, S., Saraç, Y.S. and Karakas, Ö. (2009), Effect of fluoride-containing desensitizing agents on the bond strength of resin-based cements to dentin, Journal of Applied Oral Science, Bauru School of Dentistry, University of Sao Paulo, Vol. 17 No. 5, pp. 495–500.

Sauro, S., Faus-Matoses, V., Makeeva, I., Martí, J.M.N., Martínez, R.G., Bautista, J.A.G. and Faus-Llácer, V. (2018), Effects of polyacrylic acid pre-treatment on bonded-dentine interfaces created with a modern bioactive resin-modified glass ionomer cement and subjected to cycling mechanical stress, Materials, MDPI AG, Vol. 11 No. 10, available at:https://doi.org/10.3390/ma11101884.

Sirisha, K., Rambabu, T., Ravishankar, Y. and Ravikumar, P. (2014), Validity of bond strength tests: A critical review-Part II, Journal of Conservative Dentistry, Medknow Publications, 1 September.

Sirisha, K., Rambabu, T., Shankar, Y.R. and Ravikumar, P. (2014), Validity of bond strength tests: A critical review: Part I, Journal of Conservative Dentistry, Medknow Publications.

Swartz, M.L., Phillips, R.W. and Clark, H.E. (1984), Long-term F Release from Glass Ionomer Cements, Journal of Dental Research, Vol. 63 No. 2, pp. 158–160.

Tanumiharja, M., Burrow, M.F. and Tyas, M.J. (2000), Microtensile bond strengths of glass ionomer (polyalkenoate) cements to dentine using four conditioners Journal of Dentistry, Vol. 28 No. 5, pp. 361–366.

Tay, F.R., Smales, R.J., Ngo, H., Wei, S.H. and Pashley, D.H. (2001), Effect of different conditioning protocols on adhesion of a GIC to dentin. The Journal of Adhesive Dentistry, Vol. 3 No. 2, pp. 153–67.

Vohra, F., Alghamdi, A., Aldakkan, M., Alharthi, S., Alturaigi, O., Alrabiah, M., Al-Aali, K.A., et al. (2018), Influence of Er: Cr: YSGG laser on adhesive strength and microleakage of dentin bonded to resin composite. In-vitro study, Photodiagnosis and Photodynamic Therapy, Vol. 23, available at:https://doi.org/10.1016/j.pdpdt.2018.08.002.

Yoshida, Y., VanMeerbeek, B., Nakayama, Y., Snauwaert, J., Hellemans, L., Lambrechts, P., Vannerle, G., et al. (2000), Evidence of chemical bonding at biomaterial-hard tissue interfaces, Journal of Dental Research, Vol. 79 No. 2, pp. 709–714.

Biomedical
Communication

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp-156-159

BBRC
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The Potential of Health Tourism Regarding Stimulation of Functional Capabilities of the Cardiovascular System

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ABSTRACT

The use of coronary artery bypass grafting due to its complexity and invasiveness requires effective physical rehabilitation. In this regard, inclusion of health tourism into the composition of these measures was considered to be very promising, which could significantly minimize the consequences of the operation in the late postoperative period and improve the quality of life of patients and their degree of adaptation to any form of activity. The study was conducted on 37 patients aged 45-65 years who underwent coronary artery bypass grafting 3 months ago. Patients who received rehabilitation using wellness tourism showed an acceleration and deepening of recovery. Only with its use it was possible to more fully strengthen the cardiovascular system, to optimize the overall physical fitness and ability to self-service. One can think that the use of health tourism provides a quick and pronounced healing effect in patients after coronary artery bypass grafting due to powerful stimulation of the muscular, vascular, respiratory and nervous systems, balancing the processes of anabolism and catabolism and stimulating protein synthesis throughout the body.

KEY WORDS: CORONARY ARTERY BYPASS GRAFTING, REHABILITATION, HEALTH, TOURISM, REHABILITATION.

INTRODUCTION

In the modern world there is a widespread prevalence of cardiological pathology (Medvedev et al., 2007b). Its presence in a significant part of the population, especially of working age, weakens the health of the population as a whole and creates a significant burden on healthcare facilities. This problem stimulates the development of cardiology in all countries of the world, which goes in two ways. One way is to improve the pharmacological effects on the patient's body in order to stabilize their condition. The second way is more radical - it is associated with the improvement of surgical operations on the heart and blood vessels. One of such operations is coronary artery bypass grafting, through which it is possible to increase the duration and quality of life for a large number of people with coronary heart disease. Recently, the number and quality of such operations has increased in the world (Uzhegov, 2005, Skoryatina et al., 2019).

Now coronary artery bypass grafting is considered as a priority treatment for coronary artery lesions. By coronary artery bypass grafting, the necessary blood flow in the narrowed arteries is restored, myocardial hypoxia is eliminated, myocardial infarction is prevented,

ARTICLE INFORMATION

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Received 7th Jan 2020 Accepted after revision 7th March 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/28

its contractile function is strengthened, and the quality and life expectancy of patients improves (Avaliani et al., 2005).Coronary artery bypass grafting is a complex surgical procedure that involves bones and muscles of the chest, heart muscle and blood vessels. Very important for the recovery of patients is the implementation of rehabilitation measures in the postoperative period aimed at minimizing the consequences of the operation, preventing and treating postoperative complications, and increasing the patient's adaptability to motor activity, along with the recovery time, (Mavrodiy, 2017; Mal et al 2018, Makhov et al., 2018b). The aim of the present study was to evaluate the effectiveness of the physical rehabilitation program for patients who underwent coronary artery bypass grafting based on health tourism.

MATERIALS AND METHODS

The study involved 37 patients of 45-65 years age, who underwent coronary artery bypass grafting 3 months ago. Randomly, these patients were divided into 2 equal comparable groups - experimental and experimental. An experimental group (18 people) underwent rehabilitation according to the standard program of the rehabilitation center. It included physiotherapy, classes on simulators, physiotherapy and massage. The experimental group (19 people) underwent rehabilitation according to the program, which was based on health tourism with elements of therapeutic exercises with breathing exercises, the use of Scandinavian walking and massage. In both groups, rehabilitation lasted 2 months. The control group consisted of 22 clinically healthy volunteers of 45-65 years old, examined once. Patients of the experimental and experimental groups were examined twice - when taken under observation and at the end of the rehabilitation course.

Standard research methods were used: analysis and synthesis, estimates of heart rate, respiratory rate, and blood pressure levels. A standard orthostatic test was also performed. The patient was lying for 5 minutes, and then slowly got to his feet. The Ruthier test allowed us to determine the performance of the heart during exercise. The patient was in a lying position for 5 minutes, then the pulse rate was determined for a 15-second interval (P1) at rest. He then performed 30 squats for 45 seconds. After the load, the patient lies down and his heart rate was again counted for the first 15 seconds (P2), and then for the last 15 seconds of the first minute of recovery (P3).

The Ruthier Index was calculated using the formula: 4 x (P1 + P2 + P3) - 200/10.The 6-minute walk test is carried out by determining the distance that the patient is able to walk in 6 minutes along the corridor at the

Table 1. The results of the rehabilitation studies

Indicators	Traditional technique, M±m, n=18		Author's technique, M±m, n=19		Control, M±m, n=22
	exodus	in the end	exodus	in the end	
Heart rate, beats/minute	94.2±1.23	79.0±0.87 p<0.05	90.9±0.93	67.9±0.55 p<0.01 p1<0.05	69.9±1.12
Systolic blood pressure, mmHg	141.0±1.40	133.6±0.75 p<0.05	139.8±1.14	118.0±0.22 p<0.01 p1<0.05	120.1±0.98
Diastolic blood pressure, mmHg	87.0±0.60	80.0±0.57 p<0.05	89.5±0.85	71.7±0.32 p<0.01 p1<0.05	69.5±0.37
Respiratory rate, times / minute	21.3±0.26	18.6±0.46 p<0.05	21.9±0.31	17.3±0.11 p<0.01 p1<0.05	17.1±0.24
Orthostatic test, beats / minute	23.3±0.34	19.0±0.36 p<0.05	25.7±0.47	14.2±0.20 p<0.01 p1<0.05	14.0±0.36
Roufier Index, points	12.9±0.23	8.7±0.20 p<0.01	12.8±0.37	5.2±0.18 p<0.01 p1<0.05	4.5±0.15
6-minute walk test, steps/ minute	325.6±1.68	355.4±1.10	411.3±0.98	488.2±0.62 p<0.01 p1<0.05	501.2±1.54

Legend: p - the reliability of the dynamics during rehabilitation, p1 - the reliability of differences in the results of rehabilitation between groups.

highest possible pace. According to the results, the patient belongs to a certain functional class. The first class corresponded to a distance of 425-550 meters. The second functional class included a distance of 301-425 meters passed. To the third - 151-300 meters. In the fourth functional class, the patient was able to walk less than 150 meters in 6 minutes.The results of the study were processed using Microsoft EXCEL by calculating the Student t-test (t).

RESULTS AND DISCUSSION

The results of the studies carried out in the work are presented in Table 1. No significant differences in the indicators between patients in both groups were found. Comparison of the results of using both rehabilitation options for patients who underwent coronary artery bypass grafting revealed that for all indicators between them there are significant differences in favor of the author's exposure regimen. As a result of the rehabilitation in the experimental group, the heart rate decreased by 33.9%, reaching the control level. In the experimental group, this indicator decreased by only 19.2%. Similar dynamics in the experimental group was experienced by normalized indicators of systolic and diastolic blood pressure, which decreased by 18.5% and 24.8%, respectively. The use of the traditional regimen was accompanied by less functionally beneficial dynamics of blood pressure levels. This was accompanied by a decrease in the frequency of respiratory movements in both groups, more pronounced in the experimental group (26.6%), ensuring its exit to the control level.

During rehabilitation, the orthostatic test index experienced a decrease in the experimental group by 22.6%, in the experimental group by 80.9% with its exit in the second case to the control level. At the same time, the Ruthier index also returned to normal only against the background of the use of author's rehabilitation schemes. In the experimental group, it decreased by 2.5 times, while in the experimental group it decreased by only 48.3%.When evaluating the results of the 6-minute walk test, it turned out that the author's rehabilitation scheme provided a more pronounced than the traditional scheme increase in the number of steps that the patient is able to go through during the test (by 18.7%). Moreover, the achieved changes allowed this indicator to normalize during the observation period only in the experimental group. Coronary artery bypass grafting is a complex surgical intervention that allows you to restore hemodynamics in the arteries of the heart by bypassing the site of narrowing of the coronary vessel using shunts. It is a proven surgical method for the treatment of coronary heart disease (Bokeria et al., 2016). Despite the rapid and significant improvement in hemodynamics, after this operation, the general condition of these patients improves slowly. Often, recovery can be very delayed (Mal et al., 2018).

Given the importance of accelerating the process of rehabilitation of patients after coronary artery bypass grafting and especially increasing its effectiveness, we tested two rehabilitation schemes for such patients. More preferred results were obtained in a group of patients undergoing rehabilitation based on health tourism. This was largely due to the achievement of a more pronounced improvement in the state of the cardiorespiratory system and the level of their general physical capabilities.

The obtained results give reason to believe that the property of Wellness tourism with elements of easy exercises, breathing exercises, Nordic walking and massage earlier and more fully provides in patients undergoing coronary artery bypass grafting, relief is available for typical post-operative phase syndromes: cardiac, poststenoticescuu, respiratory, hemorheological, psychopathological, geodinamicheskogo and metabolic (Mal, Kharitonov et al., 2018). Made more pronounced effect in the experimental group should be linked here optimizing complex effects on the brain, musculoskeletal system and hemodynamics of patients (Makhov et al., 2018a). The simultaneous use of all elements of the author's rehabilitation has achieved a high result is not simply due to the summation effects of all components of the rehabilitation and development of vzaimoponimanija their actions (Glagoleva et al., 2018; Vorobyeva et al., 2019).

Apparently, the advantage of the developed scheme of rehabilitation is determined by the use it health tourism, implemented in Nordic walking. The use of these components of rehabilitation led to the development of in patients the rapid increase in exercise capacity with the activation of regenerative processes in all organs of the body and primarily in the heart and blood vessels (Medvedev et al., 2007a). Under this option actively the rehabilitation of most muscle, spend a maximum of calories than when dosed walking. In addition, during the proposed rehabilitation of the removed load from the joints of the legs, and minimizes them the impact (Medvedev, 2019).

Thanks to the combination of health tourism and Nordic walking, adaptation to physical activity is accelerated, as a result of which it is easier to tolerate, and positive changes in hemostasis and blood rheology also occur. This is manifested by functionally beneficial biochemical, morphological and functional changes in the muscles, in the cardiovascular system and in most neurons (Makhov et al., 2019). Strengthening in the body of patients who underwent coronary artery bypass grafting, aerobic metabolism increases their mood, accelerates the general restoration of physical abilities, leading to their reliable return to their normal lives due to the approximation of all indicators considered by them to the level of clinically healthy volunteers.

CONCLUSION

For patients who underwent coronary artery bypass grafting, a general weakening of the body and the cardiovascular system is characteristic. The use of the traditionally used physical rehabilitation scheme, including therapeutic physical culture, training on

simulators, physiotherapy and massage is not able to provide a quick and pronounced healing effect in such patients. The proposed method of physical rehabilitation of patients who underwent coronary artery bypass grafting, based on health tourism and including elements of therapeutic exercises with breathing exercises and Nordic walking, turned out to be much more effective than the traditional healing scheme.

REFERENCES

Avaliani, V.M., Chernov, I.I. and Shonbin, A.N. (2005) Coronary surgery for multifocal atherosclerosis. Moscow: Universum Publishing, 384.

Bokeria, L.A. and Gudkova, R.G. (2016) Cardiovascular Surgery-15. Diseases and congenital malformations of the circulatory system. Moscow: Scientific Center for Cardiovascular Surgery named after A.N. Bakuleva of the Russian Academy of Medical Sciences, 212.

Glagoleva, T.I. and Medvedev, I.N. (2018) Physiological Features Of Anti-aggregational Control Of Blood Vessels Over The Shaped Elements Of Blood In Calves At The Onset Of Ontogenesis. Research Journal of Pharmaceutical, Biological and Chemical Sciences, 9(5) : 440-447.

Makhov, A.S. and Madvedev, I.N. (2019) Functional characteristics of children with Down syndrome and possibilities of their correction with the help of athletic activity in Russia. Bali Medical Journal, 8(2): 587-591. DOI: 10.15562/bmj.v8i2.1097

Makhov, A.S. and Medvedev, I.N. (2018a) Ensuring The Physiological Optimum Of The Body Using Hydro-procedures. Research Journal of Pharmaceutical, Biological and Chemical Sciences, 9(6) : 354-359.

Makhov, A.S. and Medvedev, I.N. (2018b) The Effect Of Regular Physical Activity On The Functioning Of The Nervous System. Research Journal of Pharmaceutical, Biological and Chemical Sciences, 9(6) : 794-798.

Mal, G.S., Kharitonov, E.L., Vorobyeva, N.V., Makhova, A.V. and Medvedev, I.N. (2018) Functional Aspects Of Body Resistance. Research Journal of Pharmaceutical, Biological and Chemical Sciences, 9(6) : 60-65.

Mal, G.S., Vorobyeva, N.V., Makhova, A.V., Medvedev, I.N. and Fayzullina, I.I. (2018) Features Of Physical Rehabilitation After Myocardial Infarction. Research Journal of Pharmaceutical, Biological and Chemical Sciences, 9(6) : 280-285.

Mavrodiy, V.M. (2017) Cardiology. Main problems. Moscow: Zaslavsky A.Yu., 8122.

Medvedev, I.N. (2019) Physiological peculiarities of thrombocyte activity of candidates into masters of sports in athletics of preadult age. Bali Medical Journal, 8(3): 635-639. DOI: 10.15562/bmj.v8i3.1090

Medvedev, I.N. and Kumova, T.A. (2007a) Angiotensin II receptor inhibitors: role and place in arterial hypertension and metabolic syndrome treatment. Russian Journal of Cardiology, 5 : 97-99.

Medvedev, I.N. and Kumova, T.A. (2007b) Valsartan effects on platelet activity in patients with arterial hypertension and metabolic syndrome. Russian Journal of Cardiology, 3 : 66-69.

Skoryatina, I.A. and Medvedev, I.N. (2019) Correction of aggregation level of basic regular blood elements in patients with hypertension and dyslipidemia receiving rosuvastatin and non-medicinal treatment. Bali Medical Journal, 8(1) : 194-200. DOI:10.15562/bmj.v8i1.648

Uzhegov, G.N. (2005) Diseases of the heart and blood vessels. St. Petersburg, 320.

Vorobyeva, N.V. and Medvedev, I.N. (2019) Functional activity of platelets in new-born calves of black-marked breed. Bulgarian Journal of Agricultural Science, 25(3) : 570-574.

Dental
Communication

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp-160-168

BBRC
Bioscience Biotechnology
Research Communications

Evaluation of Color Changes and Surface Topography of Different Feldspathic Ceramic Materials After Khat, *Catha edulis* Extract Immersion

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ABSTRACT

The effects of khat chewing habits on the stainability of feldspathic metal ceramic (MC) materials used in the fabrication of dental restorations remain unexplored. This study investigated the effect of khat extract (KE) on mean color changes (ΔE*) and surface topography among different feldspathic MC materials, namely, VMK VM13, VMK MASTER, and VMK 95). Sixty feldspathic MC specimens were prepared from nickel chromium alloy with the above-mentioned ceramic materials. The ΔE* of the specimens were measured using a spectrophotometer before and after immersion in KE and thermocycling for 10 days. The surface topography was captured to examine the surface of specimens after KE staining with a white light interferometric microscope. Results showed that the ΔE* values of the different types of tested feldspathic MC were influenced by KE. The lowest values of ΔE* were recorded from glazed VMK VM13, VMK MASSTER, and VMK 95 with acceptable color changes, whereas the highest and unacceptable values of ΔE* were observed from polished VMK MASTER and VMK 95. All the tested groups and subgroups obtained significant differences with p values > 0.05 in Student's t-test and ANOVA test. All the tested feldspathic MC materials had showed significant differences in the parameter of average color changes for all tested groups in form of glazed or polished specimens. Clinical significance: Re-glazing step is an essential step before final cementation and after chair-side adjustment of any feldspathic MC prosthesis for patients who chew khat..

KEY WORDS: COLOR MEASUREMENT, FELDSPATHIC PORCELAIN, KHAT, SURFACE TOPOGRAPHY.

ARTICLE INFORMATION

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Received 15th Jan 2020 Accepted after revision 19th March 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/29

INTRODUCTION

Feldspathic metal ceramic (MC) restorations are considered as a color stable. In aesthetic prosthesis, such as feldspathic ceramics, discoloration may occur due to intrinsic or extrinsic factors. Intrinsic factors include changes within the ceramic, whereas extrinsic factors involve adsorption or absorption of stains from the oral cavity and the smoothness of the prosthesis surface (Singh et al., 2015). Restoration stainability has been proven to be dependent on numerous factors, such as the brand and shade of the prosthetic material, exposure time and intensity of dissimilar food and beverages, drinking, and polishing techniques (Stawarczyk et al., 2012).One of the criteria for the success of aesthetic materials is color stability. Thus, the assessment of color changes using color measuring devices, such as spectrophotometers, has become common. In such assessments, the precision, standardization, and numerical expression of color are recorded. The data are labeled in the CIE L*a*b*system, which uses three-dimensional colorimetric quantities; L*measures the brightness of the color, a* handles the red–green content, and b* assesses the yellow–blue content. Then, the mean color changes (ΔE*) are calculated using L*a*b* (Rosentritt et al., 2015; Turgut et al., 2011). ΔE* reveals whether a change in the overall shade can be detected by a human observer (Rosentritt et al., 2015) (3).

Previous studies have considered color differences (ΔE*) greater than 3.5 unit to be clinically unacceptable (Yilmaz et al., 2011 Abdalkadeer et al., 2019; Alghazali et al., 2012, 2019). Many studies have been conducted on the color stability of different aesthetic ceramic materials in several common beverages. Some of the researchers have shown that changes in color stability are greatest in tea (Er et al., 2006; Haralur et al., 2019), coffee or Arabic Qahwa (Abdalkadeer et al., 2019; Saba et al., 2017; Sarikaya and Güler, 2011), and Coca-Cola (Alghazali et al., 2019; Sarıkaya et al., 2018). Chair-side or intraoral polishing of feldspathic ceramic restorations is efficient, easy for clinicians, and minimizes repeated laboratory procedures (Hmaidouc et al., 2014). However, careful intraoral polishing of the ground surfaces is always necessary because the final occlusal adjustments of dental prostheses have to be made after cementation. Some investigations have been made using different polishing techniques on ceramic surface instead of glazing (Raimondo et al., 1990; Wright et al., 2004). Khat (Catha edulis) is an evergreen shrub that belongs to Celastraceae family. It grows in Yemen and southern Saudi Arabia, as well as in certain East African countries (Ageely 2009).

Khat leaves are habitually chewed by people in these regions because of their psychostimulant effect, which is similar to that produced by amphetamine-like substances (Wabe 2011). These leaves are most frequently chewed in one preferred side of the mouth (usually the left side). Young fresh leaves are chewed in form of a bolus and held in the lower buccal pouch unilaterally for 3–5 hours or longer (Al-Alimi et al., 2018). Given the rampant practice of khat chewing, especially in southern region of South Africa, exploring the effect of khat on materials used for dental prosthesis fabrications is essential. However, studies on the effect of khat on oral health remain scarce. Few studies have investigated the association between khat chewing and dental ceramic materials. Moreover, the role of khat as a staining material contributing to dental ceramic discoloration and surface effect in relation to maintaining the polishability of ceramic materials has yet been studied nor documented.

A recent clinical study by Al Moaleem et al., (2020) concluded that khat chewing had a statistically significant effect on the bacterial biofilm formation on restorative materials, whereas no significant effect was found on feldspathic and all ceramic prostheses. In view of khat chewing in relation to composite restorations, a single clinical study recorded that composite filling materials may be related with demineralization of composite filling materials at the composite tooth border; this possibility may result in altered color of the composite and tooth structure (Al-Alimi et al., 2014). Moreover, in vitro studies have examined the effect of khat extract (KE) on the color of composite materials and reported that KE shows a clinically perceptible ΔE* with current types of composite filling (Al Anesi et al., 2019). The objectives of the present study were as follows: 1) to assess the effect of KE on the stainability of selected VITA feldspathic MC specimens (i.e., VMK VM(R)13, VMK MASTER, and VMK 95); 2) to measure the color changes on the basis of the basic color of VITAPAN classical shade guide (A1–D4); and 3) to evaluate the surface topography of the specimens after immersion in KE and aging using a white light interferometric microscope. The null hypothesis of this study is that KE and thermocycling can affect the color stainability of polished feldspathic MC specimens in comparison with glazed specimens. The color changes were clinically acceptable.

MATERIALS AND METHODS

Study Design: Sixty feldspathic MC specimens were prepared for the in vitro study. The specimens were used to measure the effect of KE on the color stainability of glazed or polished feldspathic MC materials. Furthermore, the surface topography of the specimens after immersion in KE was evaluated using a white light interferometric microscope. Table 1 presents the materials and devices used in this study.

Specimen Preparation and Fabrication: Sixty metal specimens with 0.4 mm thickness and 12 × 12 mm dimension were prepared and constructed from green wax. All metal specimens were invested, burned, and casted with nickel–chromium dental casting alloy (Wiron(R) 99, Bego, Germany) using the conventional manner and according to manufacturer's instructions. The casted specimens were divested, and the residual surface investment was removed by sandblasting with 250 µm aluminum oxide abrasion particles, finished by carborundum discs and metal trimmers. Finally, the specimens were adjusted to achieve a uniform thickness

(i.e., 0.4 mm). The 60 metal disc specimens were divided into three equal groups on the basis of the type of feldspathic ceramic materials used.

The feldspathic ceramic of VITA VM(R) 13 (VITA, Zahnfabrik, Germany) was used to make 20 specimens of the first group. First, two coats of paste opaque were used, and a dentine layer, in which powder and liquid were mixed according to manufacturer's instructions, was then applied over the specimens using a metallic jig. Subsequently, enamel layer was applied using the same technique for dentine layer, and the specimens were burned. Burning was performed according to manufacturer's instructions, and specimens were grinded with a diamond bur to achieve the uniform thickness. Using the same technique, 20 specimens were constructed from each of VMK MASTER and VMK95 (VITA, Zahnfabrik, Germany). Lastly, the specimens were glazed. The opaque layer was 0.3 ± 0.1 mm, whereas the body porcelain was 2.0 ± 0.3 mm in thickness (Al Moaleem et al., 2012; Jan et al., 2013).

Samples Surface Treatments: The prepared specimens of the three groups were divided into two equal subgroups with 30 specimens each. The first subgroup specimens (10 samples from each group) were obtained from the laboratory with glazed layer and without any further treatment, whereas the specimens of the second subgroup (10 specimens from each group) were polished using a porcelain polishing kit according to manufacturer's instructions to characterize the clinical condition of the prostheses. Each 10 feldspathic MC specimen was polished using a polishing kit with equal number of grits and in one direction under a constant load.

Color Measurements: The color of each specimen was recorded before and after thermocycling and immersion within KE mixture. The color of each specimen at both intervals was measured at the same position (i.e., center of the specimen) using a portable spectrophotometer (Vita Easy Shade, Vita Zahnfabrik H. Rauter GmbH & Co. KG, Bad Sackingen, Germany). A putty index was made around the tip of the device with a window of 4 mm diameter in the center to standardize the extent of color measurement (Haralur et al., 2019). The spectrophotometer was used to measure the CIE-Lab values to provide a numerical representation of 3D color measurements. Each time, the specimen color was measured twice for L*, a*, and b*, and the average value was then considered the color of the specimens before immersion, following the CIL Lab color system. The ΔE* values were calculated for the different specimens by assuming the formula: $\Delta E^* = [(L1^* - L2^*)2 + (a1^* - a2^*)2 + (b1^* - b2^*)2]1/2$, where ΔL* is the lightness of L*, Δa* is the variation of a*, and Δb* is the variation of b*.

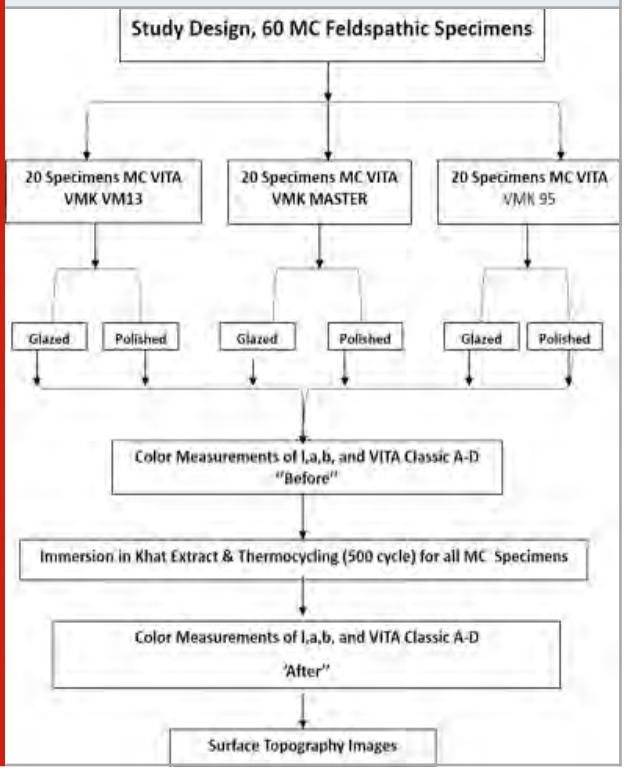
Table 1. Materials and devices used in the study

Material or Device Type	Brand Name	Composition	Manufacturer
Nickel chromium casting alloy	Wiron(R) 99	Ni 65%, Cr 22.5%, Mo 9.5%, Nb 1% Si1%, Fe 0.5%, Ce0.5%, Cmax 0.021	BEGO, Germany
Feldspathic P/L ceramic	VITA VM(R)13	Silicon dioxide, possesses a glassy matrix, and has assorted quantities of potassium, sodium, barium, or calcium	VITA Zahnfabrik, Bad Säckingen, Germany
Feldspathic P/L ceramic	VMK MASTER	Natural feldspar veneering ceramic for conventional bonding alloys	VITA Zahnfabrik, Bad Säckingen, Germany
Feldspathic P/L ceramic	VMK 95	Pure-grade potash and albite feldspar materials	VITA Zahnfabrik, Bad Säckingen, Germany
Khat	*C. edulis* plant	Alkaloids, terpenoids, flavonoids, sterols, glycosides, tannins, amino acids, v itamins, and minerals	
Spectrophotometer	Vita Easy Shade Spectrophotometer	Device used for measuring wavelength transmitted from one object at a time without being affected by subjective interferences of color	VITA Zahnfabrik H. Rauter GmbH & Co. KG, Bad Sackingen, Germany
Surface roughness and topography tester	White light interferometric microscope	3D printer of surface characteristics	Contour GT-K1, Bruker Nano GmbH, Berlin, Germany

A low ΔE* was considered good shade matching, and a score of ≥3.5 was considered an acceptable color change (Alghazali et al., 2012; Alghazali et al., 2019). The Vita Easy Shade spectrophotometer recorded the basic color of the specimens according to the VITAPAN classical shade guide A1–D4 before the immersion (Figure 1).

Figure 1: Basic VITAPAN classical shade guide on the spectrophotometer

Figure 2: Study design, steps for color measurement, and type of staining materials with thermocycling



Specimen Immersion and Thermocycling: KE was prepared and presented by the Substance Abuse and Toxicology Research Center, Jazan University. It was prepared from fresh mincing khat leaves in 100% distilled water (V/W) and then finely minced. KE was kept in –80 °C ultralow-temperature freezer until further use. It was then mixed with NaOH until its pH was similar to that of the oral cavity. All specimens were immersed in KE for 10 days, as mentioned in previous in vitro studies (Al Anesi et al., 2019; Al Moaleem et al., 2020a). The same procedures were executed daily to obtain fresh KE. During the immersion time, an aging process was conducted using a thermocycling machine, where 100 cycles were accomplished every day in 5 °C cold water and then in 55 °C hot water (1000 cycles). All specimens were dipped in distilled water, followed by the removal from immersion media. Specimens were wiped dry with a tissue paper and left in place for complete dryness.

After 10 days of KE immersion and thermocycling, the colors of the specimens were remeasured with the same shade using the spectrophotometer, and the readings were registered as after immersion. The aforementioned equation was used to calculate the average color of L, b, and a after immersion. The reading of the basic shade for VITAPAN classical shade guide was measured again as the reading after immersion. All procedures for specimens' preparation, fabrication, surface finishing and polishing, specimen immersion, and thermocycling were performed by the same operator. Color was measured for all assigned specimens by the same operator under the same settings and gray background.

Surface Topography Scanning: One specimen from each glazed or polished porcelain was scanned after KE immersion and thermocycling. The surface topography of the six specimens was characterized graphically using the white light interferometric microscope (Contour GT-K1, Bruker Nano GmbH, Berlin, Germany) under 50× magnification, with back scan and dimension parameters of 20 µm in VSI/VXI mode; this method was performed to obtain a 3D interpretation of the specimen surfaces. Vision 64 software (Bruker Nano GmbH, Berlin, Germany), which is part of the GT-K1 system, was used to replicate the surface topography constraints (Figures 3a–3f).

Data Statistical Analysis: The mean values of ΔE*, ΔL*, Δa*, and Δb* of the different feldspathic MC specimens (i.e., VMK VM13, VMK MASTER, and VMK 95) in forms of glazing and/or polishing were recorded and then compared before and after KE immersion. Changes in the basic color of VITAPAN classical shade were documented. Data were entered into Microsoft Excel 13 and analyzed using Statistical Package for Social Science version 22.0 (SPSS Inc., Chicago IL, USA) software. Descriptive statistics was intended for each parameter for the three groups. The ΔE* values were compared using one-way ANOVA test, followed by a post hoc comparison by Bonferroni test to detect any significant difference between and within the groups at $P > 0.05$. Student's t-test was used to detect the significances between each pair of the three groups.

RESULTS AND DISCUSSION

In the vitro study, 60 specimens were included, represented by letters a–f, in the analysis of results. The images were

recorded using the white light interferometric microscope, in which a and b represent glazed and polished VMK VM 13, respectively; c and d denote the glazed and polished VMK MASTER, respectively; and e and f represent glazed and polished VMK 95 after KE immersion and thermocycling of the specimens. The ΔE* values were calculated using the aforementioned equation. High ΔE* values were observed for the polished subgroups of VMK MASTER, VMK 95, and VMK VM 13, followed by the glazed VMK 95 and VMK MASTER; whereas glazed VMK VM 13 had the lowest ΔE* of 0.348. The ΔE* values were significantly differed among all tested groups, either in glazed or polished forms, with P values of >0.05. Table 2 shows the significant differences in the ΔE* values using Student's t-test. From the table, VMK MASTER had a significant P value of 0.005, whereas VMK VM13 had a nearly significant P value (0.053), and VMK95 had an insignificant P value (0.337; Table 3). Figure 3 shows the changes in the basic color of the VITAPAN classical shade guide (A1–D4) of the tested groups. The highest changes were observed for VMK MSSTER (90%), followed by 70% for VMK 95, and only less than half (40%) for VMK VM13 ceramic type.

Figure 4 shows the representative white light interferometric microscopic images of the tested shades of ceramics after KE immersion and thermocycling. The red and blue areas represent the part of the surface with the highest (i.e., the peaks) and lowest (i.e., the valleys) heights, respectively. One specimen from each subgroup showed a different high pattern of peaks and valleys across the surfaces, with no identical pattern across each surface in either glazed or polished form. Furthermore, the microscopic images for VMK VM 13 specimens obtainable a nonuniform surface with distinct sharp projections, as shown by the dotted areas with pores at different sides originating from the center (Figures 4a and 4b). VMK MASTER showed a few pores located on the border for the glazed specimens (Figure 4c), whereas pores are all over the surface for the polished specimens (Figure 4d). VMK 95 specimens demonstrated a uniformly irregular surface pattern with heights and valleys, that is, narrow at the border, irregular, and superficial scratch areas at the border (Figure 4e); however, it is crossed the entire surfaces, which causes a uniformly irregular surface for all the specimens (Figure 4f).

Table 2. ΔE* after KE immersion and thermocycling for different groups in relation to surface type based on ANOVA, followed by Bonferroni tests

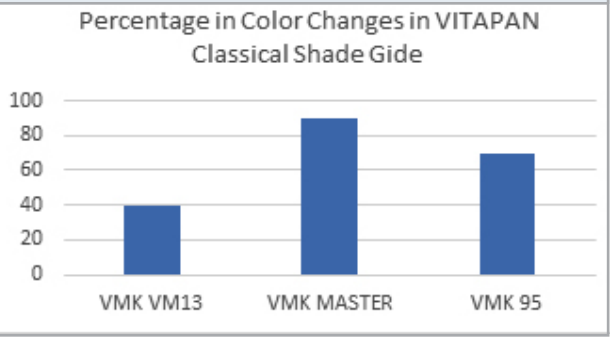
Material Surface Type	Mean and SD of (ΔE) for Each Material	VMK 13[a,b]	VMK MASSTER[c,d]	VMK 95[e,f]	P value
Glazed[a,c,e]	0.348 (0.115) VMK VM13	---	0.000	0.000	0.000*
	1.282 (0.259) VMK 95	0.000	---	0.000	
	2.881 (0.399) VMK MSSTER	0.000	0.000	----	
Polished[b,d,f]	2.963 (0.456) VMK VM13	---	0.000	0.000	0.000*
	7.256 (0.529) VMK 95	0.000	---	0.000	
	4.935 (0.569) VMK MSSTER	0.000	0.000	----	

Table 3. Comparison among different groups in relation to the surface type using Student's t-test

Ceramic Type	Surface Type	Mean	SD	Sig.
VMK 13	Glazed	0.348	0.115	0.053
	Polished	2.962	0.456	
VMK MASSTER	Glazed	1.282	0.259	0.005*
	Polished	7.258	0.529	
VMK 95	Glazed	2.881	0.399	0.337
	Polished	4.935	0.569	

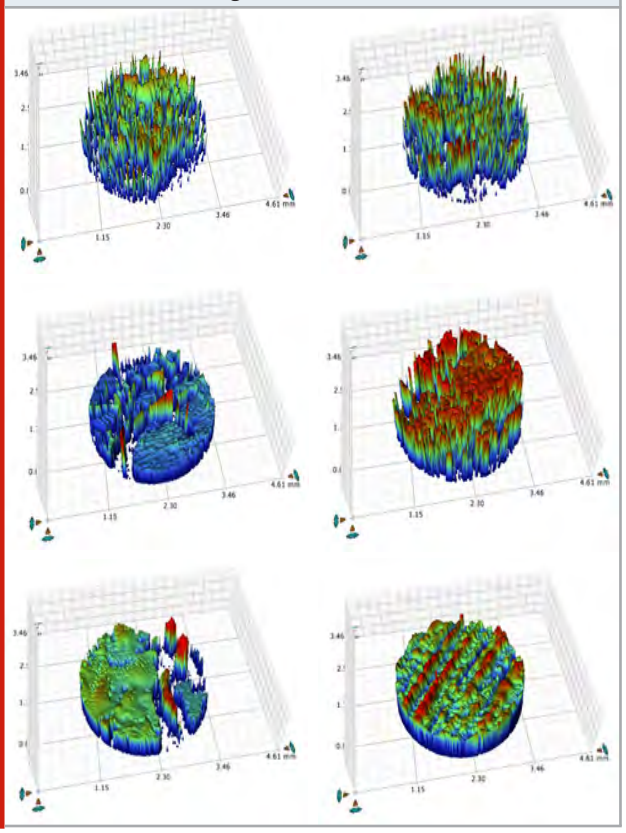
*Significant (P < 0.05)

Figure 3: Comparison between color changes in basic color shade of VITABAN classical shade for the tested specimens after KE immersion



MC prostheses are still used as restorative materials in many countries due to their metal strength, aesthetic properties, color stability, biocompatibility, marginal integrity, and easy and familiar fabrication by dental technicians (Jain et al., 2013). Exogenous staining may occur due to the capability of prostheses to adsorb or absorb stains from khat chewing in the oral cavity, which may potentially affect color stainability.

Figure 4: Characteristic images of white light interferometric microscope of tested VMK 13 (a, b), VMK MASSTER (c, d), and VMK 95 (e, f) glazed or polished ceramic specimens obtained at 50× magnification



This in vitro study investigated the effect of KE immersion with thermocycling on the stainability of different types of feldspathic MC specimens used for replacing missing/decayed coronal portions or missing tooth/teeth. The ΔE* values of glazed or polished surfaces were compared using a physical polishing set. The results of this study complement a wide range of adverse effect of KE on feldspathic MC specimens. The first null hypothesis of this study (i.e., KE and thermocycling can affect the color stainability of polished feldspathic MC specimens in comparison with glazed specimens) was accepted because the stainability of feldspathic MC material specimens in glazed or polished ceramic form were affected by KE immersion and the thermocycling. Nonetheless, the second hypothesis was at the margin because most of the tested specimens were clinically acceptable in the glazed form but mostly clinically unacceptable in the polished form.The most problematic issue that prosthodontists and dental clinicians face during their daily practice is shade selection. The reference tooth most often considered by dental clinicians is the neighboring, contralateral or the opposing tooth/teeth. However, other teeth should also be to obtain a clinically acceptable shade. Moreover, teeth should be polished with prophylaxis before selecting the appropriate shade.

The effect of KE solution and thermocycling on the color of tested materials was evident in all specimens with different degrees. Table 2 shows that the ΔE* value was the highest on polished specimens, with 2.963, 7.256, and 4.935 recorded for VMK VM 13, VMK MASTER, and VMK 95, respectively. This result agrees with the finding of Hill and Gibson., (1987), who stated that khat modifies the enamel surface of teeth, resulting in beverage collections on the surface and color change of the tooth after a period of time Al-Alimi et al., (2014), concluded that natural teeth become discolored because of the acidic and mechanical effect of KE on the tooth surfaces. Moreover, the use of large quantities of soft drinks or beverages and sugar tablets for a long period can result in cervical discoloration in the enamel and dentine, staining of teeth, attrition, and cervical caries at the chewing side (El-Wajeh and Thornhill., 2009; Al-Meshal et al., 1991).

The clinical precision of Vita Easy Shade spectrophotometer has been validated by several studies (Karagoz-Motro et al., 2012; ALGhazali et al., 2011). However, such devices may encounter problems while measuring curved surfaces because the measuring probe tip is flat. Edge loss errors are common due to the fact that the probe tip of the instrument cannot be in direct contact with the buccal surfaces of natural teeth or ceramic surfaces (Gupta et al., 2012). In addition, positioning errors of the probe tip cannot be excluded; such errors reduce the L* values recorded for such types of ceramic crown. The ΔE* values were assessed in terms of perceptibility and acceptability for small color differences because of their role as a guide control throughout the selection of ceramic materials for khat users. The ΔE* measurements using Vita Easy Shade spectrophotometer are more precise and accurate than those of other digital instruments and can thus be used in dental practice and research with some limitations (Kim-Pusateri et al., 2009; Alghazali et al. 2011). Also, the CIE Lab system was selected to evaluate the average color differences because it is well suited for the determination of small color differences (Alghazali et al., 2012; Sarikaya and Güler, 2011).

Prosthesis must not only have the dimensions, texture and contours of the teeth to be replaced but should also have similar light behavior. The color stability of the restoration is also critical for the long-term success of aesthetic restorations. Although the physicomechanical properties of ceramics have vastly improved, they remain susceptible to discoloration (Derafshi et al., 2017). Extrinsic factors, such as beverages, mouthwashes, acid solutions, toothbrushing, and high temperatures, are reported to induce surface degradation of ceramics (Kukiattrakoon et al., 2009). Hmaidouc et al., (2014) and Sarikaya and Güler., (2010) found no significant difference between the surfaces of fine glazed and

polished full-contour zirconia specimens and between feldspathic VMK 95 and Ceramco III porcelain groups, respectively. Lawson et al. (2014), recorded similar surfaces for glazed or polished feldspathic Ceramco III and lithium disilicate or zirconia. This result agrees with the findings of the present study, that is, no significant differences were observed between the glazed or polished test specimens (Table 3).

By contrast, Sarikaya and Güler (2011) and Saba et al. (2017), reported that Ceramco III feldspathic porcelain demonstrated the highest ΔE* values with no significant difference among the other groups (i.e., Mark II, Matchmaker MC, and VMK 95). The results of the present study indicated that significant differences were observed among the three types of tested feldspathic materials (Table 2). These differences could be related to the type of feldspathic material used and the technique of construction of the specimens. Alghazali et al., (2012), performed a clinical study and found that ΔE* of 2.8 is clinically acceptable. Alghazali et al., (2019); Abdalkadeer et al. (2019); Sarikaya and Güler (2011), concluded that ΔE* values between 1 and ≤3.3 can be detected by the human eyes and are clinically acceptable for feldspathic; moreover, low-fusing porcelain specimens immersed in coffee or Coca-Cola are color-stable, with average values of ΔE* at an acceptable level. Saba et al. (2017) confirmed that coffee may adversely affect color and may consequently compromise the aesthetics of feldspathic CAD/CAM blocks. All the tested specimens were clinically acceptable in ΔE* values ranging from 0.348 for glazed VMK VM13 to 2.963 for polished VMK VM13, which are clinically acceptable.

The only unacceptable ΔE* values were for feldspathic specimens of VMK MASTER and VMK 95, and this result could be related to the composition of those ceramic materials (Tables 2 and 3). Recdently, Alotaibi et al. (2019) have evaluated the crown color of extracted teeth after endodontic treatment with different endodontic sealers using a Vita Easy Shade Advance and the equation $\Delta E^* = ([\Delta L^*]^2 + [\Delta a^*]^2 + [\Delta b^*]^2)^{1/2}$ after the aging period reached 3 months. They recorded the ΔE* values ranging from 7.02 to 8.14 without significant difference between the groups. Greta et al (2020), performed a clinical study using Vita Classic shade guide with Vita Easy Shade Advance to measure the color They concluded that the color difference between the restoration and the reference tooth exceeded the perceptibility thresholds. The findings demonstrated high percentage in the color changes in relation to the basic Vita Classical shade guide and recorded 90%, 70%, and 30% changes for VMK MASTER, VMK 95, and VM VMK13, respectively (Figure 3).

Few studies have used white light interferometric microscopic images in examining the surface topography of ceramic materials after immersion in stained materials. In this study, the polished samples of VM VMK13 (Figure 4b) showed high surface topography alteration compared with other polished samples of VMK MASTER and VMK 95 (Figures 4d and 4f), indicating that the mechanical and acidic effect of KE was noticeable. KE is fibrous in nature, and this property may cause a mechanical effect on the surfaces of ceramics, especially those of polished specimens (Jorgensen et al., 2009). The glazed surfaces of VM VMK13 specimens replicated the lowest effect of KE on their surface topography (Figure 4a). The images obtained in this study were dissimilar compared with the images documented in an earlier study by Egilmez et al., (2018), but were in parallel of a previous finding in relation to KE and feldspathic samples (Al Moaleem et al., 2020b). The limitations of the current in vitro study are as follows. Thermocycling was performed with water. This condition is different from the oral cavity environment during khat chewing, which is usually associated with soft drinks and smoking. Moreover, daily brushing of teeth by patients were not simulated during the immersion and thermocycling period. During aging, the immersion solution permitted staining on both sides of the samples However, in clinical conditions, the material is cemented to a tooth structure and is visible to KE and light on the exposed one.

CONCLUSION

The following conclusions can be drawn from this in vitro study. KE showed a significant effect on glazed and polished specimens of feldspathic MC materials. It showed an acceptable ΔE* values for all glazed specimens and polished VMK 13, with significant differences among all groups and subgroups. The effects were higher and unacceptable for VMK MASSTER, followed by polished VMK 95 specimens.

ACKNOWLEDGMENTS

The authors would like to thank the Deanship of Scientific Research, Jazan University, Kingdom of Saudi Arabia for funding this study in Future Scientists Program #V under Grant number FS5-038. The authors are also grateful for Substance Abuse Research Center, Jazan University for providing the khat extract.

Conflicts of Interest: The authors declare no conflict of interest.

REFERENCES

Abdalkadeer HK, Almarshedy SM, Al Ahmari NM, et al. Influence of the Coca-Cola Drinks on the Overall Color of Glazed or Polished Porcelain Veneers Fabricated from Different Materials and Thicknesses: An In Vitro Study. J Contemp Dent Pract 2020;21(1):56–61.

Ageely HM (2009). Prevalence of Khat chewing in college and secondary (high) school students of Jazan region, Saudi Arabia. Harm Reduct J. 6:18.

Al Moaleem MM, Amit P, Al Ahmari NM, Shariff M (2020b). Oral Biofilm on Dental Materials Among khat Chewers. Curr Phar Biot. 21(3); dOi: 10.2174/1389201021666200121142300

Al Moaleem MM, Porwal A, Al Ahmari NM, Shariff M, et al (2020a). Khat Chewing Induces a Floral Shift in Dental Material-Associated Microbiota: A Preliminary Study. Med Sci Monit. 26: e918219

Al-Alimi KR, Razak AAA, Saub R (2014). Salivary caries parameters: Comparative study among Yemeni khat chewers and nonchewers. Journal of Dental Sciences. 9(4):328-31.

Al-Alimi, K. R.; Razak, A. A. A.; Saub, R. (2018). Is Khat chewing habit a risk factor for occlusal caries progression? African Health Sciences.1 8 (4), 1036-1045.

Al-Anesi WA, Madfa AA, Dubais MA and Albahari AA (2019). Effects of Khat Extract and Other Staining Media on Color Change of Composite Resins Subjected to Various Polishing Methods. Oral Biol Dent. 7:1-10

Alghazali N, Burnside G, Smith PW, Preston AJ, et al (2012). Assessment of perceptibility and acceptability of colour difference of denture teeth. J Dent. 40 supp: 10-7.

ALGhazali N, Burnside G, Smith PW, Preston AJ, Jarad FD (2011). Performance Assessment of Vita Easy Shade Spectrophotometer on Colour Measurement of Aesthetic Dental Materials. Eur. J. Prosthodont. Rest. Dent. 19(4); 168-174

Alghazali N, Hakami AA, AlAjlan GA, Alotaibi RM et al (2019).

Influence of the Arabic-Coffee on the Overall Color of Glazed or Polished Porcelain Veneers – In vitro Study. The Open Dentistry Journal. 13; 65-61.

Al-Meshal, I. A; Qureshi, S.; Ageel, A. M.; Tariq, M. (1991). The toxicity of Catha edulis (khat) in mice. Journal of substance abuse. 3 (1), 107-115

AL-Moaleem MM, Shah FK, Khan NS, Porwal A (2011): The effect of thermocycling on the bonding of different restorative materials to access opening through porcelain fused to metal restorations. J Adv Prosthodont. 4:186-189.

Alotaibi RM, Altuwaijri SM, Ahmed DR (2019). The degree of crown discoloration induced by a bioceramic and other conventional endodontic sealers. Saudi Endod J. 9:192-7.

Derafshi, R.; Khorshidi, H.; Kalantari, M.; Ghaffarlou, I (2017). Effect of mouth rinses on color stability of monolithic zirconia and feldspathic ceramic: An in vitro study. BMC Oral Heal. 17:129-35.

Egilmez F, Ergun G, Cekic-Nag I, Vallittu PK, Lassila LV (2018). Comparative color and surface parameters of current esthetic restorative CAD/CAM materials. J Adv Prosthodont. 10:32-42

El-Wajeh, Y.; Thornhill, M. (2009). Qat and its health effects. British dental journal. 206 (1), 17.

Er AU, Turker SB, Kocak A, Aktepe E (2006). Effect of five staining solutions on the colour stabilityof two acrylics and three composite resins based provisional restorations. Eur J Prosthodont Restor Dent. 14: 121-125

Greta DC, Gasparik C, Colosi HA, Dudea D (2020). Color matching of full ceramic versus metalceramic crowns - a spectrophotometric study. MEDICINE AND PHARMACY REPORTS j. 93(1); 89-96.

Gupta R, Prakash H, Shah N, Jain V (2005). Spectrophotometric evaluation of color changes of various tooth colored veneering materials after exposure to commonly consumed beverages. J Indian Prosthodont Soc. 5:72-8.

Haralur SB, Alqahtani NRS, Mujayri FA (2019). Effect of Hydrothermal Aging and Beverages on Color Stability of Lithium Disilicate and Zirconia Based Ceramics. Medicina, 55, 749

Hill CM, Gibson A (1987). The oral and dental effects of q'at chewing. Oral Surg Oral Med Oral Pathol. 63(4):433-6.

Hmaidouch R, Mu ¨ller W_D, Lauer H-C, Weigl P (2014). Surface roughness of zirconia for full-contour crowns after clinically simulated grinding and polishing. International Journal of Oral Science. 6(4); 241-6.

Jain, C.; Bhargava, A.; Gupta, S.; Rath, R.; Nagpal, A.; Kumar, P (2013). Spectrophotometric evaluation of the color changes of different feldspathic porcelains after exposure to commonly consumed beverages. Eur J Dent. 7; 172–180.

Jorgensen, E.; Kaimenyi, J. T. (1990). The status of periodontal health and oral hygiene of Miraa (Catha edulis) chewers. East African medical journal. 67 (8), 585-90.

Karagoz-Motro P, Kursoglou P, Kazazoglu E (2012). Effects of different surface treatments on stainability of ceramics. J Prosthet Dent. 108:231-7

Kim-Pusateri S, Brewer JD, Davis EL and Wee AG (2009), Reliability and accuracy of four dental shade-matching devices, J Prosthet Dent. 101: 193-199.

Koseoglu, M., Albayrak, B., Gül, P. and Bayindir, F. (2019) Effect of Thermocycle Aging on Color Stability of Monolithic Zirconia. Open Journal of Stomatology 9; 75-80.

Kukiattrakoon,B.; Junpoom,P.; Hengtrakool, C (2009). Vicker's micro hardness and energy dispersivex-rayanalysis of fluorapatite-leucite and fluorapatite ceramics cyclically immersed in acidic agents. J. Oral Sci. 51, 443–450.

Lawson N, Janyovula S, Syklawer S, McLaren EA, Burgess J (2014). Wear of enamel opposing zirconia and lithium disilicate after adjustment, polishing and glazing. J Dent. 42: 1586-91.

Raimondo RL Jr, Richardson JT, Wiedner B (1990). Polished versus autoglazed dental porcelain. J Prosthet Dent. 64(5):553-7.

Rosentritt M, Sawaljanow A, Behr M, Kolbeck C, Preis V (2015). Effect of tooth brush abrasion and thermo-mechanical loading on direct and indirect veneer restorations. Clin Oral Invest.19: 53-60.

Saba DA, Salama RA, Haridy R (2017). Effect of different beverages on the color stability and microhardness of CAD/CAM 53. hybrid vs feldspathic ceramic blocks: an in vitro study. Future Dent J. 3:61–66.

Sarikaya I, Güler AU (2010). Effects of different polishing techniques on the surface roughness of dental

porcelains. J Appl Oral Sci. 18:10-6.
Sarikaya I, Güler AU (2011). Effects of different surface treatments on the color stability of various dental porcelainsy. J Dent Science. 6(2):65–70
Sarıkaya I, Yerliyurt K, Hayran Y (2018). Effect of surface finishing on the colour stability and translucency of dental ceramics. BMC Oral Health.. 18:40-47.
Singh K, Suvarna S, Agnihotri Y, Sahoo S, Kumar P (2015). Color stability of aesthetic restorative materials after exposure to commonly consumed beverages: A systematic review of literature. Eur J Prostho. 2(1): 15-22.
Stawarczyk B, Sener B, Trottmann A, Roos M, Ozcan M, Hammerle C (2012). Discoloration of manually fabricated resins and industrially fabricated CAD blocks versus glassceramic: effect of storage media, duration, and subsequent polishing. Dent Mater. 31(3):377-383
Turgut S, Bagis B. Color stability of laminate veneers (11): An in vitro study. J Dent. 39: 57-64.
Wabe NT (2011). Chemistry, pharmacology, and toxicology of khat (Catha edulis forsk): a review. Addict Health. 3:137-49.
Wright MD, Masri R, Driscoll CF et al (2004). Comparison of three systems for the polishing of an ultra-low fusing dental porcelain. J Prosthet Dent. 92(5): 486–490.
Yilmaz B, Karaagaclioglu L (2011). In vitro evaluation of color replication of metal ceramic specimens using visual and instrumental color determinations. J Dent. 105: 21-27.
Yılmaz, C, Korkmaz T, Demirk¨opr¨ul¨u H, Erg¨un G, ¨Ozkan Y (2008). Color Stability of Glazed and Polished Dental Porcelains. Journal of Prosthodontics. 17; 20–24.

Pharmaceutical Communication

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp-169-173

Antidiarrheal and Antipyretic Activity of Ethyl Acetate and Hydro-Alcoholic Extracts of *Diplazium esculentum* Leaves

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ABSTRACT

The present study was aimed at evaluating antidiarrheal and antipyretic activities of the ethyl acetate and hydro-alcoholic extract of the leaves of *Diplazium esculentum*. Antidiarrheal and Antipyretic activity was evaluated in rodent animals at doses of 250 and 500 mg/kg B.W. The antidiarrheal activity was investigated by the effect of extracts on castor oil-induced diarrhea in rats and the activities were compared to that of loperamide. Antipyretic activity was estimated using Brewer's yeast-induced hyperpyrexia in rats and the activities were compared to that of paracetamol.Hydroalcoholic extracts showed the highest percentageinhibition of defecation (71.91%) was recorded for leaf extract (500 mg/kgb w) of *D. esculentum*. Hydroalcoholic extract at the doses of (500 mg/kg p.o.) significantly decreased the rectal temperature of the rats. The study corroborates the significant antidiarrheal and antipyretic activities of hydroalcoholic leaf extract of *D. esculentum* and raise the demand of further scientific investigation.

KEY WORDS: *DIPLAZIUM ESCULENTUM*, HYDRO-ALCOHOLIC EXTRACT, ANTIDIARRHEAL, ANTIPYRETIC.

INTRODUCTION

Diarrhea is the passage of abnormal liquid or unformed stool at increased frequency. Infectious agents, certain medications, plant and animal toxins, gastro-intestinal disorders and substances that increase gastrointestinal tract secretions may cause it. It can also be caused by the ingestion of poorly absorbable materials, or inflammatory and dysmotility problems of the gastro-intestinal tract (Palombo, 2006; Meite et al., 2009). Diarrheal diseases are one of the leading causes of morbidity and mortality in developing countries and are responsible for the death of millions of people each year. There are a number of epidemiological and experimental evidences worldwide related to acute diarrheal disease, which is one of the principal causes of death in the infants. Around 2.5 million children die each year worldwide and 80% of which are reported in developing countries (Walker et al., 2011). Diarrhea is most common in crowded living conditions coupled with poor hygiene and malnutrition (Gutiérrez et al., 2007).Antibiotics used as antidiarrheal drugs

ARTICLE INFORMATION

*Corresponding Author: rsmrpal@gmail.com
Received 6th Feb 2020 Accepted after revision 20th March 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)

*Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/30*

sometimes provoke adverse effects and microorganisms tend to develop resistance toward them. Therefore, the search for safe and more effective agents from plant origin has continued to be an important area of active research, (Junejo et al., 2018, Anand et al 2019).

Pyrexia or Fever is defined as an elevation of body temperature. It is a response due to tissue damage, malignancy, and inflammation or graft rejection. Cytokines, interleukin, interferon and Tumor Necrosis Factor α (TNF- α) are formed in large amount under this condition, which increase PGE2 which in turn triggers hypothalamus to elevate body temperature (Rajani et al., 2011).Fever is associated with symptoms of sickness behavior which consist of lethargy, depression, anorexia, sleepiness and inability to concentrate. This increase in set point triggers increased muscle tone & shivering. However antipyretic medication can be effective at lowering the temperature which may include the affected persons' comfort (Duraisankar and Ravichandran, 2012). Antipyretics are drugs which can reduce elevated body temperature. Regulation of body temperature requires a delicate balance between production and loss of heat, and the hypothalamus which regulate the set point of body temperature. Drugs like paracetamol do not influence body temperature when elevated by factors such as exercise or increase in ambient temperature (Gupta et al., 2008).

Diplazium esculentum (Retz.) Sw. (Athyriaceae) is a wild edible rhizomatous fern mainly consumed as vegetables which are probably the most consumed fern along the hill tribe of North Eastern India and Western Ghats (Archana et al., 2012). The young fronds are used in myriad of way to prepare local dishes including stir-fried and salads. The fern is believed to contain various medicinal properties and some of them are evaluated and confirmed by research. It act as mast cell stabilizer and can prevent anaphylactic shock (Das et al., 2012), decoction of the plant can be used to treat hemoptysis and cough (Rahmat et al., 2004). The plant is also reported to use traditionally for the treatment of dysentery, glandular swellings, indigestion, diarrhea and various skin infections (Lense, 2011). In context of our research endeavor, we have planned to study the antidiarrheal and antipyretic activity of ethyl acetate and hydro-alcoholic extracts of *Diplazium esculentum* leaves.

MATERIALS AND METHODS

Collection and extraction of plant material: Fresh leaves of *Diplazium esculentum* (Retz.) Sw. were collected in March 2014 from Dibrugarh forest, Dibrugarh district, Assam, India. The plant species were identified and authenticated by Botanical Survey of India, Eastern Regional Centre, Shillong, India, and a voucher specimen (BSI/ERC/2014/Plant identification/360) was deposited. Air-dried powdered material of previously collected plant, *Diplazium esculentum* was packed in a Soxhlet extractor and extracted successively with the following solvents: petroleum ether (60- 80oC), chloroform, ethyl acetate, methanol and water. Each time before extracting with the next solvent, the powdered material was air dried first and then oven dried below 50°C. Finally, the marc was macerated with chloroform water (ratio) for 24 hours to obtain the aqueous extract. Each extract was concentrated by distilling off the solvent (in a rotary vacuum evaporator) and then evaporated to dryness on the water bath (Annan et al., 2013; Junejo et al., 2018). Reagent and chemicals All chemicals used in the study were of analytical grade, manufactured by Rankem Fine Chemicals Limited (RFCL), Mumbai and Himedia Laboratories, Mumbai.

Acute oral toxicity study: Acute oral toxicity test was performed as per OECD) guidelines 423. The animals were used with the approval of the Institutional Animal Ethics Committee (Approval No.IAEC/DU/50 Dated 24.09.2013, Registration No. 1576/Go/a/11/CPCSEA dated 17.02.2012)and the study was conducted following internationally accepted principles forlaboratory animal use and care. Experiments were performed using healthy young adult wistaralbino rats(both male and female), nulliparous, non-pregnant and weighing 150 to 250 gm(Junejo et al., 2014; Junejo et al., 2017).

Antidiarrheal activity: Healthy adult Wistar albino rats of either sex (200-250gm) were used for the antidiarrheal study. The animals were obtained from the Laboratory Animal Resources, Dibrugarh University (Approval No. IAEC/DU/50 dated 24.09.2013, Registration No. 1576/Go/a/11/CPCSEA dated 17.02.2012) and acclimatized to normal laboratory conditions for one week prior to study and provided with pellet diet and tap water ad libitum. Castor oil-induced diarrhea was done according to the previously described methods(Shoba and Thomas, 2011; Uddin et al., 2005). Rats of either sex were divided into four groups of five rats each. The animals were fasted for 18 h prior to the test. Group I animals were treated with normal saline (10 ml/kg), which served as control, while Group II received loperamide (50 mg/kg). Groups III, IVand V, VI were treated with ethyl acetate and hydro-alcoholic extracts of *Diplazium esculentum* leaves at 250 mg/kg and 500 mg/kg doses respectively. The activity of each group was expressed as percent inhibition (%) of diarrhea. The percent inhibition of defecation was calculated using the formula:

$$\%\text{Inhibition of defecation} = [(A-B)/A] \times 100$$

Where 'A' indicates mean number of defecation caused by castor oil and 'B' indicates mean number of defecation caused by drug or extract.

Antipyretic activity: The antipyretic activity of the tested extracts was screened in adult albino rats (200- 250g bw) by using yeast-induced hyperpyrexia model. The animals were divided in six groups (n = 6). All groups were kept at fasting and allowed free access of drinking water. Group I received saline as control and group II received paracetamol as standard drug while III-VI groups received 250 and 500 mg/kg of ethyl acetate and hydro-Alcoholic extracts of *Diplazium esculentum* leaves. Normal temperature was recorded using digital

thermometer and then pyrexia was induced in all animals by injecting 20% aqueous suspension of Brewer's yeast (10 ml/kg s.c.). After 24 h, rectal temperature was recorded and groups 3-8 were injected with above doses. After drugs administration, rectal temperature was again recorded periodically at 1, 2, 3 and 4 h of drugs administration (Khan et al., 2014; Kang et al., 2008).

Statistical analysis: The results are expressed as the mean ± standard error of the mean (SEM). Statistical analysis has been carried out with comparison between standard and treated groups. Differences were considered to be statistically significant at $p<0.05$.

RESULTS AND DISCUSSION

Both ethyl acetate and hydro-alcoholic extracts showed considerable antidiarrheal effect in castor oil-induced diarrhea test in rats. Hydro-alcoholic extract significantly inhibited the frequency of defecation when compared with untreated control rats ($p<0.05$). Results are shown in Table 1. Both extracts decreased the total number of wet feces produced upon administration of castor oil when compared to the castor oil treated rats. Hydro-alcoholic extract showed 53.97 and 71.91% inhibition of defecation at the doses of 250 and 500 mg/kg, respectively. Standard drug loperamide (50 mg/kg) also increased onset of diarrhea and exhibited 74.67inhibition of defecation.

Table 1. Antidiarrhea activity of leaf extracts of *Diplazium esculentum*

Group	Treatment Group	Dose (mg/kg B.W)	Number of diarrheal faces in 4 hours	% Inhibition of defecation
I	Blank control	10 ml/kg	12.32 ± 1.36	---
II	Loperamide (Standard)	50 mg/kg	3.12 ± 0.95	74.67
III	Ethyl acetate Extract	250 mg/kg	10.21 ± 1.75**	17.12
IV	Ethyl acetate Extract	500 mg/kg	8.11 ± 2.15	34.17
V	Hydro-Alcoholic Extract	250 mg/kg	5.67 ± 1.81*	53.97
VI	Hydro-Alcoholic Extract	500 mg/kg	3.46 ± 1.42	71.91

Data are expressed as the means ± SEM. *p < 0.05, **p < 0.01 vs control group

Table 2. Antipyretic activity of leaf extracts of *Diplazium esculentum*

Group	Treatment Group	Dose (mg/kg B.W)	Rectal temperature (°C) After administration of drug					
			Normal	after 24h	1h	2h	3h	4h
I	Saline	10ml	37.12± 1.53	39.34± 1.37	39.35± 0.99	39.59± 1.23	39.65± 0.79	39.67± 1.45
II	Paracetamol (Standard)	150mg	37.03± 2.14	39.11± 0.97	38.65± 1.48	37.89± 1.36*	37.77± 1.68	37.22± 1.64
III	Ethyl acetate Extract	250mg	36.23± 1.46	38.89± 1.75	38.58± 1.13	38.46± 1.29*	38.47± 1.05	37.33± 1.89
IV	Ethyl acetate Extract	500mg	37.05± 1.65	39.75± 2.42**	39.58± 1.75	39.46± 0.88	38.49± 1.46	38.11± 1.18
V	Hydro- Alcoholic Extract	250mg	36.13± 0.87*	38.15± 1.53	38.11± 1.42	37.08± 2.12	37.07± 1.66**	36.77± 1.71
VI	Hydro- Alcoholic Extract	500mg	37.34± 1.02	39.44± 0.95	38.67± 1.11	38.33± 1.53	37.54± 0.83*	37.48± 1.32

Data are expressed as the means ± SEM. *p < 0.05, **p < 0.01 vs control group

The inhibition was dose dependent and remained significant up to 3h of administration as shown in Table 2. The maximum antipyretic effect of hydro-alcoholic extract was observed at 500mg/kg i.e. 37.48 ± 1.32while, the antipyretic effect of paracetamol was 37.22 ± 1.64.

Administration of 50 mg/ml dose of loperamide (standard) showed (3.12 ±0.95) diarrheal feces in 4 hours causing 74.67% inhibition of defecation. Ethyl acetate extract of *Diplazium esculentum* administered at 250 mg/kg dose showed (10.21 ± 1.75) number of diarrheal feces

in 4 hr causing 17.12% inhibition of defecation. When administered at a higher dose (500 mg/ml), anti-diarrheal activity increased to 34.17%. Similarly, the hydro-alcoholic extract at 250 mg/kg concentration showed 53.97% inhibition while at 500 mg/kg, 71.91% inhibition was observed. Thus with increasing concentration of the drug dose, antidiarrheal activity also increases. The subcutaneous injection of brewer's yeast evoked pyrexia by ultimately increasing synthesis of prostaglandin and is considered as a valuable in-vivo screening test for the assessment ofantipyretic potential (Muhammad et al., 2012; Wan et al., 2013).

Yeast-induced pyrexia is called pathogenicfever. Its etiology could be the production of prostaglandins. Theinhibition of prostaglandin synthesis could be the possible mechanism of antipyretic action as that of paracetamol and the inhibition of prostaglandin can be achieved by blocking the cyclooxygenase enzyme activity (Igbe et al., 2009). The antipyretic effect of test extract is related with several mediators which cause pyrexia especially prostaglandins (Muhammad et al., 2013). The intraperitoneal administration of *Diplazium esculentum* leaf extracts significantly attenuated rectal temperature of yeast induced febrile rats. Thus, it can be postulated that *Diplazium esculentum* leaf extracts interferes with the release of prostaglandins at any stage.

CONCLUSION

Finally we conclude that the test extract may be useful in the protection against antidiarrheal and Antipyretic diseases. In comparison with the standard drug loperamide and paracetamol, hydro-alcoholic extracts of *Diplazium esculentum* leaves showed significant antidiarrheal and antipyretic efficacy. A more detailed and in-depth phytochemical investigation is necessary to identify the novel chemical entity responsible for the bioactivity of *Diplazium esculentum* leaves.

Conflict of Interest: None

REFERENCES

Anand U., Nadia Jacobo-Herrera, Ammar Altemimi and Naoufal Lakhssassi (2019) A Comprehensive Review on Medicinal Plants as Antimicrobial Therapeutics: Potential Avenues of Biocompatible Drug Discovery Metabolites 2019, 9, 258; doi:10.3390/metabo9110258

Annan K, Dickson RA, Amponsah IK et al., (2013). Pharmacognostic evaluation and physicochemical analysis of *Paullini apinnata*L. (Sapindaceae). J Pharmacogn Phytochem, 2(2), 203-208.

Archana GN, Pradesh S, Nikhila GS et al., (2012). Conservation of vegetable fern (*Diplazium esculentum*) of Western Ghats through *in vitro* spore culture, J Aquat Biol Fish, 2(2), 43-50.

Das B, Paul T, Apte KG et at., (2012). Anti anaphylactic and mast cell stabilizing activity of *Diplazium esculentum* Retz. on sensitized wistar rats. J Ethnopharmacol, 3, 136-141.

Duraisankar M and Ravichandran V (2012). Antipyretic Potential of Polyherbal Ayurvedic Products. Asian J Pharm Clin Res, 5(2), 146-150.

Gupta M, Shaw BP and Mukerjee A (2008). Studies on Antipyretic, Analgesic and Ulcerogenic Activity of Polyherbal Preparation in Rats and Mice. Intl J Pharmacol, 4(2), 88-94.

Gutiérrez SP, Sanchez MAZ, Gonzlalez CP et al., (2007). Anti diarrhoeal activity of different plants used in traditional medicine. Afr J Biotechnol, 6 (Suppl 25), 2988-2994.

Igbe I, Ozolua R., Okpo S et al., (2009). Antipyretic and analgesic effects of theaqueous extract of the fruit pulp of *Hunteriaumbellata* K. Schum (Apocynaceae). Trop J Pharm Res, 8, 331336.

Junejo JA, Gogoi G, Islam J et al., (2018). Exploration of antioxidant, antidiabetic and hepatoprotective activity of *Diplaziumesculentum*, a wild edible plant from North Eastern region of India. Future J Pharm Sci, 4, 93-101.

Junejo JA, Mondal P, Rudrapal M et al., (2014). Antidiabetic assessment of the hydro-alcoholic leaf extracts of the plant *Tetrastigmaangustifolia*(Roxb.), a traditionally used North-Eastern Indian vegetable. Biomed Pharmacol, 7(2), 635-644.

Junejo JA, Rudrapal M, Nainwal LM et al., (2017). Antidiabeticactivity of hydro-alcoholic stem bark extract of *Callicarpaarborea*Roxb. with antioxidant potential in diabetic rats. Biomed Pharmacother, 95, 84-94.

Kang J, Khan M, Park N et al., (2008). Antipyretic, analgesic, and anti-inflammatory activities of the seaweed *Sargassum fulvellum* and *Sargassum thunbergii* in mice. J Ethnopharmacol, 116(1), 187-190.

Khan H, Khan MA, Khan A et al., (2014). Antipyretic activity ofdecoction of Joshanda and its saponin and sterol contents: validation in ananimal-based model. J Evid Based Complement Alternat Med 19, 99-103.

Lense O (2011). Biological screening of selected traditional medicinal plants species utilized by local people of Manokwari West Papua Province. Nus Biosci, 3, 145-150

Meite S, N'guessan DJ, Bahi C et al., (2009). Antidiarrheal activity of the ethyl acetate extract of *Morinda morindoides* in rats. Trop J Pharma Res, 8(Suppl 3), 201-207.

Muhammad N, Barkatullah IM, Khan H et al., (2013). In vivo screening of essential oils of Skimmi alaureola leaves for antinoci-ceptive and antipyretic activity. Asian Pac J Trop Biomed, 3, 202-206.

Muhammad N, Saeed M and Khan H (2012). Antipyretic, analgesic and anti-inflammatory activity of Viola betonicifolia whole plant. BMC Complement Altern Med, 12:59.

Palombo EA (2006). Phytochemicals from traditional medicinal plants used in the treatment of diarrhoea: modes of action and effects on intestinal function.

Phytother Res, 20 (Suppl 9), 717–724.
Rahmat A, Kumar V, Fong LM et al., (2004). Determination of total antioxidant activity in three types of local vegetables shoots and the cytotoxic effect of their ethanol extract against different cancer cell lines. Asia Pac J Clin Nutr, 13(3), 292-299.
Rajani GP, Gupta D, Sowjanya K et al., (2011). Screening of antipyretic activity of aerial parts of *Nelumbonuciferagaertn* in yeast induced pyrexia. Pharmacologyonline, 1, 1120-1124.
Shoba FG and Thomas M (2011). Study of antidiarrheal activity of four medicinal plants in castor-oil induced diarrhea. J Ethnopharmacol, 76(1), 73-76.
Uddin SJ, Sjilpi JA, Alam SM et al., (2005). Antidiarrhoeal activity of the methanol extract of the barks of *Xylocarpus moluccensis* in castor oil and magnesium sulphate induced diarrhea models in mice. J Ethnopharmacol, 101, 139-143.
Walker CLF, Friberg IK, Binkin N et al., (2011). Scaling up diarrhea prevention and treatment interventions: A lives saved tool analysis. PLoS Med, 8 (Suppl 3), e1000428.
Wan J, Gong X, Jiang R et al., (2013). Antipyretic and anti-inflammatory effects of asiaticoside in lipopolysaccharide-treated rat through up-regulation of Heme Oxygenase-1. Phytother Res, 27, 1136-1142.

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Difference of Multidisciplinary Examination and Student Objective Oral Case Analysis Scores of Indonesian Medical Students

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ABSTRACT

Learning duration is one of factors that can influence student achievement. The number of learning weeks in the Undergraduate Program, Faculty of Medicine, Universitas Padjadjaran (Program *Studi Sarjana Kedokteran Fakultas Kedokteran Universitas Padjadjaran* or PSSK FK Unpad) had changed since the 2018/2019 academic year. This study aims to investigate the differences of Multidisciplinary Examination (MDE) and Student Objective Oral Case Analysis (SOOCA) scores of students whom the number of learning weeks were different. This study was conducted using a numerical comparative bivariate analysis with cross-sectional design. The data used in this study was MDE and SOOCA scores of Endocrine and Metabolism System (EMS) and Neuro-Behavior and Special Sense System (NBSS) of medical students batch 2016 who experienced 16 weeks of learning as well as batch 2017 who had 14 weeks of learning. Mann-Whitney test shows a significant difference of median in the EMS MDE scores between batch 2016 and 2017 (Me batch 2016=77.33; Me batch 2017=63.75; $p<0.001$). A significant difference of median was also found in MDE scores of NBSS between both batches (Me batch 2016=80.20; Me batch 2017=61.43; $p<0.001$). A single SOOCA examination was used to assess both EMS and NBSS simultaneously, and the median of its score between batch 2016 and 2017 was found to have no significant difference (Me batch 2016=79.25; Me batch 2017=80.00; $p>0.05$). This study shows that different number of learning weeks might contribute to the differences in the students' achievement of MDE scores.

KEY WORDS: MDE SCORE, NUMBER OF LEARNING WEEKS, SOOCA SCORE.

INTRODUCTION

Learning achievement is an important thing to be achieved by students because it could show the level of students understanding about the material being taught. It is shown by the examination scores and the Grade Point Average (GPA). The study conducted in 2014 revealed that the factor most affecting the passing of the Indonesian Doctor Competency Examinations (*Uji Kompetensi Dokter Indonesia* or UKDI) was the GPA achieved at the undergraduate program(Utomo et al., 2014). The medical student's understanding of the material being taught could be factor that influences the level of subsequent professional misconduct(Yates and James, 2010). There are various factors that could affect the academic performance of medical students, such as the physical and mental condition, motivation, quality of life of students, pressure from parents and peers, and learning duration at university (Mandal et al., 2012, Shawwa et al., 2015 Azizollah 2016, Haque et al., 2018). The study about learning duration factor to students academic achievement is still rarely

ARTICLE INFORMATION

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Received 7th Jan 2020 Accepted after revision 25th Feb 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)
A Society of Science and Nature Publication, Bhopal India

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/31

done. The study conducted in 2013 revealed that six years extended learning duration resulted better graduates in terms of grades and time needed to graduate compared to five years learning duration (Tešija et al., 2013). The study on learning achievement differences of medical students if the length of learning duration have a difference that are not so long or only few weeks has never been done.

The number of learning weeks in several system blocks in PSSK FK Unpad had changed since the 2018/2019 academic year(FK Unpad, 2017)(FK Unpad, 2018). This was in line with the Unpad policy to uniform academic schedules throughout undergraduate study programs. The system blocks that had changes in the number of learning weeks for 2nd year medical students in odd semester were EMS and NBSS (FK Unpad, 2017)(FK Unpad, 2018). The number of learning weeks of EMS in the 2017/2018 academic year (medical students batch 2016) was six weeks, while in the 2018/2019 academic year (medical students batch 2017) reduced into five weeks (FK Unpad, 2017) (FK Unpad, 2018). The number of learning weeks of NBSS which was previously ten weeks changed into nine weeks (FK Unpad, 2017)(FK Unpad, 2018). This study aims to investigate learning achievement differences in the MDE and SOOCA scores of the EMS and NBSS between the batch 2016 and 2017 who have different number of learning weeks. The results of the study are expected to be utilized as a consideration for policy makers in FK Unpad especially and Unpad generally in making policies regarding the academic system which will be applied.

Table 1. Characteristics of research subjects

	2016 batch n = 258 (%)	2017 batch n = 271 (%)
Gender: Males	87 (33.72)	94 (34.69)
Females	171 (66.28)	177 (65.31)
Age average	18	18
Entrance selection:		
SNMPTN	126 (48.84)	104 (38.38)
SBMPTN	132 (51.16)	167 (61.62)
GPA average of 1st year study	3.44	3.45

MATERIAL AND METHODS

Ethical statement: This study was conducted after getting approval from Ethical Committee of Universitas Padjadjaran Bandung No. 885/UN6.KEP/EC/2019. Study design: This study wasa cross-sectional numerical comparative bivariate analytic using secondary data. The data used were MDE and SOOCA scores of EMS and NBSS.

Materials and/or Subjects: The population of this study were the medical students of PSSK FK Unpad batch 2016 and 2017 who had different number of learning weeks (6 and 10 learning weeks of EMS and NBSS respectively in batch 2016 and 5 and 9 learning weeks of EMS and NBSS respectively in batch 2017), accepted through Seleksi Nasional Masuk Perguruan Tinggi Negeri (SNMPTN) or Seleksi Bersama Masuk Perguruan Tinggi Negeri (SBMPTN) and did the tests for the first time.

The students who did the tests outside the first exam schedule were excluded from this study. The variables analyzed in this study were MDE and SOOCA scores of EMS and NBSS of medical students batch 2016 and 2017. The data were obtained from Academic Assessment Unit, Faculty of Medicine, Universitas Padjadjaran.

Statistics: This study used total sampling technique. Statistical analysis was performed using Mann-Whitney testing, and processed using IBM® SPSS® version 20.

RESULTS AND DISCUSSION

There were 258 students at the beginning of the 2016/2017 academic year (medical students batch 2016) and 271 students at the 2017/2018 academic year (medical students batch 2017) who were registered as new medical student in FK Unpad. In the batch 2016, 126 students entered through the SNMPTN and 132 students

Table 2. Data processing result of the EMS MDE scores

	EMS MDE	
	2016 batch (6 learning weeks, n = 228)	2017 batch (5 learning weeks, n = 265)
Median	77.33	63.75
Maximum	89.33	79.38
Minimum	49.33	43.13
p	<0.001	

Table 3. Data processing result of the NBSS MDE scores

	NBSS MDE	
	2016 batch (10 learning weeks, n = 252)	2017 batch (9 learning weeks, n = 263)
Median	80.40	61.43
Maximum	89.20	77.29
Minimum	52.40	41.68
p	<0.001	

through the SBMPTN, while in the batch 2017, 104 and 167 students entered through the SNMPTN and SBMPTN, respectively. Both of the batches had same age average, 18 years old, when the first year of study was begun. The GPA average obtained by the batch 2016 in the first year of study was 3.44, while the batch 2017 obtained 3.45. Characteristics of research subjects can be seen in table 1. There were 228 and 265 data of the EMS MDE scores of students batch 2016 and 2017 respectively, after data selection was done. The EMS MDE scores data obtained were tested using the Mann-Whitney test due to abnormal data distribution. The p value=0,000 obtained from the analysis using the IBM® SPSS® version 20 was used to reject the null hypothesis.

Thus, in the EMS MDE scores result, it can be found a significant median difference between the batch 2016 with six weeks of learning duration and batch 2017 with five weeks of learning duration. Data processing result of the EMS MDE scoresis shown in table 2. After data selection done on the NBSS MDE scores data, there were respectively 252 and 263 data scores of students batch 2016 and 2017. The NBSS MDE scores data obtained were tested using the Mann-Whitney test due to abnormal data distribution. The p value=0,000 obtained from the analysis using the IBM® SPSS® version 20 was used to reject the null hypothesis. Thus, on the NBSS MDE scores, the significant median difference can be found between the batch 2016 with ten learning weeks and the batch 2017 with nine learning weeks. Data processing result of the NBSS MDE scores can be seen in table 3.

The number of the EMS+NBSS SOOCA scores data of medical students batch 2016 and 2017 respectively were 244 and 261 after data selection conducted. The EMS+NBSS SOOCA scores data obtained were tested using the Mann-Whitney test due to abnormal data distribution. The p value=0.987 obtained from the analysis using the IBM® SPSS® version 20 using was used to accept the null hypothesis. Thus, in the EMS+NBSS SOOCA scores, no significant median difference was found between the batch 2016 and 2017. Data processing result of the EMS+NBSS SOOCA scores can be seen in Table 4.

In this study, it was found that the EMS and NBSS MDE scores achieved by medical students batch 2016 (6 learning weeks of EMS and 10 learning weeks of NBSS) were higher than batch 2017 (5 weeks learning of EMS and 9 weeks learning of NBSS), either from the median, the highest, or the lowest score. The batch 2016 medical students had one week longer than batch 2017 in learning weeks number either for EMS or NBSS blocks. The one week difference of learning duration could affect students in reading material deeperly. The MDE examines the level of students material knowledge in depth, thus one week difference of learning duration in each system blocks could influence the students ability in answering MDE questions. The previous study revealed that longer learning duration resulted graduates with better grades than shorter learning duration (Tešija et al., 2013). The difference of difficulty level of MDE questions between the batch 2016 and 2017 may also cause significant differences in the achievement of the EMS and NBSS MDE scores. The EMS+NBSS SOOCA scores achievements between the batch 2016 and 2017 were not found significant median difference.

This could be due to the same tutorial cases of EMS and NBSS blocks between the batch 2016 and 2017 and also the tutorial process that made students familiar doing presentations. The SOOCA exam is one of components of learning process assessments in FK Unpad that assesses the medical students' analytical ability by presenting cases in front of the examiners. The cases examined in SOOCA are all cases that have been studied by students in the tutorial. There are several cases studied in one block system. The students do not know which case they will get and present. It will be determined when students enter the presentation material making room. Therefore medical students tend to prepare the SOOCA in long period of time to master all the tutorial cases.

The high study motivation of students batch 2016 and 2017 to get good score on the SOOCA could also be a reason why the results of the EMS+NBSS SOOCA scores were not found significant median difference. The SOOCA score is one of the determinants of the final system block score which has big proportion. The learning strategy of the students batch 2016 and 2017 are self-directed learning, as a result of the implementation of the Problem Based Learning (PBL) system at FK Unpad(Loyens et al., 2008) (Universitas Padjadjaran, 2018). Previous study has shown that good motivation and learning strategy will result in good test scores (Azizollah et al., 2016).

This study has not been conducted before, i.e. investigating differences in learning outcome if the learning duration difference is not too long. The limitation of this study is that it did not investigate the differences of learning

Table 4. Data processing result of the EMS+NBSS SOOCA scores

	EMS+NBSS SOOCA	
	2016 batch (16 learning weeks, n = 244)	2017 batch (14 learning weeks, n = 261)
Median	79.25	80.00
Maximum	100.00	96.00
Minimum	16.00	0.00
p		0.987

achievement in other system blocks. This study did not consider the difficulty level of the MDE questions between the 2016 and 2017 batches. Previous study revealed that there was an unexpected impact of the Multiple Choice Question (MCQ) exam type in the learning process of medical students (Aras et al., 2014). The students only studied exam questions of previous batches before the exam (Aras et al., 2014), therefore when they faced new exam questions, the students can not answer it optimally. Based on these findings, the study can also be further developed by taking into account the level of difficulty of the MDE questions of 2016 and 2017 batches compared to the previous batches exam questions, since it can affect the ability of students to answer the MDE questions. Further study that aims to investigate the factors that can influence the learning achievement is required in order to obtain a comprehensive research results regarding the factors that can affect students academic achievement.

ACKNOWLEDGMENTS

We thank undergraduate program and academic assessment unit of Faculty of Medicine Universitas Padjadjaran for supporting data in this study.

Conflict of Interest: No potential conflict of interest relevant to this article was reported.

REFERENCES

Aras, I., Rahayu, G. R. & Prabandari, Y. S. 2014. Persepsi Dampak Ujian dengan MCQ terhadap Proses Belajar Mahasiswa Fakultas Kedokteran. 2014, 3, 8.

Azizollah, A., Zare, S., Shahrakipour, M. & Ghoreishinia, G. 2016. The relationship between academic achievement motivation and academic performance among medical students. 8, 12272-12280.

FK Unpad. 2017. Kalender akademik FK Unpad 2017-2018.

FK Unpad. 2018. Kalender akademik FK Unpad 2018-2019.

Haque, M., A.Rahman, D. N. I., Majumder, M. A., Izaa Rahman, N., Haque, S., Zulkifli, Z., Lugova, H., Musa, R. & Alattraqchi, A. 2018. Assessment of academic/non-academic factors and extracurricular activities influencing performance of medical students of faculty of medicine, Universiti Sultan Zainal Abidin, Malaysia.

Loyens, S., Magda, J. & Rikers, R. 2008. Self-Directed Learning in Problem-Based Learning and its Relationships with Self-Regulated Learning. Educational Psychology Review, 20, 411-427.

Mandal, A., Ghosh, A., Sengupta, G., Bera, T., Das, N. & Mukherjee, S. 2012. Factors affecting the performance of undergraduate medical students: a perspective. Indian J Community Med, 37, 126-9.

Shawwa, L., Abulaban, A., Abulaban, A., Merdad, A., Baghlaf, S., Algethami, A., Abu-Shanab, J. & Balkhoyor, A. 2015. Factors potentially influencing academic performance among medical students.

Tešija, R. A., Maslov Kružicevic, S., Banožic, A., Esteban, C. D., Sapunar, D. & Puljak, L. 2013. Impact of extended course duration and stricter study organization on attrition and academic performance of medical students. Croatian medical journal, 54, 192-197.

Universitas Padjadjaran. 2018. Buku Pedoman Penyelenggaraan Pendidikan Fakultas Kedokteran Universitas Padjadjaran Tahun Akademik 2018/2019.11.

Utomo, B., Roostantia, R. & Safitri, I. 2014. Analisis Faktor yang Berpengaruh terhadap Tingkat Kelulusan UKDI Dokter Baru Lulusan Fakultas Kedokteran Universitas Airlangga. 2014, 3, 10.

Yates, J. & James, D. 2010. Risk factors at medical school for subsequent professional misconduct: Multicentre retrospective case-control study. BMJ (Clinical research ed.), 340, c2040.

Dental
Communication

30Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp-178-186

BBRC
Bioscience Biotechnology
Research Communications

Role of Novel Biomaterial Bioactive Glass with Enamel Matrix Derivative in Regeneration: A Systematic Review

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ABSTRACT

To evaluate the efficacy of enamel matrix derivative (EMD) with bioactive glass (BG) in the management of periodontal osseous defects. The addressed focused question was "Does the use of EMD with BG, improve its efficacy in the management of periodontal defects in comparison to EMD alone?" Databases were searched up to December 2019 using different combinations of MESH words. Six randomized clinical trials were included. One study showed significantly better periodontal outcomes for BG as an adjunct to EMD as compared to EMD alone. However, in two studies, improvement in the periodontal parameters for BG application as an adjunct to EMD and EMD alone were comparable. One clinical trial indicated significant improvement in clinical periodontal measures with the use of adjunctive EMD to BG compared with BG alone. However, one study showed equal outcomes between adjunctive EMD and BG alone. One study showed significant clinical improvement for BG compared with EMD. In conclusion, it remains unclear whether the efficacy of EMD in the management of periodontal osseous defects is improved when it is used in combination with BG as compared to when EMD is used alone given that the number of selected studies was relatively low and reported parameters were inconsistent.

KEY WORDS: ENAMEL MATRIX DERIVATIVE; BIOACTIVE GLASS; PERIODONTAL OSSEOUS DEFECTS; SYSTEMATIC REVIEW.

INTRODUCTION

Periodontitis is an inflammatory condition of periodontal tissues caused by complex oral biofilms and is characterized by irreversible periodontal tissue damage, which if not treated, may lead to tooth loss (Tonetti et al., 2018; Hajishengallis, 2015). This disease affects almost 50-90% of the global population and is considered one of the most common oral diseases and is the sixth most prevalent disease in the world (Preshaw et al., 2012). The putative microorganisms that includes Porphyromonas gingivalis, Prevotella intermedia, Tannerella forsythia, and Treponema denticola are responsible in the development of chronic periodontitis and activate innate, inflammatory, and adaptive immune responses (Van der Velden, 2017). As a result, this disease creates a local proinflammatory state and secretes a plethora of cytokine production which is manifested by dysregulated immune responses and results in periodontal tissue destruction (Akram et al., 2016).

Treatment of periodontitis aims to repair, regenerate and maintain the periodontal tissues. Multiple management strategies for periodontal disease have been utilized, including scaling and root planning (SRP) (Smiley et al.,

ARTICLE INFORMATION

*Corresponding Author: ozzydey84@gmail.com
Received 15th Jan 2020 Accepted after revision 10th March 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)
A Society of Science and Nature Publication, Bhopal India

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/32

2015), administration of local and systemic antibiotics (Keestra et al., 2015), photodynamic therapy (Akram et al., 2017), probiotic therapy (Ikram et al., 2018), metformin therapy (Akram et al., 2018), surgical intervention (Akram et al., 2019) and guided tissue regeneration (GTR) (Akizuki et al., 2005). Guided tissue regeneration (GTR), which is placement of barrier membranes and bone fillers or grafts in the periodontium (Bottino et al., 2012), aims to achieve the regeneration of lost periodontal tissues. Bioactive glass (BG), in particular, has been used as a contemporary alloplastic bone substitute to restore periodontal defects. It binds to natural bone and stimulates the regeneration of periodontal tissues in the implantation site (Hench, 2006). It induces the formation of a hydroxy carbonate apatite (HCA) layer, causing migration of osteoblasts to defect area, protein adsorption, incorporation of collagen fibrils, and attachment of stem cells and, therefore, regeneration of bone (Mondal et al., 2018).

Use of BG filler to restore periodontal defects requires much simpler surgical techniques as compared to using GTR membranes (Mengel et al., 2006; Yukna et al., 2001) and have demonstrated better bone regeneration when compared to surgical interventions alone (Zhang et al., 2016). In addition, when used with autogenous bone grafts, BG fillers have shown results comparable to autogenous grafts combined with hydroxyapatite (Galindo-Moreno et al., 2008). Lately, enamel matrix derivative (EMD) has been used as an adjunct to surgical periodontics for the regeneration of lost periodontal bone (Miron et al., 2016). EMD comprises amelogenin and other proteins extracted from porcine fetal teeth and has shown to stimulate the regeneration of periodontal ligament (PDL) cells (Amin et al., 2016). In addition, it facilitates the proliferation and attachment of PDL cells such as fibroblasts, by stimulating the production and release of cyclic adenosine monophosphate levels, transforming growth factor-β, and interleukin-6 (Kawase et al., 2000; Lyngstadaas et al., 2001; Schwartz et al., 2000; der Pauw et al., 2000).

It is hypothesized that the use of EMD in combination with conventional freeze-dried allografts could produce a synergistic effect in periodontal regeneration procedures. In a study by Sculean et al. (2005a), patients with intrabony defects treated with EMD as an adjunct to BG demonstrated substantial improvement in periodontal measures compared with BG alone. However, Sculean et al., (2002) in a clinical trial comparing the effect of EMD combined with BG and EMD alone in the management of periodontal osseous defects, concluded that all patients showed comparable clinical outcomes at follow up regardless of the materials used. Hence, there appears to be a debate and contradictory results in terms of the purpose of EMD with and without BG in the treatment of periodontal defects and therefore, a systemic review is deemed necessary. This review aims to systematically evaluate the efficacy of EMD in combination with BG in the management of periodontal osseous defects.

MATERIALS AND METHODS

Systematic review question and protocol: This systematic review followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Moher, Liberati, Tetzlaff, & Altman, 2009). The focused systematic review question was "Does the use of EMD, when used with BG, improve its efficacy in the management of periodontal defects in comparison to EMD or BG used alone?"

Eligibility criteria: To conduct the systematic review, following criteria were considered: (a) Randomized controlled clinical trials (RCTs) in humans (b) Trials evaluating efficacy of EMD and BG in the treatment of intrabony defect. (c) Studies reporting pocket depth (PD), clinical attachment loss (CAL) as primary outcomes and gingival recession (REC), plaque index (PI), gingival index (GI) or bleeding on probing (BOP) as secondary outcomes and (d) English language articles only. The studies were excluded if they had in vitro or experimental design, letters to the editor, review papers and unpublished articles.

Search: The author searched the PUBMED, EMBASE, and CENTRAL databases up to December 2019 for appropriate articles addressing the focused question. A structured approach to literature searching was used to identify the relevant papers that directly compare the efficacy of EMD with or without BG in subjects with the presence of at least one intra-bony defect. Following that, reference lists of original studies were hand-searched to identify any articles that could have been missed during the initial search. Hand searching of the following journals was performed: Journal of Clinical Periodontology, Journal of Periodontology, and Journal of Periodontal Research. Different combinations of MeSH (Medical Subject Headings) terms were considered: enamel matrix derivative; enamel matrix protein; bioactive glass; bioglass; ceramics; intrabony defect; intraosseous defect.

Screening methods and data abstraction: Titles and abstracts of articles that satisfied the selection protocol were screened and checked for agreement. Thereafter, the full-text screening was done. The information from the accepted studies was tabulated according to the (1) study design, (2) demographic characteristics of study participants, (3) study groups, (4) intrabony defect, (5) assessed periodontal parameters, (6) subjects follow up, and (7) main outcome. The kappa value for the intra-assessor agreement was 0.92.

Quality of the studies: The methodological quality of the included studies according to a grading system was developed using the Jadad scale (Jadad et al., 1996).

RESULTS AND DISCUSSION

Study selection: A total of 542 studies were initially identified. Twenty-six studies which did not fulfill the eligibility criteria after full-text screening were excluded (Appendix A). In total, 6 studies (Kuru et al., 2006; Leknes et al., 2009; Sculean et al., 2002; Sculean et al.,

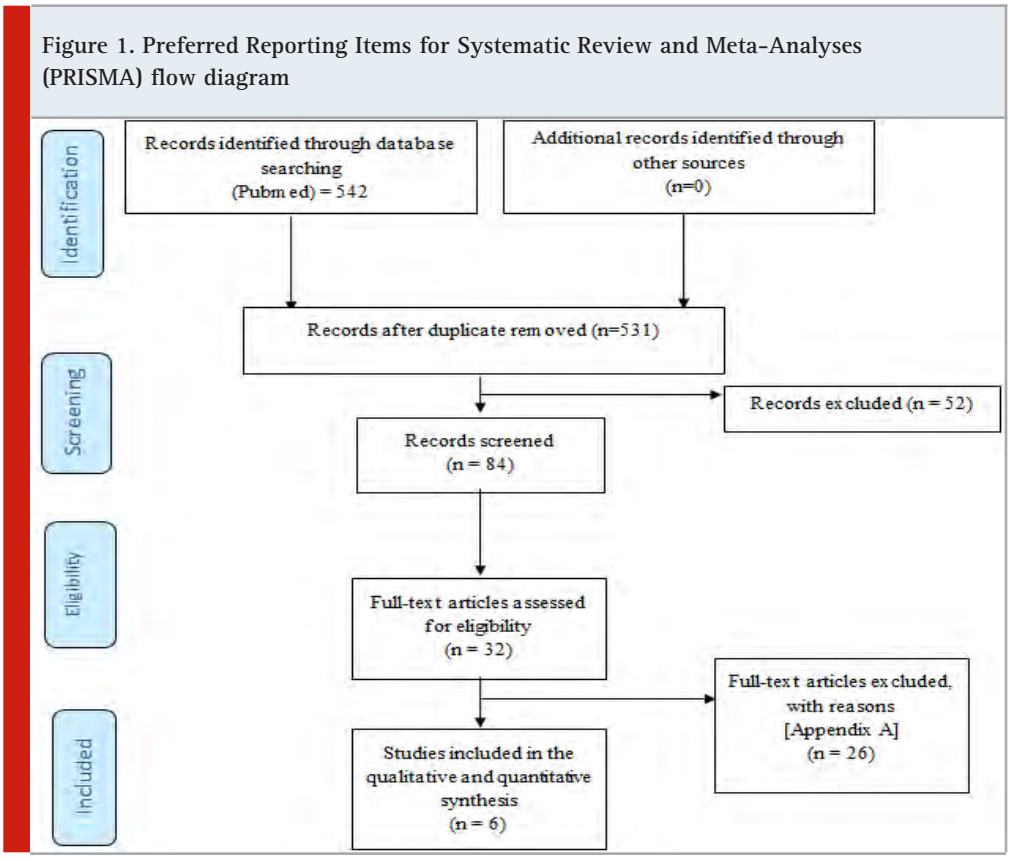
2007; Sculean et al., 2005a; Sculean et al., 2005b) were included and processed for data extraction. All studies (Kuru et al., 2006; Leknes et al., 2009; Sculean et al., 2002; Sculean et al., 2007; Sculean et al., 2005a; Sculean et al., 2005b) were performed at either universities or health care centers. Figure 1 shows the PRISMA study identification flow chart.

General characteristics of the selected articles: All 6 studies (Kuru et al., 2006; Leknes et al., 2009; Sculean et al., 2002; Sculean et al., 2007; Sculean et al., 2005a; Sculean et al., 2005b) included in the present systematic review were RCTs. The total number of patients in these clinical trials ranged between 6 and 30 individuals (Kuru et al., 2006; Leknes et al., 2009; Sculean et al., 2002; Sculean et al., 2007; Sculean et al., 2005a; Sculean et al., 2005b). Only two studies (Leknes et al., 2009; Sculean et al., 2007) reported the mean age of study participants, which was 46.1 and 52.5 (age range 38 to 74). Five clinical studies (Leknes et al., 2009; Sculean et al., 2002; Sculean et al., 2007; Sculean et al., 2005a; Sculean et al., 2005b) reported the number of female participants, which ranged from 6 to 16 individuals. Five studies (Sculean et al., 2002; Sculean et al., 2007; Sculean, et al., 2005a; Sculean et al., 2005b) used EMD and BG in the test group while one study (Leknes et al., 2009) used BG alone in the test group. In the control group, four studies (Kuru et al., 2006; Leknes et al., 2009; Sculean et al., 2007; Sculean et al., 2005b) used EMD while two studies (Sculean et al., 2002; Sculean, et al., 2005a) used BG (Table 1). In all the studies (Kuru et al., 2006; Leknes et al., 2009; Sculean et al., 2002; Sculean et al., 2007; Sculean, et al., 2005a; Sculean, et al., 2005b) the follow-up period ranged from 24 – 192 weeks. In one study (Sculean et al., 2007), 16.66% of patients dropped out of the trial. Smokers were in included in studies by Sculean et al and Leknes et al respectively (Leknes et al., 2009; Sculean et al., 2002). All the enrolled participants had a complication-free healing period with no side-effects related to BG and EMD.

Clinical periodontal parameters of included studies: All clinical studies (Kuru et al., 2006; Leknes et al., 2009; Sculean et al., 2002; Sculean et al., 2007; Sculean, et al., 2005a; Sculean et al., 2005a; Sculean et al., 2005b) described clinical periodontal measures (Table 2). In four studies (Kuru et al., 2006; Sculean et al., 2002; Sculean et al., 2007; Sculean, et al., 2005a), the mean PI ranged from 0.18 to 0.9 at follow up. In four studies (Kuru et al., 2006; Sculean et al., 2002; Sculean et al., 2007; Sculean, et al., 2005a) gingival index was reported, which was 0.17 to 0.9 at follow-up. Three studies (Sculean et al., 2002; Sculean et al., 2007; Sculean, et al.,2005a) reported BOP in percentage which ranged from 22% to 43%. In 5 studies, the mean PD ranged from 1.0 mm to 5.73 mm at follow-up.

All six studies (Kuru et al., 2006; Leknes et al., 2009; Sculean et al., 2002; Sculean et al., 2007; Sculean, et al., 2005a; Sculean et al., 2005b) reported CAL and five studies (Kuru et al., 2006; Leknes et al., 2009; Sculean et al., 2002; Sculean et al., 2007; Sculean et al., 2005a) reported REC which ranged from 6.3 mm to 13.7 mm and 2.4 mm to 6.5 mm respectively at follow-

Figure 1. Preferred Reporting Items for Systematic Review and Meta-Analyses (PRISMA) flow diagram



up. Relative bone loss was reported by only one study (Kuru et al., 2006).

The main outcome of the studies: All studies (Kuru et al., 2006; Leknes et al., 2009; Sculean et al., 2002; Sculean et al., 2007; Sculean, et al., 2005b; Sculean, et al., 2005b) that reported periodontal indices demonstrated that EMD was successful in intrabony periodontal osseous defects at follow-up. Among these clinical studies (Kuru et al., 2006; Leknes et al., 2009; Sculean et al., 2002; Sculean et al., 2007; Sculean, et al., 2005a; Sculean, et al., 2005b), one study (Kuru et al., 2006) showed significantly better periodontal outcomes for BG as an adjunct to EMD as compared to EMD alone. However, two studies by Sculean et al., (2007; 2005a).
Demonstrated equal improvement for BG as an adjunct to EMD and EMD alone. Sculean et al. (2005b), showed significantly better clinical measures for EMD as an adjunct to BG as compared to BG alone. However, one study (Sculean et al., 2002) showed equal improvement in periodontal indices for adjunctive EMD and BG alone. Leknes et al. (2009) reported significant improvement in clinical periodontal parameters with BG compared with EMD in intrabony osseous defects.

Quality of the clinical studies: All clinical studies in

Table 1. General characteristics of the selected studies

Investigator, year	Study design	Sample size (Female %)	Mean age range (in years)	Study groups		Follow-up (weeks)	Main outcome
				Test (n)	Control (n)		
Sculean et al. 2002	RCT	28 (53.5)	NA	EMD+BG (14)	BG (14)	48	Equal improvements in clinical parameters for both groups at follow-up
Kuru et al. 2006	RCT	23 (NA)	NA	EMD+BG (13)	EMD (10)	32	Clinical parameters were significantly better for test group as compared to control at follow-up
Sculean et al. 2005a	RCT	30 (53.3)	NA	EMD+BG (15)	EMD (15)	48	Improvements in clinical parameters for both groups were comparable at follow-up
Sculean et al. 2005b	RCT*	6 (100)	NA	EMD+BG	BG	24	Clinical parameters were significantly better for test group as compared to control at follow-up
Leknes et al. 2009	RCT*	13 (61.5)	52.5 (41 – 74)	BG (13)	EMD (13)	48	Clinical parameters were significantly better for test group as compared to control at follow-up
Sculean et al. 2007	RCT	25 (56)	46 (38 - 55)	EMD+BG (12)	EMD (13)	192	Improvements in clinical parameters for both groups were comparable at follow-up

RCT; randomized clinical trial, *Split-mouth technique, EMD; enamel matrix derivative, BG; bioactive glass, PD; pocket depth
NA; not available

this systematic review were RCTs. Four studies used coin toss method for randomization (Kuru et al., 2006; Leknes et al., 2009; Sculean et al., 2007; Sculean et al., 2005a). Five studies (Kuru et al., 2006; Leknes et al., 2009; Sculean et al., 2002; Sculean et al., 2007; Sculean et al., 2005a a) reported the power analysis. The quality of one study was regarded as high (Leknes et al., 2009) and hence this study received a score of 5. Three studies were graded as moderate receiving a score of 3 (Kuru et al., 2006; Sculean et al., 2007; Sculean, et al., 2005a), whereas two studies were graded as poor receiving a score of 1 (Sculean et al., 2002; Sculean et al., 2005b). A summary of the quality scoring of the studies is presented in Table 3.

The present review was based on the hypothesis of

Table 2. Clinical periodontal parameters of the included studies

Authors	PD (mm)	CAL (mm)	REC (mm)	PI	GI	BOP (%)	RBL (mm)
Sculean et al. 2002	EMD+BG Baseline: 8.07±1.14 Follow up: 3.92±0.73 BG	EMD+BG Baseline: 9.64±1.59 Follow up: 6.42±1.08 BG	EMD+BG Baseline: 1.50±1.16 Follow up: 2.50±1.08 BG	EMD+BG Baseline: 0.9±0.5 Follow up: 0.6±0.4 BG	EMD+BG Baseline: 1.8±0.9 Follow up: 0.8±0.7 BG	EMD+BG: Baseline: 60 Follow up: 40 BG:	NA
	Baseline: 8.07±1.32 Follow up: 3.85±0.66	Baseline: 9.78±1.71 Follow up: 6.71±1.89	Baseline: 1.64±0.74 Follow up: 2.92±1.85	Baseline: 0.8±0.7 Follow up: 0.7±0.4	Baseline: 2.1±1.9 Follow up: 0.9±0.6	Baseline: 58 Follow up: 43	
Kuru et al. 2006	EMD+BG Baseline: 9.77±1.01 Follow up: 5.73±0.80	NA	NA	EMD+BG Baseline: 0.30±0.05 Follow up: 0.19±0.05	EMD+BG Baseline: 0.30±0.06 Follow up: 0.17±0.05	NA	EMD+BG: Baseline: 6.24±0.78 Follow up: 2.76±0.69
	EMD Baseline: 9.47±0.81 Follow up: 5.03±0.89			EMD Baseline: 0.29±0.06 Follow up: 0.18±0.05			EMD: Baseline: 6.38±0.62 Follow up: 2.15±0.42
Sculean et al. 2005a	EMD+BG Baseline: 8.5±1.1 Follow up: 4.4±1.2	EMD+BG Baseline: 10.4±1.5 Follow up: 7.1±1.5	EMD+BG Baseline: 1.9±1.1 Follow up: 2.8±0.9	EMD+BG Baseline: 0.5±0.3 Follow up: 0.4±0.4	EMD+BG Baseline: 1.2±0.4 Follow up: 0.5±0.4	EMD+BG: Baseline: 52 Follow up: 28	NA
	EMD Baseline: 8.5±1.5 Follow up: 4.0±1.6	EMD Baseline: 10.2±2.1 Follow up: 6.3±2.2	EMD Baseline: 1.5±1.4 Follow up: 2.4±1.6	EMD Baseline: 0.4±0.2 Follow up: 0.4±0.3	EMD Baseline: 1.1±0.3 Follow up: 0.4±0.4	EMD: Baseline: 50 Follow up: 22	
Sculean et al. 2005b	NA	EMD+BG§ Baseline: 11-13 Follow up: 7-8 BG¶ Baseline: 9-14 Follow up: 7-11	NA	NA	NA	NA	NA
Leknes et al. 2009	EMD: Buccal: Baseline: 1.1±0.5 Follow up: 1.1±0.4	EMD: Buccal: Baseline: 11.2±2.5 Follow up: 12.2±2.9	EMD: Buccal: Baseline: 3.5±1.0 Follow up: 5.3±2.2	NA	NA	NA	NA

	Lingual: Baseline: 3.8±2.8	Lingual: Baseline: 12.0±3.2	Lingual: Baseline: 3.8±1.6				
	Follow up: 2.1±1.3	Follow up: 11.9±2.	Follow up: 5.0±1.6				
	Proximal: Baseline: 6.5±1.3	Proximal: Baseline: 14.2±2.4	Proximal: Baseline: 4.2±1.3				
	Follow up: 4.0±2.2	Follow up: 13.6±2.7	Follow up: 6.5±1.8				
	BG: Buccal: Baseline: 1.1±0.5	BG: Buccal: Baseline: 11.4±2.8	BG: Buccal: Baseline: 4.4±2.1				
	Follow up: 1.0±0.6 Lingual: Baseline: 3.3±1.8	Follow up: 11.6±3.1 Lingual: Baseline: 12.6±2.8	Follow up: 5.0±1.8 Lingual: Baseline: 4.5±1.6				
	Follow up: 2.5±1.5 Proximal: Baseline: 6.9±1.6	Follow up: 11.7±2.2 Proximal: Baseline: 14.9±3.0	Follow up: 5.5±1.6 Proximal: Baseline: 5.5±1.6				
Sculean et al.	Follow up: 4.3±1.6 EMD+BG Baseline: 8.6±1.0	Follow up: 13.7±2.9 EMD+BG Baseline: 10.3±1.6	Follow up: 6.2±1.3 EMD+BG Baseline: 1.7±1.0	EMD+BG Baseline: 0.8±0.4	EMD+BG Baseline: 1.7±0.5	EMD+BG: Baseline: 53	NA
2007	Follow up: 4.1±1.0 EMD Baseline: 8.6±0.9	Follow up: 6.7±1.2 EMD Baseline: 10.4±1.6	Follow up: 2.6±0.9 EMD Baseline: 1.8±1.2	Follow up: 0.9±0.4 EMD Baseline: 0.7±0.5	Follow up: 0.6±0.4 EMD Baseline: 1.8±0.8	Follow up: 34 EMD: Baseline: 49	
	Follow up: 3.9±0.6	Follow up: 6.7±1.1	Follow up: 2.8±1.0	Follow up: 0.7±0.5	Follow up: 0.8±0.6	Follow up: 36	

EMD; enamel matrix derivative, BG; bioactive glass, PD; pocket depth, CAL; clinical attachment loss, REC; recession, PI; plaque index, GI; gingival index, RBL; relative bone loss, BOP; bleeding on probing; NA; not available.
§ treatment on tooth #19, #30, #31
¶ treatment on tooth #19, #4, #3

whether EMD when used with BG, improves with efficacy in the management of periodontal osseous defects in comparison to EMD alone. All studies (Kuru et al., 2006; Leknes et al., 2009; Sculean et al., 2002; Sculean et al., 2007; Sculean et al., 2005a; Sculean, 2005b) included in the present systematic review showed that EMD was effective in the treatment of intrabony periodontal osseous defects at follow-up.Guided tissue regeneration (GTR) and grafting have been used extensively since the last few decades to restore periodontal defects (Bottino et al., 2009; Bottino et al., 2012). It has been observed in multiple RCTs that GTR is more effective than open-flap debridement in treating periodontal defects (Jepsen et al., 2002; Murphy & Gunsolley, 2003). EMD, a GTR material that is primarily composed of porcine amelogenin, is more effective in restoring clinical attachment levels and radiographic bone when compared to flap procedures (Amin et al., 2016). In addition to the clinical trials (Esposito et al.,2009), in vitro as well as in vivo studies have shown that EMD stimulates the proliferation of pre-osteoblastic cells (Boyan et al., 2000; Schwartz et al., 2000). Although a systematic review of using EMD against other types of GTR materials and periodontal treatments found it to be clinically effective, it did not include any studies which used EMD in combination with BG.

Studies included in this review assessed the clinical effectiveness of using EMD with BG and compared that with the use of BG or EMD alone. Comparable clinical periodontal parameters were observed among the studies using EMD with BG and those using BG or EMD alone

(Kuru et al., 2006; Sculean et al., 2002; Sculean et al., 2007; Sculean, et al., 2005a; Sculean et al., 2005b). However, it is appropriate to mention that there was inconsistency observed among the studies with regards to study groups included and parameters measured. A major shortcoming of these studies is the limited follow-up period of the treated patients. Only one study (Sculean et al., 2005a) followed-up patients for up to 192 weeks while the remaining studies followed up patients for a period of only 12 to 48 weeks (Kuru et al., 2006; Leknes et al., 2009; Sculean et al., 2002; Sculean et al., 2007; Sculean 2005b). In addition, smoking harms periodontal health (Preber & BergstrÖM, 1986) and quitting smoking can improve the outcomes of periodontal treatment (Patel et al., 2012). Given this, in only 2 studies included in this review, smokers were included (Leknes et al., 2009; Sculean et al., 2002). Therefore, further studies with follow up of longer duration and strict inclusion and exclusion criteria are needed to assess the long-term efficacy of using EMD with BG in the management of periodontal osseous defects.

Table 3. Assessing the quality of included RCTs using the Jadad scale.

Reference	Randomization		Blinding		An account of all patients	Total score
Sculean et al. 2002	0	0	0	0	+1	1
Kuru et al. 2006	+1	+1	0	0	+1	3
Sculean et al. 2005a	+1	+1	0	0	+1	3
Sculean et al. 2005b	0	0	0	0	+1	1
Leknes et al. 2009	+1	+1	+1	+1	+1	5
Sculean et al. 2007	+1	+1	0	0	+1	3

Only half of the studies (Sculean et al., 2002; Sculean et al., 2007; Sculean et al., 2005a) assessed in the present review reported all major clinical periodontal parameters (PD, CAL, REC, PI, GI, and BOP) and only one study (Kuru et al., 2006) assessed RBL. A comparison of only clinical parameters in the absence of RBL does not allow accurate prediction of periodontal outcomes (Haffajee et al., 1983). In addition, none of the studies assessed the subgingival microbial flora of the treated periodontal defects which is essential for an accurate surrogate assessment of periodontal recovery. Also, studies aimed at comparing the effectiveness of EMD+BG and BG or EMD through the use of biomarkers present in oral fluids are recommended (Taba., et al 2005) to accurately assess the efficacy of these GTR materials.

The study by Sculean et al. (2005b) also assessed the use of EMD+BG histologically and observed that healing around EMD+BG showed more mineralized tissue, PDL, and cementum. Conversely, when BG was used alone, more epithelial cellular growth was seen, suggesting that EMD+BG is more effective than BG. This lack of bone of formation around BG has been observed previously as well. Nevins et al observed that using BG to fill periodontal defects (Nevins et al., 2000) resulted in cellular growth that was characterized by the formation of junctional epithelium and limited formation of mineralized tissue and clinical attachment. The findings suggest that using BG alone decreases the likelihood of the formation of mineralized tissue. However, these histological observations are in contrast to the clinical findings shown in this systematic review i.e. comparable clinical outcomes for the use of EMD with and without BG in the management of periodontal osseous defects. Therefore, further randomized controlled trials aiming to assess the clinical and histological outcomes of the use of EMD with and without BG in the management of periodontal osseous defects are recommended.

CONCLUSION

It remains unclear whether the efficacy of EMD in the management of periodontal osseous defects is improved when it is used in combination with BG as compared to when EMD is used alone given that the number of selected studies was relatively low and reported parameters were inconsistent.

Conflict of interest statement: No conflict of interest

REFERENCES

Akizuki T, Oda S, Komaki M, Tsuchioka H, Kawakatsu N, Kikuchi A, Yamato M, Okano T, Ishikawa I. (2005) Application of periodontal ligament cell sheet for periodontal regeneration: A pilot study in beagle dogs. J. Periodontal Res. Vol 40: Pages 245-251.

Akram, Z., Abduljabbar, T., Hassan, A., Ibrahim, M., Javed, F., & Vohra, F. (2016) Cytokine profile in chronic periodontitis patients with and without obesity: a systematic review and meta-analysis. Dis. Markers Vol. 2016: Pages 4801418.

Akram, Z., Hyder, T., Al-Hamoudi, N., Binshabaib, M. S., Alharthi, S. S., & Hanif, A. (2017) Efficacy of photodynamic therapy versus antibiotics as an adjunct to scaling and root planing in the treatment of periodontitis: a systematic review and meta-analysis. Photodiagnosis Photodyn. Ther. Vol. 19: Pages 86-92.

Akram, Z., Vohra, F., & Javed, F. (2018). Locally delivered metformin as adjunct to scaling and root planing in the treatment of periodontal defects: A systematic review and meta-analysis. J. Periodontal Res. Vol. 53 No 6: Pages 941-949.

Akram, Z., Shafqat, S. S., Niaz, M. O., Raza, A., & Naseem, M. (2019) Clinical efficacy of photodynamic therapy and laser irradiation as an adjunct to open flap debridement in the treatment of chronic periodontitis: A systematic review and meta-analysis. Photodermatol. Photoimmunol. Photomed.

Amin, H. D., Olsen, I., Knowles, J., Dard, M., & Donos, N. (2016). Interaction of enamel matrix proteins with human periodontal ligament cells. Clin. Oral Investig. Vol 20 No 2: Pages 339-347.

Bottino, M. C., Jose, M. V., Thomas, V., Dean, D. R., & Janowski, G. M. (2009). Freeze-dried acellular dermal matrix graft: Effects of rehydration on physical, chemical, and mechanical properties. Dent. Mater. Vol 25: Pages 1109-1115.

Bottino, M. C., Thomas, V., Schmidt, G., Vohra, Y. K., Chu, T.-M. G., Kowolik, M. J., & Janowski, G. M. (2012). Recent advances in the development of gtr/gbr membranes for periodontal regeneration-a materials perspective. Dent. Mater. Vol 28: Pages 703-721.

Boyan, B. D., Weesner T. C. , Lohmann C. H., Andreacchio D., Carnes DL, Dean DD, Cochran DL & Schwartz Z. (2000). Porcine fetal enamel matrix derivative enhances bone formation induced by demineralized freeze dried bone allograft in vivo. J. Periodontol. Vol 71: Pages 1278-1286.

Chitsazi, M.-T., Shirmohammadi, A., Faramarzie, M., Pourabbas, R., & Rostamzadeh, A. N. (2011). A clinical comparison of nano-crystalline hydroxyapatite (ostim) and autogenous bone graft in the treatment of periodontal intrabony defects. Med Oral Patol Oral Cir Bucal. Vol 16: Pages 448-453.

Eppley, B. L., Pietrzak, W. S., & Blanton, M. W. (2005). Allograft and alloplastic bone substitutes: A review of science and technology for the craniomaxillofacial surgeon. J. Craniofac. Surg. Vol 16: Pages 981-989.

Esposito, M., Grusovin, M. G., Papanikolaou, N., Coulthard, P., & Worthington, H. V. (2009) Enamel matrix derivative (Emdogain®) for periodontal tissue regeneration in intrabony defects. Cochrane Database of Systematic Reviews, Vol. 4.

Galindo-Moreno, P., Ávila, G., Fernández-Barbero, J. E., Mesa, F., O'Valle-Ravassa, F., & Wang, H. L. (2008). Clinical and histologic comparison of two different composite grafts for sinus augmentation: A pilot clinical trial. Clin Oral Implants Res. Vol 19: Pages 755-759.

Hajishengallis, G. (2015) Periodontitis: from microbial immune subversion to systemic inflammation. Nat. Rev. Immunol. Vol 15 No 1: Pages 30-44.

Hench, L. L. (2006). The story of bioglass®. J Mater Sci Mater Med. Vol 17: Pages 967-978.

Ikram, S., Hassan, N., Raffat, M. A., Mirza, S., & Akram, Z. (2018) Systematic review and meta-analysis of double-blind, placebo-controlled, randomized clinical trials using probiotics in chronic periodontitis. J. Investig. Clin. Dent. Vol 9 No 3: Pages e12338.

Jadad, A. R., Moore, R. A., Carroll, D., Jenkinson, C., Reynolds, D. J. M., Gavaghan, D. J., & McQuay, H. J. (1996) Assessing the quality of reports of randomized clinical trials: Is blinding necessary? Control Clin. Trials Vol 17: Pages 1-12.

Jepsen, S., Eberhard, J., Herrera, D., & Needleman, I. (2002) A systematic review of guided tissue regeneration for periodontal furcation defects. What is the effect of guided tissue regeneration compared with surgical debridement in the treatment of furcation defects? J. Clin. Periodontol. Vol 29: Pages 103-116.

Kawase, T., Okuda, K., Yoshie, H., & Burns, D. M. (2000) Cytostatic action of enamel matrix derivative (emdogain®) on human oral squamous cell carcinoma-derived scc25 epithelial cells. J. Periodontal Res. Vol 35: Pages 291-300.

Keestra, J. A. J., Grosjean, I., Coucke, W., Quirynen, M., & Teughels, W. (2015) Non-surgical periodontal therapy with systemic antibiotics in patients with untreated chronic periodontitis: A systematic review and meta-analysis. J. Periodontal Res. Vol 50: Pages 294-314.

Kuru, B., Yılmaz, S., Argın, K., & Noyan, Ü. (2006) Enamel matrix derivative alone or in combination with a bioactive glass in wide intrabony defects. Clin. Oral Investig. Vol 10: Pages 227-234.

Leknes, K. N., Andersen, K.-M., Bøe, O. E., Skavland, R. J., & Albandar, J. M. (2009) Enamel matrix derivative versus bioactive ceramic filler in the treatment of intrabony defects: 12-month results. J. Periodontol. Vol 80: Pages 219-227.

Lyngstadaas, S. P., Lundberg, E., Ekdahl, H., Andersson, C., & Gestrelius, S. (2001) Autocrine growth factors in human periodontal ligament cells cultured on enamel matrix derivative. J. Clin. Periodontol. Vol 28: Pages 181-188.

Mengel, R., Schreiber, D., & Flores-de-Jacoby, L. (2006) Bioabsorbable membrane and bioactive glass in the treatment of intrabony defects in patients with generalized aggressive periodontitis: Results of a 5-year clinical and radiological study. J. Periodontol. Vol 77: Pages 1781-1787.

Miron, R. J., Chandad, F., Buser, D., Sculean, A., Cochran, D. L., & Zhang, Y. (2016) Effect of enamel matrix derivative liquid on osteoblast and periodontal ligament cell proliferation and differentiation. J. Periodontol. Vol 87 No 1: Pages 91-99.

Moher, D., Liberati, A., Tetzlaff, J., & Altman, D. G. (2009) Preferred reporting items for systematic reviews and meta-analyses: The prisma statement. Ann. Intern. Med. Vol 151: Pages 264-269.

Mondal, S., Hoang, G., Manivasagan, P., Moorthy, M. S., Nguyen, T. P., Phan, T. T. V., ... & Oh, J. (2018) Nano-hydroxyapatite bioactive glass composite scaffold with enhanced mechanical and biological performance for tissue engineering application. Ceramics Int. Vol 44 No 13: Pages 15735-15746.

Murphy, K. G., & Gunsolley, J. C. (2003) Guided tissue regeneration for the treatment of periodontal intrabony and furcation defects. A systematic review. Ann.

Periodontol. Vol 8: Pages 266-302.
Nevins, M. L., Camelo, M., Nevins, M., King, C. J., Oringer, R. J., Schenk, R. K., & Fiorellini, J. P. (2000) Human histologic evaluation of bioactive ceramic in the treatment of periodontal osseous defects. Int. J. Periodontics Restorat. Dent. Vol 20: Pages 458-467.
Patel, R. A., Wilson, R. F., & Palmer, R. M. (2012) The effect of smoking on periodontal bone regeneration: A systematic review and meta-analysis. J Periodontol. 83: Pages 143-155.
Preber, H., & BergstrÖM, J. A. N. (1986) Cigarette smoking in patients referred for periodontal treatment. Eur. J. Oral Sci. Vol 94: Pages 102-108. Preshaw, P. M., Alba, A. L., Herrera, D., Jepsen, S., Konstantinidis, A., Makrilakis, K., & Taylor, R. (2012) Periodontitis and diabetes: A two-way relationship. Diabetol. Vol 55: Pages 21-31.
Reynolds, M. A., Aichelmann-Reidy, M. E., & Branch-Mays, G. L. (2010) Regeneration of periodontal tissue: Bone replacement grafts. Dent. Clin. North Am. Vol 54: Pages 55-71.
Schwartz, Z., Carnes Jr, D.L., Pulliam, R., Lohmann, C.H., Sylvia, V.L., Liu, Y., Dean, D.D., Cochran, D.L. & Boyan, B.D. (2000) Porcine fetal enamel matrix derivative stimulates proliferation but not differentiation of pre-osteoblastic 2t9 cells, inhibits proliferation and stimulates differentiation of osteoblast-like mg63 cells, and increases proliferation and differentiation of normal human osteoblast NHOst cells. J. Periodontol. Vol 71: Pages 1287-1296.
Sculean, A., Barbé, G., Chiantella, G. C., Arweiler, N. B., Berakdar, M., & Brecx, M. (2002) Clinical evaluation of an enamel matrix protein derivative combined with a bioactive glass for the treatment of intrabony periodontal defects in humans. J. Periodontol. Vol 73: Pages 401-408.
Sculean, A., Pietruska, M., Arweiler, N. B., Auschill, T. M., & Nemcovsky, C. (2007) Four-year results of a prospective-controlled clinical study evaluating healing of intra-bony defects following treatment with an enamel matrix protein derivative alone or combined with a bioactive glass. J. Clin. Periodontol. Vol 34: Pages 507-513.
Sculean, A., Pietruska, M., Schwarz, F., Willershausen, B., Arweiler, N. B., & Auschill, T. M. (2005a) Healing of human intrabony defects following regenerative periodontal therapy with an enamel matrix protein derivative alone or combined with a bioactive glass. J. Clin. Periodontol. Vol 32: Pages 111-117.
Sculean, A., Windisch, P., Keglevich, T., & Gera, I. (2005b) Clinical and histologic evaluation of an enamel matrix protein derivative combined with a bioactive glass for the treatment of intrabony periodontal defects in humans. Int. J. Periodontics Restorat. Dent. Vol 25: Pages 139-147.
Smiley, C. J., Tracy, S. L., Abt, E., Michalowicz, B. S., John, M. T., Gunsolley, J., . . . Forrest, J. L. (2015) Systematic review and meta-analysis on the nonsurgical treatment of chronic periodontitis by means of scaling and root planing with or without adjuncts. J. Am. Dent. Assoc. Vol 146: Pages 508-524.
Taba, M., Kinney, J., Kim, A. S., & Giannobile, W. V. (2005) Diagnostic biomarkers for oral and periodontal diseases. Dent. Clin. North Am. Vol 49: Pages 551-571.
Tonetti, M. S., Greenwell, H., & Kornman, K. S. (2018) Staging and grading of periodontitis: Framework and proposal of a new classification and case definition. J. Clin. Periodontol. Vol 45: Pages: S149-S161.
Van der Pauw, M. T., Van den Bos, T., Everts, V., & Beertsen, W. (2000) Enamel matrix-derived protein stimulates attachment of periodontal ligament fibroblasts and enhances alkaline phosphatase activity and transforming growth factor β1 release of periodontal ligament and gingival fibroblasts. J. Periodontol. Vol 71: Pages 31-43.
Van der Velden, U. (2017) What exactly distinguishes aggressive from chronic periodontitis: is it mainly a difference in the degree of bacterial invasiveness?. Periodontol. 2000 Vol. 75 No 1: Pages 24-44.
Yukna, R. A., Evans, G. H., Aichelmann-Reidy, M. B., & Mayer, E. T. (2001) Clinical comparison of bioactive glass bone replacement graft material and expanded polytetrafluoroethylene barrier membrane in treating human mandibular molar class ii furcations. J. Periodontol. Vol 72: Pages 125-133.
Zhang, X., Zeng, D., Li, N., Wen, J., Jiang, X., Liu, C., & Li, Y. (2016) Functionalized mesoporous bioactive glass scaffolds for enhanced bone tissue regeneration. Sci. Reports Vol 6: Pages 19361.

Medical
Communication

30Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp-187-194

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Therapeutic Efficacy of Photodynamic Therapy in Oral Squamous Cell Carcinoma: a Systematic Review

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ABSTRACT

How the application of photodynamic therapy (PDT) is clinically efficacious in the rapid treatment of oral squamous cell carcinoma (OSCC)?. This focused question has been designed for the present study, which is comprised of the theme: Does PDT offer effective treatment in the regression of OSCCs ? Three indexed databases were searched (PubMed, EMBASE and CENTRAL) from May 1965 up to and including September 2019 for pertinent literature. Articles were selected if they had prospective design, published in English language and reported efficacy of PDT in the treatment of OSCCs in adult patients. Thirteen studies were included. A total of 447 patients with OSCCs were included. Their mean age ranged between 60.8 years to 69.6 years. The follow-up period of the clinical trials ranged from 3 months to 144 months. All studies showed statistically significant improvement in the complete regression of OSCCs on follow-up. Several clinical trials categorized their outcomes as complete, partial or no response to therapy. For PDT, the complete response ranged from 16% to 100% in the OSCCs. PDT shows to be a clinically efficient therapeutic modality for OSCCs. PDT is equally effective as surgery with regards to rates of recurrence.

KEY WORDS: ORAL CANCER, PHOTODYNAMIC THERAPY, PHOTOSENSITIZERS, LITERATURE REVIEW.

INTRODUCTION

Oral cancer is ranked sixth among all cancers and is considered a wide scale global health crisis distributed among diverse geographical areas (highest recorded in the South-East Asia) (Petti, Masood, & Scully, 2013). Among all the oral cancers, oral squamous cell carcinoma (OSCC) cover almost 90% of all oral malignant lesions (Johnson, Jayasekara, & Amarasinghe, 2011). Oral cancers are about twice as common in men as in women and are slightly more common in blacks than in whites (Kachuri, De, Ellison, & Semenciw, 2013). Worldwide, OSCC is a major health-care problem, and is the most frequently diagnosed cancer in some countries (Parkin, Bray, Ferlay, & Pisani, 2005). Great improvements in surgical techniques, radiotherapy, and chemotherapy have been achieved (Cooper et al., 2004), but the 5-year survival rate for OSCC is still between 40% and 60% and has not greatly improved over the last 30 years (Fonseca, 2017 Siegel et al 2019, Hung et al., 2020).

Oral squamous cell carcinoma is a malignant tumour that commonly invades the jawbone. Treatment often requires a surgical resection that compromises the patient's quality of life, function, and aesthetic. Intraorally, it occurs most commonly in the tongue (20-30%), floor of the mouth (15-20%), retro-molar and tonsillar pillar areas (15%), soft palate (10-15%), buccal mucosa (10%), gingiva (10%), alveolar bone (10%), and maxillary sinus

ARTICLE INFORMATION

*Corresponding Author: mohamkindi10@gmail.com
Received 9th Jan 2020 Accepted after revision 29th Feb 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)
A Society of Science and Nature Publication, Bhopal India

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/33

(5-10%) (Marx & Stern, 2012). About 40% of head and neck SCC mortality is due to locoregional recurrence, and about 30% develop a distant metastasis in the 5-year period following the diagnosis (de Bree, Deurloo, Snow, & Leemans, 2000).A comprehensive number of factors with probable impact on the outcome of the diseases are well-established. These include patient related factors that involve sex predilection, age, use of tobacco and alcohol, Sociodemographic conditions, diagnostic delays, organic stress and other miscellaneous factors (Massano, Regateiro, Januário, & Ferreira, 2006). Early detection and rapid treatment modality of OSCC are essential for high survival rate (Hassona, Scully, Shahin, Maayta, & Sawair, 2016 Li et al 2018, Siegel et al., 2019. Hung et al., 2020).

There is a wide range of therapeutic modalities for OSCC. The most common includes surgical excision of the lesion. Other pharmacological treatment modalities include topical application of drugs such as vitamin A, steroids, herbal medicines, aloe vera, curcumin and turmeric which requires a steady 3-5 months of healing and recovery (Singh et al., 2016; Triesscheijn, Baas, Schellens, & Stewart, 2006). In addition, there are several systemic drugs that are useful for the regression of tumor mass (Karemore & Motwani, 2012). Moreover, certain other therapeutic modalities include radiotherapy which possesses several unwanted side effects such as oral mucositis, neuro-sensory disturbances, infections and fibrosis that could compromise the patient's quality of life (Sroussi et al., 2017, Hung et al., 2020).

Nevertheless, surgery and radiation are widely used because these modalities work effectively. Photodynamic or photodynamic therapy (PDT) has gained a major popularity in the field of oral health. Such type of treatment utilizes photo/light therapy that activates a photosensitizer dye in the presence of oxygen. Several types of photosensitizers have been used for photobiomodulation depending on their mode of action (Castano, Demidova, & Hamblin, 2004). These include intravenous injections, orally ingested or topical application. The introduction of light on the photosensitizer at tumor site creates an array of destructive oxygen species including singlet oxygen and damaging free radicals producing localized cell death (Allison & Moghissi, 2013; Allison & Sibata, 2010). The illustration in Figure 1 shows the use of an injected photosensitizer in combination with a photodynamic light to treat a facial tumor.

Such modality has been widely used in a set of oral diseases including periodontal diseases, lichen planus, fungal infections, red and white leukoplakia (Akram et al., 2016; Akram et al., 2018; Baltazar et al., 2015; Li, Wang, Zheng, & He, 2018). Ample data confirms that PDT has been used to treat more robust types of cancers of head and neck origin including OSCC (Grant, Hopper, Speight, Macrobert, & Bown, 1993; Alexander Kübler, Haase, Rheinwald, Barth, & Mühling, 1998). However, it is very important to understand that OSCC lesions are of various types including primary or recurrent, invasive or non-invasive. This does put a great impact on the choice of therapy. On the other hand, research indicates that there are several number of recurrence rates found with the use of PDT (Schweitzer, 2001; Schweitzer & Somers, 2010). However, to our familiarity from the published data, no systematic review has been published that evaluated the therapeutic efficacy of PDT in the treatment of OSCC lesions in adult patients. Considering the contrary results and novel idea, the aim of the present study was to assess how the application of PDT is clinically efficacious in the rapid treatment of OSCC.

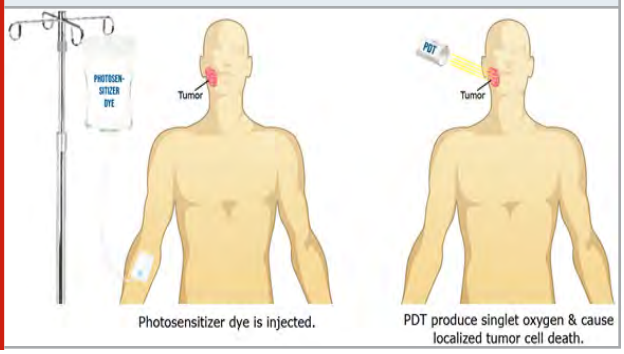
MATERIAL AND METHODS

Focused question: This systematic review was designed in accordance with the general guidelines set by Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (Moher, Liberati, Tetzlaff, & Altman, 2009). The focused question designed for the present study comprised of: "Does PDT offer effective treatment in the regression of OSCC lesions?"

Data search and eligibility criteria: Three ISI-indexed databases were searched (PubMed, EMBASE and CENTRAL) from May 1965 up to and including September 2019 for pertinent literature. Articles were selected if they had prospective design, published in English language and reported efficacy of PDT in the treatment of OSCC lesions in adult patients. Initial screening and evaluation of pertinent studies was performed and those studies not compliant with the selection criteria were omitted. The exclusion criteria involved review studies, case reports/series, in-vitro settings, animal studies and letters to the editor. Original articles were manually sought in journals including Lasers Med Sci, Photobiomodul Photomed Laser Surg, Photochem Photobiol Sci, and Photodiagnosis Photodyn Ther to recognize articles that may had missed from electronic database search.

Data search and abstraction: The combination of following key words were used to search for included literature: 'Photodynamic therapy', 'photochemotherapy', 'oral cancer', 'oral squamous cell carcinoma', 'invasive', 'non-invasive', 'primary tumors', 'recurrent tumors', 'malignancy', 'therapy', 'treatment'. Once the relevant literature search was accomplished, the articles were then subjected for data extraction. Important evidence

Figure 1. Photodynamic Therapy



from all the articles were extracted that included study design, subject demographics, cancer site, follow-up duration, final outcome, recurrence rate, laser and PDT related parameters.

Quality assessment: The appropriate method to evaluate quality in non-randomized controlled trials (NRCTs) is controversial. For the purpose of this review, we decided to use a modified scale method that allowed us to rank selected reports according to a previously established score system. The Methodological Index for Nonrandomized Studies (MINORS) is an instrument that was developed by a group of practicing surgeons in France and validated specifically for NRCT evaluation (Slim et al., 2003). Some modifications were introduced to the MINORS to meet the needs of our study.

Data analysis: Meta-analyses could not be performed due to high rate of heterogeneity in the study design methods, lasers used, and cancer sites in the oral cavity.

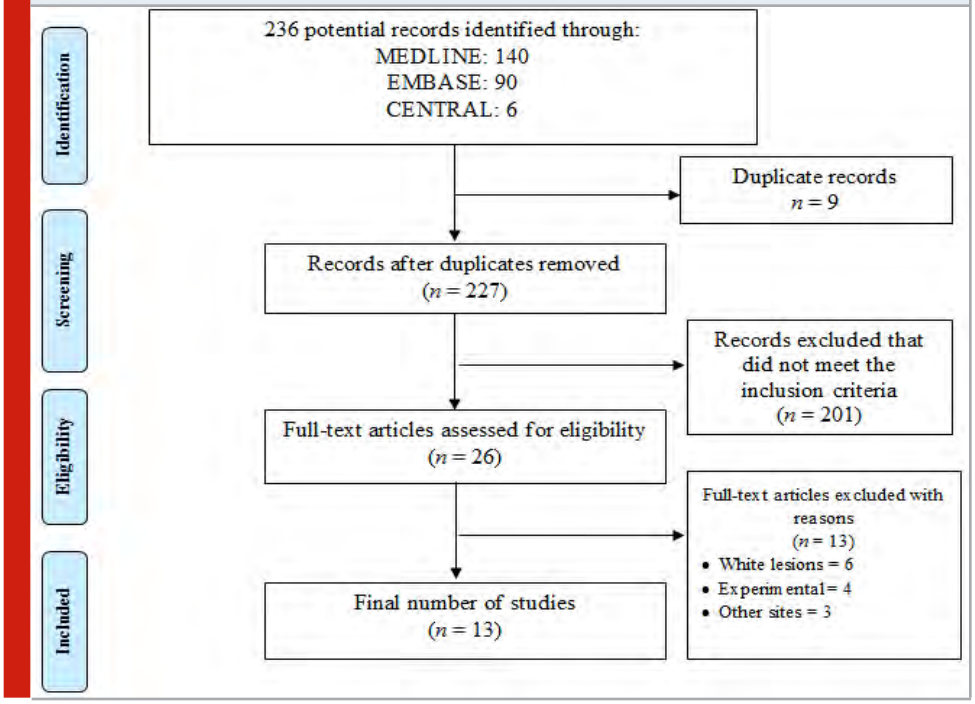
RESULTS AND DISCUSSION

Search result: Initial screening of the titles and abstracts gave a total of 236 potential articles. Removal of the duplicates (n=9) and articles that did not comply with the focused question (n=201) were later excluded from the study search. Out of twenty-six potential articles that underwent full-text reading, thirteen articles were further removed. After the final selection, thirteen studies were included and processed for data extraction (Fan et al., 1996; Fan, Hopper, Speight, Buonaccorsi, & Bown, 1997; Feyh, 1996; Grant et al., 1993; Hopper et al., 2004; AC Kübler, De Carpentier, Hopper, Leonard, & Putnam, 2001; Alexander Kübler et al., 1998; N. Rigual et al., 2013; N. R. Rigual et al., 2009; Schuller, McCaughan, & Rock, 1985; Schweitzer, 2001; Schweitzer & Somers, 2010; Toratani et al., 2016). These studies were performed either in health care setups or universities. The complete flow of study selection is illustrated in Figure 2 according to PRISMA standard.

Description of included studies: All clinical studies were prospective longitudinal trials. Five studies were performed in United States and United Kingdom, two studies were performed in Germany, while one study was performed in Japan. A total of 447 patients with OSCCs were included for the treatment of PDT. Their mean age ranging between 60.8 years to 69.6 years. Cancer sites included in the clinical trials comprised of tongue, floor of the mouth, alveolus, gingiva, buccal mucosa, lips, larynx, neck, oropharynx and palate. The follow-up period of the clinical trials ranged from 3 months to 144 months. Four studies reported about recurrence of the OSCC to be 0% to 20% only (Table 1).

Photodynamic related parameters: A total of nine studies used argon pumped dye laser, while two studies used diode laser. One study each used excimer dye laser and gold vapour laser, respectively. The wavelength ranged from 628 to 665 nanometres (nm). Energy fluence and power density were reported in ten and eight studies, that ranged from 20–150 joules per centimetre square (Jcm-2) and 100 to 250 milliwatts per centimetre square (mW cm-2), respectively. Only two studies reported the power output. Duration of irradiation was reported in four studies that ranged from 1.8 to 143 minutes (min). Optic fibre diameter was mentioned in only three studies that ranged from 400 to 600 micrometre (µm). Five studies used photofrin, two studies used hematoporphyrin

Figure 2: PRISMA flow chart representing study search.



derivative (HPD), 5-aminolevulinic acid (ALA) and Foscan as photosensitizer (PS), respectively. One study used metatetrahydroxyphenylchlorin (mTHPC) and 3-(1'-hexyloxyethyl) pyropheophorbide a (HPPH) as PS, respectively. Pre-irradiation time of PS ranged from 48 to 240 hours in the clinical studies. Dose of the PS ranged from 0.15 mg/kg to 60 mg/kg. None of the studies reported the number of laser sessions except one study that reported only once (Table 2).

Quality Assessment: In general, they suffer from methodologic drawbacks, mainly difficulties in concealing the allocation of patients and the inherent complexity of blinding between PDT and surgical cases. Older clinical trials also are limited by the small number of patients included (Table 1).

Main outcome of the studies: All PDT studies showed statistically significant improvement in the complete regression of OSCCs on follow-up. Several clinical trials categorized their outcomes as complete, partial or no response to therapy. For PDT, the complete response ranged from 16% to 100% in the OSCCs. To achieve high survival rate with sound quality of life, minimally invasive intervention is preferred over radical surgical therapy. This goes for all the type of head and neck cancers including OSCC lesions (Maxwell et al., 2014). Although surgical therapy and radiotherapy are suitable therapeutic modalities, they often compromise the significant functional roles of the oral environment. PDT is a noninvasive method that maintains speech and deglutition. The laser light application to stimulate the PS does not interrupt the sound adjacent tissue structures. Most importantly, PDT does not disrupt the underlying fibrotic structures including collagen and elastin fibres; therefore the level of scarring is reduced (Hopper et al., 2004).

Repetitive surgical therapy is mainly problematic due to limitations in the access and advanced deterioration in the tissue structures. Moreover repeating radiotherapy is generally unfeasible due to a maximum permitted dose to the areas of the head and neck (Dilkes, Benjamin, Ovaisi, & Banerjee, 2003; Hopper et al., 2004). In addition,

Table 1. General characteristics of the studies.

Author et al. Year	Country/ Patients	Sample size	Male/ Female ratio	Mean age (age range)	Cancer site	Follow-up (mos)	Recurrence (%)	Main conclusion	Quality of studies
Schuller et al.[22] 1985	United States	24	14/10	67 (NA)	FM, T, L, F, N, TO, LA, PH	NR	NA	PDT is feasible for oral cancer with well-tolerable and low toxicity.	Low
Fyeh [23] 1996	Germany	83	NA	NA	PH, F, LA	50	NA	PDT is an adequate treatment for early stage superficial cancers	Low
Grant et al.[17] 1993	United Kingdom	11	NA	NA	T, BM, A, L, P	19	0	PDT offers an effective repeatable treatment option, whether on its own or as adjunct to local excision	Moderate
Kübler et al.[18] 1998	Germany	12	11/1	NA	FM, BM	16	NA	PDT offers an effective repeatable treatment option without causing harm	Moderate
Fan et al.[24] 1996	United Kingdom	18	11/7	62.6 (NA)	BM, FM, T, A	48	NA	PDT is an adequate treatment for superficial cancers	Moderate
Hopper et al.[25] 2004	United Kingdom	114	NA	64 (30-99)	BM, T, FM, P, L, PH	24	NA	PDT offers an effective alternative treatment for early oral squamous cell carcinoma	Moderate
Schweitzer[19] 2001	United States	20	NA	NA	OC, PH, LA	6-115	20	PDT offers a curative. treatment of early stage oral cavity and laryngeal	High

Fan et al.[26] 1997	United Kingdom	20	16/4	60.8 (30-82)	BM, T, FM, P	15	NA	malignancies with minimal side effects PDT is a promising new treatment for patients with oral cancer	Low
Rigual et al.[28] 2009	United States	20	14/6	61.2 (NA)	OC, L	53	NA	PDT is an adequate treatment for oral and laryngeal cancers	Moderate
Rigual et al.[29] 2013	United States	40	28/13	65 (39-88)	P, BM, T, FM,	3	NA	PDT is safe for the treatment of early stage cancer of the oral cavity.	High
Toratani et al.[27] 2016	Japan	30	12/22	NA BM, FM,	A, T, G	6	NA	PDT is an effective treatment modality for superficial oral carcinomas, with excellent healing and minimal side effects	Low
Schweitzer and Somers[20] 2010	30 United States	15/15	NA (35-82)	OC, L, OP	3-144	20		PDT provides a surgical oncologic modality for potentially curative treatment of early stage oral cavity and oropharyngeal smalignancie	Moderate
Kübler et al.[30] 2001	United Kingdom	25	19/6	69.6 (NA)	L	3	8	PDT is an effective treatment. modality for small primary tumours of the lips	Low

A, alveolus; BM, buccal mucosa; FM, floor of mouth; G, gingiva; LA, larynx; L, lips; N, neck; OC, oral cavity; P, palate; PH, Pharynx, OP; oropharynx; T, tongue; PDT – photodynamic therapy, NA – not available

surgical intervention at an already radiation induced conveys a major possibility of higher disease rates secondary to slow healing of the wound and formation of fistula and warrants an increased doses that may cause disturbances in the angiogenic component of the cancer cells, making them low radiosensitive (Hopper et al., 2004). The additional benefit of PDT is in the procedure being a simple and PDT has the benefit of being an outpatient method. This suggests that PDT is completed within a short time, also entails a short healing time, and involving a small cost (AC Kübler et al., 2001). These features till date, characterise key factors when opting between surgical therapy and radiation therapy for other HNCs. However, the main limitation of PDT is the complexity of use with regards to its direction of phototherapy on the exposure area, that suggests its fundamental use in treating shallow and easy to reach and manageable cancers. Momentary photosensitization has highly deterring problems, although novel PS are curbing the duration of action of PDT (Fan et al., 1997; Grant et al., 1993; Alexander Kübler et al., 1998).

It should be noted from the included clinical studies that laser parameters were either missing or had meaningful differences. Characteristics related to PDT including wavelength, energy fluence, and power density either had a large variation or data not reported. It is well-known that multiple number of laser sessions has a significant effect on the clinical efficacy of phototherapy (Wang et al., 2001). In the reported studies, the number of sittings were not mentioned. It is evident that by applying a single application of PDT to sustain anti-proliferative effect of cancer, it is assumable that one laser session is equally effective. Moreover, diameter of fibre produces an effect on total power density and output that may alter the genuine energy released during the process, thereby affecting the anticancer efficacy. None of the studies described the power output of the laser used. These missing parameters of laser protocols may put some effect on the therapeutic efficacy of PDT on cancer treatment. However, since most included studies were incomplete, in terms of basic items such as drug and light dose, number of treatment sessions, recurrence rate, this proves the poor quality of the studies making a valid conclusion impossible. Therefore, future research with consistent laser dimensions are needed to interpret the efficacy of PDT in treating OSCC.

Our systematic literature review does have some limitations. Firstly, no meta-analyses could be performed to interpret the overall odds ratios across different studies. Studies being performed in different countries suggest the inclusion of different ethnic group patients whose level of severity and hence outcomes are critically affected which may have produced potential bias with regards to a high degree of selectivity. Presently, photobiomodulation for treating OSCC is only being carried out in only limited health care centres globally. Moreover, several studies

Table 2: Laser and photosensitizer related parameters of the studies.

Investigators	Type of laser	Wave length (nm)	Energy fluence (J cm^{-2})	Power output (W)	Power density (mW cm^{-2})	Duration of irra. (min)	Optic fibre diameter (µm)	Types of PS	Pre-irra. time (hours)	Dose of PS (mg/kg)	Number of laser sessions
Schuller et al.[22]	Argon pumped dye	630	NA	3	NA	NA	NA	HPD	72	3-5	NA
Fyeh[23]	Argon pumped dye	630	NA	NA	100	NA	600	HPD	48	NA	NA
Grant et al.[17]	Argon pumped dye	630	50-100	NA	150	NA	NA	Photofrin	48	2.0	NA
Kübler et al.[18]	Argon pumped dye	630	100	NA	100	16.6	NA	ALA	120	NA	NA
Fan et al.[24]	Gold vapour laser	628	NA	NA	250	143	400	ALA	150-240	60	NA
Hopper et al.[25]	LED	652	20	NA	100	3.33	NA	mTHPC	96	0.15	NA
Schweitzer[19]	Argon pumped dye	630	50-100	NA	100-500	NA	NA	Photofrin	48-60	2.0	NA
Fan et al.[26]	Argon pumped dye	652	5-20	NA	250	1.8-8.0	400	Foscan	72-96	0.15	NA
Rigual et al.[28]	Argon pumped dye	630	75	NA	NA	NA	NA	Photofrin	48	2.0	NA
Rigual et al.[29]	Argon pumped dye	665	50-140	NA	NA	NA	NA	HPPH	NA	4.0	1
Toratani et al.[27]	Excimer dye laser	630	100-150	0.16	NA	NA	NA	Photofrin	48	2.0	NA
Schweitzer and Somers[20]	Diode laser	630	50-100	NA	NA	NA	NA	Photofrin	48-60	2.0	NA
Kübler et al.[30]	Argon pumped dye	652	20	NA	100	NA	NA	Foscan	96	0.15	NA

ALA - 5-aminolevulinic acid; HPD – hematoporphyrin derivative; HPPH - 3-(1'-hexyloxyethyl) pyropheophorbide a; mTHPC - metatetrahydroxyphenylchlorin; LED – light emitting diode; nm – nanometer; J cm-2 – joules per centimetre square ; mW – milliwatts; mW cm-2 – milliwatts per centimetre square; mm – millimetre; PTC – Phenothiazine chloride; PS – photosensitizer; mg/mL – milligram per millilitre; NA – not available

on PDT included a limited number of study cohorts. For instance, the total number of studies that were included consisted only 447 patients with OSCC treated with PDT. Furthermore, to achieve a high survival rate with quality of life requires complete elimination or at least control of tumor. It was observed that PDT studies did not demonstrate these findings. Moreover, plenty of scarring occurred in several trials in which functional loss was also noted. All these measures do have an impact on the overall quality of life. With future studies with long period of follow-up, PDT could be reflected as a valid and acceptable adjunctive therapeutic modality in the future. It is well tolerable by patients, that could additionally serve as a substitute therapy for patients with medical problems who may not be able to bear the unwanted complications of radiation therapy or those patients who may be too hampered to undergo surgery. It is indicated that PDT is associated with lower morbidity rates and less side effects.

CONCLUSION

PDT shows to be a clinically efficient therapeutic modality for OSCCs. PDT is equally effective as surgery with regards to rates of recurrence. However, extreme caution should be made while interpreting the findings of this study as number of parameters including laser parameters, type of patients and number of treatment sessions may affect the overall outcome of PDT in the treatment of OSCC.

Competing interests: None declared.

REFERENCES

Akram, Z., Al-Shareef, S. A. A., Daood, U., Asiri, F. Y., Shah, A. H., AlQahtani, M. A., . . . Javed, F. (2016) Bactericidal efficacy of photodynamic therapy against periodontal pathogens in periodontal disease: a systematic review. Photomedicine and Laser Surgery Vol. 34 No 4: Pages 137-149.

Akram, Z., Javed, F., Hosein, M., Al-Qahtani, M. A., Alshehri, F., Alzahrani, A. I., & Vohra, F. (2018) Photodynamic therapy in the treatment of symptomatic oral lichen planus: A systematic review. Photodermatology, Photoimmunology & Photomedicine Vol. 34 No 3: Pages 167-174.

Allison, R. R., & Moghissi, K. (2013) Photodynamic therapy (PDT): PDT mechanisms. Clinical Endoscopy Vol. 46 No 1: Pages 24-29.

Allison, R. R., & Sibata, C. H. (2010) Oncologic photodynamic therapy photosensitizers: a clinical review. Photodiagnosis & Photodynamic Therapy Vol. 7 No 2: Pages 61-75.

Baltazar, L. M., Ray, A., Santos, D. A., Cisalpino, P. S., Friedman, A. J., & Nosanchuk, J. D. (2015) Antimicrobial photodynamic therapy: an effective alternative approach to control fungal infections. Frontiers in microbiology Vol. 6: Pages 202.

Castano, A. P., Demidova, T. N., & Hamblin, M. R. (2004) Mechanisms in photodynamic therapy: part one-photosensitizers, photochemistry and cellular localization. Photodiagnosis & Photodynamic Therapy Vol. 1 No 4: Pages 279-293.

Cooper, J. S., Pajak, T. F., Forastiere, A. A., Jacobs, J., Campbell, B. H., Saxman, S. B., . . . Rotman, M. (2004) Postoperative concurrent radiotherapy and chemotherapy for high-risk squamous-cell carcinoma of the head and neck. New England Journal of Medicine Vol. 350 No 19: Pages 1937-1944.

de Bree, R., Deurloo, E. E., Snow, G. B., & Leemans, C. R. (2000) Screening for distant metastases in patients with head and neck cancer. The Laryngoscope Vol. 110 No 3: Pages 397-401.

Dilkes, M., Benjamin, E., Ovaisi, S., & Banerjee, A. (2003) Treatment of primary mucosal head and neck squamous cell carcinoma using photodynamic therapy: results after 25 treated cases. The Journal of Laryngology & Otology Vol. 117 No 9: Pages 713-717.

Fan, K. F., Hopper, C., Speight, P. M., Buonaccorsi, G., MacRobert, A. J., & Bown, S. G. (1996) Photodynamic therapy using 5-aminolevulinic acid for premalignant and malignant lesions of the oral cavity. Cancer: Interdisciplinary International Journal of the American Cancer Society Vol. 78 No 7: Pages 1374-1383.

Fan, K. F., Hopper, C., Speight, P. M., Buonaccorsi, G. A., & Bown, S. G. (1997) Photodynamic therapy using mTHPC for malignant disease in the oral cavity. International Journal of Cancer Vol. 73 No 1: Pages 25-32.

Feyh, J. (1996) Photodynamic treatment for cancers of the head and neck. Journal of Photochemistry and Photobiology B: Biology Vol. 36 No 2: Pages 175-177.

Fonseca, R. J. (2017) Oral and Maxillofacial Surgery-E-Book: 3-Volume Set: Elsevier Health Sciences.

Grant, W., Hopper, C., Speight, P., Macrobert, A., & Bown, S. (1993) Photodynamic therapy of malignant and premalignant lesions in patients with'field cancerization 'of the oral cavity. The Journal of Laryngology & Otology Vol. 107 No 12: Pages 1140-1145.

Hassona, Y., Scully, C., Shahin, A., Maayta, W., & Sawair, F. (2016) Factors influencing early detection of oral cancer by primary health-care professionals. Journal of Cancer Education Vol. 31 No 2: Pages 285-291.

Hopper, C., Kübler, A., Lewis, H., Tan, I. B., Putnam, G., & Group, F. S. (2004) mTHPC-mediated photodynamic therapy for early oral squamous cell carcinoma. International Journal of Cancer Vol. 111 No 1: Pages 138-146.

Hung, Li Chen , Pei-Tseng Kung, Chi-Hsuan Lung, Ming-Hsui Tsai, Shih-An Liu, Li-Ting Chiu (2020) Assessment of the Risk of Oral Cancer Incidence in A High-Risk Population and Establishment of A Predictive Model for Oral Cancer Incidence Using A Population-Based Cohort in Taiwan Int. J. Environ. Res. Public Health 17(2), 665; https://doi.org/10.3390/ijerph17020665

Johnson, N. W., Jayasekara, P., & Amarasinghe, A. H. K. (2011) Squamous cell carcinoma and precursor lesions of the oral cavity: epidemiology and aetiology. Periodontology 2000 Vol. 57 No 1: Pages 19-37.

Kachuri, L., De, P., Ellison, L., & Semenciw, R. (2013) Cancer incidence, mortality and survival trends in Canada, 1970-2007. Chronic Diseases and Injuries in Canada Vol. 33 No 2: Pages 69-80.

Karemore, T. V., & Motwani, M. (2012) Evaluation of the effect of newer antioxidant lycopene in the treatment of oral submucous fibrosis. Indian Journal of Dental Research Vol. 23 No 4: Pages 524.

Kübler, A., De Carpentier, J., Hopper, C., Leonard, A., & Putnam, G. (2001) Treatment of squamous cell carcinoma of the lip using Foscan-mediated photodynamic therapy. International Journal of Oral and Maxillofacial Surgery Vol. 30 No 6: Pages 504-509.

Kübler, A., Haase, T., Rheinwald, M., Barth, T., & Mühling, J. (1998) Treatment of oral leukoplakia by topical application of 5-aminolevulinic acid. International Journal of Oral and Maxillofacial Surgery Vol. 27 No 6: Pages 466-469.

Li, Y., Wang, B., Zheng, S., & He, Y. (2018) Photodynamic therapy in the treatment of oral leukoplakia: a systematic review. Photodiagnosis & Photodynamic Therapy Vol. 25: Pages 17-22.

Marx, R. E., & Stern, D. (2012) Oral and maxillofacial pathology: a rationale for diagnosis and treatment: Hanover Park, IL: Quintessence Pub. Co.

Massano, J., Regateiro, F. S., Januário, G., & Ferreira, A. (2006) Oral squamous cell carcinoma: review of prognostic and predictive factors. Oral surgery, oral medicine, oral pathology, oral radiology, and endodontology, Vol. 102 No 1: Pages 67-76.

Maxwell, J. H., Mehta, V., Wang, H., Cunningham, D., Duvvuri, U., Kim, S., . . . Ferris, R. L. (2014) Quality of life in head and neck cancer patients: impact of HPV and primary treatment modality. The Laryngoscope Vol. 124 No 7: Pages 1592-1597.

Moher, D., Liberati, A., Tetzlaff, J., & Altman, D. G. (2009) Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. Annals of Internal Medicine Vol. 151 No 4: Pages 264-269.

Parkin, D. M., Bray, F., Ferlay, J., & Pisani, P. (2005) Global cancer statistics, 2002. CA: A Cancer Journal for Clinicians Vol. 55 No 2: Pages 74-108.

Petti, S., Masood, M., & Scully, C. (2013) The magnitude of tobacco smoking-betel quid chewing-alcohol drinking interaction effect on oral cancer in South-East Asia. A meta-analysis of observational studies. PloS One Vol. 8 No 11: Pages e78999.

Rigual, N., Shafirstein, G., Cooper, M. T., Baumann, H., Bellnier, D. A., Sunar, U., . . . Tan, W. (2013) Photodynamic therapy with 3-(1′-hexyloxyethyl) pyropheophorbide a for cancer of the oral cavity. Clinical Cancer Research Vol. 19 No 23: Pages 6605-6613.

Rigual, N. R., Thankappan, K., Cooper, M., Sullivan, M. A., Dougherty, T., Popat, S. R., . . . Henderson, B. (2009) Photodynamic therapy for head and neck dysplasia and cancer. Archives of otolaryngology–head & neck surgery Vol. 135 No 8: Pages 784-788.

Schuller, D. E., McCaughan, J. S., & Rock, R. P. (1985) Photodynamic therapy in head and neck cancer. Archives of Otolaryngology Vol. 111 No 6: Pages 351-355.

Schweitzer, V. G. (2001) PHOTOFRIN-mediated photodynamic therapy for treatment of early stage oral cavity and laryngeal malignancies. Lasers in Surgery and Medicine Vol. 29 No 4: Pages 305-313.

Schweitzer, V. G., & Somers, M. L. (2010) PHOTOFRIN-mediated photodynamic therapy for treatment of early stage (Tis-T2N0M0) SqCCa of oral cavity and oropharynx. Lasers in Surgery and Medicine Vol. 42 No 1: Pages 1-8.

Siegel, Rebecca L., Kimberly D. Miller Ahmedin Jemal (2019) CA: A Cancer Journal for Clinicians Volume 69, Issue 1 https://doi.org/10.3322/caac.21551

Singh, N., Hebbale, M., Mhapuskar, A., Ul, S. N., Thopte, S., & Singh, S. (2016) Effectiveness of Aloe Vera and Antioxidant along with Physiotherapy in the Management of Oral Submucous Fibrosis. The Journal of Contemporary Dental Practice Vol. 17 No 1: Pages 78-84.

Slim, K., Nini, E., Forestier, D., Kwiatkowski, F., Panis, Y., & Chipponi, J. (2003) Methodological index for non-randomized studies (MINORS): development and validation of a new instrument. ANZ Journal of Surgery Vol. 73 No 9: Pages 712-716.

Sroussi, H. Y., Epstein, J. B., Bensadoun, R. J., Saunders, D. P., Lalla, R. V., Migliorati, C. A., . . . Zumsteg, Z. S. (2017) Common oral complications of head and neck cancer radiation therapy: mucositis, infections, saliva change, fibrosis, sensory dysfunctions, dental caries, periodontal disease, and osteoradionecrosis. Cancer Medicine Vol. 6 No 12: Pages 2918-2931.

Toratani, S., Tani, R., Kanda, T., Koizumi, K., Yoshioka, Y., & Okamoto, T. (2016) Photodynamic therapy using Photofrin and excimer dye laser treatment for superficial oral squamous cell carcinomas with long-term follow up. Photodiagnosis Photodynamic Therapy, Vol. 14: Pages 104-110.

Triesscheijn, M., Baas, P., Schellens, J. H., & Stewart, F. A. (2006) Photodynamic therapy in oncology. The Oncologist Vol. 11 No 9: Pages 1034-1044.

Wang, I., Bendsoe, N., Klinteberg, C. A. f., Enejder, A., Andersson-Engels, S., Svanberg, S., & Svanberg, K. (2001) Photodynamic therapy vs. cryosurgery of basal cell carcinomas: results of a phase III clinical trial. British Journal of Dermatology Vol. 144 No 4: Pages 832-840.

Biotechnological
Communication

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp-195-200

Supervised Feature Reduction Technique for Biometric Recognition Using Palm print Modalities

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ABSTRACT

Technological resource assessments security is a major concerned and Biometric is one of the most robust identification techniques. The common approach for the biometric identification process is to compare the extracted feature vectors of query imposter with feature vectors of rest imposters. In biometric recognition, the datasets have very large number of imposters and this imposes the condition on the identification process. To make the identification process fast, dimensionality reduction is required at either dataset or in feature vectors. This paper proposes the palmprint identification algorithm with dimensionality reduction at datasets as it reduces feature vector size too. One Dimensional Principle component analysis (1DPCA) cannot correlate the neighbor pixels and transformation from 2-dimension-to-1-dimension increases the computation cost. Therefore, two Dimensional PCA (2DPCA) is employed to process the dataset fast in comparison with 1DPCA. For classification, Supervised learning-based classifier provides higher accuracy and hence Support Vector Machine (SVM) classifier is used for recognition. The success of the classifier depends on the extracted features to be matched. The proposed algorithm uses Histogram of Gradient (HOG) features which is the best combination with SVM. Accuracy of the proposed algorithm is compared with the accuracy of other models. The experiment results and comparative analysis on PolyU datasets reveal that the proposed algorithm achieves 96.36% accuracy which is best amongst all.

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KEY WORDS: 2D-PRINICIPAL COMPONENT ANALYSIS, HISTOGRAM OF GRADIENT, PALMPRINT RECOGNITION.

ARTICLE INFORMATION

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Received 11th Feb 2020 Accepted after revision 25th March 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)
A Society of Science and Nature Publication, Bhopal India

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/34

INTRODUCTION

Biometrics is now a day's widely used for person identification and authentication. It is a pattern recognition process based on his/ her physiological or behavioral characteristics (Zhang, 2013). The unique physiological characteristics like iris, face, fingerprint, hand geometry and voice are known as Biometrics, various researchers have explained using these biometric modalities for identification (Chen et al, 2013; Ahmad et al, 2016). Both spatial and spectral features are used for finger knuckle recognition (Kumar, 2019). Abdulbaqi et al detected fingerprint edges using active contour model of Euclidean distance transformation (Abdulbaqi et al, 2018). Fusion of face and iris at feature level and score level gives accuracy of 99.22% and 100% respectively(Alay et al, 2019). Nguyen et al explained convolutional neural network extractor of fingerprint minutiae (Nguyen et al, 2020).

The major application areas of the biometrics are ATMs, smart card, personal computers, network access, keyless entry for automobiles etc. Even though many state-of-the-art security systems are developed, the cyber-attack has exposed weaknesses of the security systems. Out of these, palm based identification have been intensively developed because of its crucial advantage over other features. The classical feature of palm region ridge ending and ridge bifurcation (minutia) are used for palmprint matching. The palm region can be easily recognized in low resolution images also. High resolution image also contains ridges and wrinkles which can be utilized as classification and matching features. The palmprint matching process consists of four steps. First is to crop region of interests. Features are extracted in the second step. The extracted features are reduced in third step and the last step is classification for individuals' identification.

The algorithms used for palmprint feature extraction are classified as: 1) structure-based approaches, 2) Statistics-based approaches, 3) Subspace-based approaches and 4) texture & transform domain frequency field feature based methods. Structure features includes lines and feature points which are sensitive to the captured resolution of palmprint and hence difficult to capture it. Frequency field features avoid the texture information causing instability in capturing of palm features. Sub-space methods include Eigen space based component analysis like PCA, ICA and LDA. Hai-feng Sang and Fang Liu (Sang et al, 2016) applied 2DPCA method for contactless defocused palmprint images over the database of 50 subjects. Euclidean distance based classifier was used for matching. However, success rate for defocused image was lower than that of clear image. To preserve the 2D information of images over 2DPCA subspace, 2D- locality preserving projections (2DLPP) is used on reduced features (Xin et al, 2007). 2DPCA is sensitive to illumination changes. Jinyu Guo et al (Guo et al. 2007) proposed a method using phase congruency with 2DPCA to tackle illumination problem. Similarly to overcome the noise in palmprint, local DCT based enhancement was integrated with 2DPCA on the enhanced images (Cui et al, 2010). Individual local mean 2DPCA is used to resolve the light illumination problem in face recognition (Hacherangchai et al, 2019).

For comparing the accuracy results of the distance measurement, PCA is used to store the iris computing process (Sari et al, 2018). They implemented this palmprint recognition algorithm on multimedia chip OMAP3530. To increase the accuracy over the illumination variation, entropy map and 2DPCA was proposed by Jinyu Guo et al (Guo et al, 2013). Arunkumar et al proposed improved histogram of oriented lines (IHoL) descriptor which is less sensitive to translation and illumination. It is also robust against small transformation variations. So the histogram values used in the work remains unchanged (Arunkumar and Valarmathy, 2016). Using PCA with improved HoL gives high recognition rates. Lie and Kim (Lie and Kim, 2016) proposed a method named Local Micro-structure Tetra Pattern (LMTrP) which obtains the advantages of direction and thickness. The superfluous features are removed using line-sharp filter. The given image is represented using single feature vector formed by concatenating local region histograms of the proposed descriptor LMTrP The dimensions are reduced by applying linear kernel based discrimination analysis. This method provides better stability against rotation and translation up to certain extent.

Palmprint features are extracted using principle component analysis and linear discriminant analysis. SVM classifier is used to for recognition. The author presented integration of palmprint features with features of other modalities to increase the accuracy. Instead of calculating histogram in spatial domain, Chaudhari (Chaudhari et al, 2012) transformed the image plane into Radon and histogram of Radon coefficients are used for matching the palmprints. The algorithm is robust to rotation and scaling invariance. Computationally simple algorithm is proposed by (Chaudhari et al,2013) ,where area and periphery of the polygon formed using the outermost coefficients of the Radon. PCA and radon transform used for face recognition (Hiremath et al, 2014). Probabilistic neural network, PCA and Radon transform gives equal error rate of 9.87% in training set of 10 images (Ooi et al, 2016). Using course to fine patchmatch for palm vein recognition approaches the state of the art results with improved time efficiency (Hernandez et al, 2019). Gumaei (Gumaei et al, 2018) proposed hybrid method for feature extraction which uses histogram of gradient and steerable guassian filter. They constructed efficient approach for palmprint recognition. For dimensionality reduction for extracted features is achieved using auto-encoder. The classification is done with regularized extreme learning machine.

Attallah et al (Atallah et al, 2018) used two different Haar wavelet decomposition components for feature extraction of palmprint image. These features are fused to give output. They used histogram of gradient and binarized statistical image features for fusion. The basic requirement for the palm print recognition

algorithm is that features extraction must be robust to the environmental conditions. In 1DPCA, 2D image is transformed and represented as a point in high-dimensional vector. However, the formation from 2D to 1D breaks the correlation between the pixels in spatial domain. In this paper we have used 2DPCA. 2DPCA offers projection in 2D space and hence reduces the conversion time. HOG has inheritable advantages of illumination invariant and extract the local information from strong and lengthy lines. Thus PCA helps to characterize the lines and HOG fulfill the robustness of the extracted features. Therefore this paper proposes integration of 2DPCAand HOG for palm print identification. Later, SVM is used over the HOG transformed features for the classification..

Proposed Algorithm: Figure1 illustrates the proposed palmprint identification system. To train the SVM classifier (i.e. enrolment), A set of features from extracted using 2DPCA and HOG from all users are used. The classifier outputs each person. For the identification process, test palmprint is passed through same feature extraction process and SVM classifier. We used median filter to remove noise, also referred as pre-processing of palmprint. To process recognition step fast, size reduction plays an important role. Multi-spectral (band) images have tendency to be redundant at certain extent when spectral are adjacent to each other. PCA de-correlates these pixel values. Therefore PCA can be employed reducing image dimension and hence the computation cost. However, 1DPCA has weakness that it requires the conversion of 2D image into 1D vector. This process losses the correlation between the neighbor pixels and also increases the computation cost of conversion process. Therefore, 2DPCA which directly calculates the eigen-vectors form image is employed instead of matrix to vector conversion process (i.e. 1DPCA). 2DPCA works in row and column to reduce the dimension. 2DPCA calculation is as follows: Let A is a random matrix of size m x n, which represents m x n image. Considering the linear projection of A on X fields $y = AX$ where X is an n dimensional unit column vector and y is the projected feature on x. Characterization of the A vector using 2DPCA is as follows.

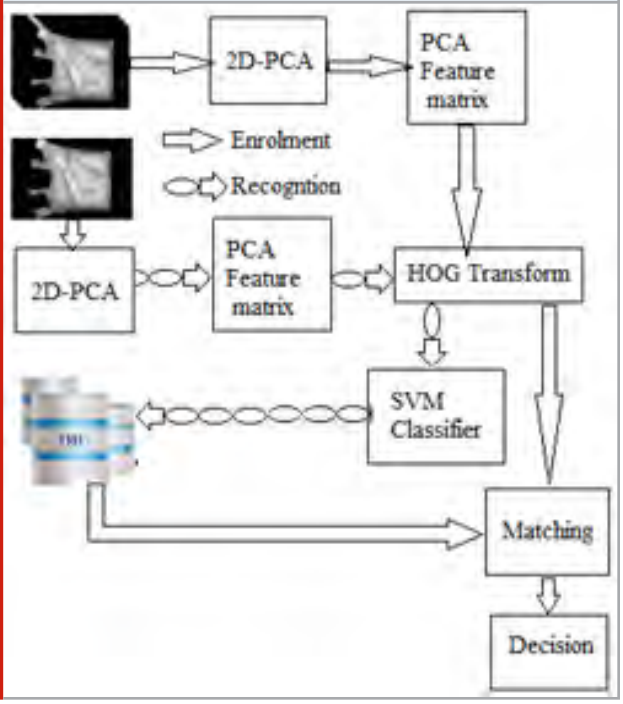
Considering that there are c pattern classes in the space $R^{m\times n}$ and the sample space includes images $\{X_1, X_2.....X_n\}$ where, $X_i \in R^{mxn}$ and each sample belongs to a class j where $j \in \{1,2,....c\}$. The total scatter matrix can be defined as $S_T = (1/N)\sum_{k=1}^{N}(X_K - \bar{X})(X_K - \bar{X})^T$, where N indicates the number of samples and $\bar{X} = (1/N)\sum_{k=1}^{N} X_k$ is the mean of all training samples.

In 2DPCA algorithm, the optical projection vector A_{opt} satisfies with

$$A_{opt} = \arg\max \left| A^T S_T A \right| = [A_1, A_2, ...A_m] \quad (1)$$

Where $\{A_i \mid i = 1,2,....m\}$ are orthogonal eigenvectors of S_T corresponding to the largest m eigen values, respectively.

Figure 1: Proposed Palmprint Matching System



In biometric recognition, the classification plays an important role. All classification algorithms analyses the various image features. These typical image features are characterized and isolated in the training phase and this partition is used to classify biometric in testing phase. Further, priori knowledge based statistical processes are used in supervised classification and clustering approach is used in un-supervised classification. Some of them are Maximum likelihood, K-means clustering, Support vector machine, Neural network, decision tree classifier etc. The choice of classifier depends on the type and size of database and features extraction method. In biometric recognition, the size of data base is too large and hence SVM is the most suitable choice for the large sets of images. In comparison with NN, SVM offers simple geometric interpretation and provides sparse solution reducing mathematical complexity. SVM with linear kernel is used as it provides fast speed. Hence SVM is used in the proposed approach.

The success of the classifier strongly depends on extracted features from the palmprint. Principle lines and wrinkle in the palmprint are the strongest features. During the palmprint acquisition process, the translation and rotation are encountered problems. HOG extracts the orientation features of the gradient (i.e. lines) in spatial domain. Further, extracted features are scale and illuminationinvariant. Therefore HOG features are the best choice for SVM based classifier. The HOG features transform for vector image X can be written as

$$\varphi_\theta(X) = Db * \left[(g_\theta * X)\Theta(g_\theta * X)\right] \quad (2)$$

Where g_θ is oriented edge filter, b is blurring function and D is the sparse selection matrix to achieve histogram. This operation is performed over the bank of edge filters and responses from each filter are concatenated to obtain final feature vector.

$$\varphi_\theta(X) = [\varphi_1(X), \varphi_2(X).....\varphi_\theta(X)] \quad (3)$$

Further equation 2 can be expressed as second order interaction (Bristow et al, 2014) in the form of

$$\varphi_\theta(X) = DBM\left(G_\theta \otimes G_\theta\right)\left(X \otimes X\right) \quad (4)$$

Where M is the selection matrix, B and G are convolutional matrices prototype. In equation 4, the $BM(G_\theta \otimes G_\theta)$ can be viewed as filter bank applied to second order statistics of image data. Thus HOG provides second order covariance and filter bank based prior information. This prior information helps SVM to classify the features successfully. In addition, the second order covariance of image is enough to discriminate one biometric image from other biometric image. Overall, these features are used as input on SVM classifier. Combining with HOG extraction with 2DPCAand SVM training, the process includes following steps: 2DPCA, features extraction using HOG, training and detection.

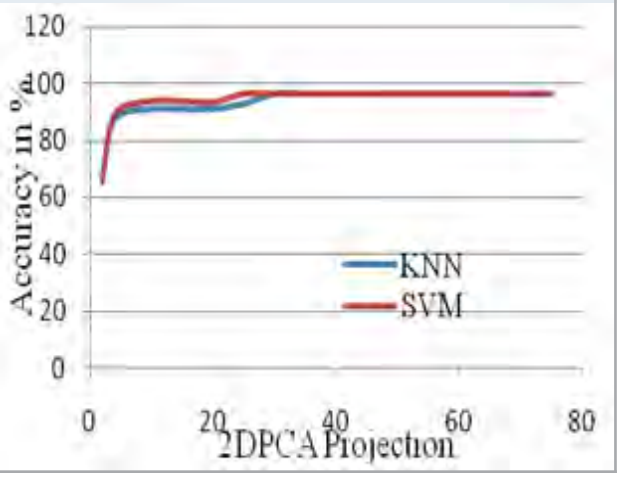
RESULTS AND DISCUSSION

We experimented with Hong Kong polyU palmprint datasets. Total 3800 gray scale palm images of 190 palms are used in the dataset. The resolution of the image is 72 pixels per inch. From total sets, 50% images are used for enrolment and rest are used to test. As pre-processing step, median filtering is applied to remove noise from palmprint. 25 largest Eigenvectors are used in 2DPCAtransformation. The HOG features are also tested with K- Nearest Neighborhood. 2DPCA is applied to 128 x 128 palmprint. The window size of 3x3 is used for feature extraction. HOG features are calculated by assembling the histogram with 9 bins and range of 20 degrees per bin. These feature vectors are normalized for SVM classification. In the experiment, verification process consist of one to one comparison and identification process consist of comparison of one to all is carried out. SVM is binary classifier. The bias value for SVM training model is kept in auto mode to get best bias value. Fig 2 presents the effect of 2DPCA projection for accuracy of identification. Large number of projections provides better accuracy and after 30 projections, the KNN and SVM both achieved same accuracy. Table 1 represents the comparison of obtained accuracy with other model. Inclusion of HOG with 2DPCA in the proposed algorithm provides better accuracy (i.e. 96.36%) in comparison with other 2DPCA or 1D PCA algorithms.

Table 1. Accuracy Comparison of the Proposed Model With Other Models

Method	Accuracy
Proposed 2DPCA + HOG + SVM	96.36
Proposed HOG + K- Nearest Neighbor	92.72
HOG +PCA + SVM (Arunkumar et al, 2010)	95
HOG + PCA (Jia et al, 2014)	95.37
2DHOG + 2DPCA (Jia et al, 2014)	95.68
Pseudo-Zernike Moments+ Naive Bayes Classification (Lakshmi et al, 2010)	79.24
Legendre Moments+ Naive Bayes Classification (Lakshmi et al, 2010)	80.45
Chebyshev Moments+Naive Bayes Classification (Lakshmi et al, 2010)	51.27
Pseudo–Zernike Moments + BBN (Lakshmi et al, 2012)	90.1
Legendre Moments + BBN (Lakshmi et al, 2012)	92.5
Chebyshev Moments + BBN (Lakshmi et al, 2012)	54.9
IHoL + PCA (Arunkumar et al, 2016)	94.2
PCA+LDA+SVM (Vinodkumar, 2016)	83.5

Figure 2: Analysis of 2dpca Projection Over Accuracy



CONCLUSION

This paper proposes the biometric recognition using HOG and SVM. To reduce the dimensionality, 2DPCA is used in comparison with 1DPCA to save time and to preserve the locality of the features. Use of HOG method improves the robustness and increases the feature distinguishing capability.SVM having inherent advantages for large set of databases is used.In addition, The HOG preserves the locality with second order statistic and it is the key

parameter for the success of SVM over KNN.Thus HOG is the perfect the feature extraction method supporting SVM. Experiments results show that the proposed system improves detection accuracy while maintaining a relatively satisfactory speed.

REFERENCES

Abdulbaqi, A.S., Mahdi, R.H. and Mosslah, A.I., (2018) March. Biometrics detection and recognition based-on geometrical features extraction. In 2018 International Conference on Advance of Sustainable Engineering and its Application (ICASEA) (pp. 59-63). IEEE.

Ahmad, M.I., Woo, W.L. and Dlay, S., (2016). Non-stationary feature fusion of face and palmprint multimodal biometrics. Neurocomputing, 177, pp.49-61.

Alay, N. and Al-Baity, H.H., A multimodal biometric system for personal verification based on different level fusion of iris and face traits. An International Peer Reviewed Open Access Journal For Rapid Publication, p.565.

Nguyen, V.H., Liu, J., Nguyen, T.H.B. and Kim, H., (2020). Universal fingerprint minutiae extractor using convolutional neural networks. IET Biometrics.

Arunkumar, M. and Valarmathy, S., (2016). Palm print identification using improved histogram of oriented lines. Circuits and Systems, 7(08), p.1665.

Attallah, B., Serir, A., Chahir, Y. and Boudjelal, A., (2017). Histogram of gradient and binarized statistical image features of wavelet subband-based palmprint features extraction. Journal of Electronic Imaging, 26(6), p.063006.

Bristow, H. and Lucey, S., (2014). Why do linear SVMs trained on HOG features perform so well?. arXiv preprint arXiv:1406.2419.

Chaudhari, J., Patil, P.M. and Kosta, Y.P., (2012). Feature extraction using Histogram of Radon Transform for Palmprint matching. International Journal of Advances in Engineering & Technology, 5(1), p.416.

Chaudhari, J.P., Patil, P.M. and Kosta, Y.P., (2013). Computation Less Radon Based Palmprint Characterization. European Journal of Scientific Research, 115(1), pp.133-140.

Chen, F., Huang, X. and Zhou, J., (2013). Hierarchical minutiae matching for fingerprint and palmprint identification. IEEE Transactions on Image Processing, 22(12), pp.4964-4971.

Cui, Y. and Li, B.N., (2010) September. A Palm-Print Recognition System Based on OMAP3530. In 2010 6th International Conference on Wireless Communications Networking and Mobile Computing (WiCOM) (pp. 1-4). IEEE.

Deepika, C.L., Kandaswamy, A., Vimal, C. and Satish, B., (2010). Palmprint authentication using modified legendre moments. Procedia Computer Science, 2, pp.164-172.

Deepika, C.L., Kandaswamy, A. and Gupta, P., (2012), July. Orthogonal moments for efficient feature extraction from line structure based biometric images. In International Conference on Intelligent Computing (pp. 656-663). Springer, Berlin, Heidelberg.

Gumaei, A., Sammouda, R., Al-Salman, A. and Alsanad, A., (2018). An effective palmprint recognition approach for visible and multispectral sensor images. Sensors, 18(5), p.1575.

Guo, J., Liu, Y. and Yuan, W., (2011), October. Palmprint recognition based on phase congruency and two-dimensional principal component analysis. In 2011 4th International Congress on Image and Signal Processing (Vol. 3, pp. 1527-1530). IEEE.

Guo, J., Liu, Y., Yuan, W. and Long, D., (2013). Palmprint Recognition Using Entropy Map and 2DPCA from a Single Image Per Person. Journal of Computational Information Systems, 9(9), pp.3415-3421.

Hancherngchai, K., Titijaroonroj, T. and Rungrattanaubol, J., (2019), July. An Individual Local Mean-based 2DPCA for Face Recognition under Illumination Effects. In 2019 16th International Joint Conference on Computer Science and Software Engineering (JCSSE) (pp. 213-217). IEEE.

Hernández-García, R., Barrientos, R.J., Rojas, C. and Mora, M., (2019). Individuals Identification Based on Palm Vein Matching under a Parallel Environment. Applied Sciences, 9(14), p.2805.

Hiremath, P.S. and Hiremath, M., (2014). RADON transform and PCA based 3D face recognition using KNN and SVM. Int J Comput Appl, pp.0975-8887.

Hussein, I.S., Sahibuddin, S.B., Nordin, M.J. and Sjarif, N.B.A., (2019). Multimodal palmprint technology: A review. Journal of Theoretical and Applied Information Technology, 97(11), pp.2882-2896.

Inass Shahadha Hussein, Shamsul Bin Hussein, I.S., Sahibuddin, S.B., Nordin, M.J. and Sjarif, N.B.A., (2019). Multimodal palmprint technology: A review. Journal of Theoretical and Applied Information Technology, 97(11), pp.2882-2896.

Jia, W., Hu, R.X., Lei, Y.K., Zhao, Y. and Gui, J., (2013). Histogram of oriented lines for palmprint recognition. IEEE Transactions on systems, man, and cybernetics: systems, 44(3), pp.385-395.

Kumar, A., (2019). Toward Pose Invariant and Completely Contactless Finger Knuckle Recognition. IEEE Transactions on Biometrics, Behavior, and Identity Science, 1(3), pp.201-209.

Li, G. and Kim, J., (2017). Palmprint recognition with local micro-structure tetra pattern. Pattern Recognition,

61, pp.29-46.
Ooi, S.Y., Teoh, A.B.J., Pang, Y.H. and Hiew, B.Y., (2016). Image-based handwritten signature verification using hybrid methods of discrete Radon transform, principal component analysis and probabilistic neural network. Applied Soft Computing, 40, pp.274-282.
Pan, X., Ruan, Q.Q. and Wang, Y.X., (2007). An improved 2dlpp method on gabor features for palmprint recognition. In 2007 IEEE International Conference on Image Processing (Vol. 2, pp. II-413). IEEE.
Sang, H. and Liu, F., (2009), November. Defocused palmprint recognition using 2DPCA. In 2009 International Conference on Artificial Intelligence and Computational Intelligence (Vol. 1, pp. 611-615). IEEE.
Sari, Y., Alkaff, M. and Pramunendar, R.A., (2018), June. Iris recognition based on distance similarity and PCA. In AIP Conference Proceedings (Vol. 1977, No. 1, p. 020044). AIP Publishing LLC.
Srikantaswamy, R., (2016), July. Fusion of fingerprint, palmprint and hand geometry for an efficient multimodal person authentication system. In 2016 2nd International Conference on Applied and Theoretical Computing and Communication Technology (iCATccT) (pp. 565-570). IEEE.
Zhang, D.D., (2013). Automated biometrics: Technologies and systems (Vol. 7). Springer Science & Business Media.

Biomedical
Communication

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp-201-205

BBRC
Bioscience Biotechnology
Research Communications

Functional Platelet Activity in Dutch Newborn Calves

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ABSTRACT

The hemostatic properties of platelets in cattle are physiologically very significant, as they affect the course of metabolic processes. This is especially significant in early ontogenesis and, apparently, strongly depends on the genetic characteristics of animals. The study was carried out on 37 Dutch calves, which were obtained from healthy cows after a normal pregnancy. All calves were examined and examined for 1-2 days, 3-4 days, 5-6 days, 7-8 days and 9-10 days of their ontogenesis. In the work were applied biochemical, hematological and statistical research methods. In the examined animals, during the neonatal phase, there was a tendency to inhibition of platelet aggregation in response to all inductors used. The number of platelet-discocytes in the blood of the examined Dutch calves in the first 10 days of their life experienced an upward trend. The amount of active platelet species they had decreased by 11.1%. The number of small and large platelet aggregates in the blood also decreased in them during the observation period. This was provided in the observed calves with a tendency to weaken the severity of synthesis in thromboxane platelets, a decrease in the level of adenosine phosphates in them and a weakening of their secretion. The level of platelet actin and myosin on the 1-2nd day in the examined calves was small and tended to decrease during the observation. Additional self-assembly of actin and myosin during platelet aggregation of the observed animals experienced some decrease during the observation period. It is clear that newborn calves of the Dutch breed are characterized by a high degree of functional sufficiency of platelets, creating physiologically favorable conditions for microcirculation processes. At the basis of these changes they have a small activity of the mechanisms that implement the hemostatic properties of platelets. Low intravascular activity of platelets in newborn calves of the Dutch breed is able to provide them with optimum perfusion and metabolic processes in all animal tissues that are necessary for the normal development of animals.

KEY WORDS: CALVES, NEWBORN PHASE, DUTCH BREED, PLATELETS, AGGREGATION, SECRETION.

ARTICLE INFORMATION

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Received 5th Jan 2020 Accepted after revision 28th Feb 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/35

INTRODUCTION

Hemostatic mechanisms are an important component of maintaining homeostasis (Zavalishina,. (2018h). It is clear that the state of the functional indicators of the hemostasis system very significantly determines the processes of hemocirculation (Usha et al., 2019). It is greatly influenced by platelets, the state of activity of which is able to regulate the course of microcirculation (Zavalishina, 2018d) in various living organisms (Kulikov et al., 2019). It is known that hemostatically significant manifestations of platelets are capable of experiencing dynamics at different ages (Zavalishina, 2018a), under the conditions of the formation of many dysfunctions (Chinarov, 2018), the development of pathological processes (Zavalishina et al., 2019) and against the background of their correction by any method (Zuev et al., 2006). However, many aspects of platelet hemostasis in cattle remain poorly understood. At present, only certain facts are known on platelet activity in these productive animals that are at separate stages of their ontogenesis (Vorobyeva et al., 2018).

There is still no complete picture of the relationship between the genetic characteristics of cattle and their platelet activity at different stages of their ontogenesis. At the same time, the efficiency of capillary blood flow, and, consequently, the level of trophism of tissues and the growth rate of all body structures and the formation of their functional characteristics (Sharkayeva et al., 2016; Zavalishina, 2018j), is largely related to the level of platelet activity in calves. Considering the presence of genetic differences between the breeds of cattle, it was of great interest to find out the peculiarities of platelet activity in calves of highly productive in terms of the volume of Dutch milk yield at the very beginning of their ontogenesis - during the neonatality phase. The goal of the present work is to find out the level of platelet activity in Dutch calves during the neonatal phase.

MATERIALS AND METHODS

Research was conducted in strict accordance with ethical principles established by the European Convent on protection of the vertebrata used for experimental and other scientific purposes (adopted in Strasbourg March 18, 1986, and confirmed in Strasbourg June 15, 2006).The work was performed on 37 calves of the Dutch breed. All calves taken into the study were obtained from healthy cows after normal pregnancy. All calves were examined and examined during the neonatal phase 5 times: 1-2, 3-4, 5-6, 7-8 and 9-10 days of life. All animals underwent an indirect assessment of the activity of synthesis in thromboxane platelets and an indirect elucidation of the enzymatic activity of cyclooxygenase and thromboxane synthetase in them using three transfer tests that evaluated platelet aggregation on a photoelectrocolorimeter. In platelets, the amount of adenosine triphosphate (ATP) and adenosine diphosphate (ADP) was evaluated, as well as the degree of their secretion in response to the contact of collagen with platelets.

The content of actin and myosin in inactive platelets and platelets that have aggregated in response to ADP has been determined (Ermolaeva et al., 1992). The expression of platelet aggregation (AP) was determined using a visual micromethod using as inducers ADP (at a dose of 0.5 x 10-4 M), collagen (at a dilution of 1: 2 from the main suspension), thrombin (at a dose of 0.125 u/ml), adrenaline (at a dose of 5.0x10-6 M) and ristomycin (at a dose of 0.8 mg/ml) in plasma which was previously standardized by the number of platelets to the level of 200x109 platelets per liter (Shitikova, 2000). The intravascular activity of platelets was determined by applying the method of phase-contrast microscopy (Shitikova, 2000). Statistical processing of received information was made with the help of a programme packet “Statistics for Windows v. 6.0”, “MicrosoftExcel”. Differences in data were considered reliable in case $P<0,05$.

RESULTS AND DISCUSSION

The Dutch calves taken under observation during the neonatal period showed a tendency to decrease the initial small platelet activity. Thus, in the examined calves, at 1-2 days of life, the AP developed in response to collagen for 37.4±0.13 s, in subsequent periods of observation it was inhibited, reaching by 9-10 days of life 38.6±0.15 s. A similar trend to slowing down the AP process was found in relation to ADP and ristomycin, which occurred at the end of observation at 47.6±0.26 s and 56.9±0.24 s, respectively. The tendency to slow the development of antibodies in response to thrombin (up to 60.1±0.20 s) and adrenaline (up to 108.2±0.15 s) has also been clarified.

The level of platelet-discocytes in the blood of the examined calves during the neonatal phase has undergone a tendency to increase. During the observation period, the sum of the activated platelet varieties experienced a slight decrease, totaling 11.1%. The number of small and large platelet aggregates in blood in the observed animals in the first 10 days of life gradually decreased by 21.7% and 2 times, respectively.It is clear that the inhibition of AP in Dutch calves during the observation period was largely due to the weakening of their synthesis in thromboxane platelets, which was indirectly indicated by a decrease in AP in a simple transfer test, the rate of which for 9-10 days of life was 24.8±0.16%. These results were provided in the observed calves due to the tendency to a decrease in the activity of both platelet enzymes for the synthesis of thromboxane, cyclooxygenase and thromboxane synthetase. The intensity of AP recovery during the collagen-aspirin test, which characterizes the level of activity in platelets of cyclooxygenase, was 73.0±0.09% by the end of the observation. The degree of AP recovery in the process of carrying out a collagen-imidazole sample, which makes it possible to indirectly estimate the level of platelet thromboxane synthetase activity, in the examined calves also decreased during the observation time and reached 34.3±0.12% on day 9-10.

Table. State of platelet activity in newborn calves of Dutch breed

Considered indicators	Calves of dutch breed, n=37, M±m				
	1-2 day	3-4 day	5-6 day	7-8 day	9-10 day
The level of platelet % aggregation recovery during the collagen-aspirin test,	75.9±0.14	75.5±0.17	74.8±0.09	73.7±0.08	73.0±0.09
The level of recovery of platelet aggregation during the collagen-imidazole test, %	36.6±0.11	36.0±0.05	35.6±0.08	35.0±0.07	34.3±0.12
The state of platelet aggregation in a simple transfer test, %	26.5±0.14	26.2±0.12	25.6±0.10	25.0±0.09	24.8±0.16
The amount of ATP in platelets prior to the start of secretion, μmol /109 platelets	5.35±0.018	5.30±0.012	5.26±0.016	5.24±0.007	5.19±0.005
The number of ADP in platelets before the start of secretion, μmol/109 platelets	3.20±0.004	3.16±0.002	3.13±0.007	3.10±0.006	3.07±0.008
secretion level ATP,%	25.5±0.10	25.3±0.12	25.0 ±0.08	24.7±0.07	24.2±0.13
secretion level ADP,%	32.8±0.08	32.6±0.11	32.3±0.07	32.0±0.13	31.5±0.10
The amount of actin in inactive platelets,% of total protein in platelets	20.9±0.14	20.6±0.10	20.0±0.05	19.7±0.06	19.3±0.04
The amount of actin in platelets with ADP-aggregation,% of total protein in platelets	32.4±0.12	32.1±0.06	31.8±0.08	31.5±0.05	31.2±0.10
The amount of myosin in inactive platelets,% of total protein in platelets	9.8±0.15	9.6±0.18	9.4±0.06	9.1±0.07	8.9±0.14 p<0.05
The amount of myosin in platelets with ADP-aggregation,% of total protein in platelets	22.3±0.10	22.1±0.16	21.7±0.05	21.5±0.10	21.2±0.15
Platelet aggregation time with ADP, s	46.7±0.20	46.8±0.17	47.0±0.24	47.3±0.18	47.6±0.26
Platelet aggregation time with collagen, s	37.4±0.13	37.7±0.20	38.0±0.14	38.3±0.19	38.6±0.15
Platelet aggregation time with thrombin, s	58.5±0.12	58.8±0.24	59.4±0.28	59.8±0.17	60.1±0.20
Platelet aggregation time with ristomycin, s	54.6±0.10	54.9±0.17	55.3±0.30	56.6±0.19	56.9±0.24
Platelet aggregation time with adrenaline, s	105.8±0.25	106.8±0.22	107.3±0.16	107.6±0.18	108.2±0.15
The number of platelet platelets, %	84.0±0.21	84.4±0.29	84.9±0.17	85.3±0.23	85.6±0.26
Total Active	16.0±0.19	15.6±0.12	15.1±0.16	14.7±0.15	14.4±0.14
Platelet Count, %				p<0.05	p<0.05
The number of small	2.8±0.09	2.6±0.05	2.4±0.03	2.3±0.04	2.3±0.06
platelet aggregates per 100 free platelets			p<0.05	p<0.01	p<0.01
The number of medium	0.10±0,017	0.09±0.012	0.07±0.014	0.06±0.010	0.05±0.018
and large platelet aggregates per 100 free platelets		p<0.05	p<0.01	p<0.01	p<0.01
Note: p - reliability of the dynamics of indicators in relation to 1-2 daily age					

Initially, a small amount of ATP and ADP in the platelets of calves in the observation process experienced a tendency to decrease, reaching 5.19±0.005 and 3.07±0.008 µmol/109 platelets towards its end. Moreover, during the observation, the levels of their secretion from the platelet granules tended to decrease by 5.4% and 4.1%, reaching 24.2±0.13% and 31.5±0.10% by the end of the observation, respectively.In inactive platelets of the examined calves, the number of molecules of actin and myosin for 1-2 days was 20.9±0.14 and 9.8±0.15% of the total protein in platelets, dropping to 19.3±0.04 by the end of observation and 8.9±0.14% of total protein in platelets. Self-assembly of actin and myosin in the course of platelet aggregation in Dutch calves during the neonatal phase also experienced a slight downward trend.

Studies on various physiological aspects of blood have been conducted for quite some time (Shitikova, 2000). They helped to gather a large amount of information, which allows finding out various aspects of regulatory mechanisms in mammals (Korepanova et al., 2015). It becomes clear that platelet activity has a greater physiological significance in the organism of animals. At the same time, its condition in young cattle of highly productive cattle breeds is still very poorly studied. In the work performed, an attempt was made to elucidate the peculiarities of platelet activity in newborn calves, a Dutch breed.

When evaluating the time of AP development in the examined calves in response to collagen and ristomycin, it was possible to note the initial small adhesive activity of their platelets, which had a tendency to weaken during the neonatal period. It is clear that these changes were based on at least two biologically important mechanisms (Zavalishina, 2018b). First, calves have a tendency to weaken platelet aggregation, which develops in response to the appearance of collagen in plasma (Zavalishina, 2018c). The basis of this phenomenon is the development of a decrease in initially low density on the membranes of platelets in calves of collagen receptors - glycoproteins Ia-IIa and VI (Vorobyeva et al, 2018). The presence of a second mechanism to ensure low platelet adhesion in calves of the Dutch breed was indicated by a tendency for AP to weaken in response to ristomycin (Zavalishina, 2018e). This biological mechanism was associated with the development of a weak decrease in the concentration of von Willebrand factor in the blood of calves during the neonatal phase and the inactive involvement of receptors to it (GPIb) on the platelet surface in the adhesive process (Zavalishina, 2018f).

In the work it was found out that for newborn Dutch calves a tendency to inhibition of initially inactive platelet aggregation is characteristic. There is reason to believe that these changes are designed to improve blood circulation processes in microvessels, (Zavalishina, 2018g). The initial low sensitivity of platelets to the stimulators of the aggregation process, which at the same time has a tendency to weaken, was also manifested by inhibition of interaction with the platelets of strong aggregation inducers - collagen and thrombin (Usha et al., 2019). Obviously, these changes were based on the weakening of the activity of the phospholipase C and the whole phosphoinositol mechanism, which were combined with a small degree of phospholation of the proteins of the actino-myosin complex (Zavalishina, 2018i). The optimally low production of inositol triphosphate in their platelets, apparently, provided a small degree of Ca2+ release from its depot and contributed to a decrease in the intensity of actomyosin self-assembly and a decrease in its reduction.

Considered weak agonists of the platelet aggregation process, ADP and adrenaline provided a low degree of severity in calves of the Dutch breed at birth, which tended to decrease during neonatality (Zavalishina, 2018k). These changes were obviously based on a low density of receptors for them on the platelet surface, a small degree of expression of fibrinogen receptors (GPIIb-IIIa), and low activation of phospholipase A2 during platelet aggregation. This provided a functional minimum of the yield of arachidonic acid from membrane phospholipids, which limited the synthesis of thromboxane A2 (Zavalishina, 2018l). At the same time, the activity of platelet cyclooxygenase and thromboxane synthetase was low in calves of the Dutch breed, which also restrained the synthesis of thromboxane A2 aggregate. This circumstance was proved by the results of carried out transfer tests, which made it possible to detect low activity of platelet cyclooxygenase and thromboxane synthetase in the blood plates of calves. A low AP level in newborn Dutch-born calves with all inductors was also ensured by their basal actin formation and myosin formation during platelet aggregation and unexpressed secretion of ATP and ADP platelets from granules (Shitikova, 2000).

Detection in the blood of Dutch calves of a low level of active forms of platelets proved in them a low sensitivity of platelets to any inducers of aggregation. The basal level of intravascular platelet activity found in them also proved the poor availability of collagen in their vascular wall due to the high endothelial integrity. This circumstance was ensured by the presence in the blood of the observed calves of a small number of active species of platelets and their aggregates. Indirectly, this confirmed the low sensitivity of Dutch-born calves' platelets to the aggregation inducers (ADP, thrombin, adrenaline) that are constantly present in their blood (Zavalishina, 2018m).

A decrease in the initially low aggregation ability of platelets in the observed calves caused a decrease in the level of their active forms and their aggregates of any size circulating in the blood. The found changes can be considered as an important mechanism for ensuring low activity of platelet hemostasis, optimum hemocirculation of capillaries and maintaining platelet vascular relationships at a physiologically beneficial level. The revealed small intravascular platelet activity in the newborn calves of the Dutch breed indicated a low activity of the adhesive and aggregation properties of

the platelets exhibited in vivo. Taking into account the data given in the literature (Kulikov et al., 2019), it can be assumed that the platelets of newborn calves of the Dutch breed have a very pronounced ability to disaggregate. This is due to the high sensitivity of their receptors to anti aggregant substances of vascular origin.

CONCLUSION

Newborn calves of the Dutch breed have a high degree of functional perfection of platelets. It largely provides physiologically favorable conditions for microcirculation and tissue metabolism. This is possible as a result of the low activity of their platelet mechanisms that ensure the flow of adhesion, aggregation and secretion. Small intravascular platelet activity in newborn calves of the Dutch breed contributes significantly to the formation of the optimum of their overall viability, which is required for their rapid growth and development.

REFERENCES

Chinarov, V.I. (2018) Evaluation of the competitiveness of dairy cattle breeds. Achievements of science and technology of the agro-industrial complex, 32(10) : 74-78.

Ermolaeva, T.A., Golovina, O.G. and Morozova, T.V. (1992) The program of clinical and laboratory examination of patients with thrombocytopathy. St. Petersburg, 25.

Korepanova, L.V., Starostina, O.S. and Batanov, S.D. (2015) Blood as an indicator of the interior characteristics of crossbred animals. Zootechny, 10 : 26-28.

Kulikov, E.V., Zavalishina, S.Yu., Abdelaziz Norezzine, Glagoleva, T.I. and Shemyakova, S.A.(2019) Optimising the effect of activated carbon on the microrheological properties of erythrocyte in rats with experimentally developed obesity. Bali Medical Journal, 8(3) : 643-649. DOI: 10.15562/ bmj.v8i3.1112

Sharkayeva, G.A. and Sharkayev, V.I. (2016) Potential breeding base of imported dairy cattle in the Russian Federation. Zootechny, 1 : 2-4.

Shitikova, A.S. (2000) Platelet hemostasis. St. Petersburg: Publishing House of St. Petersburg. State Medical University, 227.

Usha, B.V., Zavalishina, S.Y., Vatnikov, and Glagoleva, T.I. (2019) Diagnostics of early dysfunctions of anticoagulant and fbrinolytic features of rats' vessels in the course of metabolic syndrome formation with the help of fructose model. Bali Medical Journal, 8(1) : 201-205. DOI:10.15562/bmj.v8i1.923

Vorobyeva, N.V., Mal, G.S., Zavalishina, S.Yu., Glagoleva, T.I. and Fayzullina, I.I. (2018) Influence Of Physical Exercise On The Activity Of Brain Processes. Research Journal of Pharmaceutical, Biological and Chemical Sciences, 9(6) : 240-244.

Zavalishina, S.Yu. (2018a) Deficiency Of Iron As A Cause Of Dysfunction In Calves And Piglets. Research Journal of Pharmaceutical, Biological and Chemical Sciences, 9(5) : 978-983.

Zavalishina, S.Yu. (2018b) Functional Activity Of Plasma Hemostasis In Neonatal Calves With Iron Deficiency, Who Received Ferroglucin And Glycopin. Research Journal of Pharmaceutical, Biological and Chemical Sciences, 9(5) : 1186-1191.

Zavalishina, S.Yu. (2018c) Functional Antiaggregatory Properties Of Blood Vessels In Calves During Transition From Dairy To Plant Type Of Nutrition. Research Journal of Pharmaceutical, Biological and Chemical Sciences, 9(5) : 1110-1116.

Zavalishina, S.Yu. (2018d) Functional Features Of Platelets In Newborn Calves With Iron Deficiency. Research Journal of Pharmaceutical, Biological and Chemical Sciences, 9(5) : 1153-1158.

Zavalishina, S.Yu. (2018e) Functional Properties Of Anticoagulation And Fibrinolysis In Calves Of Plant Nutrition. Research Journal of Pharmaceutical, Biological and Chemical Sciences, 9(5) : 1082-1087.

Zavalishina, S.Yu. (2018f) Functional Properties Of Coagulation Hemostasis In Calves During The Phase Of Dairy-Vegetative Nutrition. Research Journal of Pharmaceutical, Biological and Chemical Sciences, 9(5) : 784-790.

Zavalishina, S.Yu. (2018g) Functional Properties Of Hemocoagulation In Calves Of Dairy Nutrition. Research Journal of Pharmaceutical, Biological and Chemical Sciences, 9(5) : 1016-1022.

Zavalishina, S.Yu. (2018h) Functioning Of Mechanisms Of Hemocoagulation Restriction In Calves At Change Of Methods Of Nutrition. Research Journal of Pharmaceutical, Biological and Chemical Sciences, 9(5) : 800-806.

Zavalishina, S.Yu. (2018i) Physiological Features Of Vascular Hemostasis In Calves Of Dairy-Vegetative Food. Research Journal of Pharmaceutical, Biological and Chemical Sciences, 9(5) : 1137-1143.

Zavalishina, S.Yu. (2018j) Physiology Of Vascular Hemostasis In Newborn Calves. Research Journal of Pharmaceutical, Biological and Chemical Sciences, 9(5) : 1037-1044.

Zavalishina, S.Yu. (2018k) Physiological Features Of Primary Hemostasis In Newborns Calves With Functional Digestive Disorders. Research Journal of Pharmaceutical, Biological and Chemical Sciences, 9(6) : 1514-1520.

Zavalishina, S.Yu. (2018l) The Dynamics Of The Physiological Properties Of Hemostasis In Newborn Calves With Functional Disorders Of The Digestion Against The Background Of Their Consumption Of Needles Extract. Research Journal of Pharmaceutical, Biological and Chemical Sciences, 9(6) : 1726-1731.

Zavalishina, S.Yu. (2018m) The Functional State Of Primary Hemostasis In Newborns Calves With Dyspepsia. Research Journal of Pharmaceutical, Biological and Chemical Sciences, 9(6) : 1543-1549.

Zavalishina, S.Y., Vatnikov, Y.A., Kulikov, E.V., Kubatbekov,T.S., Vilkovysky, I.F., Petrov, A.K., Tishchenko, A.L., Drukovsky, S.G., Zharov, A.N., Grishin, V.N. and Glagoleva, T.I. (2019) Effect of a combination of arterial hypertension and insulin resistance on hemostasis activity. Bali Medical Journal, 8(1): 211-215. DOI:10.15562/bmj.v8i1.1151

Zuev, A.V. and Osadchaya, O.Yu. (2006) Problems and solutions for creating highly productive dairy herds. Moscow, 265.

Dental
Communication

BBRC
Bioscience Biotechnology
Research Communications

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp-206-211

Influence of Er Cr Ysgg, Er Yag and Conventional Treatment on the Shear Bond Strength of Self Etch and Self-Adhesive Resin Cements

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ABSTRACT

The present study was designed to evaluate shear bond strength of dentin when bonded with self- etch and self-adhesive resin cements with ErCrYSGG laser (ErCrY) and Er-YAG (ErY) conditioning. Ninety extracted molar teeth were mounted and allocated into nine groups (n = 10) according to the dentine surface conditioning and type of cement. Three type of cement, Panavia, Rely-X and Maxcem were used in the study for comparison among the laser conditioning SBS values. All specimens were tested for shear bond strength using universal testing machine. Ten samples from each group were assessed for modes of failure. Data were assessed using analysis of variance and Tukey multiple comparisons test. The highest mean shear bond strength was observed in conventional treatment with Panavia cement application (21.51 (± 2.13) MPa) whereas the least mean shear bond strength was measured in Er-YAG conditioning along with Maxcem cement application (14.89 (± 3.48) MPa). The hypothesis was partly accepted as the influence of conditioning methods among different cements was comparable, except Panavia cement ($p > 0.05$). Moreover, the SBS values were significantly influenced by the type of resin cement rather than the type of laser used in dentinal conditioning ($P < 0.001$). Among all groups the most common type of observed failure was adhesive. The study was revealed that the type of Self-etch and self-adhesive cements exhibited significant influence on their bond strength to laser treated (ErCrYSGG and Er-YAG) dentin compared to the type of surface conditioning.

KEY WORDS: BOND STRENGTH; ERCRYSGG; ERYAG; SELF ETCH; SELF ADHESIVES; RESIN CEMENT.

INTRODUCTION

In the field of restorative dentistry, steps have been taken to simplify the use of adhesive procedures. Initially, a conventional adhesive restoration technique, a standard procedure was employed; however, due to an increased risk of excess demineralisation and salivary contamination these procedures evolved over time. Recently, self- etch, self-adhesive resin cements are introduced for the purpose of easy handling and quick bond formation (Durski et al., 2016). Using the multipurpose system presents with an opportunity for a positive bond formation in a restorative retention. Several studies demonstrated the dentin adhesive bond formation to be considered as a major factor influencing the success of restorative retention (Esteves-Oliveira et al., 2007, Gulec et al., 2018). The self -etch self- adhesive cement exhibited reduction in the need for pre-treatment of dentinal surface, minimises the procedure time and technique sensitivity; however, its viscosity limits decalcification and deep penetration into dentin, which result in compromised bond formation (Ferreira-Filho et al., 2018).

With the advancement in technology and change in the perceptions, minimal invasive treatments are

ARTICLE INFORMATION

*Corresponding Author: kalahdal@ksu.edu.sa
Received 11th Feb 2020 Accepted after revision 25th March 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

Clarivate Analytics crossref

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)
A Society of Science and Nature Publication, Bhopal India

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/36

implemented in current practices. Recently, the use laser technology has been introduced to enhance the dentin surface adhesion to the resin cement (Gulec et al., 2018). A contemporary method to enhance bond strength is the use of high intensity lasers. Erbium: yttrium-aluminum-garnet (ErYAG) and erbium, chromium: yttrium-scandium-gallium-garnet (Er, Cr: YSGG) belonging to the Erbium family have an ability to ablate the hard tissues without thermal damage, reduces dentinal hypersensitivity and exhibit bactericidal property (Bandéca et al., 2012, Acar et al., 2014). The use of these lasers increases the resistance of the dentine surface against a caries progression developing under the restoration (Harorli et al., 2015, Giray et al., 2014). The dentinal conditioning has exhibited a positive bond formation at the dentinal interface with the resin cement. The phototherapy causes the hydroxyapatite crystal as well as water to absorb wavelength energy of the laser (Er:YAG, λ= 2.94 m; Er,Cr:YSGG, λ=2.87 m) resulting in the dentin ablation and water evaporation forming imbricate irregularities on the dentin surface. The topographical change along with clearance of the smear layer initiate favourable condition for adhesive bond between resin and dentin (Esteves-Oliveira et al., 2007, Lukac et al., 2016). Moreover, phototherapy conditioned dentinal surface led to open dentinal tubules and peritubular dentin rather than intertubular dentin (Gulec et al., 2018).

Previous studies suggest that the laser irradiation modifies the dentin surface cultivating susceptible conditions for effective adhesive bond between resin and dentine (Ramos et al., 2015, Aras et al., 2016). In addition laser parameters including, wavelength, pulse-energy and power are a critical factor, in dentin conditioning outcomes for adhesive bonding to resins (Ramalho et al., 2015, Ferreira-Filho et al., 2018, Lopes et al., 2015). Esteves-Oliveira et al proclaimed that the use of self-etch adhesives is influenced by the type of erbium laser as the shear bond strength of resin to dentine was greater in the Er: YAG-laser conditioned surface compared to the Er, Cr:YSGG-laser irradiated, (Esteves-Oliveira et al., 2007, David and Gupta, 2015). This mounting interest in laser therapy encourages further investigation in laser conditioning using different type of self- etch and self -adhesive resin cements. It is hypothesized that use of Er, Cr:YSGG and Er: YAG laser for dentin conditioning presents with comparable self-etch, self- adhesive resin cement bond strength outcomes to conventional conditioning techniques. Therefore, the present study was designed to evaluate shear bond strength of dentin when bonded with self- etch and self- adhesive resin cements with ErCrYSGG laser (ErCrY) and Er-YAG (ErY) conditioning.

MATERIAL AND METHODS

The project was approved by the institutional research review board. The study compared the shear bond strength of dentin when bonded with self- etch and self- adhesive resin cements with ErCrYSGG laser (ErCrY) and Er-YAG (ErY) conditioning. A total of 90 extracted molars, with no caries, restoration or fracture were selected and stored in thymol solution (0.01%) for 1 week. Each tooth was cleansed using the chlorohexidine to remove the debris, calculus and plaque before sample preparation. The samples were shifted temporarily into a jar of distilled water at 4°C. All the specimen roots were sectioned using a diamond saw (Leitz 1600, Wetzlar, Germany) at 2mm below the cemento-enamel junction. The teeth were then mounted in acrylic resin (Meliodent; Kulzer, Hanau, Germany) and the occlusal enamel was removed to expose the mid coronal dentine. Dentine surface preparation: Each specimen was cut by the diamond saw (Leitz 1600, Wetzlar, Germany) to prepare an area of 4 mm in the mesiodistal plane followed by 400 – 600 grit carbide paper (Buehler) polish under a water coolant spray. Using the stereomicroscope the surface was closely examined for any enamel residue or pulpal tissue exposure. The total 90 extracted tooth was now divided into three categories: control (no laser treatment - standard bur cut with cylinder diamond burs with medium-sized particles), ErCrYSGG (ErCRY) laser and ErYAG (ErY) laser application.

Composite disc preparation:A putty mould (Easy Composites' Uni-Mould system, UK) combined with a wax disc was prepared with dimension of 2 mm diameter and 3mm height. Resin composite disc was prepared with a similar dimension of 2mm x 3 mm using technique of placing the putty mold on a glass slide and packing the material (MultiCore Flow bulk-fill composite Ivoclar-Vivadent, Schaan, Liechtenstein) in a mould. The excess was removed using the hand instrument and the disc was photo polymerised using the light source (Bluephase G2; Ivoclar-Vivadent) for 40 sec from top to bottom through another glass placed on the mould. The composite discs were finished followed by polishing and measured for required dimensions. Sample preparation: The dentinal surface in the all specimens were polished with a 600 grit silicon carbide waterproof abrasive paper before dentinal conditioning, creating an area of 4 mm in diameter under a water coolant spray. The samples were divided randomly into three groups, conventional, ErCrYSGG and ErYAG laser (n=30). Following the distribution experimental groups abide by the subsequent conditioning protocol

Group 1: Conventional treatment:Specimens were conditioned using a diamond bur with medium sized particles to flatten the dentine followed by polishing. No laser treatment was applied on the dentine.

Group 2: ErCr laser: Dentinal surface was conditioned by Er,Cr:YSGG (Waterlase C100; BioLase Tech, Inc., CA) laser power 4.5 W, wavelength of 2780 nm and frequency 20 Hz in a noncontact mode from a distance of 2 mm using tip (MZ = 8, 6 mm) for a interval of 60 sec.

Group 3: ErY laser: Dentinal surface was conditioned by the ErYAG laser (Kavo Key Laser 3, Kavo Dental GmbH & Co. KG,). The laser frequency 6 Hz, with a working distance of 20 mm, wavelength 2940 nm and directing 300 mJ per pulse.

After the tooth dentin conditioning, each group consisting of 30 specimens was distributed further into three sub groups based on the type of cement applied.

Group A: Panavia 2.0
Group B: RelyX Unicem
Group C: Maxcem

Total of 9 groups were created as a result as shown below:

Gp A1- Control	Gp A2- ErCr laser	G p A3- ErY laser
Gp B1-Control	Gp B2- ErCr laser	G p B3-ErY laser
Gp C1-Control	Gp C2- ErCr laser	G p C3- ErY laser

Each dentinal surface was smeared with a particular cement according to manufacturer's instructions and composite discs were cemented under a constant load of 15kg for 30 secs. The excess cement was removed using a micro brush. The cement layer was polymerized from all sides for 20 seconds (80 seconds in total). After completion of the process, tooth specimens underwent thermocycling for 30,000 cycles, under temperature of 5 to 55 ^{o}C with a dwell time of 30 sec in (Thermocycler (GMBH, Miebacher Strabe, Germany) and then stored in humid conditions (Incubator, Memmert Universal Oven, Germany) at 37 ^{o}C. The shear bond strength (SBS) was evaluated using universal testing machine (Instron 8500 Plus, Canton) through the controlled application of force at a cross-head speed of 0.5mm/min, resulting in fracture at the cement interface. The chisel-shaped probe was applied parallel to the interface on the composite disc until fracture. Shear bond strength was expressed in Megapascals (MPa). The fractured surface (bonding interface) was evaluated through the stereomicroscope and classified according to the type of failure; adhesive (the interface between dentine and cement), cohesive (within the material) and admixed (cement partly remains on dentine interference). The normality of data was assessed using Kolmogorov-Smirnov test. Mean and standard deviations (SD) of the observed data were assessed using descriptive statistics. Comparison of means and SD was performed with ANOVA and Multiple comparisons tests (Tukey-Kramer). Statistical software for social sciences (SPSS 20.0 version) was employed and p <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

The Kolmogorov-Smirnov test displayed the normality of the data to be distributed evenly. The study groups without laser conditioning are designated as A1, B1, and C1 for the Panavia, RelyX Unicem and Maxcem cement, respectively. The laser conditioning, ErCrY laser and ErYAG laser study groups were classified as A2, B2, C2 and A3, B3, C3 for the three types of cement (Panavia, RelyX Unicem and Maxcem), respectively. The highest mean shear bond strength was observed in conventional treatment with Panavia cement application (21.51 (± 2.13)) whereas the least mean shear bond strength was measured in Er-YAG conditioning along with Maxcem cement application (14.89 (± 3.48)). Table 2 presents the mean and standard deviation of shear bond strength measured in each study group. Analysis of variance (ANOVA) was performed among the study groups exhibiting statistical differences in the calculated mean SBS value (p-value <0.05) (table 2).

Two - way ANOVA and tukey HSD Post-hoc test revealed that the SBS strength is significantly influenced by the type of resin cement rather than a type of laser used in dentinal conditioning (P < 0.001)(table 2). Except for panavia, which successfully presented with a significant difference between the control group and Er-YAG conditioning (p<0.036); however, similar results were appreciated with the ERYSGG group. As for the Rely X and Maxcem, the results were comparable, each group showing no evident significance (p >0.05). A comparison between the three types of cement exhibited a significant difference between Panavia and Maxcem in each type of conditioning. Nevertheless, Rely X presented with comparable results with no significance in the study groups (p >0.05). However, tukey HSD Post-hoc test demonstrated that study groups under different circumstances also displayed a significant difference in the mean value (p<0.05) that includes an evident comparable result between panavia control to Rely X Er- YAG and Rely X Er- YSGG.

The failure modes assessment pointed out that Adhesive failure was more evident among the study group compared to cohesive and admixed failure. 100% adhesive failure was observed in the Rely-Er-Cr-YSG, Max-Control and Max-Er-Cr-YSGG groups. Cohesive failure was appreciated only in panavia control group (n=1). Admixed failure ranged from 20 – 40 % mainly among four study groups; Pan control, Pan-Er-YAG, Rely-Er-YAG and Max-Er-YAG. The analysis of the failure mode indicated Er-Cr-YSGG and Er-YAG conditioned exhibited adhesive failures compared to conventional treatment. However, all the cement study groups equally demonstrated adhesive failure except panavia control, which exhibited equal failure of adhesive and admix failure (4 specimens each) and 1 failure of cohesive type (table 3).

The present study was based on the hypothesis, that use of Er, Cr:YSGG (30 Hz 4.5 W) and Er: YAG (6 Hz 2 W) laser for dentin conditioning will show comparable self-etch, self -adhesive resin cement bond strength outcomes to conventional conditioning techniques. The influence of conditioning methods among different cements was comparable, except Panavia cement. The study's results revealed the formation of a strong shear bond strength depends upon the type of the cement used rather than the type of dentin conditioning. Therefore, the hypothesis was partly accepted in addition to a multitude of explanations can be provided in this regards including each step performed in the study for establishing a certain standard and shear bond failure in the specimens. The use of universal testing machine to measure the shear

bond strength, sets a peculiar standardization, consistency and homogeneity in the variable outcome (Jayasheel et al., 2017). To age the bonded specimen and simulate the oral functions, the specimen were placed in a thermocycler after cement application. As stated by Brunzel et al (2010) thermocycling creates favorable condition at the dentine bonding surface that instigates artificial ageing of the bonding system.The present study aimed to compare the effectiveness of dentinal conditioning between the conventional technique and two different types of lasers, ErCrYSGG and ErYAG.

Table 1. Cements used in the study and their application details

Cement/ manufacturer	Adhesive system	Component	Dentin pretreatment	Material pretreatment	Luting agent pretreatment
Panavia 2.0	ED primer II one-step self-etch	ED primer A 00252: ED Primer 2.0	combine a drop of each ED primer liquid	Conventional preparation or laser treatment. Add one drop of each	Mix universal and catalyst paste for 20 s followed by 20 s
Kuraray Inc., Tokyo, Japan		HEMA, MDP, 5-NMSA, water, accelerator ED primer B 00129: 5-NMSA, accelerator, w ater, sodium benzene sulphinate Universal paste 00269 Catalyst paste 00053	A and B for 5 s air dry for 60 s gently.	Clearfil SE primer and Porcelain Bond activator for 5 secs for bond activation.	of light cure.
RelyX Unicem 3M, ESPE, USA	No adhesive	(filler load 72% wt, particle size < 9.5 µm) Liquid: Methacrylated phosphoric esters, dimethacrylates, acetate, stabilizers, self-curing initiators, light-curing initiators	No pretreatment	No pretreatment	Mix the capsule for 15 s, light cure for 20 s
Maxcem Kerr Corporation 1717 W. Collins Ave .Orange, CA 92867-5422 U.S.A	self-etch, self-adhesive dual cure resin cement.	GPDM, co-monomers (mono-, di-, and tri-functional methacrylate monomers), proprietary self-curing redox activator, photoinitiator (CQ), stabilizer, barium glass fillers, fluoroaluminosilicate glass filler, fumed silica (filler load 67% wt, particle size 3.6 µm)	No pretreatment	No pretreatment	use the automix cartridge system and dispense the material Remove extra cement and cure for 10 seconds (self-cure for 2 – 3mins).

Table 2: Mean and standard deviation with three different types of cement-based on three different dentine conditioning methods.

Note: Dissimilar superscript small alphabets in same row denote significant difference in dentinal conditioning (p<0.05). Dissimilar superscript capital alphabets in same column denote significant difference in a different types of cement (p<0.05). * denotes a significant difference between the two different study groups.

Cement Type	No laser (Control)	Er-Cr-YSGG	Er-YAG	ANOVA P value
Panavia	21.51 (2.13)$^{a\ A*}$	19.16 (1.70)$^{ab\ A}$	18.43 (1.36)^{bA}	
Rely-X	18.24 (3.66)$^{a\ AB}$	17.39 (2.11)$^{a\ AB*}$	17.54 (3.61)a ^{AB*}	<0.001
Maxcem	16.40 (1.89)$^{a\ B}$	15.71 (2.26)$^{a\ B}$	14.89 (3.48)$^{a\ B}$	

It is proposed that laser conditioning causes the water evaporation from the surface resulting in microburst activity with increased surface tension for bonding (Cassimiro-Silva et al., 2016, Gulec et al., 2018). The low organic content in peritubular dentin is easily opened up by the laser; thus, dentinal tubules produce a cuff like appearance around it (Samad-Zadeh et al., 2011). In the present study, use of laser showed comparable outcomes of SBS to conventional conditioning technique. It is narrated that the laser conditioning facilitates the removal of the smear layer, which allows the formation of retention tags to improve the shear bond strength (Gulec et al., 2018). In addition, it is suggested that using conventional bur conditioning leads to the formation of a smear layer that limits the retention tags formation compared to laser conditioning, which ablates the interprismatic dentin and removes smear layer, permitting resin tags formation(Naranjo et al., 2015, Cassimiro-Silva et al., 2016).

Kiomarsi et al (2018), presented higher shear bond strength with Er YAG laser-treated dentin compared to the control group. The possible explanation for high SBS was the micro ablation of the dentine surface without any thermal damage to hard tissue and pulp; in addition, to antimicrobial activity (Kiomarsi et al., 2018). However, in the present study, only Panavia demonstrated similar results of high SBS in ErYAG treated dentine compared to the control group; however, other cement did not exhibit an evident difference. Appreciating the descriptive statistics, there was a slight difference between the mean values of the study groups; nevertheless, multiple comparison test proved no significant difference between conditioning treatment in Relay -X and Maxcem cement study groups. Therefore, the viscous nature of the cement can be deduced as a limiting factor for strong SBS observed. Furthermore, the slight difference in the mean value of the Er YAG and ErYSGG study groups indicates structural differences in ablated dentin after laser conditioning. As per Kiomarsi et al (2018), ErCrYSGG creates a scaled surface showing a thermal damaged surface because its wavelength is easily absorbed in the tissue leading to a rise in temperature. In contrast, Er YAG creates a surface with close resemblance to the conventional acid etched surface (Harorli et al., 2015).

Table 3: Failure mode between study groups.

Study Groups	Adhesive	Cohesive	Admixed
Pan-Control	40	10	40
Pan-Er-Cr-YSGG	70	0	30
Pan-Er-YAG	80	0	20
Rely-Control	60	0	40
Rely-Er-Cr-YSGG	100	0	0
Rely-Er-YAG	80	0	20
Max-Control	100	0	0
Max-Er-Cr-YSGG	100	0	0
Max-Er-YAG	80	0	20

In the current study, the effect of dentinal conditioning using three different types of cement; Panavia, Rely x and Maxcem was evaluated. The results displayed a significant difference between the SBS among Panavia and Maxcem specimens. However, SBS among the specimens of Rely-X and other cement groups was comparable. Naranjo, Ali, and Belles (2015) presented in their study no significance in the shear bond strength in self- etch self- adhesive resin cements. These authors recommended that conventional methods and total etch methods are more preferred compared to self- etch and self- adhesive cement. Furthermore, in the present study, no significant relation with Rely X was evident because of its low pH and low surface interaction, which leads to limited resin tags formation (Zidan et al., 2015).

Nevertheless, statistical test revealed that if dentinal conditioning was varied in addition to change in cement preference, a significant difference was observed among the Panavia controls and Rely X. Many studies' have assessed the tensile bond strength that measures the adhesive nature of the luting cement (Brunzel et al., 2010, Souza et al., 2016, Sekhri et al., 2016). This study, on the contrary, evaluated shear bond strength; the analysis of shear bond indicates the strength of the adhesive bond in an altered configuration (Brunzel et al., 2010). Few authors have reported that cohesive failures are observed in cases of the shear bond test; however, the present study displayed adhesive failure in the majority of specimens (Brunzel et al., 2010, Souza et al., 2016). Despite the effort for dentin conditioning to make the surface more retentive, the self -etch and self - adhesive cements has a limited capacity to demineralized dentine. In addition, they displayed limited inflow of viscous cement leaving a thick area of collagen mesh resulting in 100% adhesive failure (Weiser and Behr, 2015, Aguiar et al., 2014). I

t is pertinent to mention that there was no evident difference between the three dentin conditioning groups suggesting ablated surface may not influence the interfacial bond between dentine and cement. The outcomes of the study should be viewed in light of the possible limitations. The study observed an in-vitro design, with extracted teeth; in contrast, in-vivo, dentin shows fluid movement in and out of the tubules possibly challenging the adhesive bond. In addition, the variation in the morphology and composition of dentine along with directions and dimensions of dentinal tubules vary among the teeth employed in the in-vitro design. Despite these limitations, results produced were comparable to previous in vitro studies (Jayasheel et al., 2017, Cassimiro-Silva et al., 2016). However, in vivo studies under clinical conditions are essential to validate these outcomes. As conventional etch and rinse methods has shown a better dentine adhesion compared to the self-etch and self- adhesive cements (Kiomarsi et al., 2018). Therefore, to clinical trials assessing self-etch and total etch adhesives with laser treatments are recommended.

CONCLUSION

The adhesive bond strength of self-etch and self-adhesive

resin cements was not influenced by the use of ErCrYSGG and Er-YAG laser for dentin conditioning, except Panavia. The type of Self-etch and self-adhesive cements exhibited significant influence on their bond strength to laser treated (ErCrYSGG and Er-YAG) dentin.

Conflict of interest statement: No conflict of interest

REFERENCES

Acar, O., Tuncer, D., Yuzugullu, B. & Celik, C. (2014). The Effect of Dentin Desensitizers and Nd: Yag Laser Pre-Treatment On Microtensile Bond Strength Of Self-Adhesive Resin Cement To Dentin. The Journal of Advanced Prosthodontics, 6(2):88-95.

Aguiar, T. R., André, C. B., Correr-Sobrinho, L., Arrais, C. A., Ambrosano, G. M. & Giannini, M. (2014). Effect of Storage Times and Mechanical Load Cycling On Dentin Bond Strength of Conventional and Self-Adhesive Resin Luting Cements. The Journal of Prosthetic Dentistry, 111(5):404-10.

Aras, W. M., Barroso, J. S., Blay, A., Rodrigues, J. A. & Cassoni, A. (2016). Er, Cr: Ysgg Laser Irradiation Influence on Y-Tzp Bond Strength to Resin Cement. Ceramics International, 42(12):13790-5.

Bandéca, M. C., Pinto, S. C. S., Calixto, L. R., Saad, J. R. C., Barros, É. L. D. & Shelb, A. (2012). Influence of Er, Cr: Ysgg Laser on Bond Strength of Self-Adhesive Resin Cement. Materials Research, 15(4):491-4.

Brunzel, S., Yang, B., Wolfart, S. & Kern, M. (2010). Tensile Bond Strength of a So-Called Self-Adhesive Luting Resin Cement to Dentin. Journal of Adhesive Dentistry, 12(2):143.

Cassimiro-Silva, P.F., Zezell, D.M., de Melo Monteiro, G.Q., Benetti, C., de Paula Eduardo, C. and Gomes, A.S.L. (2016, February). Evaluation of microshear bond strength of resin composites to enamel of dental adhesive systems associated with Er, Cr: YSGG laser. In Lasers in Dentistry XXII (Vol. 9692, p. 96920N). International Society for Optics and Photonics.

David, C. M. & Gupta, P. (2015). Lasers in Dentistry: A Review. International Journal of Advanced Health Sciences, 2(8):7-13

Durski, M., Metz, M. J., Thompson, J., Mascarenhas, A., Crim, G., Vieira, S. & Mazur, R. (2016). Push-Out Bond Strength Evaluation Of Glass Fiber Posts With Different Resin Cements And Application Techniques. Operative Dentistry, 41(1):103-10.

Esteves-Oliveira, M., Zezell, D. M., Apel, C., Turbino, M. L., Aranha, A. C. C., Eduardo, C. D. P. & Gutknecht, N. (2007). Bond Strength Of Self-Etching Primer To Bur Cut, Er, Cr: Ysgg, And Er: Yag Lased Dental Surfaces. Photomedicine and Laser Surgery, 25(5):373-80.

Ferreira-Filho, R., Ely, C., Amaral, R., Rodrigues, J., Roulet, J., Cassoni, A. & Reis, A. (2018). Effect Of Different Adhesive Systems Used For Immediate Dentin Sealing On Bond Strength Of A Self-Adhesive Resin Cement To Dentin. Operative Dentistry, 43(4):391-7.

Giray, F. E., Duzdar, L., Oksuz, M. & Tanboga, I. (2014). Evaluation of the Bond Strength Of Resin Cements Used To Lute Ceramics On Laser-Etched Dentin. Photomedicine and Laser Surgery, 32(7):413-21.

Gulec, L., Koshi, F., Karakaya, İ., Yanardag, E. C. & Ulusoy, N. (2018). Micro-Shear Bond Strength of Resin Cements To Er, Cr: Ysgg Laser and/or Acid Etched Enamel. Laser Physics, 28(10):105601

Harorli, O., Barutcugil, C., Kirmali, O. & Kapdan, A. (2015). Shear Bond Strength Of A Self-Etched Resin Cement To An Indirect Composite: Effect Of Different Surface Treatments. Nigerian Journal of Clinical Practice, 18(3):405-10.

Jayasheel, A., Niranjan, N., Pamidi, H. & Suryakanth, M. B. (2017). Comparative Evaluation of Shear Bond Strength of Universal Dental Adhesives-An In Vitro Study. Journal of Clinical and Experimental Dentistry, 9(7):e892

Kiomarsi, N., Arjmand, Y., Fard, M. J. K. & Chiniforush, N. (2018). Effects of Erbium Family Laser on Shear Bond Strength of Composite to Dentin after Internal Bleaching. Journal of Lasers in Medical Sciences, 9(1):58.

Lopes, R. M., Trevelin, L. T., Da Cunha, S. R. B., De Oliveira, R. F., De Andrade Salgado, D. M. R., De Freitas, P. M., De Paula Eduardo, C. & Aranha, A. C. C. (2015). Dental Adhesion to Erbium-Lased Tooth Structure: A Review of the Literature. Photomedicine and Laser Surgery, (8):393-403.

Lukac, N., Suhovršnik, T., Lukaİ, M. & Jezeršek, M. (2016). Ablation Characteristics of Quantum Square Pulse Mode Dental Erbium Laser. Journal of Biomedical Optics, 21(1):015012.

Naranjo, J., Ali, M. & Belles, D. (2015). Comparison Of Shear Bond Strength Of Self-Etch And Self-Adhesive Cements Bonded To Lithium Disilicate, Enamel And Dentin. Texas Dental Journal, 132(11):914-21.

Ramalho, K. M., Hsu, C.-Y. S., De Freitas, P. M., Aranha, A. C. C., Esteves-Oliveira, M., Rocha, R. G. & De Paula Eduardo, C. (2015). Erbium Lasers for the Prevention of Enamel and Dentin Demineralization: A Literature Review. Photomedicine and Laser Surgery, 33(6):301-19.

Ramos, T. M., Ramos-Oliveira, T. M., De Freitas, P. M., Azambuja, N., Esteves-Oliveira, M., Gutknecht, N. & De Paula Eduardo, C. (2015). Effects of Er: Yag And Er, Cr: Ysgg Laser Irradiation On The Adhesion To Eroded Dentin. Lasers in Medical Science, 30(1):17-26.

Samad-Zadeh, A., Harsono, M., Belikov, A., Shatilova, K. V., Skripnik, A., Stark, P., Egles, C. & Kugel, G. (2011). The Influence of Laser-Textured Dentinal Surface on Bond Strength. Dental Materials, 27(10):1038-44.

Sekhri, S., Mittal, S. & Garg, S. (2016). Tensile Bond Strength Of Self Adhesive Resin Cement After Various Surface Treatment Of Enamel. Journal of Clinical and Diagnostic Research: Jcdr, 10(1):ZC01.

Souza, E. M., De Munck, J., Pongprueksa, P., Van Ende, A. & Van Meerbeek, B. (2016). Correlative Analysis of Cement–Dentin Interfaces Using an Interfacial Fracture Toughness and Micro-Tensile Bond Strength Approach. Dental Materials, 32(12):1575-85.

Weiser, F. & Behr, M. (2015). Self-Adhesive Resin Cements: A Clinical Review. Journal of Prosthodontics, 24(2):100-8.

Zidan, S., Peeran, S. W. & Ramalingam, K. (2015). Rely-X Unicem Self-Adhesive Universal Resin Cement. Dentistry and Medical Research, 3(1):3.

Biomedical
Communication

BBRC
Bioscience Biotechnology
Research Communications

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp-212-218

Effect of Four Indigenous Medicinal Plants on Dengue Fever, Vector *Aedes aegypti* from Jeddah, Saudi Arabia

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ABSTRACT

Aedes aegypti is the vector mosquito of dengue fever, which is an endemic disease in Saudi Arabia, Jeddah. The use of conventional insecticides in water sources introduces risks to people and/or the environment. In this study, mosquitocidal activity and adult development inhibition were investigated using four medicinal plants (*Rhazya stricta, Lantana camara, R. chalepensis,* and *Punica granatum*) against *Ae. aegypti* under laboratory conditions. A laboratory bioassay was conducted to assess larvicidal activity of leaves and peels crude acetonic extract of *L. camara*, *Ruta chalepensis*, *Rh. stricta* and *P. granatum*. Under controlled laboratory conditions, late 3rd or early 4th instar larvae were exposed to various concentrations – ranging from 150ppm to 1000 ppm – of the extracts to obtain the median lethal concentration (LC50) values for each plant extract tested. Mortalities were observed to increase with the increase in concentrations. Acetone extract of *Rh. Stricta* revealed high activity against *Ae. aegypti* compared to the rest of the extracts. The larval mortality rates of mosquito larvae ranged from 25 to 97% for *Rh. stricta*, 23 to 94% for *L. camara*, 17 to 96% for *R. chalepensis*, and 10 to 72% for *P. granatum*. In terms of effects on adult emergence, the inhibition percentage of the mosquito ranged from 26 to 100% for *Rh. stricta*, 13 to 100% for *L. camara*, 10 to 100% for *R. chalepensis*, and 22 to 92% for *P. granatum*. The evaluated medicinal plants seemed to be a better alternative to synthetic insecticides for controlling *Ae. aegypti*.

KEY WORDS: *AEDES AEGYPTI,* BIOASSAY, DENGUE FEVER, *LANTANA CAMARA,* MEDICINAL PLANTS.

ARTICLE INFORMATION

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Received 9th Jan 2020 Accepted after revision 27th Feb 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)
A Society of Science and Nature Publication, Bhopal India

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/37

INTRODUCTION

Dengue fever is an arthropod-borne viral disease found in many tropical and subtropical countries throughout the world (Gubler, 2018; Hosseini et al., 2018). *Aedes aegypti* (L) is presently considered the main vector of dengue, yellow fever, Zika, and chikungunya viruses in many parts of the world (Kraemer et al., 2015; Mayer et al., 2017). For many decades, synthetic pesticides have been employed to successfully control the infection cycle. However, the development of insect resistance to synthetic pesticides has also been documented globally, (Smith et al., 2016, Goindin et al., 2017; David et al., 2018). High operational costs and environmental pollution have created the need for developing alternative approaches for controlling vector-borne disease (Ohia and Ana, 2015). In early historical time, humans used plant extracts and concentrated compounds to control certain pests and disease-causing insects (Pavela, 2016). Overall, the search for such compounds has been directed extensively at the plant kingdom, (Veer and Gopalakrishnan, 2016,; Ninan et al., 2017 Lakshmi et al., 2018).

Medicinal plants are common sources of bioactive compounds, especially in tropical and subtropical countries (Van Wyk and Wink, 2017). The use of phytochemicals is one such strategy that may be suitable for mosquito control. The first, commonly used plant extract against adult mosquitoes was the flower extract of *Chrysanthemum cinerariaefolium* in South Africa and India, (Maheswaran and Ignacimuthu 2012 and Govindarajan, 2016) Many authors across the world started large screening activities for using extracts of medicinal and herbaceous plants to control mosquitoes. Extracts and oils from leaves, flowers, and roots of plants were found to display mosquito larvicidal activity (Chansang et al., 2018; Pavela et al., 2018).

Several researches from all over the world have documented the toxic effect of plant extracts such as *L. camara* and *Rh. stricta* and those of essential oils in controlling *Ae. aegypti* L mosquito (Ghosh et al., 2012; Hari and Mathew, 2018; Kalita et al., 2012; Kumar and Maneemegalai, 2008; Mappau and Ganing, 2018; Muangmoon et al., 2018; Ved et al., 2018). *Lantana camara* Linn. (Verbenaceae) is a hardy, evergreen, straggling shrub with a characteristic odor; it has antibacterial, antifungal, and insecticidal properties, and its oil provides protection from *Aedes* mosquitoes (Mossa et al., 1987). *R. chalepensis L. (Rutaceae)*, commonly known as "ruda" is a perennial herb, which is widely distributed in the Mediterranean region and the Kingdom of Saudi Arabia area (Verma, 2018). The toxic effect of the R. chalepensis extract against insects, particularly mosquitoes, have been reported previously, (Morsy et al., 1998, Al-Myah et al., 2011; Madkour et al., 2012). It is an ancient medicinal plant still used in the traditional medicine of many countries as recently reported by Bedini et al., (2018).

There is a scarcity of literature regarding the insecticidal potential of *P. granatum*. Its reported larvicidal activity was observed against the instar larvae of *Chrysomya albiceps* decades ago in Egypt (Sameeh et al., 2010). Moreover, its larvicidal activity was also reported recently against the larvae of *Anopheles pharoensis* (Eidi et al., 2005). To the best of the author's knowledge, this is the first study to address the larvicidal properties of *P. granatum* and *Rh. stricta* against *Ae. aegypti*. In this study, mosquitocidal activity and adult development inhibition were investigated using four medicinal plants (*Rh. stricta, L. camara, R. chalepensis,* and *P. granatum*) against the dengue fever vector *Ae. aegypti* under laboratory conditions.

MATERIALS AND METHODS

Mosquito strain source and rearing: *Ae. aegypti* (Diptera; Culicidae) was obtained from the mosquito laboratory at Al-Amana, Jeddah, Saudi Arabia. The *Ae. aegypti* (L) larvae were reared under laboratory conditions for over 12 generations using diet media fish food. Larvae from the culture were used for the different treatments described in this study. The stock colony was maintained at room temperature (27±1°C) and relative humidity of 70±5% with a 14:10 (LD) photoperiod throughout the study. The adults were fed on a 10% sugar solution.

Plant collection and extraction: The medicinal plants *L. camara, R. chalepensis, Rh. stricta,* and *P. granatum* were collected from the campus of King Abdulaziz University, Jeddah and were identified by an experienced botanist at the department of Botany. Fresh leaves from the plants were washed and shade-dried at room temperature for one week. They were then prepared to extract the effective ingredients according to the published standard method (WHO, 2005). Forty to sixty gm of leave tissues were finely ground and loaded into a 250 ml glass stoppered Soxhlet apparatus. Absolute Acetone (200 ml) was added to the glass and the extraction was performed for 6 hours. The rotary evaporator was used to concentrate the extracts to turn them into a semi-dry material. The extracted components were kept at –10°C until use for testing against selected insect stages.

Bioassay of plant extract against *Ae. aegypti* (L): The larvicidal bioassay followed the WHO standard protocols (Finney, 1972). The stock solution of each plant extract was prepared by adding 1 ml of re suspended extracts to 99 ml of distilled water containing 0.3% dimethyl sulphoxide (DMSO). From this stock solution, concentrations of 150, 250, 500, 800, and 1000 ppm were prepared in distilled water. Larval treatments were carried out by exposing the late 3rd or early 4th instar larvae continuously to various concentrations of the plant extracts. Tests were performed in groups of 250

ml glass beakers containing 100 ml tap water. Four replicates of 25 larvae for each concentration and control trail were assembled. The larvae were given the usual larval food during these experiments. After 24 hours of exposure time, the percent of mortality was recorded. The cumulative mortalities of larvae and pupae were recorded daily for the tested plants. Live pupae were transferred to untreated water in new beakers for further observation. X Actelic 50% EC (Primiphosmethyle) was tested as a positive control, while acetone was the negative control.

Statistical Analysis: This study used a completely randomized design (CRD) in a factorial experiment. The collected data were statistically analyzed using analysis of variance (ANOVA) tools, and the means were compared by LSD at $p \leq 0.05$ using the SAS software program [SAS Institute (2006) version 9.3]. IC50 and IC95 were calculated according to the Probit analysis program (Finney 1972, Abbott, 1925). The 95% confidence intervals, values, the $\chi 2$ goodness of fit test, and regression equations were estimated by a computerized log-probit analysis. The mortalities were corrected for control mortality using Abbott's formula (Tisgratog et al., 2016).

RESULTS AND DISCUSSION

The screening of herbal bioactive insecticides is a rapidly emerging research field that aims to find potential mosquito control agents with advantages such as low costs, ease of administration, and risk-free properties that can overcome the resistance to synthetic insecticides, and safety to the environment (Remia and Logaswamy, 2010). In the present study, crude leaves / peel extracts of *Rh. stricta, L. camara, R. chalepensis* and *P. granatum* showed larvicidal activity against *Ae. aegypti* when the larvae were exposed to concentrations of the plant extract ranging from 150 to 1000 ppm for 24 hours. The detailed results of the larvicidal activity of, *Ae. aegypti* larvae are presented in Tables 1 and 2 and depicted graphically in Figs. 1–4.

Table 1. Toxicity effect of four plant extracts against different stages of *Ae. aegypti*.

Plant extract	Conc. (ppm)	Larval Mortality (%)[A]	Pupation %	Adult emergence	
				Total	Inhibition (%)[B]
Rh. stricta	150	25	75	67	26
	250	42	58	50	44
	500	77	23	17	80
	800	90	10	10	89
	1000	97	3	0	100
Control	8	92	90	10	
L. camara	150	23	77	69	27
	250	44	66	38	60
	500	74	26	23	76
	800	86	14	7	93
	1000	94	6	0	100
Control	3	97	95	5	
R. chalepensis	150	17	83	77	33
	250	39	61	40	60
	500	72	28	21	79
	800	87	13	8	92
	1000	96	4	0	100
Control	2	98	96	4	
P. granatum	150	10	90	72	22
	250	23	77	51	47
	500	41	59	36	61
	800	60	40	20	78
	1000	72	28	7	92
	Control	5	95	92	8
Positive control	2	100			

A Four replicates of 25 larvae each B Corrected with Abbott's formula [35]

Table 2. Statistical parameters of plant extract against *Ae. aegypti*.

Statistical parameters	*Rh. stricta*	*L. camara*	*R. chalepensis*	*P. granatum*
LC_{50} (ppm)	275	286	312	585
Inhibition of (%) adult development	48	68	75	71
95% (*F. L)	233–298	251–320	280–345	550–696
LC95 (ppm)	729	1203	1095	2050
95% (*F. L)	648–842	990–1560	931–1149	1628–2873
Slope	2.8	2.63	3.02	2.43
Calculated $(Chi)^2$	1.26	0.87	1.3	1.04
Tabulated $(Chi)^2$	7.8	7.8	7.8	7.8
R^2	0.96	0.90	0.92	0.98

Figure 1: The relationship between concentrations of *L. camara* extract and mortality percentage of *Ae. aegypti* larvae.

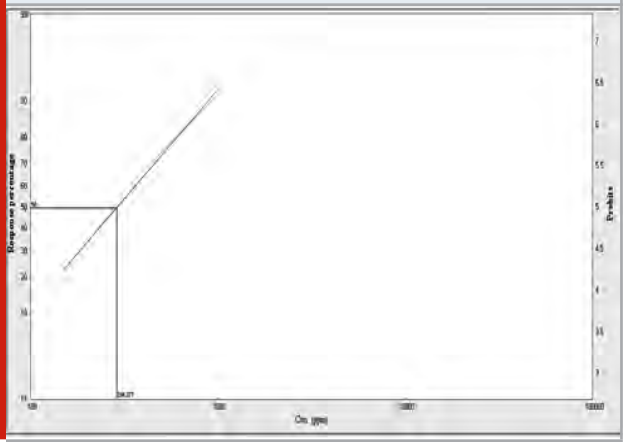
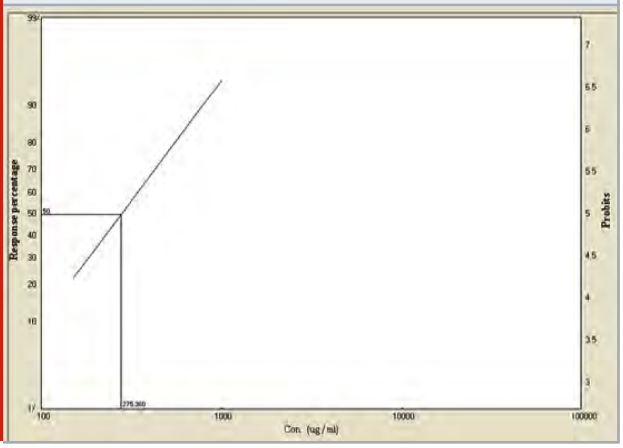


Figure 2: The relationship between concentrations of *Rh. stricta* extract and mortality percentage of Ae. aegypti larvae.



The mortality rates of the larvae exposed to various concentrations of the extracts were 25–97% (*Rh. stricta*), 23–94% (*L. camara*), 17–96% (*R. chalepensis*), and 10–72% (*P. granatum*).

Figure 3: The relationship between concentrations of *R. chalepensis* extract and mortality percentage of *Ae. aegypti* larvae.

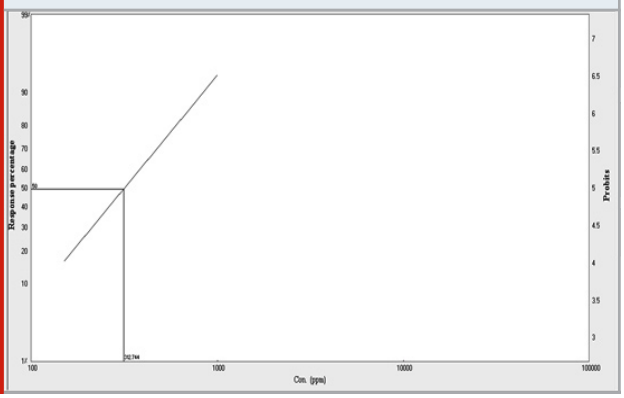
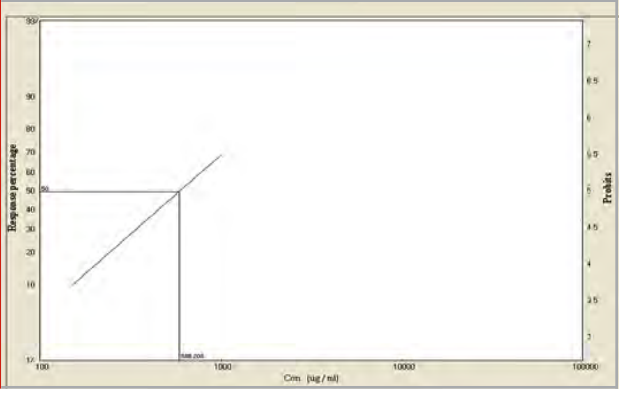


Figure 4: The relationship between concentrations of *P. granatum* extract and mortality percentage of *Ae. aegypti* larvae.



The acetone extract of *Rh. stricta* showed a significantly high larvicidal activity against *Ae. aegypti* compared with the other three extracts. The LC50/ LC95 for the four plant values were 275/729, 286/1203, 312/1095 and 585/2050 ppm for *Rh. stricta, L. camara, R. chalepensis,* and *P. granatum* respectively.

The findings of the present study are comparable to several earlier studies, (Remia and Logaswamy, 2010, Ghosh et al., 2012; Mappau and Ganing, 2018). Indeed, Ghosh and associates reported that the LC50 value of the *Rh. stricta* plant extract against mosquitoes was 251 ppm, while Remia and Logaswamy reported that the highest mortality of acetone extracts of *L. camara* leaves against Ae aegypti larvae with LC50 value was 230.7 ppm (Ghosh et al., 2012; Remia and Logaswamy, 2010). However, a higher value for LC50 (468.5 ppm) was reported by Indian scholars (Jayaraman et al., 2015). On the other hand, a higher mortality rate (85% at 150 ppm conc.) of *Ae. aegypti* 4th instar larva was reported in a recent study (Hemalatha et al., 2015).

Currently, the larvicidal mechanisms of phytochemicals are poorly understood. Recently, it has been reported that some alkaloids inhibit in vitro activity of glutathione S-transferase (GST) and acetylcholinesterase (AChE) of *Lymantria dispar* and the octopaminergic system / receptor in *Ae. aegypti* (Wang et al., 2019; Zou et al., 2017). However, subsequent studies revealed that protein carbonylation mediates an oxidative damage to the proteins involved in the energy metabolism of *Ae. aegypti* larvae (Rodríguez-Cavallo et al., 2018). The presence of certain phytochemicals (alkaloids, steroids, and phenolics) in plant extracts might be the reason for their larvicidal activity (Lakshmi et al., 2018). Unfortunately, scientific literature is unavailable regarding the efficacy of *R. chalepensis and P. granatum* against *Ae. aegypti*. However, their effects against other species of mosquitoes have been tested. For instance, *P. granatum* has been evaluated for its larvicidal activities against *Culex gelidus* and *Culex quinquefasciatus mosquitoes*. Methanol extracts showed absolute mortality of the larvae at 500 µg/ml after 24 hours (Kamaraj and Rahuman, 2010). Strikingly, a recent Libyan study found the acetone extract of R. chalepensis' aerial parts to demonstrate powerful larvicidal activity (LC50 at 1.08 ppm) against *Culex pipiens* (El-Bokl, 2016).

The cumulative mortalities during larval development to pupae and adults were recorded for the evaluation of the tested plants. The inhibition percentage of adult emergence were 26–100%, 27–100%, 33–100%, and 22–92% for *Rh. stricta, L. camara, R. chalepensis,* and *P. granatum* respectively (Table 1). Furthermore, mortality rates were observed at both the pupal stage and incomplete adult emergence. This could be due to morphological aberrations leading to failure of emergence from the exuviae of the pupal stage (Yu et al., 2015). The *R. chalepensis* extract exhibited the highest inhibition of adult emergence (75%) at LC50 (275 ppm), whereas the Rh. stricta extract showed the lowest activity (48%) at LC50 value (585 ppm) (Table 2). As shown in Table 2, when compared with earlier studies, these results revealed that the experimental plant extracts were effective in controlling *Ae. aegypti* and showed promising results (Ghosh et al., 2012; Morsy et al., 1998). The activity of plant extracts is often attributed to the complex mixture of active compounds (Kumar et al., 2012). The high efficacy of *Rh. stricta* and *L. camara* extracts may be due to the presence of chemical components including furanonaphthaquinones regioisomers and camaric acid as reported previously (Kamaraj et al., 2011). However, the biological effect of R. chalepensis against mosquitoes might be attributed to its high content of biologically active alkaloids (Sousa and Costa, 2012).

CONCLUSION

The acetone extract of *Rh. stricta, L. camara, R. chalepensis,* and *P. granatum* could comprise other approaches to be utilized for controlling the dengue vector mosquito, *Ae. aegypti*. These results could encourage the search for new active natural compounds offering a suitable alternative to insecticides synthesized from other medicinal plants; these alternatives would be safer and easier to handle than synthetic insecticides.

Authors' Contributions: A Al-Azab performed the experiments, analyzed the data, and wrote and revised the manuscript. Both A Zaituon and K Al-Ghamdi conceptualized the research idea designed the study, coordinated the work and wrote and revised the manuscript. F. Abd Al Galil assisted in collecting the literature search, read and revised the final manuscript. All authors read and approved the final manuscript for publication

ACKNOWLEDGMENTS

We gratefully acknowledge the King Abdulaziz City for Science and Technology (KACST) for their financial support under grand No. A-S-10-0013.

Competing interests: The authors declare that they have no competing interests.

REFERENCES

Abbott, W.S., (1925). A Method of Computing the Effectiveness of an Insecticide. Journal of Economic Entomology, 18, 265–267.

AL-Myah, A., Al-Mansour, N., Al-Dhahir, A., (2011). Effect of some plant extracts on the mortality of the larval mosquitoes *Culex pipiens* molestus Forskal. Basrah Journal of Science, 1, 47–61.

Bedini, S., Flamini, G., Ascrizzi, R., Venturi, F., Ferroni, G., Bader, A., Girardi, J., Conti, B., (2018). Essential oils sensory quality and their bioactivity against the mosquito *Aedes albopictus*. Scientific Reports, 8. 17857

Chansang, A., Champakaew, D., Junkum, A., Amornlerdpison, D., (2018). Potential of natural

essential oils and cinnamaldehyde as insecticides against the dengue vector *Aedes aegypti* (Diptera: Culicidae). Southeast Asian Journal of Tropical Medicine and Public Health, 1, 6–22.

David, M.R., Garcia, G.A., Valle, D., Maciel-de-Freitas, R., (2018). Insecticide Resistance and Fitness: The Case of Four *Aedes aegypti* Populations from Different Brazilian Regions. Biomed Research International, 2018, https://doi.org/10.1155/2018/6257860

Eidi, M., Eidi, A. and Zamanizadeh, H., 2005. Effect of Salvia officinalis L. leaves on serum glucose and insulin in healthy and streptozotocin-induced diabetic rats. Journal of Ethnopharmacology, 100, 310-313.

El-Bokl, M.M., (2016). Toxicity and bioefficacy of selected plant extracts against the mosquito vector *Culex pipiens* L. (Diptera: Culicidae). Journal of Entomology and Zoology Studies, 4, 483–488.

Finney DJ (1972) Probit Analysis, 2nd ed. Cambridge University Press, New York.

Ghosh, A., Chowdhury, N., Chandra, G., 2012. Plant extracts as potential mosquito larvicides. Indian Journal of Medical Research, 135, 581.

Goindin, D., Delannay, C., Gelasse, A., Ramdini, C., Gaude, T., Faucon, F., David, J.-P., Gustave, J., Vega-Rua, A., Fouque, F., (2017). Levels of insecticide resistance to deltamethrin, malathion, and temephos, and associated mechanisms in *Aedes aegypti* mosquitoes from the Guadeloupe and Saint Martin islands (French West Indies). Infectious Diseases and Poverty, 6, 38.

Govindarajan, M., (2016). Mosquito larvicidal potential of medicinal plants. In: Herbal Insecticides, Repellents and Biomedicines: Effectiveness and Commercialization. Pp 25–61, Springer India.

Gubler, D.J., (2018). Pandemic yellow fever: a potential threat to global health via travelers. Journal of Travel Medicine, 25.

Hari, I., Mathew, N., (2018). Larvicidal activity of selected plant extracts and their combination against the mosquito vectors *Culex quinquefasciatus* and *Aedes aegypti*. Environmental Sciences and Pollution Research, 25, 9176–9185.

Hemalatha, P., Elumalai, D., Janaki, A., Babu, M., Velu, K., Velayutham, K., Kunyil Kaleena, P., (2015). Larvicidal activity of *Lantana camara aculeata* against three important mosquito species. Journal of Entomology and Zoology Studies, 3, 174-181

Hosseini, S., Oliva-Ramírez, J., Vázquez-Villegas, P., Rodriguez-Garcia, A (2018). Dengue Fever: A Worldwide Threat An Overview of the Infection Process, Environmental Factors for a Global Outbreak, Diagnostic Platforms and Vaccine Developments. Current Topics in Medicinal Chemistry, 18, 1531–1549.

Jayaraman, M., Senthilkumar, A., Venkatesalu, V., (2015). Evaluation of some aromatic plant extracts for mosquito larvicidal potential against *Culex quinquefasciatus, Aedes aegypti*, and *Anopheles stephensi*. Parasitology Research, 114, 1511–1518.

Kalita, S., Kumar, G., Karthik, L., Rao, K.V.B., (2012). A Review on Medicinal Properties of Lantana camara Linn. Research Journal of Pharmacy and Technology, 6, 711.

Kamaraj, C., Bagavan, A., Elango, G., Zahir, A.A., Rajakumar, G., Marimuthu, S., Santhoshkumar, T., Rahuman, A.A., (2011). Larvicidal activity of medicinal plant extracts against Anopheles subpictus & Culex tritaeniorhynchus. Indian Journal of Medical Research, 134, 101–6.

Kamaraj, C., Rahuman, A.A., (2010). Larvicidal and adulticidal potential of medicinal plant extracts from south India against vectors. Asian Pacific Journal of Tropical Medicine, 3, 948–953.

Kraemer, M.U.G., Sinka, M.E., Duda, K.A., Mylne, (2015). The global distribution of the arbovirus vectors *Aedes aegypti* and *Ae. Albopictus*. Elife 4, e08347.

Kumar, M., Maneemegalai, S., (2008). Evaluation of Larvicidal Effect of *Lantana camara Linn* Against Mosquito Species *Aedes aegypti* and *Culex quinquefasciatus*. Advances in Biological Research, 2, 39–43.

Kumar, S., Wahab, N., Mishra, M., Warikoo, R., (2012). Evaluation of 15 Local Plant Species as Larvicidal Agents Against an Indian Strain of Dengue Fever Mosquito, *Aedes aegypti* L. (Diptera: Culicidae). Frontiers in Physiology, 3. 104.

Lakshmi, K. V., Sudhikumar, A. V., Aneesh, E.M., (2018). Larvicidal activity of phytoextracts against dengue fever vector, *Aedes aegypti* - A review. Plant science Today, 5, 167–174.

Madkour, M.H., Zaitoun, A.A., Singer, F., (2012). No TitleEfficacy of three plant species extracts in the control of Trogoderma granarium Everts (Coleoptera: Dermestidae). Journal of Food Agriculture and Environment, 2, 1200–1203.

Maheswaran, R., Ignacimuthu, S., (2012). A novel herbal formulation against dengue vector mosquitoes *Aedes aegypti* and *Aedes albopictus*. Parasitology Research, 110, 1801–1813.

Mappau, Z., Ganing, A., (2018). The Effect of Tembelekan leaves extract (*Lantana camara L*) as electrical rechargeable of mosquitoes repellent to eliminate *Aedes Aegypti*. Journal of Physiotherapy and Occupational Therapy-An International Journal, 12, 261.

Mayer, S. V., Tesh, R.B., Vasilakis, N., (2017). The emergence of arthropod-borne viral diseases: A global prospective on dengue, chikungunya and Zika fevers. Acta Tropica, 166, 155-163.

Morsy, T.A., Mazyad, S.A., el-Sharkawy, I.M., (1998). The larvicidal activity of solvent extracts of three medicinal plants against third instar larvae of *Chrysomyia albiceps*. Journal of the Egyptian Society of Parasitology, 28, 699–709.

Mossa, J., Al-Yahya, M., Al-Meshal, I., (1987). Medicinal plants of Saudi Arabia. King Saud University Press, Riyadh. 212-225.

Muangmoon, R., Junkum, A., Chaithong, U., Jitpakdi, A., Riyong, D., Wannasan, A., Somboon, P., Pitasawat, B., (2018). Natural larvicides of botanical origin against dengue vector *Aedes aegypti* (Diptera: Culicidae). Southeast Asian Journal of Tropical Medicine and Public Health 2, 227–239.

Ninan, S., Krishnakumar, K., Dineshkumar, B., (2017). Larvicidal activity of herbals: a review. Journal of drug Discovery and Therapeutics 5, 10–14.

Ohia, C., Ana, G.R.E., (2015). Pesticide toxicology and ameliorative interventions View Project Safety and Toxicological evaluation of African Medicinal Plants. Journal Biology, Agriculture, and Healthcare, 5, 22–26.

Pavela, R., (2016). History, presence and perspective of using plant extracts as commercial botanical insecticides and farm products for protection against insects - A review. Plant Protection Science, 52, 229–241.

Pavela, R., Maggi, F., Lupidi, G., Mbuntcha, H., Woguem, V., Womeni, H.M., Barboni, L., Tapondjou, L.A., Benelli, G., (2018). Clausena anisata and Dysphania ambrosioides essential oils: from ethno-medicine to modern uses as effective insecticides. Environmental Science and Pollution Research, 25, 10493–10503.

Remia, K.M., Logaswamy, S., (2010). Larvicidal efficacy of leaf extract of two botanicals against the mosquito vector *Aedes aegypti* (Diptera: Culicidae). Indian Journal of Natural Products Resources, 1, 208–212.

Rodríguez-Cavallo, E., Guarnizo-Méndez, J., Yépez-Terrill, A., Cárdenas-Rivero, A., Díaz-Castillo, F., Méndez-Cuadro, D., (2018). Protein carbonylation is a mediator in larvicidal mechanisms of Tabernaemontana cymosa ethanolic extract. Journal of King Saud University- Science, 31, 464-471.

Sameeh, A.M., Reda, F.B., Latha, S.H., Reman, I.M., (2010). Toxic and Synergistic Properties of Several Botanical Extracts against Larval and Adult Stages of the Mosquito, Anopheles pharoensis. Biopesticides international, 2, 129–145.

Smith, L.B., Kasai, S., Scott, J.G., (2016). Pyrethroid resistance in *Aedes aegypti* and *Aedes albopictus:* Important mosquito vectors of human diseases. Pesticide biochemistry and physiology, 133, 1-12.

Sousa, E.O., Costa, J.G.M., (2012). Genus Lantana: Chemical aspects and biological activities. Brazilian Journal of Pharmacognosy, 22, 1155–1180.

Tisgratog, R., Sanguanpong, U., Grieco, J.P., Ngoen-Kluan, R., Chareonviriyaphap, T., (2016). Plants traditionally used as mosquito repellents and the implication for their use in vector control. Acta Tropica, 157, 136-144.

Van Wyk, B., Wink, M., (2017). Medicinal Plants of the World, Revised Ed. ed. CABI.

Ved, A., Arsi, T., Prakash, O., Gupta, A., (2018). A Review on phytochemistry and pharmacological activity of *Lantana camara Linn*. International Journal of Pharmaceutical Sciences and Research, 1, 37–43.

Veer, V., Gopalakrishnan, R., (2016). Herbal insecticides, repellents and biomedicines: Effectiveness and commercialization. Springer India.

Verma, S., 2018. Medicinal potential of *Lantana camara*: Verbenaceae. Journal of Drug Delivery and Therapy, 8. 62-64

Wang, Z., Perumalsamy, H., Wang, X., Ahn, Y.J., (2019). Toxicity and possible mechanisms of action of honokiol from *Magnolia denudata* seeds against four mosquito species. Scientific Reports, 9. 411.

WHO (2005). Guidelines for laboratory and field testing of mosquito larvicides. Geneva.

Yu, K.X., Wong, C.L., Ahmad, R., Jantan, I., (2015). Larvicidal activity, inhibition effect on development, histopathological alteration and morphological aberration induced by seaweed extracts in *Aedes aegypti* (Diptera: Culicidae). Asian pacific Journal of Tropical Medicine, 8, 1006–1012.

Zou, C.S., Lv, C.H., Wang, Y.J., Cao, C.W., Zhang, G.C., (2017). Larvicidal activity and insecticidal mechanism of *Chelidonium majus* on *Lymantria dispar*. Pesticide biochemistry and physiology, 142, 123–132.

Dental
Communication

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp-219-224

Knowledge of Tooth Avulsion Management Among Emergency Room Physicians in Saudi Arabia

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ABSTRACT

Tooth avulsion is the displacement of an intact tooth out of the bony socket. This study evaluates the knowledge of Emergency Room physicians about tooth avulsion management, and determine the demographic factors associated with knowledge. A cross-sectional observational study was conducted on ER physicians. Major public hospitals were conveniently selected and used as clusters; test subjects were conveniently approached. Data were collected through a validated, self-administered questionnaire. Levels of knowledge were assessed in certain fields of avulsion management through selected questions. A total of 244 medical practitioners in emergency departments participated in the study. When asked about the importance of immediate management and critical extra-alveolar time of avulsed teeth, 35.4% of the respondents responded correctly. As for the importance of not replanting primary teeth, 46.3% of the respondents reported correctly. The majority of physicians knew the proper handling and proper cleaning technique of avulsed teeth with 78.5% and 79.3% correct responses respectively. The overall knowledge levels of physicians were poor in 61% and only 39% showed good knowledge. Results showed that ER physicians have demonstrated poor knowledge level regarding tooth avulsion management. Therefore, training programs would be helpful for timely dental referral of the patient presented with tooth avulsion in ER.

KEY WORDS: DENTAL TRAUMA, EMERGENCY, PHYSICIANS, TOOTH AVULSION.

ARTICLE INFORMATION

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Received 13th Jan 2020 Accepted after revision 20th March 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)

*Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/38*

INTRODUCTION

Traumatic dental injuries (TDI) are an impact type of injuries that occur to teeth and/or the associated hard and soft tissues of the oral cavity. And it is well documented in the literature that traumatic dental injuries are the most common injuries of the orofacial region, and it varies from simple enamel chipping to complete loss of the tooth. Tooth avulsion can be defined as the displacement of an intact tooth out of the bony socket. It is a serious condition, the most serious among all traumatic dental injuries. In a systematic review of literature the findings were that the prevalence of TDI is high worldwide. In Riyadh, it was measured that the prevalence of dental trauma among Saudi boys aged 5-6 years was found to be 33% with tooth avulsion being the second most common cause of trauma, while among Saudi 12-14 year old boys the prevalence was 34% with tooth avulsion comprising 3% (Ai-Majed, Murray, et al., 2001, Holan and Shmueli, 2003, Andersson, et al., 2007, Glendor, 2008, Lam, 2016, Aren et al., 2018).

TDIs are the most common type of injuries among all facial injuries, with tooth avulsion ranging between 1-16% among all dental traumas (Flores, Andersson, et al., 2007). In a review by (Glendor, 2009), the possible etiologies and risk factors of traumatic dental injuries were presented as: oral related factor, intentional TDI or unintentional TDI, with the latter being the most common cause; and it included falls, collisions, struck by an object, sports and road traffic accidents (Glendor, 2009). According to the guidelines of the International Association of Dental Traumatology, better prognosis of the avulsed tooth can be achieved by appropriate emergency management and adherence to the recommended time of sixty minutes (Flores, Andersson, et al., 2007). Furthermore, the consequences encountered from an avulsion injury will have a negative impact on the patient's biology, psychology and emotions (Bendo, Paiva, et al., 2010, Glendor, 2008).

In a study that investigated the association between treating/non-treating TDIs and the impact on patient's quality of life, it was concluded that children who have experienced TDI are more likely to have an impact than those without TDI, and that treatment did not eliminate the impact, though it reduced it (De Souza Cortes, Marcenes, et al., 2002; Ramos-Jorge, Bosco, et al., 2007; Fakhruddin, Lawrence, et al., 2008; Bendo, Paiva, et al., 2010). From an economic point of view, tooth loss due to trauma would require prosthetic therapy to replace the tooth, such therapies imply a high cost (Borum and Andreasen, 2001 Aren et al., 2018).

Avulsion is considered a public health problem, as of its high frequency, occurrence at early age, high cost and long follow-up which usually persists for the rest of the patient's life (Glendor, 2008). It's a serious condition, with unfavorable, avoidable consequence (De Souza Cortes, Marcenes, et al., 2002; Flores, Andersson, et al., 2007; Ramos-Jorge, Bosco, et al., 2007; Fakhruddin, Lawrence, et al., 2008; Bendo, Paiva, et al., 2010). It's prevalence have increased over the past decade, and it's continuing to increase (Glendor, 2008). Those injuries would require immediate medical attention (Flores, Andersson, et al., 2007) and often medical practitioners are the first to provide patients suffering from trauma with primary care. With that in mind, literature suggests that knowledge regarding avulsion and other traumatic dental injuries among medical practitioners is poor (Díaz, Bustos, et al., 2009; Subhashraj, 2009; Kumar, Sajjanar, et al., 2017; Aren, et al., 2018). The aim of this study was to determine the level of knowledge of medical practitioners in treatment of tooth avulsion in the city of Riyadh, Saudi Arabia.

MATERIAL AND METHODS

The current study is a cross-sectional observational study of emergency room physicians working in public hospitals. Ethical approval was obtained from the Institutional Review Board of King Abdullah International Medical Research Center (KAIMRC). Data was collected through a validated, self-administered questionnaire previously developed by (Abu-Dawoud, Al-Enezi, et al., 2007), the questionnaire consisted of sixteen close-ended questions and five open-ended ones regarding personal information; questions were divided into three parts. In the first part, personal information questions were included, in the second and third parts, questions to assess knowledge were included. An informed consent was obtained prior to participants' enrollment in the study, and confidentiality was strictly maintained as no identifiers were required. A representative sample size was estimated at 223 subjects based on an assumed prevalence of knowledge from previous research studies, a significance level of 5% and a precision of 5%. Six Major public Hospitals' Emergency Rooms in Riyadh were conveniently selected as clusters of samples, test subjects within each cluster were conveniently approached.

Males and females, Saudi and non-Saudi medical practitioners working in ER of hospitals in the public sector as residents, specialist, registrars and consultants were included. Analysis of results were done, and knowledge was evaluated by scoring the participants' knowledge level with a standardized method. Fields of knowledge that were assessed include: (1) importance of immediate management of avulsed teeth, (2) the importance of not replanting primary avulsed teeth, (3) knowledge regarding proper cleaning technique of grossly contaminated avulsed teeth, (4) knowledge regarding the proper handling technique and (5) knowledge regarding proper storage media. These knowledge fields were tested through nine questions. Each of the knowledge questions were given a score of one point, based on a score range of zero to nine points, zero being no knowledge and nine being full knowledge. Then, in between the two extremes, two levels existed, a poor knowledge score (0 - 5.9), and a good knowledge score (6.0 - 9.0); if all the nine questions were answered correctly, the participant would score a 9/9. The guidelines of the International Association of Dental Traumatology were used as a reference for correct answers (Andersson, Andreasen, et al., 2012; DiAngelis,

Andreasen, et al., 2012; Malmgren, Andreasen, et al., 2012). Statistical analysis was performed using SAS software, Version 9.4 of the SAS System for windows. Copyright (c) 2002-2012 by SAS Institute Inc., Cary, NC, USA. All variables were summarized as means, standard deviation and percentages. Factors associated with knowledge were tested using chi-square. Values were considered significant when P<.05.

RESULTS AND DISCUSSION

A total of 244 medical practitioners working in emergency departments gave their consent to participate in this study and filled the questionnaire, the mean age was 33.19 (8.18) of those 162 (66.7%) were males and 81(33.3%) were females. The study included 49.4% residents, 30% specialists and 20.6% consultants in hospital emergency rooms. The mean of years of experience was 6.82 (6.82). Among the participants, 66.5% have obtained their medical degree from Saudi Arabia, while 33.5% from other countries. The majority of the practitioners 69.5% reported that dental health education was not covered during medical school; in addition, 63.1% of physicians did not have any first aid course in the management of dental trauma. Whereas, 71.7% of physicians have received information on management of tooth avulsion in form of lectures, seminars, posters, from peers or personal reading (Table. 1).

Table 1. Participants Demographics and Characteristics

Variables	Sample (n=244)
Age mean (S.D.)	33.19 (8.18)
Gender n (%)	
Male	162 (66.67)
Female	81 (33.33)
Level of Education n (%)	
Resident	120 (49.38)
Specialist	73 (30.04)
Consultant	50 (20.58)
Years of Experience mean (S.D.)	6.82 (6.82)
Country of Graduation n (%)	
Saudi Arabia	157 (66.53)
Others	79 (33.47)
Dental health education during medical school n (%)	
Yes	73 (30.54)
No	166 (69.46)
First aid training in “Management of Dental Trauma” n (%)	
Yes	90 (36.89)
No	154 (63.11)
Received information on avulsion management n (%)	
Yes	175 (71.72)
No	69 (28.28)

In the questionnaire, the participants were asked about the importance of immediate management and critical extra-alveolar time of avulsed teeth, 35.4% of the respondents answered correctly (Fig. 1). As for the importance of not replanting primary teeth, 55.5% of the respondents reported correctly (Fig. 1). The majority of physicians knew the proper handling and proper cleaning technique of avulsed teeth with 78.5% and 79.3% correct responses respectively (Fig. 1).

Participants were given a total of 14 possible storage media, among them are correct and incorrect options, and participants had the choice of choosing multiple options simultaneously. The correct answers have been selected 64.3% of the time, of which, “milk” had the highest score 66.80%, followed by “patient’s mouth” with a score of 37.3%, and “saline” coming in third place with a 34% of overall storage media correct answers. The most selected incorrect answers were “tap water” 20.1%, followed by “gauze” with a score of 9.4%. Approximately 39.6% of the participants felt confident about replanting the tooth, while 27.9% felt that they lack the knowledge and training about replanting avulsed teeth (Table. 2).

Table 2. Responses to a Question Regarding Willingness to Replant an Avulsed tooth

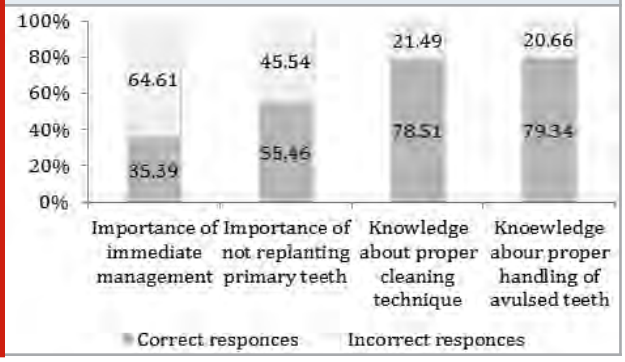
If you were at a site where someone knocked out a tooth, you would	n (%)
Not take action because of lack of knowledge and training	67 (27.9)
Not take action because of medico-legal consequences	41 (17.1)
Be confident and replant the tooth	95 (39.6)
Not be confident but replant the tooth anyway	37 (15.4)

Table 3. Associated and Unassoliated Factors with Levels of Knowledge

Age	0036*
Gender	.1138
Level of experience	.0014*
Years of experience	.0196*
Country of graduation	.8763
‘First Aid’ course covering ‘ Management of Dental Trauma’	.3801
Receiving information on management	<.0001*
Dental health educational course during medical school	.0696
*Statistically significant	

Based on the analyzed data, the overall knowledge levels of physicians were poor in 61%, while 39% showed good knowledge. To compare knowledge levels across individual's demographics, we implemented a chi-square test, and factors were considered significant when (P<.05), all factors analyzed are shown in (Table. 3). Variables such as age (P=.0036), level of experience (P=.0014), years of experience (P=.0196) and "received information on what to do if a tooth is knocked-out" (P<.0001) were found to be statistically significant factors. Other participants' characteristics including gender, country of graduation, "first aid course in dental trauma management" and "covering dental health education in medical school" were found to be insignificant (P>.05).

Figure 1. Participants Overall Scores of the Tested Fields of Knowledge



Literature revealed that the number of children visiting the emergency departments due to orofacial trauma is high (Aren, Sepet, et al., 2013). In the study of (Al-Malik, 2009) who studied the types of oral injuries attending a hospital in Jeddah, Saudi Arabia, the prevalence of avulsion to permanent teeth was found to be 16%, on the other hand, it was found that the prevalence of avulsed primary teeth was 3%. Similarly, (Aren, et al., 2018) reported that in the age group of 6 - 10 years, the prevalence of orofacial trauma was 23.7% and 18.9% for age group 11 - 18 years. In the present study, the prevalence of emergency room physicians who encountered an avulsion type of dental trauma was found to be 18.4%. Ergo, due to the high prevalence of orofacial trauma, tooth avulsion in particular, this study aimed to evaluate the level of knowledge of emergency department (ED) physicians of proper management of tooth avulsion. Díaz, Bustos, et al., (2009) reported that 90.2% of participants have not had any formal training on treatment of dental injuries, and that the majority of the participants had poor knowledge on the matter. In a study by (Trivedy, Kodate, et al., 2012), they concluded that among the participants lack of confidence in managing dental trauma was found, and that among all dental emergencies, the lowest confidence observed was for tooth avulsion.

Other studies by (Ulusoy, Onder, et al., 2012) and (Aren, Erdem, et al., 2018) revealed that the participants' perceived knowledge of dental emergencies treatment was insufficient and that the majority were willing to undergo training on the subject. Similarly, (Abu-Dawoud, Al-Enezi, et al., 2007) reported in their study which compared dentists and physicians that the knowledge level of physicians was low in 26.6% and average in 73.3%. In our study, our findings presents better results than that of (Abu-Dawoud, Al-Enezi, et al., 2007) who showed that none of the physicians have showed good knowledge, while, more than one third of the participants in our study have demonstrated good knowledge, the reason for that may be the sample of their study, in which, they included young physicians who have only graduated recently. Thus, the experience and the amount of exposure to such cases could be the controlling factor in the observed differences of knowledge.

The management of avulsed teeth differ between primary and permanent teeth (Andersson, Andreasen, et al., 2012; Malmgren, Andreasen, et al., 2012). In a survey by (Ulusoy, Onder, et al., 2012), participants were asked about their opinion on replantation of primary avulsed teeth, only about one third opted not to replant the tooth under any circumstances, while, about two thirds of them had no opinion or would replant the tooth in specific situations; their findings suggest that knowledge about avulsion management in case of primary teeth is insufficient. Similarly, these findings are consistent with that of (Erdem, et al., 2018) in which, they revealed that physicians' knowledge of traumatic dental injuries to permanent teeth were higher than that of primary teeth. On the other hand, (Needleman, Forbes, et al., 2012) found that there were no differences in the responses regarding primary and permanent teeth avulsion management, although, a significant difference were found for the luxation and uncomplicated fractures injuries. In the present study, a little more than half of the respondents had answered questions regarding knowledge of avulsion management in primary teeth correctly, and participants' knowledge were higher when compared to the previously cited literature. This could be explained by the fact that most of the emergency departments of the hospitals visited had a separate pediatric ER department.

The prognosis of avulsed teeth depends on several factors, extra-alveolar time, proper storage and transportation media, and care in handling and cleaning to preserve vitality of the periodontal ligament. The guidelines of the IADT recommends an extra-alveolar time of 60 minutes (Andersson, Andreasen, et al., 2012), beyond this, the prognosis of replantation decreases greatly as the cells of the periodontal ligament are non-viable. Thus, immediate management within this time period would increase chances of survival of the avulsed tooth up to 90%. In our study, such knowledge was lacking among the majority of the participants 64.6%, most of them chose "within few hours" and "before 24 hours have elapsed".

Similar to extra-alveolar time, handling of the avulsed tooth and appropriate initial procedures prior to re implantation is a critical factor in prognosis of the treatment provided. In the present study, our results indicate that three thirds of the physicians have a

good knowledge regarding the proper cleaning and handling of avulsed teeth. Nearby results were obtained by (Kumar, Sajjanar, et al., 2017), they found that 78% of the respondents preferred the appropriate cleaning technique, and that 48.7% would handle the avulsed tooth appropriately. Most of the previously cited studies obtained conflicting results, (Holan and Shmueli, 2003) found that only 4% of physicians would have appropriately provided initial treatment, in their study, only those who have answered all questions regarding the initial steps correctly would be included in the list of those who provided appropriate initial treatment.

Similarly, in a study by (Aren, Erdem, et al., 2018) about 10% of physicians chose the appropriate management technique. Midway results were found by (Ulusoy, Onder, et al., 2012) that about half of the physicians had no knowledge of the appropriate steps of replanting avulsed teeth. The reason for these conflicting results could be the tools used in each study or response bias of the participants, where they could be tempted to select certain answers. Therefore, further investigations should be performed to accurately determine knowledge levels of handling and cleaning techniques. Storing the tooth in a proper media would preserve the vitality of the periodontal ligament and increase prognosis. In the present study, 66.8% of the physicians chose milk as the proper transport medium. Contrary to the results of (Aren, Erdem, et al., 2018) where only 4% thought that milk is an appropriate medium. Also, in our study, 37.3% of participants recognized that intraoral transportation of avulsed teeth is appropriate.

While, Aren et al., (2018) found that 8.7% deemed intraoral transportation as the best transport medium. Similarly, in the study of (Lin, Levin, et al., 2006) 13.2% of the respondents chose saliva as the best transport medium. In our study, almost one third 27.9% of the respondents felt that they lacked sufficient training and knowledge about replanting an avulsed tooth, and 17.1% would not replant the tooth because of medicolegal consequences. Similarly, (Aren, Erdem, et al., 2018) showed that 44.4% thought that their knowledge were insufficient, and that 27.8% of emergency room physicians would not replant an avulsed tooth because of medicolegal issues. Reports by (Hamilton, Hill, et al., 1997) and (Addo, Parekh, et al., 2007) highlighted that physicians were frightened of possibly hurting the child and of legal implications. Therefore, establishing guidelines on management of tooth avulsions would be of great benefit. In conclusion, first aid management of traumatic dental injuries are usually provided by medical professionals, the results of this study suggest that emergency room physicians' knowledge is poor. Therefore, further education and training in management of dental trauma should be emphasized in both undergraduate and postgraduate studies.

Conflict of Interest: There are no conflicts of interest to declare.

ACKNOWLEDGEMENTS

This project was carried out in King Abdullah International Medical Research Center (KAIMRC).

REFERENCES

Abu-Dawoud, M., Al-Enezi, B., Andersson, L., 2007. Knowledge of emergency management of avulsed teeth among young physicians and dentists. Dent Traumatol 23, 348–55.

Addo, M., Parekh, S., Moles, D., Roberts, G., 2007. Knowledge of dental trauma first aid (DTFA): the example of avulsed incisors in casualty departments and schools in London. Br Dent J 202, E27.

Al-Majed, I., Murray, J., Maguire, A., 2001. Prevalence of dental trauma in 5–6- and 12–14-year-old boys in Riyadh, Saudi Arabia. Dent Traumatol 17, 153–8.

Al-Malik, M., 2009. Oral injuries in children attending a hospital in Saudi Arabia. J Maxillofac Oral Surg 8, 34–9.

Andersson, L., Andreasen, J., Day, P., Heithersay, G., Trope, M., DiAngelis, A., Kenny, D., Sigurdsson, A., Bourguignon, C., Flores, M., Hicks, M., Lenzi, A., Malmgren, B., Moule, A., Tsukiboshi, M., 2012. International Association of Dental Traumatology guidelines for the management of traumatic dental injuries: 2. Avulsion of permanent teeth. Dent Traumatol 28, 88–96.

Aren, A., Erdem, A., Aren, G., Sahin, Z., Tolgay, C., Çayırcı, M., Sepet, E., Güloglu, R., Yanar, H., Sarıbeyoglu, K., 2018. Importance of knowledge of the management of traumatic dental injuries in emergency departments. Ulus Travma Acil Cerrahi Derg 24, 136–44.

Aren, G., Sepet, E., Erdem, A., Tolgay, C., Kuru, S., Ertekin, C., Güloglu, R., Aren, A., 2013. Predominant Causes and Types of Orofacial Injury in Children Attending Emergency Department. Ulus Travma Acil Cerrahi Derg 19, 246–50.

Bendo, C., Paiva, S., Torres, C., Oliveira, A., Goursand, D., Pordeus, I., Vale, M., 2010. Association between treated/untreated traumatic dental injuries and impact on quality of life of Brazilian schoolchildren. Health Qual Life Outcomes 8, 114.

Borum, M., Andreasen, J., 2001. Therapeutic and economic implications of traumatic dental injuries in Denmark: an estimate based on 7549 patients treated at a major trauma centre. Int J Paediatr Dent 11, 249–58.

De Souza Cortes, M., Marcenes, W., Sheiham, A., 2002. Impact of traumatic injuries to the permanent teeth on the oral health-related quality of life in 12–14-year-old children. Community Dent Oral Epidemiol 30, 193–9.

DiAngelis, A., Andreasen, J., Ebeleseder, K., Kenny, D.,

Trope, M., Sigurdsson, A., Andersson, L., Bourguignon, C., Flores, M., Hicks, M., Lenzi, A., Malmgren, B., Moule, A., Pohl, Y., Tsukiboshi, M., 2012. International Association of Dental Traumatology guidelines for the management of traumatic dental injuries: 1. Fractures and luxations of permanent teeth. Dent Traumatol 28, 2–12.

Díaz, J., Bustos, L., Herrera, S., Sepulveda, J., 2009. Knowledge of the management of paediatric dental traumas by non-dental professionals in emergency rooms in South Araucanía, Temuco, Chile. Dent Traumatol 25, 611–9.

Fakhruddin, K., Lawrence, H., Kenny, D., Locker, D., 2008. Impact of treated and untreated dental injuries on the quality of life of Ontario school children. Dent Traumatol 24, 309–13.

Flores, M., Andersson, L., Andreasen, J., Bakland, L., Malmgren, B., Bernett, F., Bourguignon, C., DiAngelis, A., Hicks, L., Sigurdsson, A., Trope, M., Tsukiboshi, M., Von Arx, T., 2007. Guidelines for the management of traumatic dental injuries. II. Avulsion of permanent teeth. Dent Traumatol 23, 130–6.

Glendor, U., 2009. Aetiology and risk factors related to traumatic dental injuries – a review of the literature. Dent Traumatol 25, 19–31.

Glendor, U., 2008. Epidemiology of traumatic dental injuries – a 12 year review of the literature. Dent Traumatol 24, 603–11.

Hamilton, F., Hill, F., Mackie, I., 1997. Investigation of lay knowledge of the management of avulsed permanent incisors. Dent Traumatol 13, 19–23.

Holan, G., 2003. Knowledge of physicians in hospital emergency rooms in Israel on their role in cases of avulsion of permanent incisors. Int J Paediatr Dent 13, 13–9.

Kumar, S., Sajjanar, A., Athulkar, M., Sajjanar, J., Shewale, A., Wasnik, M., Dhongde, P., Moon, A., 2017. The Status of Knowledge Related to the Emergency Management of Avulsed Tooth amongst the Medical Practitioners of Nagpur, Central India. J Clin Diagn Res 11, ZC21–ZC24.

Lam, R., 2016. Epidemiology and outcomes of traumatic dental injuries: a review of the literature. Aust Dent J 61, 4–20.

Lin, S., Levin, L., Emodi, O., Fuss, Z., Peled, M., 2006. Physician and emergency medical technicians' knowledge and experience regarding dental trauma. Dent Traumatol 22, 124–6.

Malmgren, B., Andreasen, J., Flores, M., Robertson, A., DiAngelis, A., Andersson, L., Cavalleri, G., Cohenca, N., Day, P., Hicks, M., Malmgren, O., Moule, A., Onetto, J., Tsukiboshi, M., 2012. International Association of Dental Traumatology guidelines for the management of traumatic dental injuries: 3. Injuries in the primary dentition. Dent Traumatol 28, 174–82.

Needleman, H., Stucenski, K., Forbes, P., Chen, Q., Stack, A., 2013. Massachusetts emergency departments' resources and physicians' knowledge of management of traumatic dental injuries. Dent Traumatol 29, 272–9.

Ramos-Jorge, M., Bosco, V., Peres, M., Nunes, A., 2007. The impact of treatment of dental trauma on the quality of life of adolescents – a case-control study in southern Brazil. Dent Traumatol 23, 114–9.

Subhashraj, K., 2009. Awareness of management of dental trauma among medical professionals in Pondicherry, India. Dent Traumatol 25, 92–4.

Trivedy, C., Kodate, N., Ross, A., Al-Rawi, H., Jaiganesh, T., Harris, T., Anderson, J., 2012. The attitudes and awareness of emergency department (ED) physicians towards the management of common dentofacial emergencies. Dent Traumatol 28, 121–6.

Ulusoy, A., Önder, H., Çetin, B., Kaya, S., 2012. Knowledge of medical hospital emergency physicians about the first-aid management of traumatic tooth avulsion. Int J Paediatr Dent 22, 211–6.

Parasitological Communication

BBRC Bioscience Biotechnology Research Communications

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp-225-232

Prevalence of Three Mosquito Vectors: *Anopheles, Culex* and *Aedes* in Some Areas of Hooghly West Bengal, India

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ABSTRACT

Several mosquito genera such as *Anopheles, Culex* and *Aedes* serve as vectors of numerous deadly diseases throughout the world. Prevalence of these diseases strongly coincides with the prevalence of their respective vectors. During the present study adult mosquitoes of three genera (*Anopheles, Culex* and *Aedes*) were collected from selected four cattle sheds and four human habitations in both rural and urban areas of Hooghly district, West Bengal through hand collection method employing four man hours (two for human habitations and two for cattle sheds) in the first week of every month throughout the year (Jan'18 - Dec'18). Environmental temperature, humidity were recorded and GPS location of the collection points were plotted on the study area map. Altogether 4754 *Anopheles* (56.36%), 3312 *Culex* (39.26%) and 369 *Aedes* (4.37%) mosquitoes were collected from rural areas and 3766 *Anopheles* (50.16%), 3302 *Culex* (44.38%) and 372 *Aedes* (5%) mosquitoes were collected from urban areas. *Anopheles* and *Culex* mosquitoes showed higher prevalence in cattle sheds than human habitations in both rural and urban areas, whereas reverse situation is shown by Aedes mosquitoes. Monthly prevalence of different species of *Anopheles* also showed a significant difference between rural and urban areas ($p<0.01$). This study indicated that *Anopheles* and *Culex* mosquitoes are more prevalent in cattle sheds besides human habitations. Present study will illuminate about the resting-habitats in relation to mosquito ecology so that proper management strategies may be taken with a view to prevent the mosquito borne diseases in the endemic areas of Hooghly District.

KEY WORDS: MOSQUITOES; PREVALENCE; HUMAN HABITATIONS; CATTLE SHEDS; DISEASE.

INTRODUCTION

Mosquitoes belonging to several genera mainly *Anopheles*, *Culex* and *Aedes* serves as nuisance vectors of numerous deadly diseases like malaria, filaria, dengue etc. occurring throughout the world (Mondal et al, 2015). Several species of *Anopheles* mosquitoes severs as a vector of different types of malaria in tropical and subtropical countries. *Anopheles gambiae* and *Anopheles funestus* serves as the main malarial vector in African countries (Lindh et al, 2005).Whereas *Anopheles culicifacies* serves as the main malarial vector in Asian countries (Chatterjee and Chandra, 2000;Chavshin et al, 2014; Lindh et al, 2015; Seal et al 2018).

It was reported that in some rural areas of Hooghly district, West Bengal *Anopheles subpictus* Grassi serves as the main malarial vector (Chatterjee and Chandra, 2000). Some *Culex* species mostly Culex *vishnui* group and *Culex quinquifasciatus* serves as a vector of Japanese encephalitis, lymphatic filariasis in many regions of India

ARTICLE INFORMATION

*Corresponding Author: soumen.microbiology@gmail.com
Received 12th Jan 2020 Accepted after revision 28th Feb 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/39

(Kanojia et al 2003) and West Bengal also (Chandra et al, 2007; Azmi et al, 2015), whereas some species of *Aedes* mosquitoes like *Aedes aegypti* and *Aedes albopictus* globally transmits dengue fever (Gubler 2002). In all the cases adult female mosquitoes are responsible for the transmission of disease causing pathogens. So, the prevalence of these diseases is strongly coincides with the prevalence of two factors: one is the abundance of the respective vectors and second one is the introduction of the disease causing pathogens in the suitable host, which are mostly dependent on environmental conditions like temperature, humidity, rainfall, availability of suitable breeding grounds, (Khan et al, 2017).

For the purpose of management of diseases, adult control strategies are mostly used today, which uses several insecticides like DDT, malathion, pyrethroid to control the adult mosquitoes. In this regard it is very important to have a better knowledge about the ecology of mosquitoes especially their resting site preference and biting behavior throughout the different times of a year (Alten et al, 2000). Reports on prevalence of different mosquitoes in rural and urban areas of Hooghly district, West Bengal are very scanty. So, the present study has been aimed to determine the month wise prevalence of three genera of adult mosquito vectors like *Anopheles, Culex* and *Aedes* in some rural and urban areas of Hooghly district, West Bengal, India along with the species composition of anopheline mosquitoes and to find out their correlations with some environmental factors like temperature and humidity throughout the year. This study would have a great impact on better understanding of mosquito ecology and their proper management strategies in the study areas.

MATERIALS AND METHODS

The entire study was performed monthly for one year starting from January'2018 to December'2018.The entire study was carried out in some rural and urban areas of Hooghly district, West Bengal, India. For the collection of adult mosquitoes four human habitations and four cattle sheds were fixed in both rural and urban areas. The location of the study areas were recorded with a hand held GPS (Germin Etrex201 model) and thereafter these way points were superimposed on study area map and satellite image (Fig.1).

The adult mosquitoes were collected by hand collection method from four fixed human habitations and four cattle sheds in each of rural and urban areas of Hooghly. Four man hours (two for human habitations and two for cattle sheds) were employed for the collection of the adult mosquitoes and the collection was done in three different time periods (morning: 6 am - 8 am; afternoon: 4 pm - 6 pm and night: 10 pm-12 am) of the same day in the first week of every month. The duration of collection time was fifteen minutes in each place. Both the indoor biting and resting mosquitoes were collected in test tubes with a small piece of net plugged at mouth of the tubes and on the next day they were brought to the Parasitology and Microbiology Research Laboratory for their identification. The environmental temperature and humidity of the collection points were recorded by Fischer Scientific machine. In laboratory the mosquitoes were anesthetized by applying chloroform to cotton and plugging on the opening of the tubes containing adult mosquitoes.

Table 1: Month wise prevalence of three genus of mosquitoes (*Anopheles, Culex* and *Aedes*) in human habitations and cattle sheds in rural areas of Hooghly District (Day time: 6a.m-8a.m from Jan'2018-Dec'2018)

Month	Temperature (°C)	Humidity (%)	Number of collected Mosquitoes					
			Anopheles		*Culex*		*Aedes*	
			H.H	C.S	H.H	C.S	H.H	C.S
January	14.0°C	58.1%	12	27	10	15	0	0
February	18.1°C	51.5%	36	88	20	28	08	04
March	27.4°C	62.1%	42	86	32	52	10	02
April	28.2°C	63.2%	48	64	62	94	05	08
May	29.4°C	67.7%	28	52	36	68	09	03
June	30.0°C	76.2%	36	64	48	74	18	02
July	29.2°C	79.7%	68	112	76	132	13	04
August	28.2°C	81.8%	82	146	92	156	18	03
September	28.7°C	77.0%	84	124	68	96	20	06
October	23.4°C	70.3%	40	56	36	48	28	15
November	22.6°C	66.7%	22	32	30	44	11	05
December	15.2°C	61.2%	15	18	37	42	0	0

*H.H- Human Habitation, C.S- Cattle Shed

Then the mosquitoes were shorted morphologically according to the genus under a dissecting binocular. The total number of *Anopheles, Culex* and *Aedes* mosquitoes were noted down. Then the collected *Anopheles* mosquitoes were sorted out according to the species following Nagpal and Sharma (1995) and their numbers were noted down. Statistical analysis was performed following Zar, (1999) using SPSS software version 16.0.

RESULTS AND DISCUSSION

The locations of cattle sheds and human habitations in rural and urban study areas are given in Figure 1. The distance between rural and urban areas was 37.72km (Figure 2). Total 4754 *Anopheles* (56.36%), 3312 Culex (39.26%) and 369 Aedes (4.37%) mosquitoes were collected from rural areas and 3766 *Anopheles* (50.16%), 3302 *Culex* (44.38%) and 372 *Aedes* (5%) mosquitoes were collected from urban areas throughout the year. Month wise prevalence of three genera of adult mosquitoes (*Anopheles*, *Culex* and *Aedes*) in human habitations and cattle sheds in rural areas and urban areas are given in Table 1-3 and in Table 4-6 respectively. During the whole study period *Anopheles* and *Culex* mosquitoes showed higher prevalence in cattle sheds (81.42±7.14 and 77.67±7.02 respectively) than human habitations (50.64±4.58 and 50.92±4.2 respectively) in rural areas which was statistically significant ($p<0.01$).

In case of urban areas the mean density of *Anopheles* and *Culex* mosquitoes were also significantly higher ($p<0.01$) in cattle sheds (62.53±5.23 and 63.25±4.6 respectively) than human habitations (42.08±3.90 and 43.14±3.41 respectively). Whereas in both rural and urban areas *Aedes* mosquitoes showed more prevalence ($p<0.01$) in human habitations (7.25±1.24 and 7.0±1.08 respectively) than cattle sheds (3.0±0.72 and 3.33±0.83 respectively) in the study areas. There is no significant difference in the prevalence of total number *Anopheles* ($p=0.289$), *Culex* ($p=0.058$) and *Aedes* ($p=0.949$) mosquitoes between rural and urban areas. Prevalence of these three genus of mosquitoes showed positive correlation with environmental temperature and humidity. On the other hand *Anopheles* and *Culex* mosquitoes showed higher prevalence during night time in human habitations and cattle sheds in both rural and urban areas (Figure 3 & Figure 4) whereas prevalence of *Aedes* mosquitoes in night time is significantly lower than the day time in the study areas (Figure 5).

Species composition of *Anopheles* mosquitoes showed a significant difference ($p<0.01$) in different months of the year, which also differ among rural and urban regions of the study area (Figure 6 & Figure 7). Four species of *Anopheles* mosquitoes were recorded in both rural and urban areas: *An. barbirostris*, *An. subpictus*, *An. annularis* and *An. vagus*. Monthly prevalence of different species of *Anopheles* also showed a significant difference between rural and urban areas ($p < 0.01$). In case of rural areas the peak month of prevalence of *An. barbirostris* was recorded during the month of January whereas in case of urban areas the prevalence of this species was found to be very low throughout the year. *An. subpictus* was more prevalent in rural areas than urban sites. This mosquito species showed two peaks, one during June-July and another during September-October in rural areas. Prevalence of *An. annularis* and *An. vagus* found to be high in urban areas in comparison with rural areas.

During the winter season starting from November to

Table 2: Month wise prevalence of three genus of mosquitoes (*Anopheles, Culex and Aedes*) in human habitations and cattle sheds in rural areas of Hooghly District (Dusk time: 4p.m-6p.m from Jan'2018-Dec'2018)

Month	Temperature (°C)	Humidity (%)	Number of collected Mosquitoes					
			Anopheles		*Culex*		*Aedes*	
			H.H	C.S	H.H	C.S	H.H	C.S
January	18.0°C	59.3%	19	32	15	21	0	0
February	21.4°C	56.6%	56	96	28	44	05	0
March	28.7°C	60.4%	68	124	38	56	09	0
April	29.8°C	68.7%	68	82	82	120	05	07
May	30.7°C	62.5%	34	58	44	72	10	05
June	32.1°C	71.4%	44	76	56	80	16	03
July	30.8°C	73.5%	72	122	82	144	10	18
August	28.7°C	78.2%	84	136	86	132	0	0
September	29.8°C	75.0%	96	132	72	98	17	09
October	24.0°C	66.4%	48	64	56	64	21	04
November	23.2°C	66.6%	24	38	36	56	08	07
December	21.6°C	62.7%	14	22	40	46	0	0

*H.H- Human Habitation, C.S- Cattle Shed

January there is a sharp decline in the prevalence of all of the three genera of mosquitoes which reflects to the fact that there are some strong correlation between the mosquito oviposition to egg hatching with the environmental temperature.

In a field study at Gorakhpur district of Uttar Pradesh, Kanojia et al (2003) observed that there was a short peak of *Culex quinquifasciatus* in the month of March and a long peak of *Culex tritaeniorhynchus* in the month of September. They concluded that abundance of this species was related with rice cultivation as because they are mainly paddy field breeders. The present study also recorded higher abundance of *Culex* and *Anopheles* mosquitoes in the month of August, which may be due to the much availability of breeding sites during this time. Reports of several studies indicated that *Culex quinquifasciatus* as the most house frequenting mosquitoes, their prevalence in human habitations is higher than the cattle sheds (Chandra et al, 2013; Azmi et al, 2015).

Table 3: Month wise prevalence of three genus of mosquitoes (*Anopheles, Culex* and *Aedes*) in human habitations and cattle sheds in rural areas of Hooghly District (Night time: 10p.m-12a.m from Jan'2018-Dec'2018)

Month	Temperature (°C)	Humidity (%)	Number of collected Mosquitoes					
			Anopheles		*Culex*		*Aedes*	
			H.H	C.S	H.H	C.S	H.H	C.S
January	12.5°C	57.2%	16	21	15	18	0	0
February	20.1°C	58.7%	40	108	24	40	0	0
March	29.0°C	63.6%	86	146	36	112	03	0
April	30.1°C	65.4%	56	76	70	102	02	0
May	30.5°C	69.2%	48	72	56	80	0	0
June	32.2°C	78.7%	54	84	56	88	02	01
July	30.4°C	73.8%	84	146	96	172	04	02
August	28.9°C	76.5%	94	152	100	156	0	0
September	28.3°C	72.0%	112	146	88	108	04	0
October	23.2°C	60.6%	56	68	48	56	05	0
November	23.7°C	62.2%	28	46	32	44	0	0
December	19.8°C	56.9%	09	15	30	38	0	0

*H.H- Human Habitation, C.S- Cattle Shed

Table 4: Month wise prevalence of three genus of mosquitoes (*Anopheles, Culex and Aedes*) in human habitations and cattle sheds in urban areas of Hooghly District (Day time: 6a.m-8a.m from Jan'2018-Dec'2018)

Month	Temperature (°C)	Humidity (%)	Number of collected Mosquitoes					
			Anopheles		*Culex*		*Aedes*	
			H.H	C.S	H.H	C.S	H.H	C.S
January	13.8°C	55.9%	14	27	19	29	0	0
February	19.8°C	57.6%	28	40	16	36	0	0
March	28.4°C	60.1%	38	56	48	42	10	0
April	31.4°C	66.5%	32	56	40	68	8	0
May	29.6°C	66.2%	22	36	28	56	11	04
June	30.2°C	68.7%	24	46	42	56	12	03
July	29.5°C	75.2%	48	88	56	96	12	02
August	29.1°C	74.2%	66	112	82	106	12	06
September	26.7°C	75.2%	72	96	56	68	09	0
October	26.6°C	66.2%	36	48	28	36	16	08
November	24.7°C	62.4%	20	28	26	34	21	12
December	20.3°C	58.5%	08	16	25	38	0	0

*H.H- Human Habitation, C.S- Cattle Shed

Table 5: Month wise prevalence of three genus of mosquitoes (*Anopheles, Culex* and *Aedes)* in human habitations and cattle sheds in urban areas of Hooghly District (Dusk time: 4p.m-6p.m, Jan'2018-Dec'2018)

Month	Temperature (°C)	Humidity (%)	Number of collected Mosquitoes					
			Anopheles		*Culex*		*Aedes*	
			H.H	C.S	H.H	C.S	H.H	C.S
January	15.9°C	59.2%	18	29	16	36	0	0
February	23.3°C	57.8%	44	72	24	44	0	0
March	32.3°C	62.2%	58	96	28	46	0	0
April	30.5°C	62.7%	58	68	52	66	12	6
May	31.7°C	63.4%	28	48	38	62	12	8
June	33.7°C	72.3%	36	68	42	64	14	10
July	32.1°C	77.3%	64	96	72	104	8	16
August	29.6°C	76.8%	76	114	88	112	16	18
September	28.0°C	78.7%	64	84	58	88	16	09
October	27.7°C	64.6%	44	56	40	56	20	10
November	25.9°C	58.2%	24	36	32	46	04	0
December	20.7°C	55.9%	16	30	30	51	0	0

*H.H- Human Habitation, C.S- Cattle Shed

Table 6: Month wise prevalence of three genus of mosquitoes (*Anopheles, Culex* and *Aedes*) in human habitations and cattle sheds in urban areas of Hooghly District (Night time: 10p.m-12a.m, Jan'2018-Dec'2018)

Month	Temperature (°C)	Humidity (%)	Number of collected Mosquitoes					
			Anopheles		*Culex*		*Aedes*	
			H.H	C.S	H.H	C.S	H.H	C.S
January	11.6°C	58.9%	11	17	19	18	0	0
February	21.2°C	59.4%	24	36	16	28	0	0
March	30.0°C	63.5%	64	106	42	82	03	0
April	29.8°C	66.5%	52	74	62	92	09	0
May	31.9°C	66.6%	42	66	48	72	06	03
June	30.3°C	68.7%	46	66	38	72	0	0
July	28.6°C	76.2%	72	92	66	112	0	0
August	29.7°C	72.6%	84	122	92	128	08	0
September	27.8°C	75.6%	104	116	72	88	0	0
October	24.2°C	68.4%	44	64	36	48	04	05
November	23.5°C	62.5%	24	28	34	48	09	0
December	19.9°C	60.5%	10	18	42	49	0	0

*H.H- Human Habitation, C.S- Cattle Shed

The present study recorded higher prevalence of *Culex* and *Anopheles* mosquitoes in cattle sheds than human habitations in both rural and urban areas of Hooghly, West Bengal. It may be due to their resting habitat preferences in cattle sheds or zoophilic nature. As in many areas mosquito control strategy involves the control of adult mosquitoes by spraying insecticides, so it is very much necessary to have a better knowledge about the mosquito ecology, their biting behavior and resting habitat preferences.

The present study found that in the rural and urban areas of Hooghly district, West Bengal, among three mosquito genera, the abundance of *Anopheles* and *Culex* is more than the *Aedes* throughout the year. Besides this although the prevalence of *Aedes* mosquitoes was less but they prefer to rest in human habitations than cattle sheds which throw a light on their anthropophilic behavior. On the other hand *Culex* and *Anopheles* mosquitoes prefer to rest more in cattle sheds than human habitations in both rural and urban areas which indicated that they also prefer to feed cattle blood besides human blood.

Species composition of *Anopheles* mosquitoes were different in case of rural and urban areas of Hooghly. *An. subpictus* is more dominant species in the rural areas where as *An. vagus* found to be more prevalent in urban regions of Hooghly. *Anopheles subpictus* Grassi has been incriminated as a primary vector of malaria in rural areas of Hooghly, West Bengal (Chatterjee et al, 2000). The present study recorded two peak months of prevalence of this vector, one during June-July and another during September-October, which strongly coincides with the emergence of malaria in the study areas.

Figure 1: Location map of the study areas

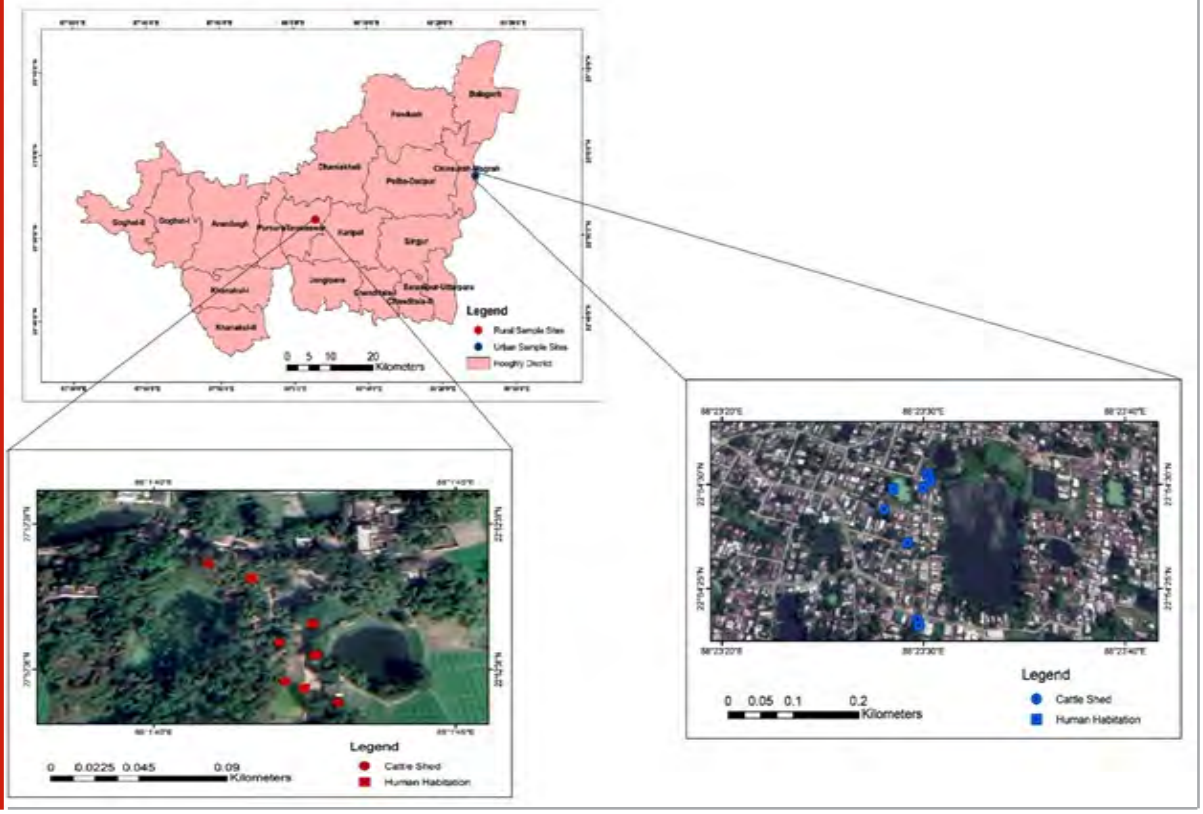


Figure 2: Distance between rural and urban sample sites

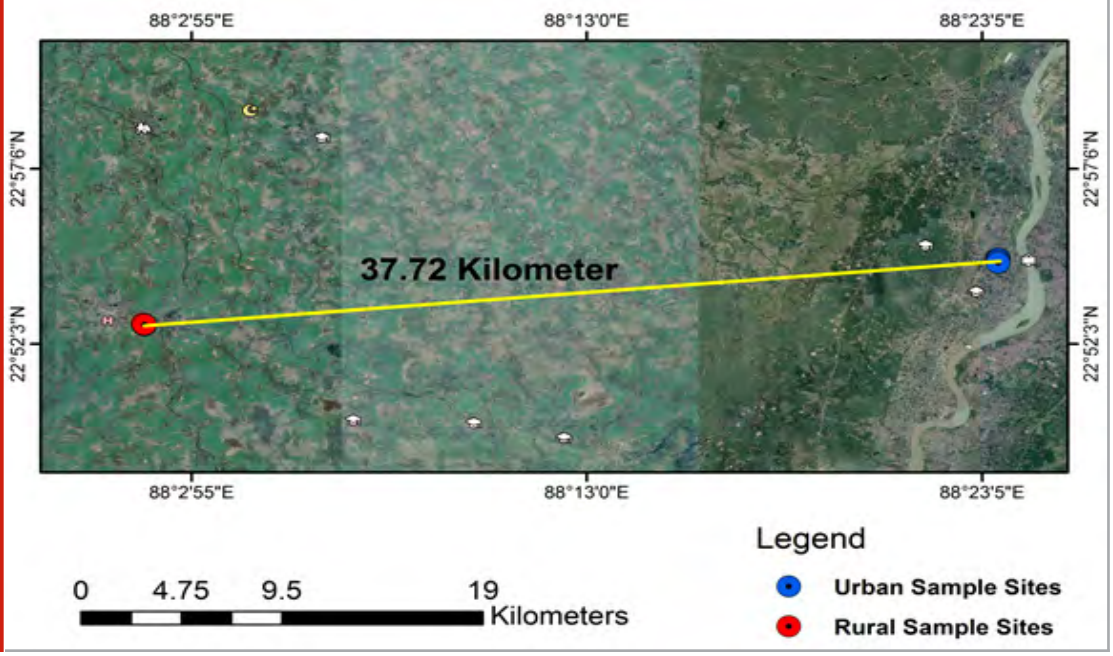


Figure 3: Percentage of *Anopheles* mosquitoes collected in three different times from Human habitations and Cattle sheds of Rural & Urban areas of Hooghly District (Jan'2018-Dec'2018).

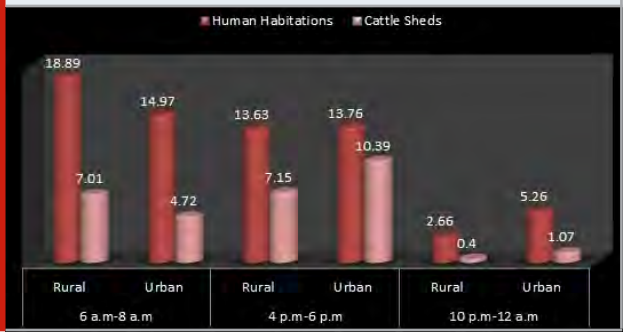


Figure 4: Percentage of *Culex* mosquitoes collected in three different times from Human habitations and Cattle sheds of Rural & Urban areas of Hooghly District (Jan'2018-Dec'2018).

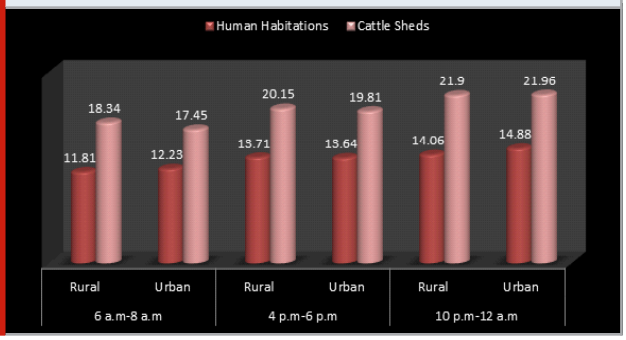
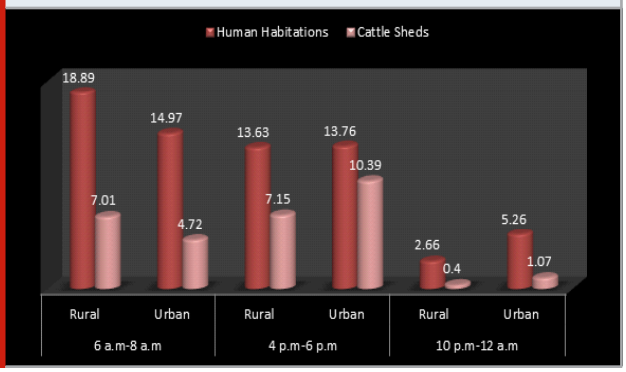


Figure 5: Percentage of *Aedes* mosquitoes collected in three different times from Human habitations and Cattle sheds of Rural & Urban areas of Hooghly District (Jan'2018-Dec'2018).



CONCLUSION

Present findings are highly significant in understanding the mosquito ecology in the study areas and to have a better knowledge on their resting habitat preferences. This study has indicated that *Anopheles* and *Culex* mosquitoes are more prevalent in cattle sheds besides human habitations. So, much care must be taken in the cattle sheds also during the application of insecticides to control the adult mosquitoes. Present study will illuminate about the resting-habitats of three genera of mosquitoes in relation to mosquito ecology so that proper management strategies may be taken with a view to prevent the mosquito borne diseases in the endemic areas of Hooghly District.

Figure 6: Month wise species composition of *Anopheles* mosquitoes in rural areas of Hooghly district (Jan'2018-Dec'2018).

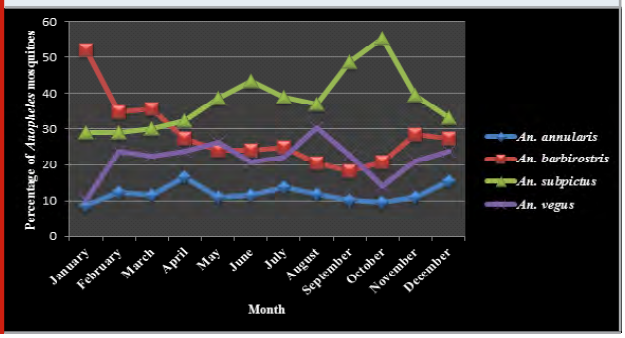
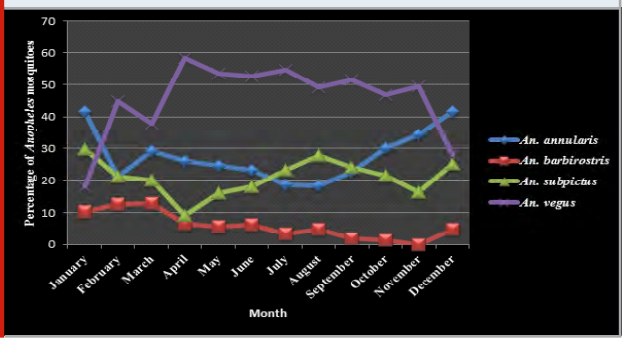


Figure 7: Month wise species composition of *Anopheles* mosquitoes in urban areas of Hooghly district (Jan'2018-Dec'2018)



ACKNOWLEDGEMENTS

The authors are thankful to University Grants Commission (UGC) for providing financial support and to The University of Burdwan for providing proper infrastructure to carry out this work.

Conflict of interest: The authors declare that they have no conflict of interest.

REFERENCES

Alten B, Bellini R, Caglar SS, Simsek FM and Kaynas S (2000). Species composition and seasonal dynamics of mosquitoes in the Belek region of Turkey. J Vector Ecol 25:146-154.

Azmi SA, Das S and Chatterjee S (2015). Seasonal prevalence and blood meal analysis of filarial vector *Culex quinquefasciatus* in coastal areas of Digha, West Bengal, India. J Vector Borne Dis 52(3):252-256.

Chandra G, Paramanik M, Mondal SK and Ghosh AK (2013). Comparative Studies of Different Indices Related

to Filarial Vector of a Rural and an Urban Area of West Bengal, India. Trop Med Surg 1(1):1000104.http://dx.doi.org/10.4172/2329-9088.1000104H

Chandra G, Chatterjee SN, Das S and Sarkar N (2007). Lymphatic filariasis in the coastal areas of Digha, West Bengal, India. Trop Doct 37(3):136-139.

Chatterjee S and Chandra G (2000). Role of *Anopheles subpictus* as a primary vector of malaria in an area in India. Jpn. J. Trop. Med. Hyg 28(3):177-181.

Chavshin AR, Oshaghi MA, Vatandoost H, Pourmand MR, Raeisi A and Terenius O (2014). Isolation and Identification of culturable bacteria from wild *Anopheles culicifacies*, a first step in a paratransgenesis approach. Parasite Vector 7(419):4-8.

Gubler DJ (2002). The global emergence/resurgence of arboviral diseases as public health problems. Arch Med Res 33(4):330-342.

Kanojia PC, Shetty PS and Geevarghese G (2003). A long-term study on vector abundance & seasonal prevalence in relation to the occurrence of Japanese encephalitis in Gorakhpur district, Uttar Pradesh. Indian J Med Res 117:104-110

Khan Y and William SJ (2017). Influence of some environmental factors on the abundance of adult mosquito species in a salt pan area near Ennore creek, Chennai. Int. J. Adv. Res. Biol. Sci 4(7):22-26. https://doi.org/10.22192/ijarbs

Lindh JM, Terenius O and Faye I (2005). 16S rRNA Gene-Based Identification of Midgut Bacteria from Field-Caught *Anopheles gambiae* SensuLato and *A. funestus* Mosquitoes Reveals New Species Related to Known Insect Symbionts. Appl. Environ. Microbiol 71(11):7217–7223.doi:10.1128/AEM.71.11.7217–7223.2005

Lindh JM, Okal MN, Herrera-Varela M, Borg-Karlson AK, Torto B, Lindsay SW and Fillinger U (2015). Discovery of an oviposition attractant for gravid malaria vectors of the *Anopheles gambiae* species complex. Malar J 14(1):119. https://doi.org/ 10.1186/s12936-015-0636-0

Mondal R, Devi NP and Jauhari RK (2015). Characterization of bacterial isolates found in breeding habitats of mosquitoes in Dehradun City, Uttarakhand. J. Adv. Res. MicroBiol 2(1):1-7.

Nagpal B and Sharma VP (1995). Indian Anophelines. Science Publishers, New York.

GIS Mapping and Breeding Habitat Characterization of Anophelines Occurring in Malaria

Seal, M., D. Pahari Nimai and C. Saha (2018) Proceedings of the National Academy of Sciences, India - Section B: Biological Sciences 89(4):1-14 DOI: 10.1007/s40011-018-0981-1

Zar JH (1999). Biostatistical Analysis. New Delhi, India: Pearson Education Singapore Pte. Ltd (Indian Branch).

Biotechnological Communication

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp-233-242

BBRC Bioscience Biotechnology Research Communications

Molecular Identification and Response Surface Methodological (RSM) Approach for Optimized Production of Amylase from *Bacillus altitudinis* GVK38

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ABSTRACT

Amylases are a class of starch degrading enzymes catalyzing the hydrolysis of internal glycosidic bonds in polysaccharides and plays a crucial role with potential industrial and commercial applications. In the present study five bacterial isolates have been evaluated for the production of extracellular amylase and one potent isolate was selected based on maximum starch hydrolysis. *Bacillus altitudinis* (KY777585) has been used for the production of amylase under submerged fermentation. *B. altitudinis* GVK38 showed maximum enzyme production of 728 U/mL at 45°C for 48 hours at pH 9.0. Further enhanced production was obtained by supplementing 1.0 % maltose as carbon source, 1.5 % Yeast extract as nitrogen source and 2.0 % salt concentration. The results of RSM reveals that F-value of 1.44, Lack of Fit F-value of 0.33, coefficient of determination (R2) for enzyme activity calculated as 0.9231, value of the adjusted determination coefficient, R2 was 0.2822. RSM depicts that the optimal level of the significant variables for the maximum amylase production were: Incubation period 72 hrs, pH – 8.00, temperature 45°C, NaCl Conc. -1.25%, maltose conc. – 1.25% and yeast extract conc. – 1.25%. The results of the study show that the isolate can be further exploited for commercial production of amylase.

KEY WORDS: AMYLASE, *BACILLUS ALTITUDINIS*, 16S rRNA, RSM, SUBMERGED FERMENTATION.

ARTICLE INFORMATION

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Received 7th Feb 2020 Accepted after revision 15th March 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)
A Society of Science and Nature Publication, Bhopal India

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/40

INTRODUCTION

Enzymes are biological catalysts, which regulate specific biochemical reactions. They speed up a reaction without being used up in the reaction. In recent past, chemical catalysts had been replaced by enzymes in various industrial applications (Keshavamurthy et al., 2018). Amylases are enzyme which hydrolyses starch molecules to give diverse products including dextrin and progressively smaller polymers composed of glucose units. Among the industrially important enzymes, amylases are considered to be the most prominent enzyme due to its wide area of potential application. Amylases are used in detergents, textiles, starch, baking and animal feed are the main industries, which use about 75% of industrially produced enzymes (Panneerselvam and Elavarasis, 2015). To meet the higher demands of these industries, low cost production of amylase is necessary. *Bacillus sp*. are one among the industrially important microorganisms used due to their rapid growth rates that lead to shorter fermentation cycles, their capacity to secrete proteins into extra cellular medium and general handling safety (Pandey 2000). In recent years, the potential of using microorganisms as biotechnological sources for the production of industrially relevant enzymes has stimulated interest in the exploration of newer and potential isolates (Alva et al., 2007). Amylases are widely distributed in nature and can be derived from various sources such as plants, animals and microorganisms (Reddy et al., 2003; Gopinath et al., 2017, Keshavamurthy et al., 2019).

Microbial enzymes have been generally favored for their easier isolation in high amounts, low-cost production in a short time, and stability at various extreme conditions, and their compounds are also more controllable and less harmful (Pandey et al., 2000). Amylases, biosynthesized by the bacteria, show unique characteristics such as thermophilic, thermotolerant, alkaline and acidophilic properties (Konsoula and Liakopoulou-Kyriakides 2007). The major advantage of using microorganisms for the production of amylases is the economical bulk production capacity and the fact that microbes are easy to manipulate to obtain enzymes of desired characteristics. Many microorganisms used in α-Amylases and β-amylases production include *Bacillus subtilis, B. cereus,* B. polmyxa*, B. amyloliquefaciens, B. coagulans, B. subtilis, Lactobacillus, Escherichia, Proteus, B. lincheniformis, B. steriothermophilu, B,megaterium, Strepotmyces sp., and Pseudomonas sp*. etc., (Gupta et al., 2003; Elmansy et al., 2018, Keshavamurthy et al., 2019).

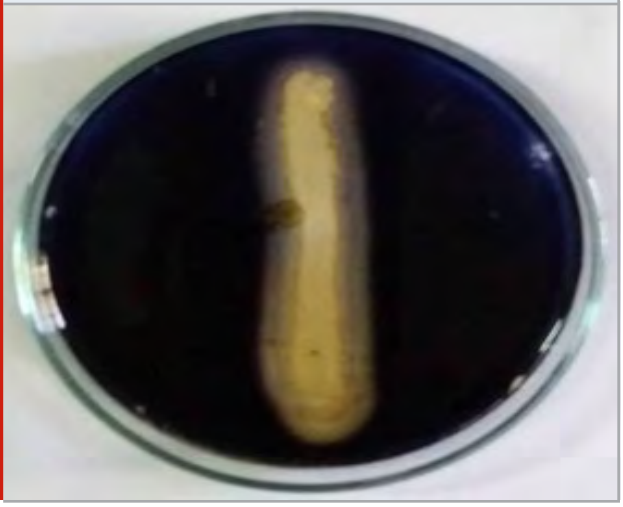
Amylase substrates are widely available from cheap plant sources, rendering the potential applications of the enzyme more plentiful in terms of costs. To obtain maximum yield of an enzyme, development of a suitable medium and culture conditions is obligatory (Srivastava and Baruah, 1986). Starch or other sugars as a carbon source and ammonium salts or complex organic compounds as a nitrogen source are needed for bacterial growth and enzyme production (Ellaiah, 2002). Optimization of the various parameters and manipulations of media are one of the most important techniques used for the enhanced production of amylase in large quantities. To meet industrial demands, production of amylase in bacteria is known to depend mostly on metabolic state of the culture. Various physical and chemical factors such as temperature, pH, incubation period, carbon and nitrogen sources have been known to affect the production of amylase (Gangadharan, 2008).

The production of amylase by submerged fermentation (SMF) and solid-state fermentation (SSF) has been thoroughly investigated by many researchers and is affected by a variety of physiochemical factors. To meet the growing demands in the industry, it is necessary to improve the performance of the system and thus increase

Table 1. Morphological and biochemical characteristics of *B. altitudinis* strain GVK38

Morphological Characteristics	Results
Gram staining	Positive rods
Colour	Creamish white
Motility test	Motile
Spore	Spore former
Physiological characteristics	
Catalase	Positive
Indole	Negative
Methyl red	Negative
Voges Proskauer	Positive
Citrate utilization	Positive
Oxidase reaction	Positive
Casein hydrolysis	Positive
Gelatin liquefaction	Positive
Starch hydrolysis	Positive
Nitrate reduction	Positive
Growth at 4°C	-
Growth at 45°C	+

Figure 1. Starch hydrolysis on starch agar plate by bacterial isolate *B. altitudinis* GVK38.



the yield without increasing the cost of production. Among the wide range of microbial species that secrete amylase, its production from bacteria is cheaper and faster than other microorganisms. The statistical experimental designs and mathematical methods have wide application in the field of microbial biotechnology. Response surface methodology (RSM) is one such method that is applied for modelling problems with the aim to optimize responses that were influenced by multiple variants. Performing statistically designed experiments, filling experimentally determined response data into a quadratic model, predicting response and checking significance of the model are the major steps involved in this process (Keshavamurthy et al., 2019).

RSM is advantageous for industrial purposes as it requires fewer numbers of experimental trials for prediction and quantification of combined interactions between the variables and hence eases the process of optimization. The 3D plots for response surface enables visualization of parameter interaction and it was often applied to satisfactory optimization of microbial enzyme production (Keshavamurthy et al., 2019). Hence to overcome all these challenges and to meet the demand a study was undertaken with the main aim of isolating the potent strain from the extreme environment and to use it in the production of amylase under submerged fermentation. Further the optimization of process parameter and media constituents were performed for enhanced production.

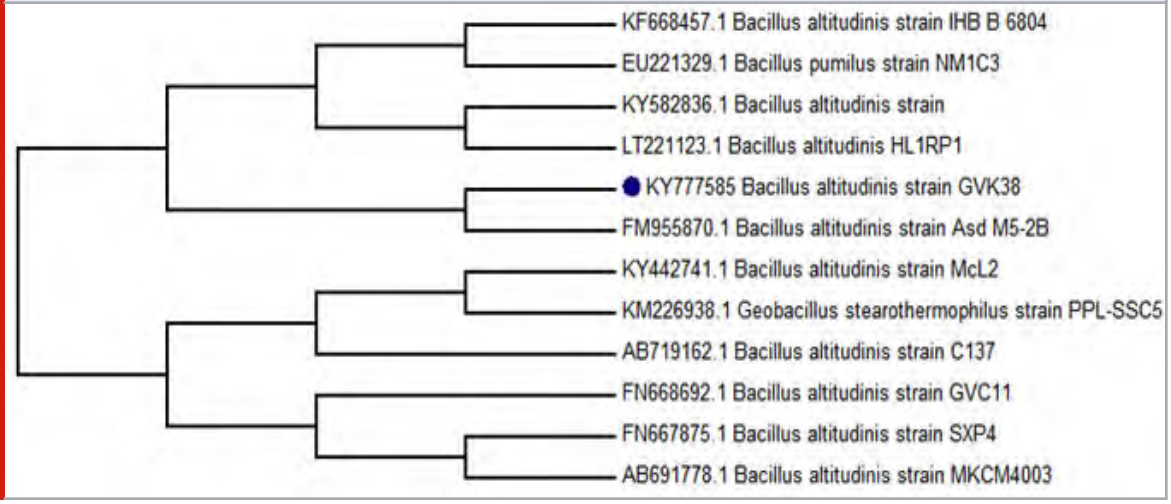
MATERIALS AND METHODS

Sample collection and Isolation of Bacteria: Soil sample was collected from the Hutti gold mines (16° 11' 45" North Latitude and 76° 38' 31" East Longitude), Raichur district of Karnataka state, India. The bacteria were isolated by serial dilution plating method. The samples were inoculated onto nutrient agar plates and incubated at 37°C for 24 h. The colonies obtained after incubation were further sub cultured and preserved under 4°C for further use. Screening and selection of strain for Optimization:The isolates were screened for amylase production bacterial colonies were screened on starch agar medium [g/L: Starch -10.0, Peptone - 5.0, NaCl - 2.0, MgSO4 - 0.2, Agar – 20.0, and pH 9.0. Inoculated plates were incubated at 37 °C for 24 h. After incubation, the plates were flooded with iodine solution [g/L: Potassium iodide - 2.5, Gram's iodine - 0.125]. Amylase positive bacterial strains were identified and recorded based on the clear zone formation around the bacterial growth. Out of five isolates, one strain of bacteria which produced maximum clear zone of hydrolysis for extracellular amylase, which was selected for further experimental studies.

Molecular identification of bacteria: Amylase positive strain *Bacillus sp*. GVK 38 was subjected for basic microscopic, biochemical, physiological and cultural characterization based on biochemical characteristics as per Bergey's Manual of Systematic Bacteriology (Niall and Paul, 2009) The bacterial isolate was further identified by 16S rDNA sequence analysis using universal primers and genomic DNA as template. The genomic DNA of the isolate was extracted as described by (Roohi et al., 2012). The PCR amplified product was sequenced at Microbial Ecology Laboratory, National Centre for Cell Science, Pune. After DNA sequencing, the obtained results were subjected to BLAST analysis to compare with the sequence similarities. Phylogenetic tree was constructed with MEGA 6.0 software using neighbor-joining method (Tamura et al., 2011). Duly annotated partial nucleotide sequences of the novel bacterial strain was deposited with NCBI Genbank and accession number was obtained.

Production of amylase by submerged fermentation: Amylase producing bacteria was grown on the starch production media [g/L: Peptone – 5.0, Starch 10.0, NaCl-2.0, MgSO4-0.2, pH 9.0, incubated on a rotary shaker for 48 hours at 37°C. Enzyme was extracted by centrifuging the incubated broth at 8,000 rpm for 10 min at 4°C. Supernatant was used as crude enzyme

Figure 2. Phylogenetic analysis of 16S rDNA gene sequence data of isolate *B. altitudinis* GVK38 and of a number of related strains



source. The experiment was carried out in 250 mL plugged Erlenmeyer flasks, each containing 100 mL sterile starch broth medium and inoculated with 1% of standard inoculum (2.3 × 106 CFU ml–1) for the tested bacterial isolate which was incubated at 45 °C on rotary shaker at 160 rpm for 48 h. The fermented medium was centrifuged at 10,000 rpm for 10 min in order to determine periodically the cell dry weight and amylases activity in the precipitate and supernatant, respectively (Yassien and Asfour, 2012).

Enzyme Assay: The amylase assay was measured by following the methodology proposed by Bernfeld (1955). Amylase activity was assayed in the reaction mixture containing 0.5 mL enzyme, 1mL of 1% starch as substrate, 1mL phosphate buffer and incubated at room temperature for 15 minutes. The reaction was arrested by adding 1mL DNS reagent. The inactivated reaction mixture was incubated on water bath for 10 minutes and, made up to 10 mL and absorbance was measured at 540 nm. Blank was prepared by immediate addition of 1mL DNS reagent to 0.5 mL enzyme, followed by the addition of 1mL starch and 1mL phosphate buffer. One unit of amylase activity is defined as the amount of enzyme required to release 1µg of reducing sugar (maltose) per ml per min under the above assay conditions.

Optimization of culture conditions and medium components for amylase production: One Variable at a Time Approach (OVAT): Optimization of production of amylase by *Bacillus altitudinis* GVK38 was checked by varying the following physical and chemical parameters using one-variable-at-a-time-approach: Amylase production by *B. altitudinis* GVK38 was carried out in basal medium with different combinations of carbon and nitrogen sources (1% w/v) and 1% inoculum size at 37 °C for 72 h in a rotary shaker (120 rpm). The initial pH of the medium was adjusted to 7.0. Parametric optimization was performed with respect to incubation period (24 to 120 h), pH (6 to 10), temperature (30 to 60oC) were studied by using various levels of the test parameter and keeping the other parameters constant. The bacterium was grown in the production medium supplemented with different carbon sources (1% w/v) such as arabinose, fructose, glucose, lactose and maltose and nitrogen sources (1% w/v) like peptone, yeast extract, beef extract and ammonium chloride. At the end of the fermentation, the fermentation broth was centrifuged at 8,000 rpm for 10 min. The supernatant obtained by the centrifugation was used for measuring the amylase activity. The un-inoculated flasks served as controls. All the experiments were done in triplicates and the average of enzyme activity was taken for statistical analysis to know the significance of each factor.

Optimization of amylase production using Response surface methodology approach: Response surface methodology (RSM) is a well-accepted statistical technique which is able to design and optimize the experimental process that involves choosing the optimal experimental design and estimate the effect of the several factors independently and also their interactions simultaneously. RSM combined with Central Composite Design (CCD) was established using Design Expert software (Version 11.0, Stat-Ease Inc., Minneapolis, USA) to analyze and plot the response surface graphs. Five factors, namely, pH, temperature, maltose, yeast extract and NaCl were optimized for enhanced production of amylase using the isolate *B. altitudinis* GVK38. Based on CCD, the factors were analysed at two levels: -1, for low level, and +1, for high level. A total of twenty-nine (29) runs were performed to optimize the process parameters, and experiments were performed according to the experimental design matrix. The results were evaluated by applying the coefficient of determination (R2), analysis of variance (ANOVA) and response plots. Employing

Figure 3. Effect of incubation period on amylase production

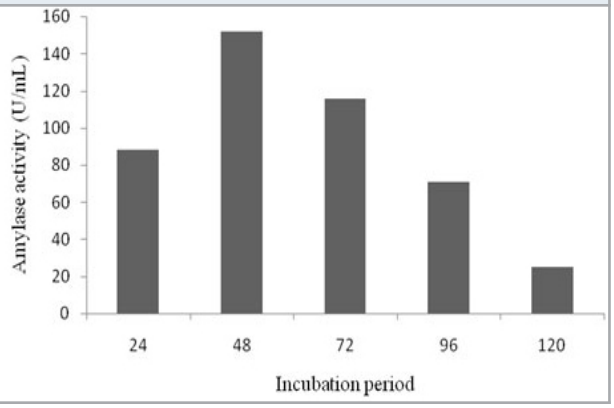


Figure 4. Effect of pH on amylase production.

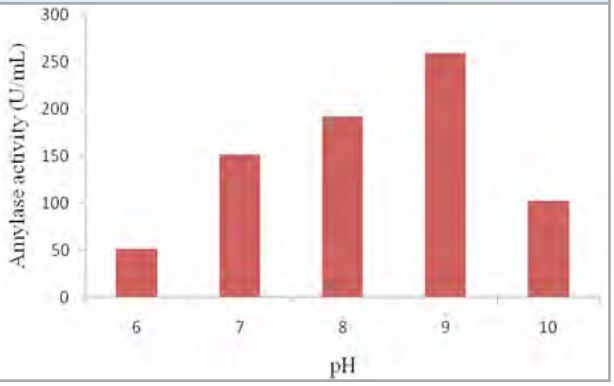
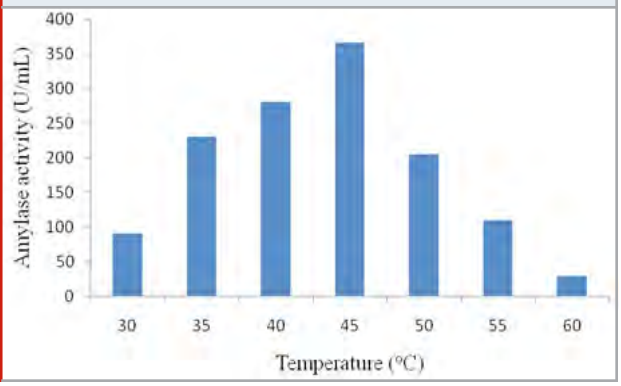


Figure 5. Effect of temperature on amylase production.



RSM, the most widely used second-order polynomial equation was developed to fit the experimental results and identify the relevant model terms:

$$Y=\beta 0\ \Sigma\beta\iota Xi+\Sigma\beta\iota X\beta ij+\Sigma XiXj \quad (1)$$

where Y is the predicted response; b0, bi, and bij are constant regression coefficients of the model; and Xi and Xj represent independent variables. The experimental design helps in investigating linear, quadratic and cross product effects of these factors and also centre points for replication (Kathiresan and Manivannan, 2006).

RESULTS AND DISCUSSION

In the current study, 52 individual bacterial isolates were isolated from the soil sample collected from mining area. Amylase producing ability of five *Bacillus species* were checked on starch media and all were found to be positive, but *Bacillus altitudinis* GVK 38 was found to be the best amylase producer (fig. 1). Verma et al., (2011) found the maximum amount of amylase production in B. subtilis followed by *B. megaterium, among nine strains tested which included B. cereus, B. megaterium and B. subtilis*. But later during screening it was found that only three strains showed amylase activity on agar plate. The maximum amylase producing *Bacillus altitudinis* GVK 38 was taken for optimization studies through submerged fermentation by varying the temperature, pH, incubation period, carbon and nitrogen source, since the production of amylase enzymes are influenced by diverse physico-chemical and biological factors (Raj and Hemashenpagam, 2012). The strain GVK38 is gram positive rod, motile, spore former and was tentatively identified as Bacillus sp. based on its morphological and biochemical characteristics (Table 1). Identification of selected *Bacillus altitudinis* GVK 38 strain was identified on the basis of standard biochemical tests according to Bergey's Manual of determinative Bacteriology. The occurrence of amylolytic organisms from the soil agrees with earlier reports of Rehana et al., (1989).

The comparison of the 16S rRNA gene nucleotide sequence (1465 bp) of the strain *Bacillus altitudinis* GVK 38 with other 16S rRNA genes sequences of closely related strains from NCBI database showed that this isolate has 99 % sequence homology with *B. altitudinis* Asd M5-2B (Accession No. FM955870) (fig. 2). The phylogenetic tree, constructed by the neighbor-joining method indicated that the strain *B. altitudinis* GVK38 is affiliated with the genus *Bacillus* and closely related to B. altitudinis strain IHBB 6804 - Accession No. KF668457. The obtained nucleotide sequence of *B. altitudinis* GVK 38 was submitted to GenBank database and the accession number assigned is KY777585 (https://www.ncbi.nlm.nih.gov/ nuccore/KY777585). The enzyme production by the bacterial strain *B. altitudinis* GVK38 was studied in submerged fermentation. The significant amylase yield was obtained for the incubation period 48 h. It was observed that the enzyme production from the bacterium was found maximum at 48 h (152 U/mL). An increase in the enzyme production was observed from 24 h to 48 h. After 48 h of incubation a decreasing trend of enzyme activity was observed (fig. 3). The results are similar to the study conducted on α-amylase production by B. altitudinis in shake flask for different intervals of time (0 to 144 h) (Kumar et al., 2014). The production of enzyme was reached maximum at 48 h after inoculation. Further increase in incubation period however, did not show any significant increase in enzyme production rather it was decreased. This is because the cells would have reached decline phase with lowered enzyme synthesis. It might be also due to the depletion of the nutrients, death phase of organism or due to the production of amylase in the medium. This result was also supported by Oyeleke and Oduwole (2009) where the optimum incubation period on the yield of amylase enzyme was found at 48 h. Our result was also in agreement with the similar findings obtained for production of α-amylase from B. amyloliquefaciens (Gangadharan et al., 2008).

The result revealed that after 48 h incubation decreased in enzyme yield might be due to the denaturation of enzyme caused by interaction with other components in the medium. The pH of the production medium plays an important role in microbial growth and hence influences the enzyme production. Previous reports on amylase production indicate greater influence by pH (Parbat and Singhal, 2011; Sankaralingam et al., 2012). In the present investigation the enzyme activity for different pH ranging from 6.0-10.0 were determined by keeping the

Figure 6. Effect of sodium chloride on amylase production.

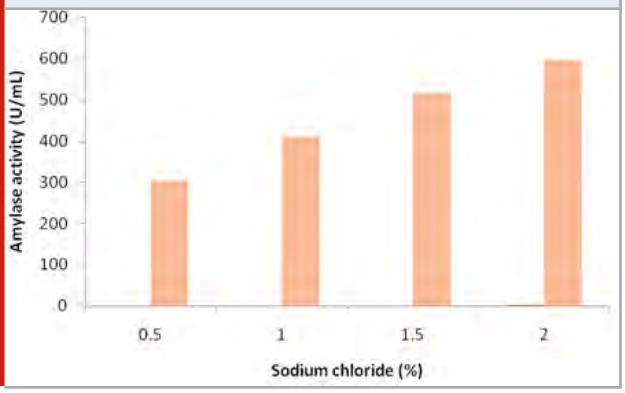
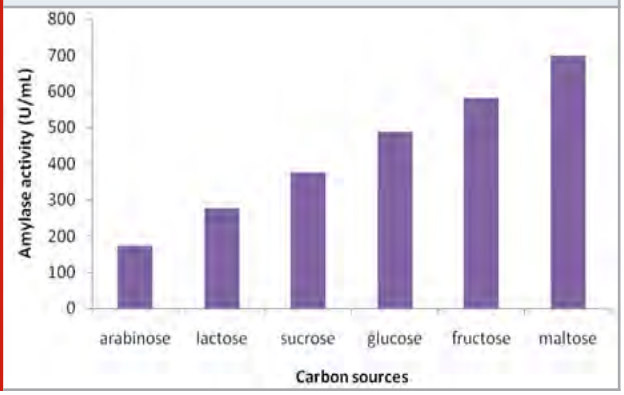


Figure 7. Influence of different carbon sources on amylase production.



optimum incubation period and temperature conditions. The maximum amylase production was found at pH 9.0. Further increase in the pH decreased the activity of amylase. Amylase production by *B. altitudinis* GVK38 was found to be maximum at pH 9.0 (259.5 U/ml) (fig.4). When pH is altered below or above the optimum, the activity appears to be decreased or becomes denatured (Devi et al., 2012). Different organisms have different pH optima and decrease or increase in pH on either side of the optimum value results in poor microbial growth (Sankaralingam et al., 2012).

In this present work the amylase activity was studied for the temperature from 30°C - 60°C. It was observed in the present study that the maximum enzyme production from the bacterium was found to be maximal after 48 hours of time at 45°C (367 U/ml) (fig. 5). Previous report on optimum production of amylase by B. marini at 40°C indicates that as the temperature increased or decreased, there was gradual decrease in the enzyme activity (Ashwini et al., 2011). At 60°C, the production of amylase was extremely low. This might be due to inhibition of bacterial growth at high temperature and hence, enzyme formation was also prohibited. Similar finding was also reported by Riaz et al., (2003) when study was carried out on production of amylase by B. subtilis GCUCM-25 at 30 - 60°C. The production of the enzyme decreased with increased temperature. The effect of temperature on activity of amylase produced by B. megaterium was found to be maximum at 40°C followed by a sharp decrease in amylase activity at 50°C reported by Oyeleke and Oduwole (2009) which is similar to the present study. Jogezai et al., (2011) also reported the maximum production of α-amylase at 40°C by using B. subtilis. As the incubation temperature was increased, the production of the enzyme was decreased. Liu and Xu, (2008) reported that the strain *B. aquimaris* VITP4 exhibited maximum enzyme production at 40°C.

Although growth was observed in the temperature range 30°C to 60°C, it was found maximum at 40°C indicating one-to-one correlation between enzyme production and biomass, which clarifies that the enzyme production is growth dependent. Sodium chloride (NaCl) is an important nutrient factor for growth and physiological activities. Various concentrations of NaCl such as 0.5%, 1.0%, 1.5%, and 2.0% were used to supplement the production media. Among the various concentrations the maximum amylase production (596 U/mL) was induced when the media was supplemented with 2.0 % (fig. 6). Vijayabaskar et al., (2012) reported 3% NaCl concentration was suitable for the amylase production . Ashwini et al., (2011) reported enzyme production at different concentrations of NaCl and found optimum enzyme yield at 4.5% NaCl concentrations. Further there was gradual decrease in enzyme production as the NaCl concentration was increased or decreased. Kokab et al., (2003) reported amylase production from B. subtilis having medium containing 2.0 % concentration of NaCl.

The effect of carbon sources on amylase production from *B.altitudinis* GVK38 was studied using different carbon sources such as arabinose, fructose, glucose, lactose, maltose and sucrose (1%) as supplement in the production media. The maximum amylase production was found when production medium was supplemented with maltose (698.5 U/mL) followed by fructose (582 U/mL) (Fig. 7). Least growth and amylase production have been recorded by lactose and arabinose. The addition of carbon source in the form of either monosaccharide or polysaccharides may influence the production of amylase enzyme. In this present study, the influence of maltose was found best carbon source than the other carbon sources. Similar finding was observed by Ashwini et al., (2011) when amylase production was optimized using different sugars at 1% (w/v) concentration. *B. marini*

Figure 8. Effect of different concentration of maltose on amylase production.

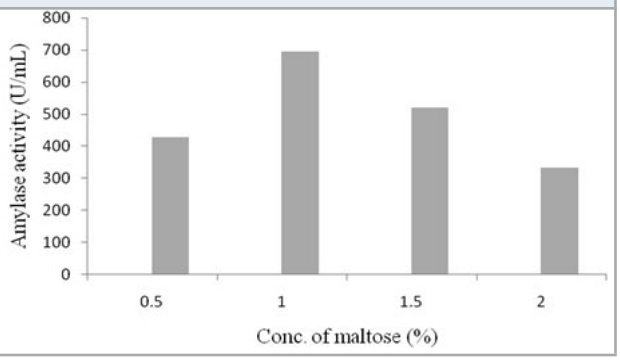


Figure 9. Influence of different nitrogen sources on amylase production

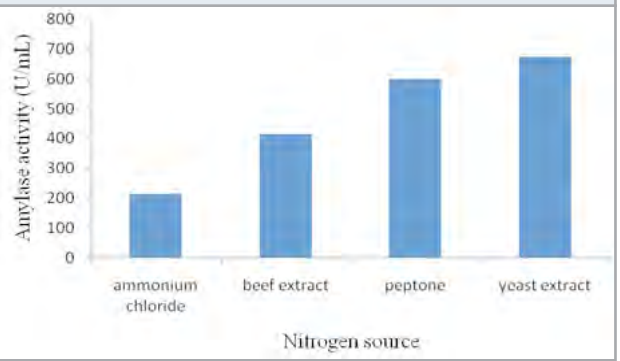
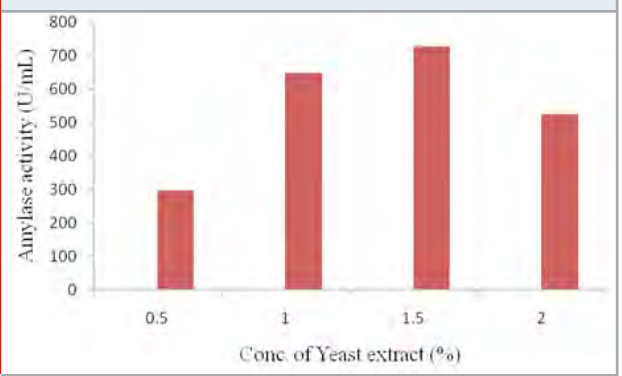


Figure 10. Effect of different concentration of yeast extract on amylase production.

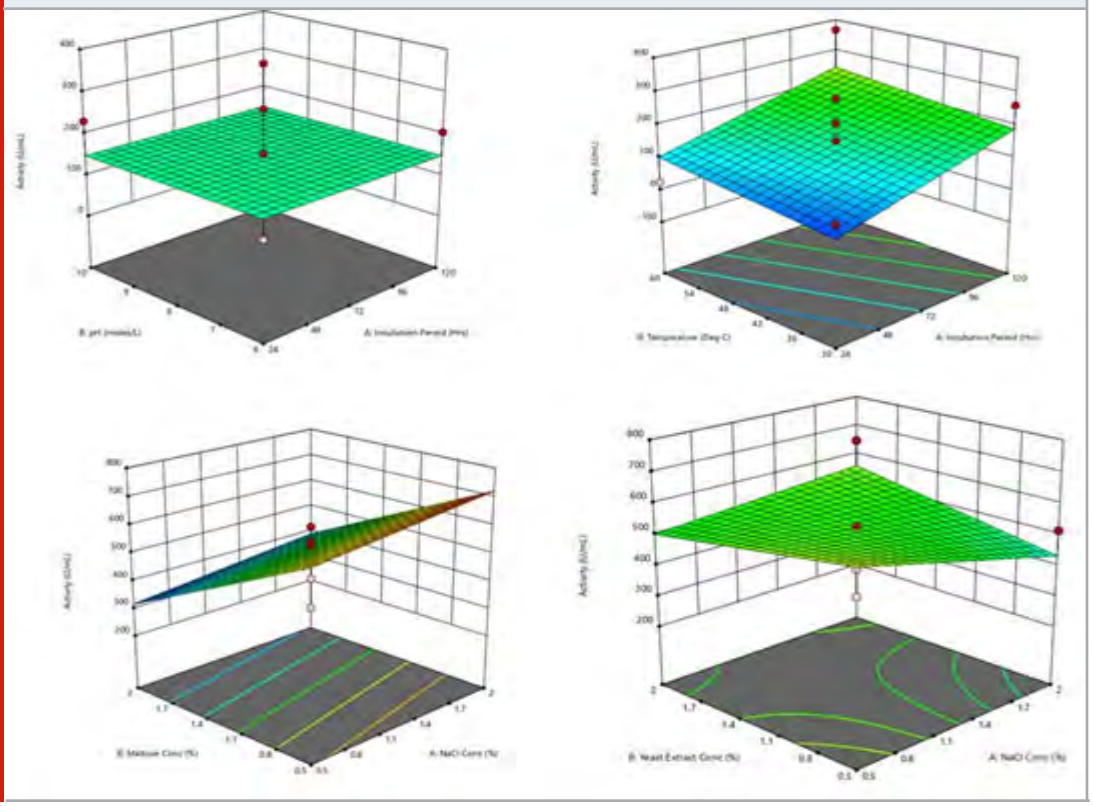


showed the maximum enzyme activity in the presence of starch as carbon source, whereas, the minimum enzyme activity was observed in the presence of dextrose. The decrease in the production of enzyme with other carbon sources may be due to catabolite repression. The finding in the present study was in agreement with earlier report where starch was observed as the best carbon source utilized by the organism Rameshkumar and Sivasudha, (2011). Similar result was also found by Goyal et al., (2005) that the soluble starch as the best carbon source supplement for amylase production by *B. lichenifomis* and *Bacillus sp*.I-3.

In the present study the supplementation of nitrogen sources on amylase production showed that yeast extract was found to be a better nitrogen source for the production of amylase (728 U/mL) (fig. 9). Yeast extract was the best nitrogen source for amylase production, probably due to its high content in minerals, vitamins, coenzymes and nitrogen components and when yeast extract was used as nitrogen source for *B. stearothermophilus* and *S. albidoflavus* respectively (Narayana and Vijayalakshmi, 2008; Roohi et al., 2011). The improvement of the nutritional value in the medium by the supplementation of organic and inorganic sources will also improve the growth of the bacterial culture and subsequently in the enzyme production. Similarly, enzyme production was more efficient in medium containing organic nitrogen sources, especially yeast extract as compared with inorganic nitrogen sources (Santos and Martins, 2003; Sourav et al., 2011). Optimization using Response Surface Methodology (RSM): To examine combined effect of the independent variables starch (A), Incubation period (B), pH (C), Temperature (D), NaCl Conc. (E) Maltose conc. (F) Yeast extract on the activity of amylase from Bacillus altitudinis GVK38 under submerged fermentation, an experiment of 54 runs were designed and performed with an incubation period of 120 hrs. The Model F-value of 1.44 implies the model is not significant relative to the noise. There is a 43.58% chance that an F-value this large could occur due to noise. P-values less than 0.0500 indicate model terms are significant. In this case there are no significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The Lack of Fit F-value of 0.33 implies the Lack of Fit is not significant relative to the pure error. There is a 77.76% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good. The coefficient of determination (R2) for enzyme activity was calculated as 0.9231.

This indicated that the statistical model explained 92.31% of the variability in response and only 07.69% of variance was not explained by the model. Value of R2 near to 1.0 indicated that the model was strong and it can predict the response in a better and this supports our results (Box et al., 1978; Keshavamurthy et al, 2019). The adjusted R2 value corrects the R2 value for the sample size and for the number of terms in the model. The value of the adjusted determination coefficient, R2 was 0.2822. This implied a higher significance of the model applied for analysing the data (Cochran and Cox, 1957; Khuri and Cornell 1987). In the present study, the adjusted R2 value (0.2822) was lesser than the R2 value (0.9231). The smaller value of

Figure 11. Response surface plots of combined effects of two variables on the production of amylase.



adjusted R2 as compared to that of the R2 may be due to the presence of multiple terms but small sample size. The interactive effects of independent variables on enzyme were studied by plotting 3D surface curves. The 3D curves of the calculated enzyme activity for the interactions between the variables are shown in Fig. 11. Optimal level of the significant variables for the maximum amylase production were: Incubation period 72 hrs, pH – 8.00, temperature 45°C, NaCl Conc. - 1.25%, maltose conc. – 1.25% and yeast extract conc. – 1.25%.

RSM mediated optimization of amylase production from *Bacillus sp*. has previously been reported by many researchers. This method was previously used to evaluate the effect of pH, temperature and inoculum size on production of amylase from *Bacillus sp* by applying a full factorial central composite design (Zambare, 2011). In another report by Tanyildizi et al., (2005) on optimization of α-amylase production by *Bacillus sp* using RSM, combined interaction of starch, glycerine, peptone and yeast extract on the production of enzymes was evaluated. Optimized level of variables for the maximum α-amylase yield from Bacillus subtilis 168 were starch 2.55 g/l, yeast extract 8.4 g/l, sodium chloride 8.1% and 48 h of incubation (Samreen, 2011).

CONCLUSION

Bacillus altitudinis GVK38 (KY777585) isolated from mining environment showed a good amylolytic activity. The optimum yield of amylase was found at 40°C and 48 h and pH 9.0 under submerged fermentation using OVAT approach. Among the different carbon sources, starch supported highest amylase production followed by maltose under submerged fermentation. Yeast extract supported maximal amylase production from different nitrogen sources. A NaCl (2.0 %) concentration was observed for highest amylase production under submerged fermentation. Amylase produced by B. altitudinis GVK 38 was found to be potent since it could be active at a wide range of pH, temperature, carbon, nitrogen sources and sodium chloride concentrations under submerged fermentation. Therefore the selected strain of *B. altitudinis* GVK38 could be beneficial and further can be exploited for industrial purposes at large scale production.

ACKNOWLEDGMENTS

Authors wish to acknowledge Dr. Yogesh Souche, Scientist, National Centre for Cell Science, Govt of India, Pune for sequencing and identification of Bacteria and Dr. Suresh Prabhu for RSM analysis.

Conflict of Interest: Authors declare that they have no conflict of interest in the publication.

REFERENCES

Alva, S., Anupama, J., Savla, J., Chiu, Y.Y., Vyshali, P., Shruti, M., Yogeetha B.S., Bhavya, D., Purvi, J., Ruchi, K., Kumudini, B.S., 2007. Production and characterization of fungal amylase enzyme isolated from Aspergillus sp. JGI 12 in solid state culture. African Journal of Biotechnology, 6, pp. 576-582.

Ashwini, K., Gaurav, K., Karthik, L., Bhaskara Rao, K.V., 2011. Optimization, production and partial purification of extracellular α-amylase from *Bacillus sp*. marini. Archives of Applied Science Research, 3, pp. 33-42.

Bernfeld, P., 1955. Amylases: and Method in Enzymology. Academic Press USA. 1: 149.

Box, G.E., Hunter, W.G., Hunter, J.S., 1978. Statistics for experimenters. John Wiley & Sons. New York.

Cochran, W.G., and Cox, M.E., 1957. Experimental design. Second edition. John Wiley and Sons. New York.

Devi, B., Unni, B.G., Wann, S.B., and Samanta, R., 2012. Immobilization of Partially Purified Alpha Amylase Enzyme Produced by Soil Borne *Bacillus sp*. Advances in applied Science research, 3, pp. 2739-44.

Elmansy, E.A., Asker, M.S., El-Kady, E.M. 2018. Production and optimization of α-amylase from thermo-halophilic bacteria isolated from different local marine environments. Bulletin of the National Research Centre, 42, pp. 31

Ellaiah, P., Adinarayana, K., Bhavani, Y., Padmaja, P., Srinivasulu, B., 2002. Optimization of process parameters for glucoamylase production under solid state fermentation by a newly isolated *Aspergillus species*. Process Biochemistry, 38, pp. 615-620.

Gangadharan, D., Sivaramakrishnan, S., Nampoothiri, K.M., Sukumaran, R.K., Pandey, A., 2008. Response surface methodology for the optimization of alpha amylase production by *Bacillus amyloliquefaciens*. Bio resource technology, 99, pp. 597-602.

Gopinath, S.C., Anbu, P., Arshad, M.K., Lakshmipriya, T., Voon, C.H., Hashim, U., Chinni, S.V., 2017. Biotechnological processes in microbial amylase production. BioMed research International, 5, pp. 235-245.

Goyal, A., Bandopadhyay, R., Sourdille, P., Endo, T.R., Balyan, H.S., Gupta, P.K., 2005. Physical molecular maps of wheat chromosomes. Functional and Integrative genomics. 5, pp. 260-3.

Gupta, R., Gigras, P., Mohapatra, H., Goswami, V.K., Chauhan, B., 2003. Microbial α-amylases: a biotechnological perspective. Process biochemistry, 38:1599-616.

Jogezai, N., Raza, A., Abbas, F., Bajwa, M., Mohammad, D., Kakar, W., Saeed, M., Awan, A., 2011. Optimization of cultural conditions for microbial alpha amylase production. Journal of Microbiology and Antimicrobials, 3:pp. 221-7.

Kandra, L., 2003. α-Amylases of medical and industrial importance. Journal of Molecular Structure: Theochem, 666, pp. 487-98.

Kathiresan, K., and Manivannan, S., 2006. Amylase production by Penicillium fellutanum isolated from mangrove rhizosphere soil. African Journal of Biotechnology, 5(10).

Keshavamurthy, M., Vishwanatha, T., Suresh Kumar, M., and Subhaschandra, M Gaddad., 2018. Enhanced production of alkaline protease from novel bacterium *Bacillus cereus* GVK21 under submerged fermentation. Bioscience and Biotechnology Research Communication, 11(3), pp. 416-425.

Keshavamurthy, M., Vishwanatha, T., Suresh Kumar, M., Subhaschandra, M. Gaddad., 2019. Enhanced Production of Extracellular Alkaline Protease by *Bacillus cereus* GVK21 by Optimized formulations. International Journal of Pharmacy and Biological Science, 9(1), pp.1103-1113.

Khuri, A.I., and Cornell, J.A., 1987. Response Surfaces: Design and Analysis. New York USA.

Konsoula, Z., and Liakopoulou-Kyriakides, M., 2007. Co-production of α-amylase and β-galactosidase by *Bacillus subtilis* in complex organic substrates. Bio Resource Technology. 98, pp.150-157.

Kokab, S., Asghar, M., Rehman, K., Asad, M.J., Adedyo, O., 2003. Bio-processing of banana peel for α-amylase production by *Bacillus subtilis*. International Journal of Agriculture and Biosciences. 5, pp. 36-9.

Kumar, D.M., Rejitha, R., Devika, S., Balakumaran, M.D., Rebecca, A.I., Kalaichelvan, P.T., 2012. Production, optimization and purification of lipase from *Bacillus sp*. MPTK 912 isolated from oil mill effluent. Advances in Applied Science Research, 3:pp. 930-8.

Kumar, Y., Singh, P.K., Singh, A.K., Masih, H., Peter, J.K., Benjamin, J.C., Rath S., 2014. Production optimization of alpha amylase from *Bacillus altitudinis*. International Journal of Scientific engineering & Technology Research, 3, pp. 0564-73.

Liu, X.D., and Xu ,Y., 2008. A novel raw starch digesting α-amylase from a newly isolated *Bacillus sp*. YX-1: purification and characterization. Bioresourse Technology, 99: pp. 4315-20.

Narayana, K.J., and Vijayalakshmi, M., 2008. Production of Extracellular a-Amylase by Streptomyces albidoflavus. Asian Journal of Biochemistry, 3: pp. 194-197.

Niall, A.L., and Paul, D.V., 2009. Genus Bacillus. Bergey's manual of systematic bacteriology. Second edition. Springer, New York, 21-128.

Oyeleke, S.B., Oduwole, A.A., 2009. Production of amylase by bacteria isolated from a cassava waste dumpsite in Minna, Niger State, Nigeria. African Journal of Microbiological Research, 3, pp. 143-6.

Pandey, A., Nigam, P., Soccol, C.R., Soccol, V.T., Singh, D., Mohan, R., 2000. Advances in microbial amylases. Biotechnology and Applied biochemistry, 31, pp.135-52.

Pandey, A., Soccol, C.R., Mitchell, D., 2000. New developments in solid state fermentation: International bioprocesses and products. Process biochemistry, 35, pp.1153-69.

Panneerselvam, T. and Elavarasis., 2015. Isolation of alpha - Amylase producing *Bacillus subtilis* from soil. International Journal of Current Microbiology and Applied Science, 4, pp. 543 – 552.

Parbat, R, and Singhal, B., 2011. Production of glucoamylase by Aspergillus oryzae under solid state fermentation using agro industrial products. International Journal of Microbiology Research, 2, pp. 204-7.

Raj, V., and Hemashenpagam, N., 2012. Production and medium optimization of amylase by *Bacillus* using fermentation methods. Journal of Microbiology and. Biotechnology Research, 2(4), pp. 481-84.

Rameshkumar, A., Sivasudha, T., 2011. Optimization of nutritional constitute for enhanced alpha amylase production using by solid state fermentation technology. International Journal of Microbiology Research, 2: pp. 143-8.

Reddy, N.S, Nimmagadda, A., and Rao, K.S., 2003. An overview of the microbial α-amylase family. African Journal of Biotechnology, 2, pp. 645-648.

Rehana, F., Venkatasubbaiah, P., Nand, K., 1989. Preliminary studies on the production of thermostable alpha-Amylase by a mesophilic strain of *Bacillus licheniformis*. Chemie, Mikrobiologie, Technologie der Lebensmittel, 12, pp. 8-13.

Riaz, N., Haq, I., Qadeer, M.A., 2003. Characterization of α-amylase by *Bacillus subtilis*. International Journal of Agricultural and Biological Engineering, 5, pp. 249-52.

Roohi, M. K., and Ahmad, I.Z., Arif, J.M., 2011. Production of cold-active extracellular a-Amylase by newly isolated microbacterium foliorum GA2 from gangotri glacier, Western Himalaya, India. Asian Journal of Biotechnology, 3: pp. 449-59.

Roohi, A., Ahmed, I., Iqbal, M., Jamil, M., 2012. Preliminary isolation and characterization of halotolerant and halophilic bacteria from salt mines of Karak, Pakistan. Pakistan Journal of Botany, 44, pp.365-370.

Samreen., Ahmad, W., Ijaz, B., Sarwar, M.T., Gull, S., Kausar, H., 2011. African Journal of Biotechnology, 10, pp. 2119-212.

Sankaralingam, S., Shankar, T., Ramasubburayan, R., Prakash, S., Kumar, C., 2012. Optimization of culture conditions for the production of amylase from *Bacillus licheniformis* on submerged fermentation. American-Eurasian Journal of Agricultural & Environmental Sciences, 12, pp.1507-13.

Santos, E. D., and Martins, M.L., 2003. Effect of the medium composition on formation of amylase by *Bacillus sp*. Brazilian Archives of Biology and Technology, 46, pp.129-34.

Sourav, B., Shilpi, B., Arijit, D., Sanchita, A., 2011. Utilization of sugarcane bagasse for solid-state fermentation and characterization of α-amylase from Aspergillus flavus isolated from Muthupettai Mangrove, Tamil Nadu, India. Australian Journal of Basic and Applied Science, 5, pp. 1012-22.

Srivastava, R.A., Baruah, J.N., 1986. Culture conditions

for production of thermostable amylase by *Bacillus stearothermophilus*. Applied Environment Microbiology, 52, pp.179-84.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular biology and evolution, 28, pp. 2731-9.

Tanyildizi, M.S., Ozer, D., and Elibol, M., 2005. Process Biochemistry, 40: pp. 2291-2296.

Verma, V.M., Shekar Avasthi, A., Raj Gupta., Monika Singh., Kushwaha, A., 2011. Isolation, Screening and Characterization of Amylolytic Microorganisms. European Journal of Experimental Biology, 1, pp. 90-96.

Vijayabaskar, P., Jayalakshmi, D., Shankar, T., 2012. Amylase production by moderately halophilic *Bacillus cereus* in solid state fermentation. African Journal of Microbiology Research, 6: pp. 4918-26.

Yassien, M.A., Asfour, H.Z., 2012. Improved production, purification and some properties of α-amylase from Streptomyces clavifer. African Journal of Biotechnology,11:14603-11.

Zambare, V.P., 2011. Optimization of Amylase Production from *Bacillus sp*. using Statistics based Experimental Design. Emirates Journal of Food and Agriculture, pp. 37-47.

Agricultural Communication

BBRC Bioscience Biotechnology Research Communications

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp-243-250

Optimization of Pre-Milling Treatments for Pigeon Pea Dhal Recovery using CIPHET Mini Dhal Mill

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ABSTRACT

The effect of different pre-milling treatments on dehulling fractions, dehulling efficiency and dehulling loss were studied using CIPHET mini dhal mill. Pre-milling trials were conducted using different levels of oil (0%, 0.3% and 0.5%) and water treatments (0%, 25% and 50%) on pigeon pea. Response surface method based on a single factor and three-level design was used to study the effect of the independent variables and to optimize process conditions. Overall best values of dehulling fractions and parameters were observed for 50% water treatment which was found similar to the predicted data.

KEY WORDS: DEHULLING EFFICIENCY; DEHULLING FRACTIONS; DEHULLING LOSS; PRE-MILLING TREATMENT

INTRODUCTION

India stands top in worldwide pulse production as well as for its highest consumption. Pigeon pea (*Cajanas cajan L.*) is one of the highly produced and consumed pulses throughout India after chickpea and pea. Pulses have an essential role in human nutrition and are found more prevalent in vegetarian people diet as one of the major protein sources (Anon, 1984). The Annual production of total pulses in India in 2017-2018 was 25.23 Million MT from which Pigeon pea accounts for 4.25 Million MT and in 2018-19 it is estimated around 3.68 Million MT (DES, DAC Report; 2017-18 & 2nd Adv Est for 2018-19).The per capita availability of pulses was approximately 35g as against the requirement of 70g per day as per the recommended dietary allowances (Roy et al., 2017). Generally, pulses are consumed in India after being converted into dhal, the dehulled splits. It is estimated that about 80% of pulses produced in the country are converted to dhal (Deshpande, 1990 and Tiwari et al., 2017). The recovery of dhal varies from 60 to 75%, depending upon the type of pulses and techniques adopted by the millers such as methods of pre-milling treatment and milling machinery used (Chavan et al., 1983; Deshpande et al., 2007 & Jennifer et al., 2011).

Dehulling is the most crucial operation of post-harvest handling of pulses. The removal of the seed coat is imperative because it is indigestible and bitter. Since in most of the pulses, husk is tightly attached with cotyledons, (Kulkarni, 2002; Gupta, 2013); therefore, a pre-treatment before milling is desirable for loosening seed coats. Singh and Ilyas (1994) and Chunilal (2017) reviewed the various pre-treatments used in pigeon pea. Edible oil treatment (up to 1%) is used in commercial mills to loosen the husk of pulses that are difficult to mill (Singh, 1995; Sokhansanj & Patil, 2003). Tiwari et al. (2007) observed an 85.5% dehusking loss and a 6.98 % powdering loss in black gram at 0.8% oil treatment with 90 °C drying temperature. Erskine studied the effect of seed size and different pre-treatments on splitting

ARTICLE INFORMATION

*Corresponding Author: er.ashutoshchavan@gmail.com
Received 13th Feb 2020 Accepted after revision 21th March 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)
A Society of Science and Nature Publication, Bhopal India

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/41

and dehulling of lentil (Lens culinaris) and reported that dehulling efficiency was highest with low seed moisture content, (Phirke & Bhole, 1999; Wang, 2005). Kurien (1968) investigated that dehulling of pigeon pea can be rendered easier by prolonged soaking in water for 12hours or more, but the dhal so obtained remains uncooked and tough even with prolonged boiling. Hence, it is necessary to optimise the oil treatment of pigeon pea to enhance recovery (Phirke & Bhole, 1999). Therefore the present study was undertaken to study milling characteristics for the CIPHET dhal mill for dehulling of pigeon pea using different pre-treatments.

MATERIALS AND METHODS

Raw material: Pigeon pea grains (Variety: Pigeon pea-407) used in the study were obtained from the agro-processing Centre of Central Institute of Post Harvest Engineering and Technology (CIPHET) Ludhiana.

Equipments used: Experiments were conducted using CIPHET developed mini dhal mill at Food Grain & Oil Processing Division, CIPHET, Ludhiana which has overall dimensions of 1000 mm × 555 mm × 1225 mm, abrasive circle surface perimeter of 1100 mm and feed rate capacity of 100 kg/h. It was driven by 3 hp electric motor rotating at 1580 rpm speed, and dhal mill shaft rotates at 615 rpm. In this machine, all three carborundum materials were laminated on a single roller, and two stoppers were provided on the screen to increase the retention time of pigeon pea (Sahay & Bisht, 1988; Mangaraj et al. 2004).

Sample Preparation: As per the conventional method of milling, cleaning of grains was done by using pedal cum power operated grain cleaner with 500-800 kg/h capacity in agro-processing centre of CIPHET, Ludhiana. Further separation of stones, pebbles, soil particles were done by using Destoner of capacity 100-200 kg/h. After cleaning and grading of pigeon pea grains, the moisture content of the grain was determined using the hot air oven drying method (AOAC, 2000).

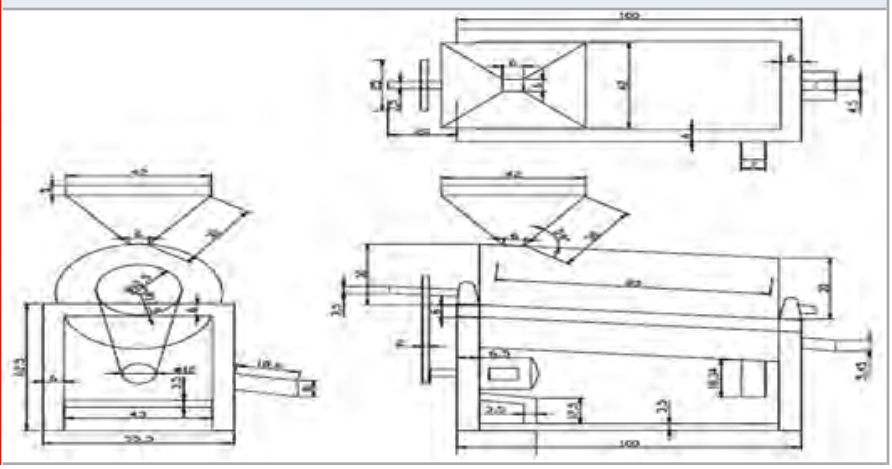
Pre-milling treatments: Pre-milling trials were conducted on pigeon pea using different levels of refined soybean oil (0%, 0.3% and 0.5%) and by using water (0%, 25% and 50%). Total 18 experiments were run each having an equal sample size of 2 kg. In the case of water pre-treatment, grain was finally dried to 8% and 12% moisture content on a wet basis for 25 % and 50% water treatment respectively. After addition of water, grains were kept in airtight plastic containers for 12 hours conditioning. The reduction of moisture content to the desired level was achieved by the conventional method of drying, i.e. by sun-drying (Mazza & Campbell, 1985; Ante et al., 2014). While in case of oil treatment, refined soya bean oil of 0.3% and 0.5 % (v/w) was added to pigeon pea samples, and the grains were mixed thoroughly to ensure all grains were uniformly coated. The oil pre-treated grains were also heaped in airtight plastic containers for 36 hours for tempering (Sokhansanj & Patil, 2003; Hiregoudar, 2014).

Drying: The pre-treated pulses were then dried in open sunlight to the desired moisture content of 9-10% (d.b) (Kurien and Parpia, 1968; Kurien, 1981; Goyal et al., 2008).

Milling: Based on preliminary trials conducted at CIPHET and optimization of milling time for dehulling pigeon pea, 12 seconds residence time was observed to be optimum. So, a complete unit for dehulling pigeon pea grain has been developed taking residence time as one of the design parameters. Thus after pretreatment, grains were milled using CIPHET dhal mill (as described above) & fractional analysis was carried out to study the effect of pre-treatment on the recovery of quality dhal. Various fractions such as dehulled grains i.e. dhal, partially dehulled grains, unhusked grains, husk and chuni were obtained, which through manual sorting weighted separately to find out the total recovery of dhal.

Dehulling efficiency, η in % and dehulling loss, ξ in % in terms of broken grains and powder were calculated using the following equation (Saxena, 1985, Singh et al. 2004 and Goyal et al., 2008).

Figure 1. Orthographic view of CIPHET mini dhal mill



$$\eta = \left(1 - \frac{TUG}{TG}\right)\left(\frac{TDG}{TDG + TBG + TP}\right) \times 100 \quad (1)$$

$$\xi = \left(\frac{TBG + TP}{TG}\right) \times 100 \quad (2)$$

Experimental design: Optimization of the milling process was done using response surface methodology (RSM) to study the effect of different pre-milling treatments on dehulling parameters and dhal recovery. The reason behind the selection of RSM was due to its suitability to find the ideal process settings to achieve optimal performance. The independent variables used in this design were oil treatment (Xi) and water treatment (Xj), each at three levels (0, 25 & 50%) for water and (0, 0.3, &0.5%) for oil treatment. The coded levels of independent variables were -1, 0 and+1 (Table 1a & 1b).

These levels were selected based on previous experiments conducted at CIPHET, Ludhiana. Since two different pre-milling treatments,i.e. using oil and water were studied, experiments were conducted separately. Thus, one factor RSM design was used for the linear model, and statistical analysis was done separately for each independent parameter. The experiments were taken in a randomized manner to reduce the effect of unexpected variations in observed responses. Considering independent variables affect the total amount of dehulled grains (TDG),unhulled grains (TUG), broken grains (TBG), powder (TP), dehulling efficiency (η)and dehulling loss (ξ) hence these were considered as responses. For this purpose, Design Expert software (Version 11) developed by Stat-Ease Inc., Minneapolis, USA,was used. Experimental data values were analyzed using one factor RSM methodand fitted in following second-degree polynomial equation as given below.

$$Y = \beta_0 + \Sigma \beta_i X + \Sigma \beta_i X^2 \quad (3)$$

Here Y is predicted response, β_0 is constant regression coefficient, β_i is the linear regression coefficient, and X is coded independent variable. To optimize process parameters, the independent variable was kept within the experimental range for maximizing TDG as well as dehulling efficiency and minimizing TUG, TBG, TP as well as dehulling loss.

Table 1a. Coded levels of independent variables used in oiltreated dhal milling

Independent variable			Coded level	
	Notation	-1	0	+1
Oil treatment (%)	X_i	0	0.3	0.5

Table 1b. Coded levels of independent variables used in watertreated dhal milling

Independent variable	Notation		Coded level	
		-1	0	+1
Water treatment (%)	X_j	0	25	50

Experimental justification: Dehulling experiments using different pre-milling treatments were conducted at optimum conditions prescribed by Design Expert software to analyze results. Observations were replicated three times and examined for any remarkable difference from predicted data values.

RESULTS AND DISCUSSION

The experimental results for different pre-milling treatments are presented in terms of the total amount of dehulled grains (TDG), unhulled grains (TUG), broken grains (TBG), powder (TP), dehulling efficiency and dehulling loss in Table 2a & 2b.

Dehulling fractions: From ANOVA for different dehulling fractions such as total dehulled grains (TDG), total unhulled grains (TUG), total broken grains (TBG) and total amount of powder (TP) are shown in Table 3a & 3b. It was observed that both pre-milling treatments were significant. In most of the cases, the quadratic model was found significant expect TP in oil treatment and TUG & TP in water treatment, where a linear model was found significant. Linear regression equations obtained for given suitable linear or quadratic model of first or second degree respectively in terms of coded factor X are shown below.

For oil treatment-

$$\begin{aligned} TDG &= 50.37 + 1.15X - 6.03X^2 \\ TUG &= 45.53 + 2.42X + 4.76X^2 \\ TBG &= 3.82 - 3.36X + 1.24X^2 \\ TP &= 0.31 - 0.22X \end{aligned} \quad (4)$$

For water treatment-

$$\begin{aligned} TDG &= 59.57 + 12.51X - 3.87X^2 \\ TUG &= 38.53 - 8.58X \\ TBG &= 3.16 - 3.69X + 1.57X^2 \\ TP &= 0.28 - 0.24X \end{aligned} \quad (5)$$

From Table 3a & 3b, it was found thatfor each response, R2 value and adjusted R^2 value were within approximately 0.20 of each other, i.e. in reasonable agreement. Therefore given models predicted response values very well. Also, high R^2 values proved that selected first and second degree models were sufficient. Since only one factor was involved, representation of the response surface for dehulling fractions showed by using two-dimensional graphs. A radar chart is a unique graphical method to represent multivariate or multilevel data in the form of two-dimensional graphs. Hence, the data obtained using three different levels of each pre-milling treatment viz. oil (0%, 0.3% & 0.5 %) and water (0%, 25% & 50%)

treatment were shown as three distinct axes points in separate radar charts as Figure 2a & 2b below. Such type of model representation helps in understanding the effect of various factors. In case of oil treatment, TDG initially increased and then decreased as oil content goes beyond 0.3% while TBG was continuously decreased with the increase in oil percentage, i.e. from 0 to 0.5% (Figure 2a). TDG was increased with an increase in water percentage, i.e. from 0 to 50% while TBG was decreased respectively with it (Figure 2b).

Kurian & Ramakrishnaiah (1983) mentioned results indicating a high amount of water content cause rupture of mucilage-gums which was tightly bonded to the hull and cotyledon layers while higher oil percentage cause difficulty in dehulling the grains properly. TP first slightly decreased with an increase in both the cases, i.e. in both water and oil treatment, but remained nearly constant for a prolonged treatment period. Similar studies were done by Goyal et al. (2008) & by Tiwari et al. (2010) reported that total loss of powder was influenced by drying temperature and water content for pigeon pea.

Dehulling parameters: The experimental results for dehulling efficiency as well as dehulling loss are represented in Table 2a & 2b. The analysis of variance for dehulling efficiency (η) and dehulling loss (ξ) was shown in Table 3a & 3b. It was seen that both pre-milling operations were quite significant. Here, as a result, the quadratic model was suggested to study the effect of different pre-milling treatments except for dehulling loss in oil treatment in which linear model was found to be significant. Regression equations in terms of coded factor X for the suitable linear model of first degree or quadratic model of the second degree are shown as following.

For oil treatment-

$$\eta = 50.34 + 1.17X - 6.01X^2$$
$$\xi = 4.99 - 3.65X \quad (6)$$

For water treatment-

$$\eta = 59.57 + 12.53X - 3.87X^2$$
$$\xi = 3.40 - 3.93X + 1.63X^2 \quad (7)$$

The model F-values from Table 3a &t3b implies only models which were found significant, and there was only a 0.01% chance that a model F-value this large could occur due to noise. P-values less than 0.05 indicated that model terms were significant. Hence both oil treatment & water treatment have significant model terms which have

Table 2a. Experimental design matrix for coded values & responses in oil treated dhal milling

Run order	Oil treatment (X_i)	TDG (%)	TUG (%)	TBG (%)	TP (%)	Dehulling efficiency (%)	Dehulling loss (%)
1	+1	44.2	54.3	1.4	0.1	44.2	1.5
2	-1	45.2	44.7	9.5	0.6	45.2	10.1
3	0	50.3	45.4	3.9	0.3	50.3	4.3
4	+1	46.4	51.4	2.1	0.1	46.4	2.3
5	-1	41.7	50.4	7.4	0.5	41.7	7.9
6	0	51.4	45.3	3.0	0.3	51.4	3.2
7	+1	45.9	52.4	1.6	0.1	45.9	1.7
8	0	49.3	47.9	2.6	0.1	49.3	2.8
9	-1	42.6	48.5	8.4	0.5	42.6	8.9

Table 2b. Experimental design matrix for coded values & responses in water treated dhal milling

Runorder	Water treatment (X_j)	TDG (%)	TUG (%)	TBG (%)	TP (%)	Dehulling efficiency (%)	Dehulling loss (%)
1	-1	42.6	48.5	8.4	0.5	42.6	8.9
2	0	62.6	33.5	3.6	0.3	62.6	3.9
3	-1	41.7	50.4	7.4	0.5	41.7	7.9
4	+1	67.4	31.9	0.7	0.0	67.4	0.7
5	+1	68.1	30.8	1.1	0.1	68.1	1.1
6	0	57.5	39.6	2.7	0.2	57.5	2.9
7	-1	45.2	44.7	9.5	0.6	45.2	10.1
8	+1	69.2	29.3	1.4	0.1	69.2	1.5
9	0	58.6	38.0	3.1	0.2	58.6	3.4

a substantial effect on the responses. Since the Prob>F values from Table 3a & 3b were obtained very small (less than 0.05), curvatures were found significant which means that the predicted value at the centre point was significantly different from the value that was obtained when running the centre point conditions. Also, R^2 values and adjusted R^2 values for every response were found within the reasonable agreement, i.e. approximately 0.20 of each other. Therefore given models predicts response values very well. Also, high R^2 values proved those first and second degree models selected were adequate. One factor response surface diagrams for dehulling efficiency and dehulling loss represented as two-dimensional radar charts (Figure 3a & 3b) which help in identifying the effect of various responses. In oil treatment, dehulling efficiency was increased initially and then decreased as oil content goes beyond 0.3% (Figure 3a).

Dehulling efficiency in water treatment was increased with an increase in water percentage, i.e. from 0 to 50% (Figure 3b). Increase in water pre-treatment percentage of pigeon pea grains from 0 to 50% showed a sharp increase in dehulling efficiency from 43.2 to 59.6% initially, and then it gradually increased up to 68.2%. These results found contrary to the results obtained by Kurien & Ramakrishnaiah (1983) because the moisture content of pigeon pea grains was less than 13%. The results obtained found in agreement with Tiwari et al. (2010) who denoted increased dehulling efficiency with an increase in water content, reaching a maximum after approximately 10 minutes of treatment. Whereas, dehulling losses found to be decreased with increase inboth oil and water treatment levels. Increase in the level of oil treatment beyond 0.3%, although not significant for dehulling efficiency, yet decrease in dehulling losses (0.5-2.8%) were obtained. Hence, oil pre-treatment assists in dehulling of pigeon pea by not increasing losses during dehulling (Goyal et al., 2008).

Numerical optimization of experimental conditions: Design Expert 11software was used to achieve optimal conditions for dehulling studies of pigeon pea obeying certain conditions as given in Table 4a & 4b for oil and water pre-treatment, respectively. The experimental conditions were optimized to obtain maximum TDG and dehulling efficiency with minimum TUG, TBG, TP and dehulling loss. Lower and higher limit values of factors and responses were obtained from experimental readings. It was predicted that oil pre-treatment of0.3% gave 50.2% TDG, 46.6% TUG, 3% TBG, 0.2% TP, 50.2% dehulling efficiency and 3.9% dehulling loss as optimum values with the most convenient desirability of 0.81. Similarly, water treatment of 50% gave 68.2% TDG, 29.9% TUG, 1% TBG, 0.0% TP, 68.2% dehulling efficiency and 1.1% dehulling loss as optimum values with the most convenient desirability of 0.97.

Predicted model accuracy of oil treated samples was 50.2 ± 0.8% for TDG, 46.6 ± 1.1% for TUG, 3 ± 0.4% for TBG, 0.2 ± 0.03% for TP, 50.2 ± 0.8% for dehulling efficiency and 3.9 ± 0.4% for dehulling loss. While for water treated samples accuracy of 68.2 ± 1.1% for TDG, 29.9 ± 1.4% for TUG, 1 ± 0.4% for TBG, 0.0 ± 0.04% for TP, 68.2 ± 1.1% for dehulling efficiency and 1.1 ± 0.4% for dehulling loss

Table 3a. Analysis of variance for oil treatment on response variables

Response	Significant model	SS	df	MS	F-value	p-value Prob > F	R^2	Adj. R^2
TDG	Quadratic	80.49	2	40.24	21.67	0.0018	0.8784	0.8378
TUG	Quadratic	68.39	2	34.19	8.04	0.0201	0.7282	0.6376
TBG	Quadratic	74.65	2	37.33	66.17	< 0.0001	0.9566	0.9422
TP	Linear	0.30	1	0.30	63.53	< 0.0001	0.9007	0.8866
η	Quadratic	80.17	2	40.08	20.96	0.0020	0.8748	0.8331
ξ	Linear	80.96	1	80.96	80.04	< 0.0001	0.9196	0.9081

Table 3b. Analysis of variance for water treatment on response variables

Response	Significant Model	SS	df	MS	F-value	p-value Prob > F	R^2	Adj. R^2
TDG	Quadratic	969.09	2	484.55	130.63	< 0.0001	0.9776	0.9701
TUG	Linear	441.96	1	441.96	61.90	0.0001	0.8984	0.8839
TBG	Quadratic	86.53	2	43.27	91.48	< 0.0001	0.9682	0.9577
TP	Linear	0.35	1	0.35	66.98	< 0.0001	0.9054	0.8919
η	Quadratic	972.41	2	486.20	128.74	< 0.0001	0.9772	0.9696
ξ	Quadratic	98.16	2	49.08	90.70	< 0.0001	0.9680	0.9573

were obtained. In order to check the validity of optimal conditions, experiments were conducted in triplicate manner and the average TDG, TUG, TBG, TP, dehulling efficiency and dehulling loss were observed to be 50.3 ± 1.1%, 46.2 ± 1.5%, 3.2 ± 0.7%, 0.2 ± 0.1%, 50.3 ± 1.2 and 3.4 ± 0.8 respectively for oil treated samples and similarly, 68.2 ±0.9 %, 30.7 ± 1.3%, 1.1 ± 0.4%, 0.1 ± 0.06, 68.2 ± 0.9 and 1.1 ± 0.4 were obtained for water treated samples respectively. Since, both predicted and average values were quite similar hence it confirms the optimum conditions.

CONCLUSION

With the help of Response surface method (RSM) design, the relationships between one or more measured responses and the vital input factors were quantified for optimizing pre-treatment conditions (oil and water treatment) for milling studies of pigeon pea. These optimized parameters could be used to design integrated mini dhal mills which will help to small commercial scale milling industries. The best optimal conditions among all pre-treatments were obtained for 50% water

Figure 2a & b. Dehulling fractions relationship for oil (2a) & water treatment (2b) respectively

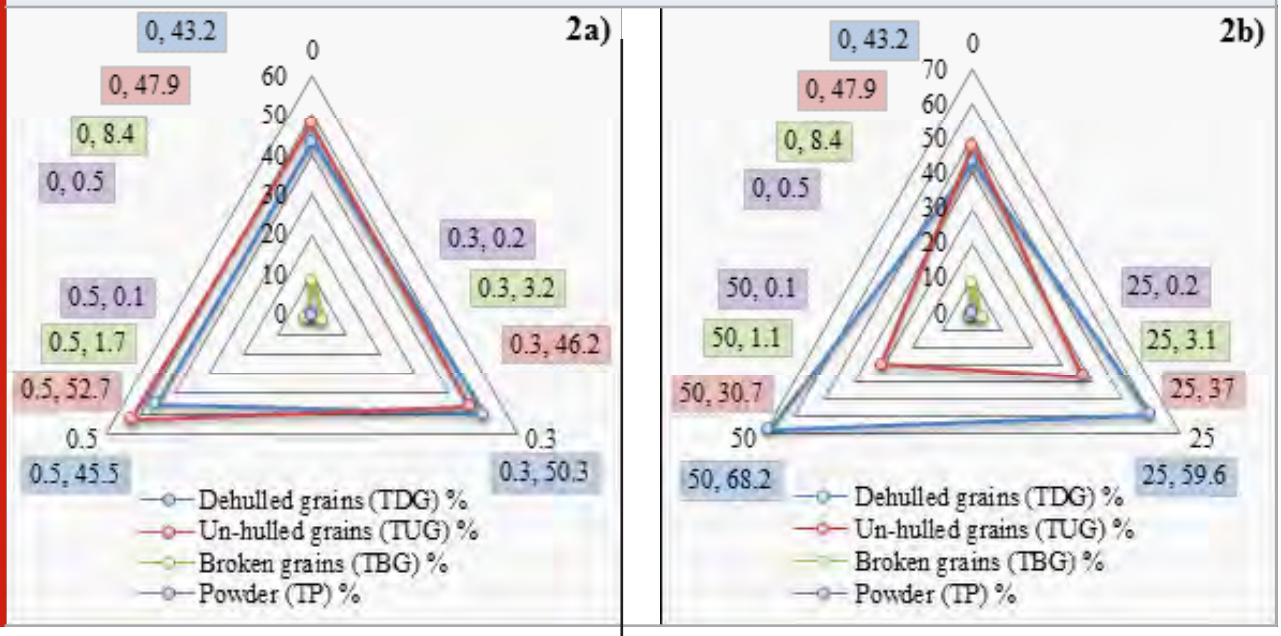


Figure 3a & b. Dehulling parameters relationship for oil (3a) & water treatment (3b), respectively

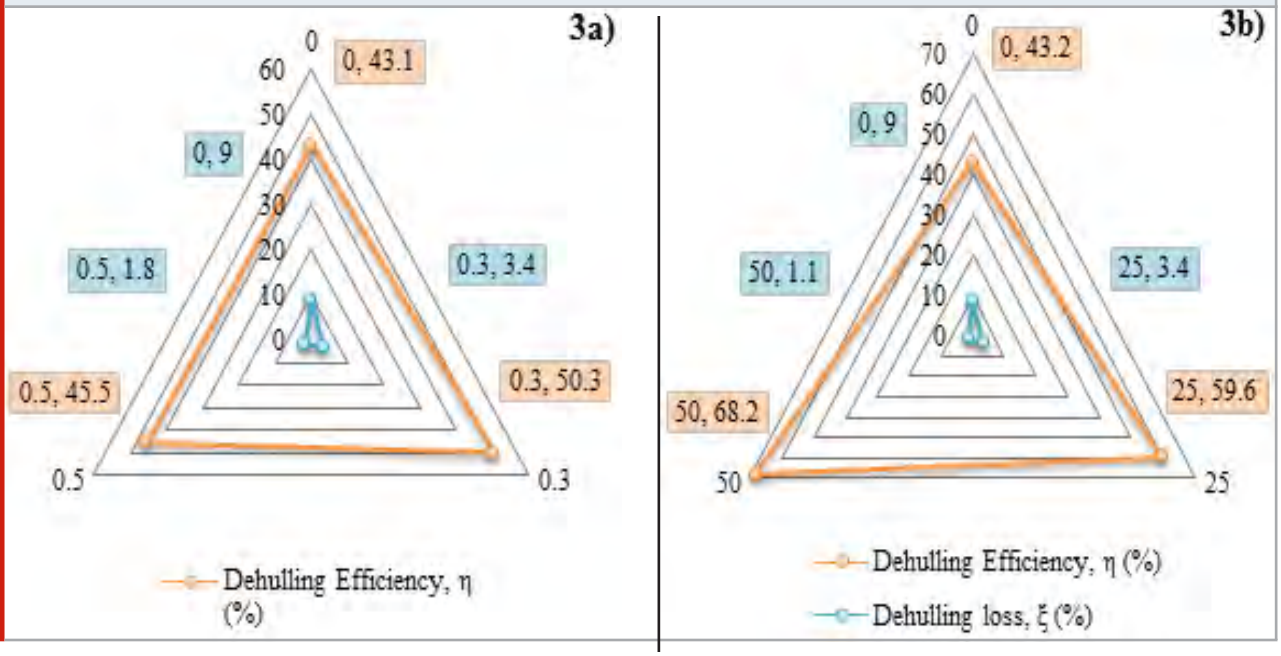


Table 4a. Optimization criteria for dehulling fractions & parameters of oil pre-treated pigeon peas

Name	Goal	Lower Limit	Upper Limit	Importance
Oil Pre-treatment	in range	0	0.5	* * *
TDG	maximize	41.7	51.4	* * *
TUG	minimize	44.7	54.3	* * *
TBG	minimize	1.4	9.5	* * *
TP	minimize	0.1	0.6	* * *
Dehulling efficiency	maximize	41.7	51.4	-
Dehulling loss	minimize	1.5	10.1	-

Table 4a. Optimization criteria for dehulling fractions & parameters of oil pre-treated pigeon peas

Name	Goal	Lower Limit	Upper Limit	Importance
Water Pre-treatment	in range	0	50	* * *
TDG	maximize	41.7	69.2	* * *
TUG	minimize	29.3	50.4	* * *
TBG	minimize	0.7	9.5	* * *
TP	minimize	0.1	0.6	* * *
Dehulling	maximize	41.7	69.2	-
efficiency	minimize	0.7	10.1	-
Dehulling loss				

pre-treated sample indicating highest 0.97 desirability value. Yet the amount of dehulled grains remained after milling is a bit higher as compared to commercial mill outputs. It was due to the small sample size and single time feeding of samples. Besides this, different varieties of pigeon pea, their shape, size and season of harvest also affect milling studies. Hence to promote its further application, pre-treatments should be tested considering the above different aspects.

ACKNOWLEDGEMENT

First two authors acknowledge their sincere thanks to Dr R. T. Patil, Ex. Director, Central Institute of Post Harvest Engineering & Technology, Ludhiana (Punjab) for giving opportunity and providing the facility to conduct experiments at CIPHET, Ludhiana and both also are grateful to Dr D.M. Kadam, (now Principal Scientist in Agricultural Process Engineering Division at CIRCOT, Mumbai) who guided them during their studies.

REFERENCES

Anonymous. 1984. Recommended dietary intakes for Indians. ICMR, New Delhi: 58-59.

Ante, G. Pliestic, S.Jovic, F.; Nenadic, K. & Jovic, A. 2014. An energy efficient corn grain drying process. Energetski Ucinkovit Proces Susenja Kukuruznog Zrna, Tehnicki Vjesnik 21 (6), 1395-1401.

AOAC. 2000. In: Official Methods of Analysis of the Association of Official Analytical Chemists (Horwitz W, ed), 17th eds. AOAC International, Maryland, USA.

Chavan, J. K.; Jawale; H. K.; Shore, D. M.; Jadhav, S. J.; & Kadam, S. S. 1983. Effects of pre-soak treatments on the cooking quality of legume dhal. Indian Food Packer: 78-80.

Chunilal, C. V. 2017. Enzymatic pre-treatments on pigeon pea (BSMR-736) for better milling quality of dhal. Department of Processing & Food Engineering, College of Agricultural Engineering and Technology, Junagadh Agricultural University, Junagadh- 362001.

DES, DAC. 2017-18 & 2018-19. DES-Directorate of Economics and Statistics, DAC- Department of Agriculture & Cooperation Annual report 2017-2018 and 2nd advance estimates report 2018-2019.

Deshpande, S. D. 1990. Studies on some engineering aspects for processing and utilization of soya bean. Ph. D. Thesis, Indian Institute of Technology, Kharagpur, India.

Deshpande, S. D.; Srivastava, S.; & Manoj, K. 2007. Application of oil-water and sodium bicarbonate pre-milling treatments for increased dhal recovery. Journal of Food Science & Technology, 44(1): 22-25.

Goyal, R. K.; Vishwakarma, R. K.; & Wanjari, O. D. 2008. Optimisation of the pigeon pea dehulling process. Biosystems Engineering, 99: 56–61.

Gupta, S. 2013. Process Optimization for Milling of Moong Beans (Vigna radiata) Thesis Submitted to Department of Processing and Food Engineering, College of Agriculture Engineering & Technology, Punjab Agricultural University Ludhiana-141004.

Hiregoudar, S.; Sandeep, T. N.; Nidoni,U.; Shrestha, B.; & Medha, V. 2014. Studies on dhal recovery from pre-treated pigeon pea (Cajanus cajan L.) cultivars. Journal of Food Science Technology. 51(5): 922–928.

Jennifer, A. W.;& Malcolmson, L. 2011. Pulse milling technologies. In: Brijesh K. Tiwari, Aoife Gowen and Brian Mckenna, Editors: Pulse Foods, Oxford: Academic Press; p. 193-221.

Kulkarni, S.D. 2002. Energy use assessment and demand projections for pulse processing industry in India. Agricultural Engineering Today, 24(3):13-21.

Kurien, P. P. 1981. Advances in milling technology of pigeon pea, In: Proceeding of an international workshop on pigeon pea. International Crop Research Institute for Semi-Arid Tropics, Patancheru, India.

Kurien, P. P.; & Parpia, H. A. B. 1968. Pulse milling in

India. Journal of Food Science & Technology, 5: 203-210.

Mangaraj, S.; Agrawal, S.; Kapur, T.; & Kulkarni, S. D. 2004.Effect of pre-milling treatment and abrasive rollers on milling of pulses. Journal of Agricultural Engineering 41: 10-15.

Mazza, G; & Campbell, C. G. 1985. Influence of water activity and temperature on dehulling of buckwheat. Cereal Chemistry, 62: 31–34.

Phirke, P. S; & Bhole, N. G. 2000. Pretreatments on pigeon pea grain for improvement of dehulling characteristics. International Journal of Food Science & Technology, 35: 305-313.

Phirke, P. S; & Bhole, N. G. 1999. The effect of pretreatment on the strength and dehulling properties of pigeon pea grains. International Journal of Food Science & Technology, 34: 107-113.

Ramakrishnaiah, N.; & Kurien, P. P. 1983. Variabilities in the dehulling characteristics of pigeon pea (Cajanus cajan L.) cultivars. Journal of Food Science Technology, 20: 287-291.

Roy D.; Joshi, P. K.; & Chandra, R. 2017. Report on Pulses for Nutrition in India Changing Patterns from Farm to Fork. International Food Policy Research Institute Washington, DC.

Sahay, K. M.; & Bisht, B. S. 1988. Development of a small abrasive cylindrical mill for milling pulses. International Journal of Food Science Technology, 23: 17-22.

Saxena, R. P. 1985. Milling of pigeon pea (Cajanus cajan) and associated aspects. PhD Thesis, G.B. Pant University of Agriculture & Technology, Pantnagar, India.

Singh, A.; & Ilyas, S. M. 1994. Energy audit of pulse milling technologies in India. IN: energy management and Conservation in Agriculture Production and Food Processing.

Singh, S. K.; Agarwal, U. S.; & Saxena, R. P. 2004. Optimization of process parameters for milling of green gram (Phaseolus aureus).Journal of Food Science and Technology, 41(2): 124–130.

Sokhansanj, S; &Patil, R. T. 2003. Dehulling and splitting pulses. In: Handbook of Post Harvest Technology (Chakraverty, A.et al., eds). Marcel Dekker, New York.

Tiwari, B. K.; Jagan Mohan, R.; Venkatachalapathy, N.; Anand, M. T.; Surabi, A.; & Alagusundaram, K. 2010. Optimization of hydrothermal treatment for dehulling pigeon pea. Food Research International, 14(6): 487-495.

Tiwari, B. K.; Jagan Mohan, R.; & Vasan, B. S. 2007. Effect of heat processing on milling of black gram and its end product quality. Journal of Food Engineering, 78: 350-360.

Tiwari, A. K.; & Shivhare, A. K. 2017. Report on pulses in India: Retrospect & prospects. Government of India Ministry of Agriculture & Farmers Welfare (Department of Agriculture, Cooperation and Fw) Directorate of Pulses Development Vindhyachal Bhavan, Bhopal-462004, (M.P.)

Wang, N. 2005. Optimization of a laboratory dehulling process for lentil (Lens culinaris). Grain Research Laboratory, Canadian Grain Commission, Winnipeg, MB, Canada. https://doi.org/10.1094/CC-82-0671.

Biotechnological Communication

BBRC Bioscience Biotechnology Research Communications

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp-251-260

Effect of Probiotic Strain *Bacillus firmus* CAS 7 As Feed Supplement for Growth, Survival and Colour Enhancement of Smoke Angelfish *Apolemichthys xanthurus*

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ABSTRACT

The effect of dietary management of probiotic micro organism, *Bacillus firmus* cas 7 on increase, survival and colour enhancement of smoke angelfish *Apolemichthys xanthurus* become evaluated. within the gift take a look at, fishes have been fed with basal diet supplemented with probiotic *B. firmus* cas 7 at 50, 100 and 150 mg kg-1 and manage basal food regimen containing no probiotic. the outcomes discovered that the basal weight-reduction plan supplemented with probiotic at one hundred fifty mg kg-1 produced considerably better weight benefit (80.95 ± 1.5g), precise increase price (0.675), survival charge (one hundred%) and feed conversion ratio (zero.ninety one) than diets supplemented with different concentrations and manage. furthermore, carotenoid content material was relatively better in fishes fed feed supplemented with probiotic in amount a hundred and fifty mg kg-1 (6.24 mg g-1) than a hundred and 50 mg kg-1 and manipulate (2.ninety one mg g-1). the experimental end result proves that the probiotic bacteria b. firmus cas 7 substantially stepped forward growth and color of smoke angelfish *Apolemicthys xanthurus* which might be useful in ornamental fish industry as feed complement. further, studies concerning the synthesis of carotenoid and appropriate dose of probiotic must be executed earlier than the use of probiotics on a massive scale to prevent any undesired consequences.

KEY WORDS: PROBIOTIC; ANGEL FISH; *BACILLUS FIRMUS*; GROWTH; CAROTENOIDS.

ARTICLE INFORMATION

*Corresponding Author: raji.shwehari@gmail.com
Received 8th Jan 2020 Accepted after revision 28th Feb 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)
A Society of Science and Nature Publication, Bhopal India

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/42

INTRODUCTION

Modern aquaculture demands alternatives that can sustain a healthy environment with best production practices (Ai, Xu, Mai, Xu, Wang, & Zhang, 2011). Probiotics in aquaculture have been reported to provide helpful property and the use of probiotics is an important management tool in fish culture (Balcazar, De Blas, Ruiz-Zarzuela, Cunningham, Vendrell, & Muzquiz,, 2006). The probiotics in aquaculture is use full for reduction of the use of harmful antimicrobial compounds, particularly antibiotics, and also improved the growth performance of the farmed species in an eco-friendly and sustainable manner (Wang, Xu, & Xia, 2005). Probiotics as feed supplements benefit to host with improving the value of feed, enzymatic role to digestion, inhibition of pathogenic microorganisms, medical properties, growth promoting factors and increasing immune response (Harikrishnan, Balasundaram, & Heo, 2010; Gupta, Gupta, & Dhawan, 2014; Gupta, Geetika, & Paromita, 2016). Several reports suggest that probiotics supplementation can cut down the cost of culture by improving the fish growth and feed utilization efficiency. The most typically used probiotics in aquaculture belong to gram-positive spore forming *bacillus spp.* (Wang et al., 2008; Gupta & Dhawan 2011, 2013; Gupta et al., 2014, 2016).

The genus Bacillus as putative probiotics has been used substantially as aquaculture feed components, due to its resistance to excessive temperature and excessive stress (Rengpipat, Rukpratanporn, Piyatiratitivorakul, & Menasa-veta, 2000). Nutritional supplementation of Bacillus spp. advanced the boom performance, immunity and ailment resistance of fish (Ai et al., 2011; Geng, Dong, Tan, Yang, Chi, Liu, & Liu, 2012) and giant freshwater prawn (*Macrobracium rosenbergii* (Gupta & Dhawan 2011, 2012). In rainbow trout (*Oncorhynchus mykiss*), significant development of feed conversion ratio (fcr), specific growth price (sgr) and protein performance ratio (in line with) turned into observed when the fish was fed with diets containing bacillus spp. (Merrifield et al., 2010; Gupta et al., 2014), as color of decorative species is taken into consideration as an essential factor for marketing of the product (Gouveia & Empis 2003; Gouveia & Rema 2005).

Color changes in fish are regularly related to environmental strain, and illumination might be a number one factor regulating pigment distribution via hormone regulation (Van der Salm, Martnez, Flik, & Wendelaar Bonga, 2004). Shade of fish pores and skin is predominantly depending on the presence of chromatophores containing colored pigments (Fox, 1957). The colour of fish pores and skin is generated via the absorption, mirrored image, and scattering of mild through the pigments and microstructures within the fish integument (Fujii, 2000). carotenoids are evidently going on pigments that variety in shades from yellow to crimson (Hill, 2002) which are lipid soluble pigments, are answerable for pores and skin coloration of ornamental fish, and might determine their commercial feed. The prosperity of the decorative fish industry has precipitated the indiscriminate use of antibiotics and chemo therapeutants for improvement in fitness and vitamins, and this therefore has led to the development of drug resistant traces of pathogenic microorganisms (Amabile-Cuevas, Cardenas-Garcia, & Ludgar,1995). Fishes are unable to perform de novo synthesis of carotenoids (cd) like other animals and consequently rely on nutritional supply to gain their herbal pigmentation (Goodwin, 1984).

Below in depth farming conditions and aquarium rearing, ornamental fish are fed completely with compound feeds, which ought to consequently be supplemented with carotenoids (Wang, Li, & Lin, 2008). The yellow, orange and red colors discovered in fish pores and skin are the end result of the group of carotenoid pigments, each carotenes and xanthophylls (Simpson, Katayama, & Chichester, 1981). Spore-forming bacillus species able to synthesizing carotenoid pigments and the biochemical evaluation at the carotenoids answerable for the yellow/ orange pigmentation found in bacillus sp. has been performed and the identification of the carotenoids was elucidated (Perez-Fons, Steiger, Khaneja, Bramley, Cutting, Sandmann, & Fraser, 2011).

The most typically found pigments had been yellow, orange and red. isolates have been nearly usually participants of the bacillus genus and in maximum cases have been related with recognised species consisting of *B. marisflavi*, *B. indicus, B. firmus, B. altitudinis and B. safensis*. 3 types of carotenoids were found with absorption maxima at 455, 467 and 492 nm, corresponding to the visible colors like yellow, orange and pink, respectively (Perez-Fons et al., 2011). A total of 1471 species of ornamental fish are traded globally (Wabnitz, Taylor, Green, & Razak, 2003) of which, marine angelfishes (Pomocanthidae) are the following maximum crucial organization than damsel and anemone fish (33%), consisting of approximately 25% of the overall change. *Pomacanthus* angelfish are the various most high prized of the coral reef fish (Frische, 1999; Debelius, Tanaka, & Kuiter, 2003). The marine angelfish circle of relatives pomacanthidae consists of 8 genera and 82 species global (Nelson, 2006).

Marine angelfish, *Apolemichthys xanthurus* (Bennett, 1833) are extensively dispensed at some point of the Indian ocean, in regions such as the Maldives and the east coast of India. these fish tend to stay in coral-rich areas singly or in pairs, often found outer reef facet and reef slopes at depths of 15 m with the most size approximately 25 cm, feeds mainly on sponges, sea squirts and small amounts of copepods (Rajeswari, & Balasubramanian,

2014) to the fine of our understanding, very little statistics is available on its replica and early ontogeny, possibly because of the problems of field observations because of low populace densities and massive domestic stages, and also because of the issue in breeding such species in small aquaria (Moyer, 1987). But, there was no tries had been made on increase, survival, coloration enhancement, herbal spawning and larval rearing of *Apolemichthys xanthurus* in captivity. Even though the software of probiotics has been diagnosed in aquaculture by means of some researchers global, no concerted tries have been made on these marine angel fishes to enhance boom and color till date. therefore an attempt turned into made to analyze the impact of probiotic bacteria B. firmus cas 7 on boom overall performance, survival and color enhancement of smoke angelfish *Apolemichthys xanthurus* that's one of the maximum appealing and commercially essential marine angel fish species.

MATERIAL AND METHODS

Isolation and identification of probiotic bacteria Bacillus firmus CAS7: Samples were collected from marine sediments of Parangipettai, Tamil Nadu, India and brought to the laboratory aseptically at 4 C. in nutrient broth sea water (NBSW) (salinity 34 g/l and pH 7.89), speared on nutrient marine agar 2216 (Himedia, USA) and incubated at room temperature for 24 h at 30 C. Colonies were spread on nutrient marine agar plates. After Gram staining and catalase testing, only one strain (CAS7) were showed Gram and catalase positive rods were retained. Retained strains were enriched for 24 h at 30 C in NBSW. Colonies were re-isolated on nutrient marine agar and preserved in the laboratory. The purity of such cultures was routinely checked during this work. Stock cultures were frozen at 80 C with 20% (vol/vol) glycerol.

Table 1. Morphological, physiological and biochemical characteristics of the probiotic strain CAS 7

Characteristics	Results
Shape	Rod
Gram stain	Positive
Spore formation	+
Motility	+
Glucose	+
Mannitol	+
Xylose	+
Starch Hydrolysis	+
Gelatin Hydrolysis	+
Fat Hydrolysis	+
Casein Hydrolysis	+
Catalase activity	-
Nitrate reduction	+
Indole	+
Citrate	+

Characterization of the potent strain: Pure cultured colonies were biochemically characterized (Himedia, Mumbai). The identification was confirmed by partial sequencing the 16 S rDNA by kumaran et al., 2009. The isolated bacterial DNA were extracted and 16s rDNA sequences were amplified by polymerase chain reaction (PCR) using universal primers of 8f (3'-AGAGTTTGATCCTGTGCTCAG 5') and 1490r (5'-GACTTACCAGGGTATCTAATCC-3'). The PCR products were electrophoresed on 1% agarose gel and visualized via ultraviolet transillumination. After that the PCR product was purified to remove the primer, primer-dimers and low molecular weight DNA fragments generated by nonspecific amplification. Five volume of binding buffer was mixed with one volume of PCR product and loaded into the purification column. The nucleotide sequences of the PCR products were determined by using the automated DNA sequence with forward and reverse primers (Bio serve pvt. Bio Technologies, India). The sequences was compared with all 16S rRNA gene sequence data stored NCBI by nucleotide BLAST.

Table 2. Formulation and Proximate composition of basal diet

Ingredients	g kg-1
Fish meal	600
Shrimp meal	160
Soybean meal	20
Wheat flour	140
Fish oil	40
Soybean phospholipids	20
Vitamin mineral mix	10
Proximate composition Dry matter	941
Crude protein	491
Crude lipids	98
Ash	96

Vitamin mineral mix (EMIX PLUS, Mumbai, India) (Quantity per kg)

Vitamin A: 22 00 000 IU; Vitamin D3: 4 40 000 IU; Vitamin B2: 800 mg; Vitamin E: 300 mg; Vitamin K: 400 mg; Vitamin B6: 400 mg; Vitamin B12: 2.4 mg; Calcium Pantothenate: 1000 mg; Nicotinamide: 4 g; Choline Chloride: 60 g; Mn: 10 800 mg; I: 400 mg; Fe: 3000 mg; Zn: 2000 mg; Cu: 800 mg; Co: 180 mg; Ca: 200 g; P: 120 g; L L-lysine: 4 g; DL-Methionine: 4 g; Selenium: 20 ppm.

Culture of probiotic bacteria B. firmus CAS 7: The strain *B. firmus* CAS 7 was cultured and prepared as described by Sun et al., (2010). 500 mL of fresh nutrient broth was seeded with 1% inoculum (1.50 x 106 CFU mL-1) and kept in a shaker incubator (200 rpm) at pH 7.5, temperature 28 oC, and salinity 30 PSU for 48 h. After incubation

period, the cells were harvested by centrifugation at 5000 xg for 10 min, washed twice with phosphate-buffered saline (pH 7.5) and re-suspended in same PBS buffer for addition to the basal diet and this was administrated for probiotic study. Cell growth was estimated by measuring optical density at 600 nm from the aliquots withdrawn at every 6 h intervals.

Preparation of control and probiotic feed: The control basal diet was formulated using the ingredients such as fish meal, shrimp meal, soya bean meal, wheat flour, fish oil and vitamin mineral mix (Table1) (Sun, Yang, Ma, & Lin, 2010).All the ingredients were dried overnight at 80° C in a hot air oven and powdered. The powdered ingredients were sieved through a fine-meshed screen (0.5 mm diameter) and mixed well. The dough was prepared by adding required amount of water with the ingredients, sterilized (autoclave at 121° C for 15 min) and incorporated with 3% (v/w) commercial vitamin mineral mix (EMIX PLUS, Mumbai, India) and pelletized using hand pelletizer to obtain 1 mm pellets. The pellets were initially sun dried and then oven dried at 60 ± 5°C for 12 hours to get moisture content. Further, they were manually broken into smaller bits and stored at room temperature in an air tightened sterile polypropylene containers.

The test feeds for the experiments were prepared by gently spraying the required amount of bacterial suspension on the control diet and mixing it part-by-part in a drum mixer to obtain a final concentration of 50, 100 and 150 mg kg-1 probiotic based on preliminary experiments in our laboratory. The probiotic cell suspensions (108cells mL-1) were added to the control diet after the dosage had been autoclaved and subsequently cooled, before pelletizing. The proximate composition (moisture, protein, ash, lipid and fibre) of all probiotic feeds and control feed were determined by the standard procedures of AOAC (1990). The viability counts (CFU/g) of the probiotic incorporate feed before and after drying was enumerated using a pour plate technique using the method described by Reddy et al., (2009) and expressed in % of survival. The probiotic strain-incorporated feeds were packed in sterile polypropylene containers and stored at 4°C for further studies.

Experimental design: The experiment was conducted at Aquaculture breeding center, Centre of Advanced Study in Marine Biology, Annamalai University, India. The juveniles of smoke angelfish, *Apolemichthys xanthurus* were obtained from MAV Breeders (Mandabam, Tamil Nadu, India) and acclimatized for 4 weeks before the trial. The feeding experiment was conducted in (20 L) rectangular fibre glass tanks, with temperature ranging from 26 - 30oC, salinity 28 -30 PSU, pH 7.4 - 7.8; and dissolved oxygen (DO) 4.2 to 5.6 mg L-1. A total of 30 fish seeds were maintained in each tank throughout the experiment (12.89 ± 0.41 g mean weight) and each treatment was conducted in triplicate. In control, the fishes were feed with prepared pellet feed alone. The probiotic bacteria of 50, 100 and 150 mg kg-1 were mixed with experimental feed. The feeding rate was about 3% of biomass per day provided in equal rations at 8.00 AM, 1.00 PM, 6.00 PM for 120 days and the excess diet was collected and dried at 60 °C, put in room temperature for 3 days to restore the natural moisture and then weighed. Feeding rate was adjusted every fortnight during sampling by batch weighing of fish in each tank after a 24 h period of starvation. At the same time, fish survival was also determined by counting the individuals in each aquarium. Experimental tanks were cleaned and water exchange was done once a week.

Growth indices: The growth parameters such as weight gain, specific growth rate (SGR), survival rate and feed conversion ratio (FCR) were assessed at 30, 60, 90 and 120 days. The weight gain (WG), specific growth rate (SGR) and feed conversion ratio (FCR) was evaluated based on standard formula as follows.

Weight gain (g) = (Final body weight (g) – Initial body weight (g)

Specific growth rate (SGR) = 100 (loge final body weight - loge initial body weight) / culture period (days)

Feed conversion ratio (FCR) = total dry feed fed (g) / total live weight gain (g)

Colour enhancement and carotenoid content estimation

The color enhancement was monitored by visual examination and estimation of carotenoid content in the skin of experimental fishes. The carotenoid content of the experimental fish skin was extracted according to the method of Torrissen & Naevdal (1984). Three fishes from each experiment were randomly sampled, anesthetised using clove oil (dissolved in 95% ethanol at 1:10) and used for carotenoid content analysis in triplicate. Briefly, 2 mg of skin were collected from both sides between the abdominal and dorsal regions of the fish and then transferred to 10 mL of pre - weighed glass tubes after the fat layer had been removed from the skin and ground well with acetone containing anhydrous sodium sulphate and made up to 10 mL with acetone. The samples were stored for 3 days at 4oC in a refrigerator, and then extracted three times till no further colour could be obtained and centrifuged at 5000 xg for 5 min. The total carotenoid content of the samples was determined using spectrophotometer (Shimadzu, UV mini 1240) using extinction coefficients (E1%, 1 cm) of 2000 for astaxanthin (Hata, & Hata, 1971) at 475 nm, and 2500 for carotenoids from alfalfa at 450 nm (Schiedt, & Jensen, 1995).

Data analysis: In the present study, all the data were analyzed by statistical methods. The two way analysis of variance (ANOVA) was performed using SPSS (Statistic Package for social science) version 11.5 software to determine the significant differences among means. For all tests, a criterion of P<0.01 was used to determine statistical significance.

RESULTS AND DISCUSSION

Based on the morphological, physiological, and biochemical characteristics, the strain CAS 7 is a Gram-positive and endospore-forming bacillus, with catalase but without oxidase, which grows in both aerobic and anaerobic environments (Table 1). Further, 16S rRNA gene sequencing and BLAST analysis confirmed that the strain belongs to *B. firmus* and designated as *B. firmus* CAS 7 (GenBank accession no. HQ116805).The 16S rRNA gene sequences of the probiotic strain CAS 7 obtained from the present study was deposited in NCBI with accession number HQ116811.

The results revealed that the weight of fishes increased with the increase in days of culture in all the experimental groups (Table.2). At the end of the experiment, it was found that there was significant weight gain in all the three experiments where fishes were fed with probiotic-supplemented basal feed. The weight gain was comparatively less in the control group. The weight gain was the highest in group 150 mg kg-1(80.95 g) followed by group 100 (67.26 g), 50 (57.37 g) and control groups (45.81 g). The specific growth rate (SGR) was significantly (P<0.01) higher in 150 mg kg-1 group (0.675) compared with fishes in the group of 100 mg kg-1 (0.527), 50 mg kg-1 (0.457) and control (0.382). Furthermore, the most advantageous value for conversion ratio (FCR) classified as follows: 150 > 100 > 50 > control group (Table 3). The survival rate of the fishes was 80 - 100% (in control and 150 mg kg-1group, respectively) (P<0.01).

The results of the carotenoid analysis revealed that the initial carotenoid content of the fish skin varied between 1.24 and 1.28 mg g-1 in all experimental groups and control and increased gradually with increasing days of culture. At the end of the experimental period, carotenoid content was 2.91 - 6.24 mg g-1 (in control and 150mg kg-1 group) (Table 3) (P<0.01) (Table.3). Thus, these results depicted that the carotenoid content of fish group fed with feed was supplemented with 150 mg kg-1 of probiotic was the highest. The research on the use of probiotics in aquatic animals has received heightened attention with the demand for environment friendly health management in aquaculture (Geng et al., 2011). A growing number of studies have demonstrated the ability of probiotics to increase the growth rate and / or feed utilization of farmed aquatic animals (Carnevali et al., 2004; Wang et al., 2008). The benefits of probiotics in fish farming are improvements of growth performances, immunity and pathogen exclusions (Qi, et al., 2009; Sun et al., 2010). Most of the probiotic studies have focused on use of *Lactobacillus* and *Bacillus spp.* (Lee, 2013).

Bacillus species are gaining more and more importance and are widely used in aquaculture due to their longer stability, easy preparedness, antagonistic effects on pathogens and enhancement of immunity (Hong et al., 2005; Gupta et al., 2014). Earlier studies suggested that the Bacillus species significantly improved the growth in Catla catla (2 × 105 B. circulans PB7 cells per 100 g feed) (Bandyopadhyay & Mohapatra ,2009), Labeo rohita (1.5 x 105 *B. circulans*) (Ghosh et al., 2003), Macrobrachium rosenbergii (*Artemia salina nauplii* with *B. subtilis* (108cells mL-1) (Keysami et al., , 2007) and Penaeus monodon (Rahiman, et al.2010). Although several studies have been undertaken to study the beneficial effects of probiotic bacteria on growth and survival rate of various organisms, no studies have been attempted on marine ornamental fishes, especially on marine angel fish, *Apolemichthys xanthurus*. Hence, an attempt was made in this study to ascertain the effect of probiotic, Bacillus firmus CAS 7 as feed supplement on growth, survival and colour enhancement in marine angelfish *Apolemichthys xanthurus*.

In the present study, the fishes were fed basal diet supplemented with probiotic *B. firmus* CAS 7 at 50, 100 and 150 mg kg-1 and the growth performance such as weight gain, survival rate and color enhancement was evaluated for 120 days. Results suggested that the growth, weight gain and SGR were comparatively higher in fish group fed with feed supplemented 150 mg kg-1 of probiotic than other experimental groups and control. Other reports also reported that the probiotic (Bacillus *sp.*) supplemented diet (bio encapsulated *Artemia* nauplii by 3×108 CFU/ L) significantly increased the weight, length and SGR of fish when compared to the control diet. Several studies suggested that the probiotic supplementation has significantly increased the weight gain and SGR in *Rachycentron canadum* (Geng et al., 2012), Labeo rohita (Giri, Sukumaran, & Oviya, 2013) (Giri et al., 2013), *Oreochromis niloticus* (Aly et al., 2008), *Epinephelus coioides* (Sun et al., 2010) and *Larimichthys crocea* (Ai et al., 2011).

The results suggested that the FCR was lower in the fish group fed with feed supplemented 150 mg kg-1 probiotic followed by 100 and 50 150 mg kg-1 and control. It seems that the reduction in FCR of fishes in experimental groups revealed dietary nutrients were utilized more efficiently when the diet was supplemented with probiotics. Similarly, Neja, Rezaei, Takami, Lovett, Mirvaghefi, & Shakouri (2006) reported that *Bacillus spp.* could be used to increase the digestive enzyme activity, survival and growth in the Indian white shrimp.Fishes

are incapable of biosynthesizing carotenoids, so diet is their sole source as only plants, bacteria, fungi and algae have the capacity for its synthesis (Geng et al., 2011). Many reports have demonstrated that skin color change over time depended on the level of carotenoid in the diet and differed among species (Chatzifotis, Pavlidis, Jimeno, Vardanis, Sterioti, & Divanach, 2005; Dharmaraj, & Dhevendaran, 2011; Ho, Zong, & Lin, 2014). The results of the study on color enhancement suggested that the carotenoid content in the skin of fishes fed with probiotic maintained coloration during periods of social interaction, suggesting that the probiotic Bacillus firmus CAS7 may play important roles in maintaining fish skin coloration. Steiger, (2012)& Liu et al., (2009) were suggested that carotenoids are commonly found in pigmented bacteria which are known to have a positive role in the intermediary metabolism of fish that could enhance nutrient utilization.The research on the use of probiotics in aquatic animals has received heightened attention with the demand for environment friendly health management in aquaculture (Geng et al., 2011).

A growing number of studies have demonstrated the ability of probiotics to increase the growth rate and / or feed utilization of farmed aquatic animals (Carnevali et al., 2004; Wang et al., 2008). The benefits of probiotics in fish farming are improvements of growth performances, immunity and pathogen exclusions (Qi et al., 2009; Sun et al. 2010). Most of the probiotic studies have focused on use of Lactobacillus and Bacillus spp. (Cabello et al., 2013; Lee, Kim, Song, Oh, Cha, Jeong, Heo, Kim, & Lee, 2013). Bacillus species are gaining more and more importance and are widely used in aquaculture due to their longer stability, easy preparedness, antagonistic effects on pathogens and enhancement of immunity (Hong, Le Duc, Cutting, 2005; Gupta et al., 2014).

Table 3. Growth performance and survival rate of smoke angel fish Apolemichthys xanthurus fed with basal diet (control -without probiotic) and basal diet supplemented with 50, 100 and 150 mg kg-1 of probiotic strain B. firmus CAS7. Values are presented in mean ± SD, n = 3, FCR - feed conversion ratio; SGR - specific growth rate (% d-1)

Days of culture	Growth Parameters	Control	50 mg kg^{-1}	100 mg kg^{-1}	150 mg kg^{-1}
0 - 30	Initial weight (g)	12.33±0.38	12.57±0.29	12.63±0.32	12.89±0.41
	Final weight(g)	20.27±1.60	25.94±1.20	29.93±1.40	30.13±1.10
	Weight gain (g)	7.94±1.60	13.37±0.29	17.3±0.30	17.24±1.20
	SGR	0.265±0.03	0.446±0.04	0.577±0.02	0.641±0.03
	FCR	2.33±0.04	1.38±0.03	1.07±0.03	1.07±0.02
	Survival rate (%)	80	90	90	100
	Carotenoid content (mg g-1)	1.24±0.06	1.28±0.08	1.25±0.04	1.25±0.06
0 - 60	Initial weight (g)	12.33±0.38	12.57±0.29	12.63±0.32	12.89±0.41
	Final weight(g)	32.83±1.50	39.92±1.60	45.57±1.10	51.56±1.30
	Weight gain (g)	20.5±1.50	27.35±1.20	32.94±1.40	38.67±1.40
	SGR	0.342±0.03	0.456±0.02	0.549±0.01	0.763±0.02
	FCR	1.80±0.05	1.35±0.02	1.12±0.03	0.96±0.05
	Survival rate (%)	80	90	100	100
	Carotenoid content (mg g-1)	2.54±0.03	3.14±0.03	3.79±0.05	4.38±0.06
0 - 90	Initial weight (g)	12.33 ± 0.38	12.57 ± 0.29	12.63 ± 0.32	12.89 ± 0.41
	Final weight(g)	46.24±0.80	55.32 ±1.50	61.02 ±1.30	72.56 ± 0.90
	Weight gain (g)	33.91±1.60	42.75 ±1.50	48.39±1.30	59.67±0.90
	SGR(% day-1)	0.377±0.04	0.475±0.02	0.552±0.05	0.730±0.02
	FCR	1.64±0.05	1.30±0.03	1.15±0.06	0.93±0.01
	Survival rate (%)	80	90	90	100
	Carotenoid content (mg g-1)	2.76±0.05	3.55±0.07	4.01±0.04	5.69±0.004
0 - 120	Initial weight (g)	12.33±0.38	12.57±0.29	12.63±0.32	12.89±0.41
	Final weight(g)	58.14±1.3	69.94±1.20	79.89±0.90	93.84±1.40
	Weight gain (g)	45.81±1.4	57.37±1.30	67.26±1.40	80.95±1.50
	SGR	0.382±0.03	0.457±0.03	0.527±0.02	0.675±0.04
	FCR	1.61±0.03	1.29±0.03	1.10±0.04	0.91±0.02
	Survival rate (%)	80	90	90	100
	Carotenoid content (mg g-1)	2.91±0.08	4.26±0.06	5.74±0.03	6.24±0.05

Earlier studies suggested that the Bacillus species significantly improved the growth in Catla catla (2 × 105 B. Circulans PB7 cells per 100 g feed) (Bandyopadhyay & Mohapatra ,2009), Labeo rohita (1.5 x 105 *B. circulans*) (Ghosh et al., 2003), Macrobrachium rosenbergii (*Artemia salina nauplii* with *B. subtilis* (108cells mL-1) (Keysami et al., 2007) and *Penaeus monodon* (Rahiman et al., 2010). Although several studies have been undertaken to study the beneficial effects of probiotic bacteria on growth and survival rate of various organisms, no studies have been attempted on marine ornamental fishes, especially on marine angel fish, *Apolemichthys xanthurus*. Hence, an attempt was made in this study to ascertain the effect of probiotic, *Bacillus firmus* CAS 7 as feed supplement on growth, survival and colour enhancement in marine angelfish *Apolemichthys xanthurus*.

In the present study a *B. firmus* CAS 7 isolated from marine environment was cultured in nutrient broth for 48 h and used as feed supplement to evaluate the improvement in growth and colour enhancement on marine smoke angel fish. The growth kinetic study revealed that the maximum cell growth of the probiotic bacterium was achieved at logarithmic phase (24th h). Elayaraja et al., (2011) studied the effect of amylase produced by *B. cereus* on the growth of polycheate and maximum cell growth as well as enzyme production was found at late logarithmic phase (36th h). The short incubation period for achieving maximum growth makes this a potential probiotic candidate species which could be used in the aquaculture industry. The dietary supplementation of *B. firmus* CAS 7 in the present study exhibited good growth performance and significantly increased survival rate of *Apolemichthys xanthurus*. In a previous study, Bacillus administration has also been shown to increase survival by enhancing resistance to pathogens by acting both cellular and humoral immune defence in shrimp and prawn (Rengpipat et al., 2000; Gupta, & Dhawan, 2013).

Abraham et al., (2007) suggested that the probiotics greatly helps in achieving natural resistance and high survivability of fish. In the present study, the fishes were fed basal diet supplemented with probiotic *B. firmus* CAS 7 at 50, 100 and 150 mg kg-1 and the growth performance such as weight gain, survival rate and color enhancement was evaluated for 120 days. Results suggested that the growth, weight gain and SGR were comparatively higher in fish group fed with feed supplemented 150 mg kg-1 of probiotic than other experimental groups and control. Jafaryan et al., (2008) also reported that the probiotic (*Bacillus sp*.) supplemented diet (bio encapsulated *Artemia nauplii* by 3×108 CFU/ L) significantly increased the weight, length and SGR of fish when compared to the control diet. Several studies suggested that the probiotic supplementation has significantly increased the weight gain and SGR in *Rachycentron canadum* (Geng et al., 2012), Labeo rohita (Giri et al., 2013), *Oreochromis niloticus* (Aly et al., 2008), *Epinephelus coioides* (Sun et al., 2010) and *Larimichthys crocea* (Ai et al., 2011).

The results suggested that the FCR was lower in the fish group fed with feed supplemented 150 mg kg-1 probiotic followed by 100 and 50 150 mg kg-1 and control. It seems that the reduction in FCR of fishes in experimental groups revealed dietary nutrients were utilized more efficiently when the diet was supplemented with probiotics. Similarly, Nejad et al., (2006) reported that Bacillus spp. could be used to increase the digestive enzyme activity, survival and growth in the Indian white shrimp. Fishes are incapable of biosynthesizing carotenoids, so diet is their sole source as only plants, bacteria, fungi and algae have the capacity for its synthesis (Geng et al., 2011). Many reports have demonstrated that skin color change over time depended on the level of carotenoid in the diet and differed among species (Chatzifotis et al., 2005; Dharmaraj, & Dhevendaran, 2011; Ho et al., 2014). The results of the study on color enhancement suggested that the carotenoid content in the skin of fishes fed with probiotic maintained coloration during periods of social interaction, suggesting that the probiotic Bacillus firmus CAS7 may play important roles in maintaining fish skin coloration. Steiger, (2012) & Amar et al., (2001) were suggested that carotenoids are commonly found in pigmented bacteria which are known to have a positive role in the intermediary metabolism of fish that could enhance nutrient utilization and may ultimately result in improved growth. in improved growth.

CONCLUSION

The results confirmed that Apolemicthys xanthurus fed with 150 mg kg-1 probiotic bacteria B. firmus CAS 7 exhibited significantly improved growth performance, survival and colour enhancement. Therefore, 150 mg kg-1 of probiotic B. firmus CAS 7 should be used for formulating nutritionally balanced diet of marine angel fish for its better growth and survival. The mode and mechanism of actions about carotenoid content increase in the fish skin and carotenoid production by this probiotic strain need further investigation.

ACKNOWLEDGEMENTS

The authors are greatly thanked to the authorities of Sathyabama Institute of Science and Technology and Annamalai University for providing the facilities. The author, M.V. Rajeswari is greatly acknowledged to University Grants Commission (UGC), Government of India for financial support through UGC-Postdoctoral fellowship for women.

REFERENCES

Abraham, T.J., Babu, C.H.S., Mondal, S., & Banerjee, T. (2007). Effects of dietary supplementation of commercial human probiotic and antibiotic on the growth rate and content of intestinal microflora in ornamental fishes. Bangladesh Journal of Fisheries Research, 11, 57–63. http://dx.doi.aquaticcommons.org/id/eprint/18902

Ai, Q., Xu, H., Mai, K., Xu, W., Wang, J., & Zhang, W. (2011). Effects of dietary supplementation of Bacillus subtilis and fructooligo saccharide on growth performance, survival, non-specific immune response and disease resistance of juvenile large yellow croaker, Larimichthys crocea. Aquaculture, 317, 155-161. http://dx.doi.org/10.1016/j.aquaculture.2011.04.036

Aly, S.M., Mohamed, M.F., & John, G. (2008). Effect of probiotics on the survival, growth and challenge infection in Tilapia nilotica (Oreochromis niloticus). Aquaculture Research, 39, 647-656. https://dx.doi.org/10.1111/j.1365-2109.2008.01932.x

Amabile-Cuevas, C.F., Cardenas-Garcia, M., & Ludgar, M. (1995). Antibiotic resistance. Animal Science, 83, 320-329. https://dx.doi.org/10.1111/j.1365-2109.2008.01932.x

Amar, E.C., Kiron, V., Satoh, S., & Watanabe, T. (2001). Influence of various dietary synthetic carotenoids on bio-defence mechanisms in rainbow trout, Oncorhynchus mykiss (Walbaum). Aquaculture Research, 32, 162-163. https://dx.doi.org/10.1046/j.1355-557x.2001.00051.x

AOAC (1990). Official Methods of Analysis of the Association of Official Analytical Chemists, Vol. 1, 14th edn. 1102 pp.

Balcazar, J.L., De Blas, I., Ruiz-Zarzuela, I., Cunningham, D., Vendrell, D., & Muzquiz, J.L. (2006). The role of probiotics in aquaculture. Veterinary Microbiology, 114, 173-186. https://dx.doi:10.1016/j.vetmic.2006.01.009

Bandyopadhyay, P., & Mohapatra, P.K.D. (2009). Effect of a probiotic bacterium Bacillus circulans PB7 in the formulated diets: on growth, nutritional quality and immunity of Catla catla (Ham.). Fish Physiology and Biochemistry, 35, 467-478. https://dx.doi 10.1007/s10695-008-9272-8

Carnevali, O., Zamponi, M.C., Sulpizio, R., Rollo, A., Nardi, M., Orpianesi, C., Silvi, S., Caggiano, M., Polzonetti, A. M., & Cresci, A. (2004). Administration of probiotic strain to improve seabream wellness during development. Aquaculture International, 12, 377-386. https://dx.doi:10.1023/b:aqui.0000042141.85977.bb

Chatzifotis, S., Pavlidis, M., Jimeno, C.D., Vardanis, G., Sterioti, A., & Divanach, P. (2005). The effect of different carotenoid sources on skin coloration of cultured red porgy (Pagrus pagrus). Aquaculture Research, 36, 1517 – 1525. https://dx.doi:10.1111/j.1365-2109.2005.01374.x

Debelius, H., Tanaka, H., Kuiter, & R. H. (2003). Angelfishes: A Comprehensive Guide to Pomacanthidae. TMC Publishing, Chorleywood, UK, 208 pp.

Dharmaraj, S., & Dhevendaran, K. (2011). Application of microbial carotenoids as a source of colouration and growth of ornamental fish Xiphophorus helleri. World Journal of Fish and Marine Sciences, 3(2),137-144. Retrieved form https://idosi.org/wjfms/wjfms3(2)11/10.pdf

Duc, L. H., Fraser, P., & Cutting, S.M. (2006). Carotenoids present in halotolerant Bacillus spore formers. FEMS Microbiology Letter, 255, 215-224. https://dx.doi:10.1111/j.1574-6968.2005.00091.x

Ege, (1939). A revision of the genus Anguilla Shaw, a systematic, phylogenetic and geographical study, Dana Report, 16, 256 pp

Elayaraja, S., Annamalai, N., Murugesan, P., Mayavu, P., & Balasubramanian, T. (2011). Effect of amylase on growth, survival and proximate composition of the Polychaete, Perinereis cultrifera (Grube, 1840). Aquaculture Nutrition, 17, 627-633. http://dx.doi.10.1111/j.1365-2095.2011.00864.x

Fox, D.L. (1957). The pigments of fishes. Academic Press Inc, New York, USA.

Frische, J. (1999). Marine Ang7elfishes. TFH Publications, Neptune, NJ, USA, 64 pp.

Fujii, R. (2000). The regulation of motile activity in fish chromatophores. Pigment Cell Research, 13, 300-319. http://dx.doi.10.1034/j.1600-0749.2000.130502.x

Geng, X., Dong, B., Tan, Q., Yang, S., Chi, H., Liu. (2011). Effects of dietary chitosan and Bacillus subtilis on the growth performance, non-specific immunity and disease resistance of cobia, Rachycentron canadum. Fish Shellfish Immunology, 31, 400 – 406. http://dx.doi.10.1016/j.fsi.2011.06.006

Geng, X., Dong, X, H., Tan, B.P., Yang, Q. H., Chi, S.Y., Liu, H.Y., & Liu, X.Q. (2012). Effects of dietary probiotic on the growth performance, non-specific immunity and disease resistance of cobia, Rachycentron canadum. Aquaculture Nutrition, 18, 46-55. https://dx.doi.org/10.1111/j.1365-2095.2011.00875.x

Ghosh, K., Sen, S.K., Ray, A.K. (2003). Supplementation of an isolated fish gut bacterium, Bacillus circulans, in formulated diets for rohu, Labeo rohita, fingerlings. Israeli J. Aquaculture Nutrition, 55, 13-21. http://dx.doi.10.1111/j.1365-2095.2011.00875.x

Giri, S.S., Sukumaran, V., & Oviya, M. (2013). Potential probiotic Lactobacillus plantarum VSG3 improves the growth, immunity, and disease resistance of tropical freshwater fish Labeo rohita. Fish Shellfish Immunology, 34, 660-666. http://dx.doi.org/10.1016/j.fsi.2012.12.008

Gouveia, L., & Empis, J. (2003). Relative stabilities of microalgal carotenoids in microalgal extracts, biomass and fish feed: effect of Storage Condition. Innovative Food Science Emerging Technology, 4, 227-233. http://dx.doi.10.1016/S1466-8564(03)00002-X

Gouveia, L., & Rema, P. (2005). Effect of microalgal biomass concentration and temperature on ornamental goldfish (Carassius auratus). Aquaculture Nutrition, 11, 19 – 23. http://dx.doi.10.1111/j.1365-2095.2004.00319.x

.Gupta, A., & Dhawan, A. (2011). A Effect of

supplementing probiotics (Prosol) on the performanceof giant freshwater prawn (Macrobrachium rosenbergii) juveniles. Indian Journal of Animal Nutrition, 28, 457-63. http://dx.doi:10.1111/j.1365-2109.2007.01787.x

Gupta, A., Dhawan, A. (2012). Effect of dietary probiotic Improval (Lactobacillus sporogenes and Saccharomyces cerevisiae) on growth and feed utilization of Macrobrachium rosenbergii post larvae. Animal Nutrition Feed Techechnology, 12, 209-17. Retrived by https://www.indianjournals.com/ijor.aspx?target=ijor:anft&t volume=12&t issue=2&article=007

Gupta, A., Dhawan, A. (2013). Probiotic based diets for freshwater prawn Macrobrachium rosenbergii (de Man). Indian Journal of Fisheries, 60, 103–109. Retrieved from http://epubs.icar.org.in/ejournal/index.php/IJF/article/view/ 17914/12929

Gupta, A., Gupta, P., Dhawan, A. (2014). Dietary supplementation of probiotics affects growth, immune response and disease resistance of Cyprinus carpio fry. Fish and Shellfish Immunology, 41, 113–119. http://dx.doi.10.1016/j.fsi.2014.08.023.

Gupta, A., Geetika, V., Paromita, G. (2016). Growth performance, feed utilization, digestive enzyme activity, innate immunity and protection against Vibrio harveyi of freshwater prawn,Macrobrachium rosenbergii fed diets supplemented with Bacillus coagulans. Aquaculture international, http://dx.doi.10.1007/s10499-016-9996-x.

Harikrishnan, R., Balasundaram, C., & Heo, M.S. (2010). Lactobacillus sakei BK19 enriched diet enhances the immunity status and disease resistance to streptococcosis infection in kelp grouper. Epinephelus bruneus. Fish and Shellfish Immunology, 29, 1037-1043. http://dx.doi.10.1016/j.fsi.2010.08.017

Hata, M., & Hata, M. (1971). Carotenoid pigments in goldfish (Carassius auratus) II. Colour change and carotenoid pigment composition. International Journal of Biochemistry, 2, 182-184. http://dx.doi.10.1016/0020-711X(71)90210-2

Hill, G.E. (2002). A Red Bird in a Brown Bag: The Function and Evolution of Colorful Plumage in the House Finch. Oxford University Press, New York. 35 pp

Ho, A.L.F. C., Zong, S., & Lin, J. (2014). Skin color retention after dietary carotenoid deprivation and dominance mediated skin coloration in clown anemonefish, Amphiprion ocellaris. AACL Bioflux 7(2), 103-115. Retrieved by https://www.bioflux.com.ro/docs/2014.103-115.pdf

Hong, H.A., Le Duc, H., Cutting, S.M. (2005). The use of bacterial spore formers as probiotics. FEMS Microbiol Reviews, 29, 813–835. http://dx.doi.10.1016/j.femsre.2004.12.001

Keysami, M.A., Saad, C.R., Sijam, K., Daud, H.M., & Alimon, A. R. (2007). Effect of Bacillus subtilis on growth development and survival of larvae Macrobrachium rosenbergii (de Man) Aquaculture Nutrition, 13, 131-136. http://dx.doi- 10.1111/j.1365-2095.2007.00463.x

Lee, B.J., Kim, S.S., Song, J.W., Oh, D.H., Cha, J.H., Jeong, J.B., Heo, M.S., Kim, K, W., & Lee, K. J. (2013). Effects of dietary supplementation of citrus by-products fermented with a probiotic microbe on growth performance, innate immunity and disease resistance against Edwardsiella tarda in juvenile olive flounder, Paralichthys olivaceus (Temminck and Schlegel). Journal of Fish Diseases, 36, 617–628. http://dx.doi. 10.1111/jfd.12035

Matsuno., T. (2001). Aquatic animal carotenoids. Fisheries Science, 67, 771–783.

Moyle, P.B., Cech, J.J. (2004). Fishes, an introduction to ichthyology. , Upper Saddle River, New Jersey, USA. Prentice-Hall, 590 pp.

Merrifield, D. L., Dimitroglou, A., Bradley, G., Baker, R.T.M. & Davies, S. J. (2010). Probiotic applications for rainbow trout (Oncorhynchus mykiss Walbaum) I. Effects on growth performance, feed utilization, intestinal microbiota and related health criteria. Aquaculture Nutrition, 16, 496-503. http://dx.doi.10.1111/j.1365-2095.2009.00689.x

Moyer, J.T. (1987). Social organization and protogynous hermaphroditism in marine angelfishes (Pomacanthidae). In: Sex Change in Fishes (ed. byA. Nakazono and T. Kuwamura), Tokai University Press, 120-147 pp.

Nejad, S., Rezaei, M. H., Takami, G.A., Lovett, D.L., Mirvaghefi, A.R., & Shakouri M. (2006). The effect of Bacillus spp. bacteria used as probiotics on digestive enzyme activity, survival and growth in the Indian white shrimp Fenneropenaeus indicus. Aquaculture, 252, 516-524. http://dx.doi.10.1016/j.aquaculture.2005.07.021

Perez-Fons, L., Steiger, S., Khaneja, R., Bramley, P.M., Cutting, S.M., Sandmann, G., & Fraser, P.D. (2011). Identification and the developmental formation of carotenoid pigments in the yellow/orange Bacillus spore-formers. Journal of Biochimica et Biophysica Act, 1811, 177-185. http://dx.doi.10.1016/j.bbalip.2010.12.009

Qi, Z.Z., Zhang, X.H., Boon, N., & Bossier, P. (2009). Probiotics in aquaculture of China-current state, problems and prospect. Aquaculture, 290, 15-21. http://dx.doi.10.1016/j.aquaculture.2009.02.012.

Rahiman, K. M. M., Jesmi, Y., Thomas, A.P., & Hatha, A.A.M. (2010). Probiotic effect of Bacillus NL110 and Vibrio NE17 on the survival, growth performance and immune response of Macrobrachium rosenbergii (de Man). Aquaculture Research, 41, 120-134. http://dx.doi.10.1111/j.1365-2109.2009.02473.x.

Rajeswari, M.V., & Balasubramanian, T. (2014). Distribution, diversity and taxonomy of marine angelfishes (Pomacanthidae) of Tamil Nadu, Southeast coast of India. International Journal of Fisheries and Aquaculture, 6, 20-31. http://dx.doi. 10.5897/ijfa12.069

Rengpipat, S., Rukpratanporn, S., Piyatiratitivorakul, S., & Menasa-veta., P. (2000). Immunity enhancement on black tiger shrimp (Penaeus monodon) by a probiont bacterium (Bacillus S11). Aquaculture, 191, 271-

288. Retrieved from http://www.elsiever.ni/locate/aquaonline/2000

Schiedt, K., Jensen, S.L. (1995). Isolation and analysis. In: Britton, G., Liaaen-Jensen, S., Pfander, E. (Eds.), Carotenoids: Isolation and Analyses, vol. 1A. Birkha¨user, Basel, Switzerland, 108 pp

Simpson, K.L., Katayama, T., & Chichester, C.O. (1981). Carotenoids in fish feeds. In: Carotenoids as Colorants and Vitamin A Precursors (Bauernfeind, J.C. ed.) volume 4: Academic Press, New York. 463 pp

Steiger, S., Perez-Fons, L., Fraser, P.D. & Sandmann, G. (2012). Biosynthesis of a novel C30 carotenoid in Bacillus firmus isolates, Journal of Applied Microbiology, 113, 888-895. http://dx.doi.10.1111/j.1365-2672.2012.05377.x

Storebakken, T. P., Foss, K., Schiedt, Austreng, E., & Jensen , S.L. (1987). Carotenoids in the diets for salmonids IV. Pigmentation of Atlantic salmon with astaxanthin, astaxanthin dipalmitate and canthaxanthin. Aquaculture,65(3-5), 279-292. https://dx.doi.org/10.1016/0044-8486(87)90241-9

Sun, Y.Z., Yang, H.L., Ma, R.L, & Lin, W.Y. (2010). Probiotic applications of two dominant gut Bacillus strains with antagonistic activity improved the growth performance and immune responses of grouper Epinephelus coioides. Fish Shellfish Immunology, 29, 803-809. http://dx.doi.10.1016/j.fsi.2010.07.018

Van der Salm, A.L., Martnez, M., Flik, G., & Wendelaar Bonga, S.E. (2004). Effects of husbandry conditions on the skin colour and stress response of red porgy, Pagrus pagrus. Aquaculture, 241: 371-386.http://doi.10.1016/j.aquaculture.2004.08.038.

Wabnitz, C., Taylor, M., Green, E., Razak, T. (2003). From ocean to aquarium. UNEP-WCMC, Cambribge, U.K. 64 pp

Wang, Y.B., Xu, Z.R., & Xia, M.S. (2005). The effectiveness of commercial probiotics in Northern White Shrimp (Penaeus vannamei L.) ponds. Fisheries Science, 71, 1034-1039. http://dx.doi.10.1111/j.1444-2906.2005.01061.x

Wang, Y.B., Li, J.R., & Lin, J. (2008). Probiotics in aquaculture: challenges and outlook. Aquaculture, 281, 1-4. http://dx.doi.10.1016/j.aquaculture.2008.06.002

Highlights

The fishes were fed basal diet supplemented with probiotic of B. firmus CAS 7 at 50, 100 and 150 mg kg-1

The growth performance such as weight gain, survival rate and color enhancement was evaluated for 120 days.

The probiotic effect of fish growth and disease resistant were showed promising activity.

Dental
Communication

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp-261-273

Techniques for Management of Supraerupted Teeth Prior to Prosthetic Treatment: Updated Review

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ABSTRACT

The increasing number of partially dentated patients is the result of changes in the patterns of teeth loss and the ageing of the population. The extent and type of occlusal change may compromise the prostheses of the unopposed tooth and teeth bounding the extraction area. Undesirable tooth movements may be prevented. The purpose of this systematic review is to summarize the different techniques used for the management of unilateral or bilateral supraerupted tooth/teeth in the anterior/posterior and maxillary/mandibular regions. Electronic and manual searches were conducted by an independent reviewer to identify relevant articles, case reports, and case series published up to November, 2019. A pilot checklist consisting of 10 items was implemented to methodologically assess the relevant published studies. Only 30 studies and case reports were included after the exclusion of all irrelevant papers. The majority of cases were related to the supraeruption of maxillary teeth, whereas few cases were related to mandibular supraeruption, and only two cases were related to anterior maxillary and mandibular supraeruption. Most clinical cases of all types of teeth supraeruption were managed semi conservatively by mini-implants or miniscrews, and only a few cases were treated with endocrowns, ameloplasty, removable or fixed appliances, or nonconservative surgical orthodontics. Dentists will obtain a brief idea of the various treatment modalities for managing supraerupted tooth/ teeth discussed in this review.

KEY WORDS: AMELOPLASTY, MINISCREW IMPLANT, SUPRAERUPTED TEETH, TEETH INTRUSION.

ARTICLE INFORMATION

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Received 3rd Jan 2020 Accepted after revision 26th Feb 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)
A Society of Science and Nature Publication, Bhopal India

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/43

INTRODUCTION

If the extraction side is not restored immediately following tooth/teeth loss, the opposing dentition will erupt, and the vertical dimension for prosthetic treatment decreases (Ataoglu et al., 2002). In this case, securing insufficient intermaxillary space for either a fixed or removable prosthesis can be an extremely valuable point in establishing treatment plans (Ataoglu et al., 2002; Joshi et al., 2010). Supraerupted posterior tooth/teeth are common clinical findings during daily dental practice. Postponing the replacement of the extracted tooth/teeth often leads to the extrusion of the opposing tooth/teeth into the edentulous space; this effect will lead to masticatory insufficiency and temporomandibular disorders. Re-establishing a functional posterior occlusion requires a comprehensive dental treatment plan when a fixed or removable prosthesis is planned for the opposing edentulous arch (Mahoorkar et al. 2010).

In adult patients, if the dentoalveolar extrusion is not severe, the space can be recaptured by performing coronoplasty and intentional endodontic treatment of the supraerupted tooth. When the extrusion is moderate, orthodontic intrusion can be conducted, and if the extrusion is severe, prosthetic rehabilitation is impossible and the removal of the teeth is often proposed (Mahoorkar et al., 2010; Shillingburg et al., 2012; Rosenstiel et al., 2015). The teeth most likely to become unopposed, and therefore susceptible to supraeruption, are those occluding against edentulous parts. Clinically, the most commonly missing posterior teeth are the first permanent maxillary molars (Djemal et al., 2004; Basutkar et al., 2018; Prakash et al., 2014; Salazar et al., 2018; Al-Fraidi & Zawawi 2010) and teeth in the mandibular arch (Patil et al., 2016; Arslan et al., 2010; Tiago et al., 2016; Baeg et al., 2016), followed by the maxillary premolars (Djemal et al., 2004); the mandibular premolars are rarely missing. The mandibular posterior teeth are more likely to be extracted than the other teeth, and with increasing age, the posterior teeth are more likely to be extracted and lost bilaterally than the other types of teeth (Marcus et al. 1991; Meskin & Brown 1988). Other studies found that maxillary teeth are the most commonly extracted teeth (Craddock et al., 2007a).

The unopposed bilateral maxillary first molars in healthy mouths and in mouths with some periodontal pathology present were studied by Compagnon and Woda 1991. They found that the majority of supraeruption occurs in the early years following opposing tooth/teeth extraction. At later years, the loss of periodontal support may be superimposed on the area. However, in healthy individuals, the gingival margin remains at its original level on the tooth/teeth during occlusal tooth movements. After 10 years of remaining unopposed, periodontal migration reverses and root exposure occurs. This phenomenon was described as passive eruption (Craddock & Youngson 2004). Most published articles related to the management of supraerupted teeth are mainly in a form of case reports, case series, or clinical and technical reports only. Thus, the aim of this review is to collect these publications in an organized manner and to simplify the methods or techniques for treating cases that may face similar conditions during daily dental practice.

Methodology for Data Collection: This review conformed to the PRISMA review protocol. The database of PubMed/Medline, ScienceDirect, and Scopus was searched using the terms "overerupted teeth, supraerupted tooth, treatment of overerupted teeth, case report, case series, review of literature." The investigators then screened the titles, cases, and abstracts using English language. Studies that reported the treatment of overeruption through orthodontics only and did not mention prosthetic treatment were excluded. Case reports and case series were mainly shortlisted; full-text articles were then collected, and the same criteria were applied. Related Definitions (GPT 9., 2017). The supraeruption or overeruption of tooth/teeth is defined as the movement of a tooth or teeth above the normal occlusal/incisal plane.

Occlusal analysis: A systematic examination of the occlusion with special consideration of the interocclusal relations of mounted casts. Occlusal contact (deflective occlusal contact/initial occlusal contact): The touching of opposing teeth on the elevation of the mandible or any contact relation of the opposing teeth.

Coronoplasty, enameloplasty, or occlusal adjustment: Occlusal or aesthetic reshaping. It can be performed through occlusal correction, which is occlusal adjustment or reshaping.

Aesthetic reshaping: The physical modification of the surfaces of the teeth to improve appearance. These modifications can be performed through axial or occlusal reduction.

Crown lengthening: A surgical procedure designed to increase the extent of supragingival tooth structure for restorative or aesthetic purposes.

Crown–root ratio: The physical relationship between the portions of tooth not within the alveolar bone as determined by a radiograph compared with the portion of tooth within the alveolar bone.

Endocrowns: Indirect monoblock restorations that use the pulp chamber of the ETT for retention (Tzimas et al., 2018).

Osteotomy: The surgical cutting of a bone; this term is also frequently used to describe smoothing, leveling, or altering the external contours of the bone and may include alveolectomy and alveoplasty.

Posterior segmental osteotomy: A surgical procedure for drastically restoring previously diminished vertical dimension following the extraction of opposing molar/

molars; dentists recommend this procedure as a treatment method to facilitate prosthetic restoration (Baeg et al., 2016).

Corticotomy: A selective alveolar bone cutting technique using ultrasonic bone surgery to enhance tooth movements (Grenga & Bovi 2013).

Objective of the Occlusal Management of Supraerupted Teeth: In general, all the needed diagnostic data must be prepared and ready for interpretation. These data include X-ray views (preapical, panoramic, and cephalometric). In addition, a maxillary and mandibular cast mounted on an appropriate articulator is necessary before designing the treatment plan needed for a particular case. The objectives of the treatment of supraeruption are as follows (Mahoorkar et al., 2010; Shillingburg et al., 2012; Rosenstiel et al., 2015):

1. To direct the occlusal forces along the long axis of the teeth.
2. To attain the simultaneous contact of all teeth in centric relation and maximum intercuspation.
3. To eliminate any occlusal interference contact on inclined planes to enhance the positional stability of the teeth.
4. To coincide centric relation with the maximum intercuspation position during different mandibular movements.
5. To arrive at the occlusal scheme selected for a particular patient (Rosenstiel et al., 1998).

Prevalence of Supraerupted Teeth: Craddock et al. (2007a) stated that the average amount of overerupted tooth/teeth ranges from 1.68 mm to 3.99 mm of tooth/teeth without opposing, in which 1.03 and 1.91 mm were in mandible and maxilla, respectively. Kiliaridis et al. (2000) found that 24% of unopposed tooth/teeth had more than 2 mm of supraeruption among 82% of the examined subjects with unopposed tooth/teeth. A conclusion from another study stated that the occlusal change in unopposed teeth is mostly within 2 mm (Faggion et al., 2011).

Causes and Sequences of Supraeruption: Supraerupted posterior tooth/teeth are one of the most common clinical findings in dental practice. The delayed replacement of lost tooth/teeth often leads to the extrusion of the opposing teeth into the edentulous space; this effect leads to masticatory insufficiency and TMJ disorders (Mahoorkar et al., 2010). When the prosthesis is planned on the opposing edentulous area, re-establishing a functional posterior occlusion requires a comprehensive dental treatment plan. The tooth/teeth opposing the extracted sites are usually tilting lingually in the mandibular teeth and buccally in the maxillary teeth. In addition, the teeth adjusent to the extracted tooth/teeth usually drifted to the mesial and distal side. (Shillingburg et al., 2012; Rosenstiel et al., 2015). If the dentoalveolar extrusion is mild, the space can be recaptured by performing coronoplasty and intentional endodontic treatment of the supraerupted tooth. Orthodontic intrusion can be done when the extrusion is moderate, but if the extrusion is severe, prosthetic rehabilitation is impossible, and the removal of the teeth is often proposed (Shafad et al., 2017).

Prevention of Supraeruption: Supraeruption of tooth/teeth can be prevented by the light forces generated during chewing against an antagonist (Gierie et al., 1999). Thus, the replacement of the lost tooth/teeth with a fixed or removable prosthesis that prevents the overeruption of its antagonist is mandatory, and it should be fabricated before vertical movement had occurred (Davenport et al., 1988). Extraction is another option for dealing with an unopposed tooth, provided this tooth is not the key tooth for future restorative options (Kayser 1981). Solnit et al. (1988) demonstrated the use of an etched metal splint to bond the unopposed tooth to its adjacent opposed tooth. This approach may eliminate supraeruption provided that the bond between teeth remains intact. Jepson and Allen (1999) recommended the use of adhesive distal cantilever bridges to stabilize tooth position following tooth removal and to prevent undesirable eruptive movement.

Considerations during the evaluation of supraerupted tooth/teeth (Craddock & Youngson 2004):

1. Is the tooth a key for future prosthesis options?
2. Has the tooth remained unopposed for a time without signs of supraeruption?
3. Could the supraeruption of the tooth/teeth present prosthetic or occlusal difficulties?
4. What is the overall state of the dentition?
5. Patient preferences and tolerance in form of aesthetic considerations and the other planned restorative treatments for the unopposed tooth/teeth and the extracted spaces.

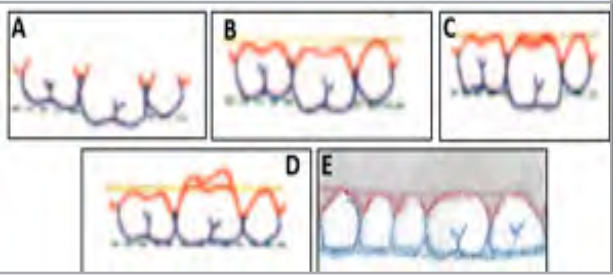
Classification of Supraerupted Teeth: Supraeruption can be classified according to the length of the overerupted tooth/teeth from the occlusal plane (Craddock & Youngson 2004; Graddock et al., 2007a; Carranza et al., 2018) into the following categories (Figs. 1A–1C): Mild, in which the supraerupted tooth extends between 0.1–1.5 mm; moderate, wherein the supraeruption of the occlusal surface is between 1.6–3.5 mm; and severe, wherein the amount of supraerupted tooth/teeth exceeds 3.5 mm in relation to the level of the occlusal plane.

Moreover, supraerupted teeth can be classified in accordance with physiological changes in the position of the gingival margins. The appearance of supraeruption may have several components, including passive and

Figure 1. Mild (A), Moderate (B), Severely overerupted teeth (C)

active eruption, periodontal growth, or physiological wear. Compagnon and Woda (1991) described periodontal growth as passive eruption that is distinct from active eruption wherein the tooth/teeth is/are in continuous movement in an occlusal direction in the absence of periodontal growth. Periodontal growth is a process in which the attachment apparatus moves in an occlusal direction with the tooth, whereas in active eruption, the tooth erupts and the attachment apparatus comes to lie apically to its original position. Wear is a physiological sequence that occurs with increasing age (Craddock & Youngson 2004; Craddock et al., 2007a) (Figs. 2A–2E). When a tooth wears down, the occlusal level may remain constant with the alveolar bone becoming closer to the occlusal plane (Fig. 2E) as described by Compagnon and Woda 1991.

Figure 2. Modified classification of overerupted teeth. (A) Tooth erupted beyond the occlusal plane. (B) Gingival margins follow tooth eruption (periodontal growth). (C) Gingival margins remain at original level (active eruption). (D) Gingival margins recede, whereas the tooth remains parallel to the actual plane (passive eruption). (E) Gingival margins remain at the original level but occlusal wear is present (relative wear) (Compagnon & Woda 1991).

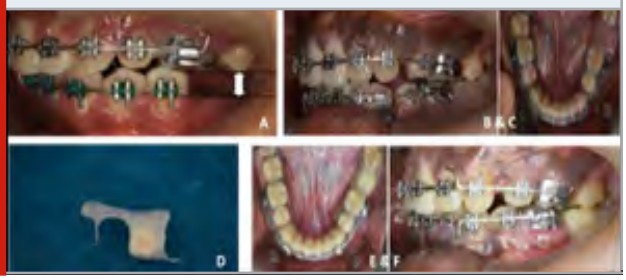


Techniques for the Management of Supraerupted Tooth/Teeth Prior to Prosthetic Treatment: Before any correction of supraerupted tooth/teeth, examination and diagnosis must be conducted throughly. A diagnostic cast or model should be made then mounted on an articulator using facebow transfer, and a trial adjustment of occlusion on cast should be done first. Many materials, including occlusal registration strips, occlusal indicator wax, marking ribbon, articulating papers, and T-scan, can be used to examine, diagnose, and detect occlusal interferences (Mahoorkar et al. 2010; Shillingburg et al 2012; Rosenstiel et al., 2015). Supraerupted tooth/teeth are preferably adjusted after the treatment of an existing disease, such as gingival inflammation, healing of a pathogenesis, and treatment of trauma from occlusion (Carranza et al., 2018; Rosenstiel et al., 2015). Treatment modalities differ and depend on whether the supraeruption involves a single tooth or a group of teeth, whether it is unilateral or bilateral or in the maxillary or mandibular arch, and on the degree or amount of the occlusal/incisal supraeruption (Basutkar et al., 2018). The types and techniques for the treatment of supraerupted teeth can be divided into three main categories:

1.Conservative: In this group, the supraerupted tooth/teeth will not be reduced from the occlusal or incisal plane. They are usually extended beyond the occlusal/incisal plane from 0.1 mm to 4 mm. They can be corrected in the following ways:

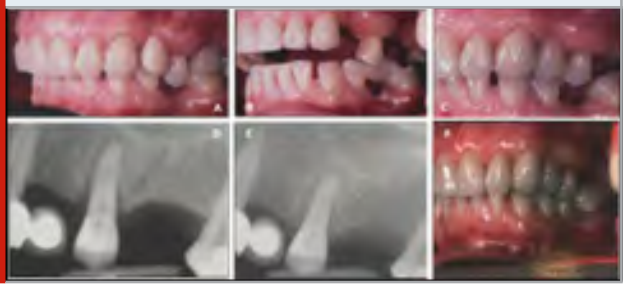
Removable acrylic appliance: In this approach, the supraerupted tooth/teeth can be corrected using a removable appliance, such as a modified posterior bite plane appliance (MPBP), which is fabricated for retention with a ball-end clasp to manage a supraerupted maxillary molar along with ongoing fixed orthodontic treatment (Shafad et al., 2017). MPBP appliances have numerous advantages. For example, their physiological force generates minimal root resorption, and it is noninvasive and cost-effective compared with mini-implant-assisted intrusion. Moreover, it reduces buccal flaring compared with occlusally placed brackets for intrusion. It may minimize discomfort to patients, is easy to remove and clean with slight bulkiness, and reduces overall treatment time (Figs. 3A–3F). It can intrude more than one tooth at the same time without the need for the special retention of the intruded molar as the same bite plane appliance and can be modified by adding an acrylic tooth to the appliance. Thus, the same appliance can act as a retention appliance, a space maintainer, and a removable partial denture. Finally, the appliance can be reactivated by increasing the height of the appliance by adding self-curing acrylic on its occlusal surface if the required intrusion is increased. However, sometimes the use of removable appliances or splint for the management of supraerupted teeth in a temporomandibular dysfunction-free patient is not preferable because it might exacerbate signs and symptoms related to appliance wearing rather than increasing the occlusal vertical dimension (Abduo & Lyons 2012).

Figure 3. (A) Overeruption measurement. (B and C) Modified Removable Posterior Bite Plane Appliance. (D) MRPBPA with acrylic tooth. (E and F) Appliance in function after months (Shafad et al., 2017).



Fixed composite bite plane: In the case of a small posterior space, the correction of a supraerupted tooth can be adjusted using a reinforced direct composite plate to intrude a maxillary premolar tooth on the basis of the Dahl concept (Dahl et al., 1975). Subsequently, the space can be restored by an FP (Djemal et al., 2004). This type of treatment can be extended for up to a year or more (Figs. 4A–4F).

Figure 4. (A) Supraerupted tooth # 25. (B) Composite occlusal plate on teeth # 34 and 36. (C) RBB replacing tooth # 24 and composite occlusal stop on # 34. (D and E) Radiograph of tooth # 25 showing funneling of coronal crestal bone at end of tooth movements. Complete treatment after year. (F) At the 5th year, tooth # 25 is being held in the occlusal plane by an opposing bridge (Djemal et al., 2004).



Intrusion only: This type of treatment can be performed using orthodontic intrusion only or in combination with mini-implants for anchorage. This treatment depends mainly on temporary skeletal anchorage devices and miniscrew and microscrew implants for skeletal anchorage. It can be accomplished after orthodontic treatment (Salazar et al., 2018), without orthodontic treatment (Al-Fraidi & Zawawi 2010), or with segmental orthodontic treatment as edge-wise arches (Acar & Ates 2016). The intrusion can be on a single tooth or a group of teeth, unilateral (Foat et al., 2015), or bilateral (Prakash et al., 2014; Salazar et al., 2018; Al-Fraidi & Zawawi 2010). It is an interdisciplinary approach based on the use of orthodontic, periodontal, restorative, and implant therapy (Arslan et al., 2010). It is considered as a better treatment option for intruding tooth/teeth than prosthodontic reduction or extraction of extruded teeth (Prakash et al., 2014). It demonstrates a low, continuous, well-controlled manner of force without causing reciprocal movements of other teeth in direction and magnitude and creates an adequate interocclusal space for replacing missing tooth/teeth by construction of FPs (Acar & Ates 2016; Faot et al., 2015; Al-Fraidi & Zawawi 2010). It can be applied for the treatment or correction of the supraerupted tooth/teeth of the maxillary arch (Acar & Ates 2016; Prakash et al., 2014; Salazar et al., 2018; Faot et al., 2015; Al-Fraidi & Zawawi 2010) as shown in Figs. 5A–5K and Figs 6A–6I, or in the mandibular arch (Arslan et al., 2010; Tiago et al., 2016) as seen in Figs. 7A–7F and Figs. 8A–8H, or in the anterior region (Faot et al., 2015) as presented in Figs. 9A–9D).

Figure 5. (A–C) Preoperative and panoramic views. (D–G) Segmental archwire and miniscrew. (H and K) Views after the removal of minscrew and appliances (Acar & Ates 2016).

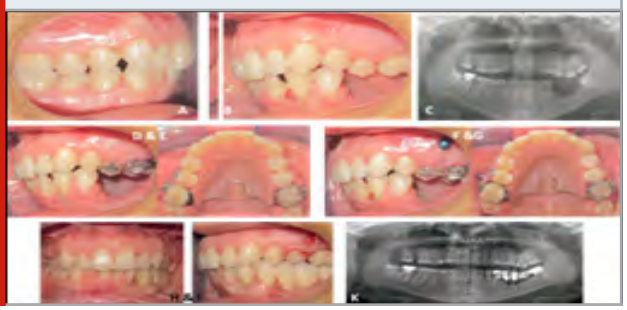


Figure 6. (A–C) Preoperative and panoramic views. (D–F) Intrusion of bilateral maxillary molars using miniscrews with alloy spring. (G–I) Postoperative intraoral views (Faot et al., 2015).

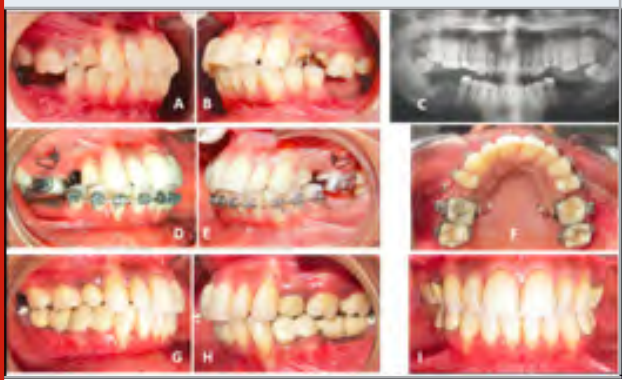


Figure 7. (A) Supraerupted mandibular 2nd molar. (B) Over-trimmed occlusal surfaces of the left mandibular 2nd premolar and 1st molar. (C) Postoperative. (D) Miniscrews and mini-implants attached to the mandibular 2nd molar by coil spring and elastic thread. (E) Partial fixed appliance on mandibular 1st and 2nd premolars and 1st molar to correct the position of the mandibular 2nd molar. (F) Intruded left mandibular 2nd molar (Arslan et al., 2010)

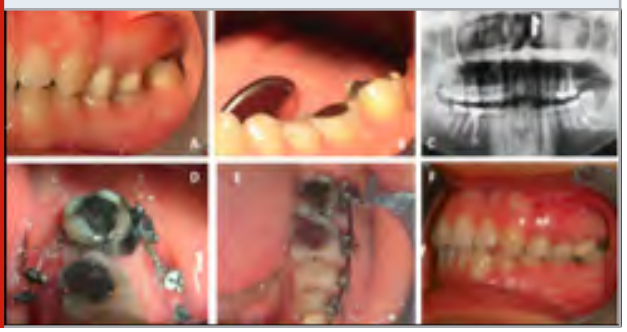


Figure 8. (A) Extrusion of tooth # 47. (B) Orthodontic mini-implant. (C and D) Radiographic of mini-implant and orthodontic abutment cemented and archwire. (E and F) View of intrusion and verticalization. (G) After intrusion and stabilization. (H) With rehabilitation (Tiago et al., 2016).

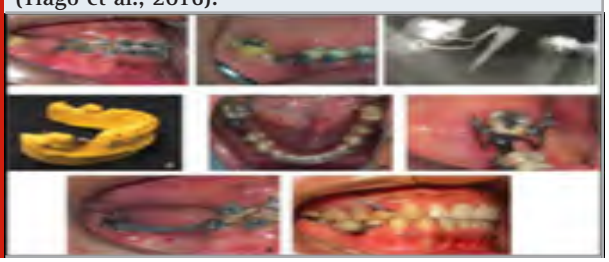
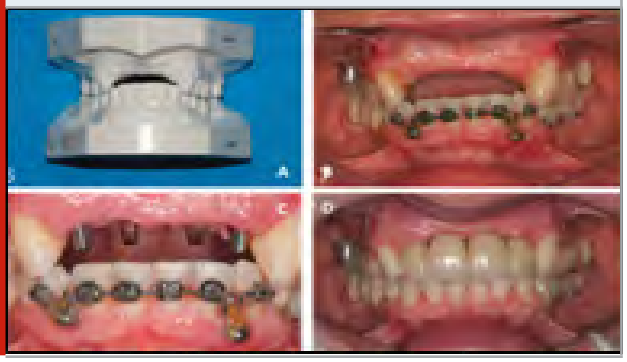


Figure 9. (A) Model of anterior region of maxilla. (B) Frontal view of clinical evaluation. (C) Interocclusal space available after prosthetic abutment's selection. (D) Frontal view of final rehabilitation (Faot et al., 2015).



The advantages of this type of treatment are that it is noninvasive and inexpensive. It allows the immediate application of forces to intrude the supraerupted tooth/teeth, requires in-office installation, and stimulates patient cooperation (Choi et al., 2005; McGuire et al., 2006; Mizrahi & Mizrahi 2007). Moreover, this system shows a good biological response of the tooth/teeth and the bony structures and soft tissues surrounding the intrusion. Tooth/teeth appear normal and acceptable, and the vitality and periodontal health of the area surrounding the treatment area are maintained (Arslan et al., 2010; Faot et al., 2015). The overall outcome of this treatment is excellent during different follow-up intervals. In general, orthodontic intrusion techniques are suitable methods for the management of supraerupted tooth/teeth and are unaccompanied by reciprocal effects on anchorage units (Hakami Z 2006).

2. Semiconservative Treatment: This type of treatment for supraerupted tooth/teeth is mainly dependent on the amount of supraeruption. If the supraeruption is between 0.1–2 mm, it can be managed by enamloplasty or coronoplasty. If the amount of supraeruption exceeds 1.5 mm, it can be corrected by intentional root canal treatment (RCT) followed by endocrown; by intrusion followed by RCT; or by the reduction of the supraerupted tooth/teeth followed by intentional RCT in the maxillary arch or mandibular arch. Finally, it can be assembled by a combination of crown lengthening (CL) and crowning. These approaches are described below:

Enameloplasty or coronoplasty: This approach is a selective reduction of occlusal interferences to influence mechanical contact conditions and the neural pattern of sensory input. It is a direct and irreversible change in the occlusal scheme. The complete function of occlusal adjustment is to eliminate the harmful action of occlusal forces. It provides the functional stimulation necessary to preserve periodontal health during activities (Carranza et al., 2018; Rosenstiel et al., 2015; Shillingburg et al., 2012; Mahoorkar et al., 2010). The objectives of coronoplasty are to mechanically eliminate all occlusal supra contacts in function and parafunction habits. In addition, it prevents trauma from occlusion, provides a stable occlusion and occlusal schema after adjustment, and improves the functional relationship between teeth (Mahoorkar et al., 2010 and Malathi et al., 2014).

Clinical steps of coronoplasty/enameloplasty or dentoplasty: This method can effectively reduce occlusal discrepancy in a slightly to moderately extruded tooth. Approximately 0.1–1.5 mm of enamel can be reduced or reshaped in different clinical situations. This reduction can be polished or reshaped with composite restoration and can be created by the reshaping of a single cusp for improving the occlusal plane (Stewart et al., 1983; Mahoorkar et al., 2010; Malathi et al., 2014). It can be performed through the following methods:

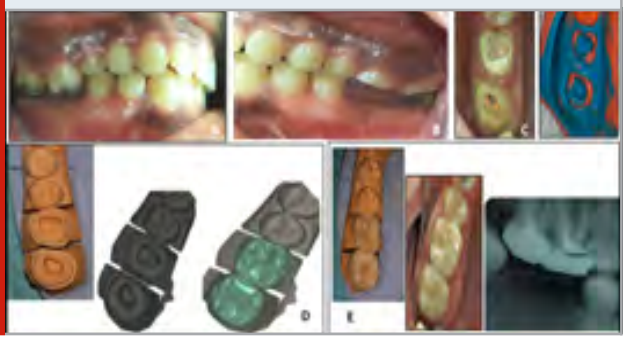
1. Removing retrusive prematurities and eliminating the deflective shift from the retruded cuspal position to the intercuspal position (ICP).
2. Adjusting the ICP to achieve stable, simultaneous, multipointed, and widely distributed contacts.
3. Testing for excessive contacts (fremitus) on incisor teeth.
4. Removing posterior protrusive supra contacts and establishing contacts that are bilaterally distributed on anterior teeth.
5. Removing or lessening mediotrusive (balancing) interferences.
6. Reducing excessive cusp steepness on the laterotrusion (working) contacts.
7. Eliminating gross occlusal disharmonies.
8. Rechecking tooth contact relationships.
9. Polishing all rough surfaces (Carranza et al., 2018; Rosenstiel et al., 2015).

In some cases, the clinician may slightly increase reduction; thus, the minimum amount of dentine should be removed and recovered with a layer of composite or glass ionomer restoration in the usual manner (Mahoorkar et al., 2010). Coronoplasty/enameloplasty or dentoplasty can be performed following a previously reported form (Carranza et al., 2018; Stewart et al., 1983) as illustrated in (Figs. 10A–10C). Grooving is usually conducted using a tapered diamond and results in the restoration of the depth of developmental grooves. Spheroiding is performed using a light paint brush stroke; this process restores the original tooth contour while reducing supra contacts. Pointing entails restoring cusp point contours.

Figure 10. Types of enamloplasty or coronoplasty, A, Grooving; B, Spheroiding; C, Pointing (Stewart et al., 1983).

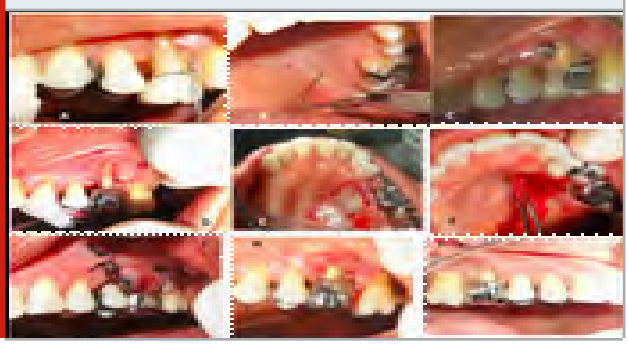
Endocrowns with intentional RCT: Results of clinical trials showed that clinicians can restore teeth by using endocrowns to improve long-term restorations for patients (Sun et al., 2019). Endocrowns after intentional RCTs are a good treatment option for restoring supraerupted endodontically treated posterior tooth/teeth. Its use, especially for endodontically treated teeth with short clinical crowns and for teeth where radicular anatomy eliminates the use of post and core, can also be justified (Basutkar et al., 2018) as shown in Figs. 11A–11E. New enhanced bonding mechanisms and advances in ceramic technology and adhesive techniques have increased the reliability of endocrowns as treatment options (Basutkar et al., 2018; Bindle & Mormann 1999; Sevimli et al., 2015).

Figure 11. (A) Supraerupted tooth # 16 and 17 with ↓ interocclusion. (B) After RCT, occlusal reduction was performed to restore occlusal plane and regain interarch space resulting in short clinical crown. (C) After RCT with shoulder finish line supragingivally and preparation inside the pulp chamber for endocrown. (D) Dies of scanned endocrowns. (E) Cemented endocrowns (Basutkar et al., 2018).



Intrusion with RCT and crowning: In some cases, the successful treatment of overerupted tooth/teeth with severe gingival recession may necessitate a multidisciplinary approach. After initial nonsurgical periodontal therapy and intentional RCT, maxillary molar extrusion can be corrected using a microimplant followed by covering the gingival recession and fixed prostheses to replace the extracted tooth/teeth; intentional RCT is then recommended as seen in Figs. 12A–12I (Mehta et al., 2018).

Figure 12. (A) Supraerupted tooth # 26. (B) Bone checking with periodontal probe. (C) Microimplant placement and postorthodontic intrusion. (D–J) Post endodontic and flap surgery. (H and I) Cemented crown (Mehta et al., 2018).



Reduction of teeth with intentional RCT and crowning: The supraerupted teeth in arches are reduced, followed by intentional RCT, CL, and crowning. These steps are done to create a stable, long term aesthetic results. The long term stability of the marginal bone levels, gingival levels, and status of the teeth subjected to endodontic therapy followed by CLP and final restorations are evaluated. Cases that underwent CL and endodontic therapy for corrections of the supraerupted teeth to regain the lost interocclusal spaces were retrieved, and cases with a complete set of clinical data and radiographs were obtained. The cases were followed by clinical and radiographical recording. The amount of interocclusal space regained was adequate to restore the missing teeth in the opposing arch. CL is a predictive procedure for the correction of supraerupted teeth in the maxilla (Figs. 13A–13E) or in the mandibular arches (Figs. 14A–14F) with a survival rate of 100% over 24–96 months (Patil et al., 2016).

Figure 13 . (A and B) Preoperative views of supraerupted tooth # 16, 17, and 18. (C) Intentional RCT of tooth #16 and 17 and extraction of tooth #18. (D) Crowning of tooth # 16 and 17 and implants with crowns of mandibular molars. (E) Postoperative view after 96 months (Patil et al., 2016).

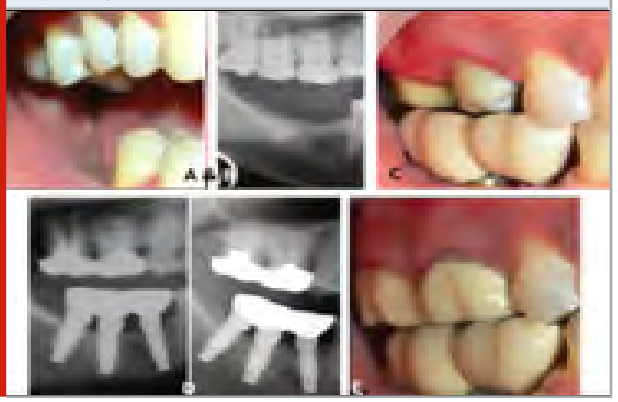
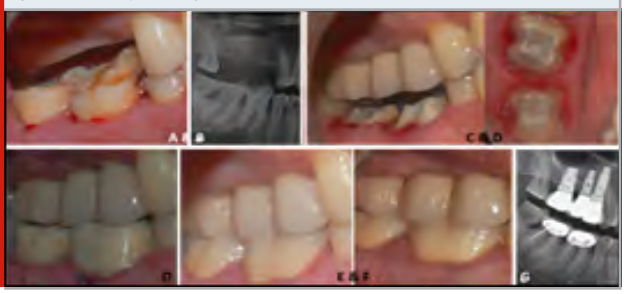


Figure 14. (A and B) Preoperative view of tooth # 46 and 47. (C and D) After occlusal reduction and intentional RCTs. (D) Maxillary and mandibular teeth with final prosthesis. (E–G) Postoperative views after 56 months (Patil et al., 2016).



Crown lengthening with crowning: Tooth/teeth with short clinical crowns and have undergone RCT followed by osseous CL resection and final prostheses with equigingival margins survive for long durations with stable gingival tissue and bone levels. This type of treatment may be predictably used to correct overerupted tooth/teeth and to obtain the necessary interarch space for prosthetic purposes. This treatment results in a favorable long-term prosthesis outcome (Patil et al., 2016).

3. Nonconservative Treatment: In this category, supraerupted tooth/teeth may be extended by 4 mm or more from the occlusal plain and can be corrected by orthognathic surgery only or removal. This category involves the following treatments

Orthognathic surgical treatment: In this approach, posterior maxillary segmental osteotomy (PMSO) can be effective in correcting the supraeruption problem by a dentoalveolar extrusion (Meningaud et al., 2006; Punde 2013). This technique is simple, safe, and quick but strict that can achieve a good surgical outcome but a poor final occlusion. Some distortions can occur at any stage of surgery. Thus, using a surgical guide in the form of an acrylic splint is mandatory to achieve an acceptable final occlusion. The placement of an orthodontic arch wire or/ and interim denture may prevent the risk of movement in the transverse and vertical dimensions (Erverdi et al., 2006). The PMSO technique is recommended for providing adequate interarch space in the presence of posterior supraerupted tooth/teeth and is usually conducted under general anesthesia. Through this technique, approximately 4 mm or more of interocclusal clearance can be achieved from the existing preoperative clearance; this effect allows the prosthetic restoration of dentition in the opposing arch. The outcome of this technique is stable without complications over 16–18 months postoperatively as presented in Figs. 15A–15H (Punde 2013). Moreover, PMSO can be bilateral (Baeg et al., 2016) as shown in Figs. 16A–16F.

Figure 15. (A) Supraerupted maxillary teeth. (B and C) Preoperative views. (D) Mock surgery and posterior segment repositioned for splint fabrication. (E and F) Osteotomy cuts 5 mm above the apices of molars and superiorly placed segment. (G and H) Postoperative after 6 months (Punde 2013).

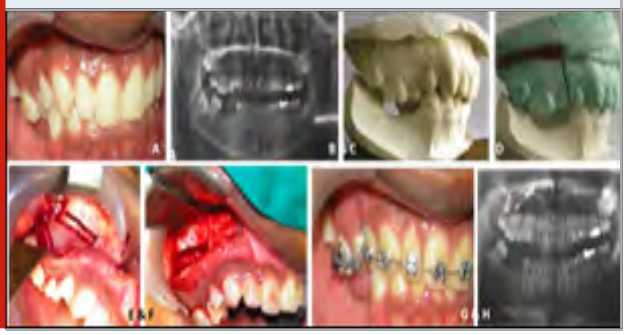
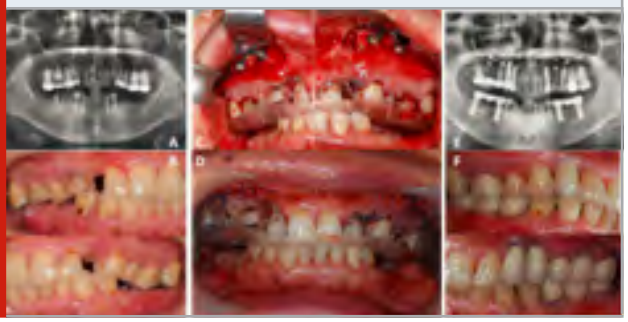
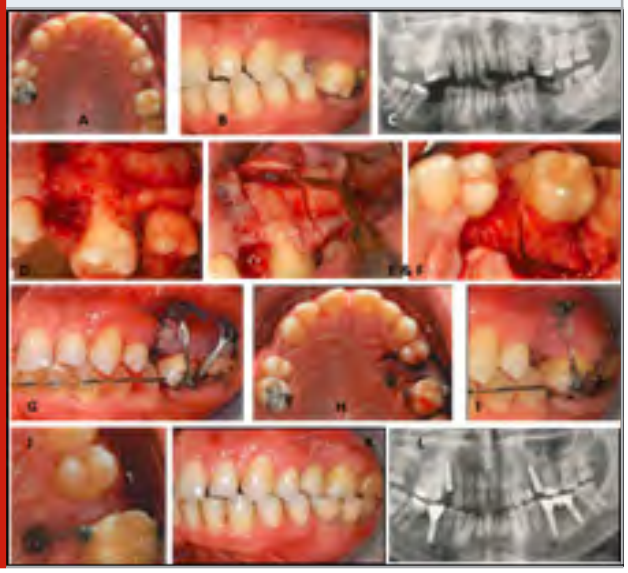


Figure 16. (A and B) Preoperative views showing the supraeruption of teeth with 0 mm interdental space. (C and D) During PMSO. (E and F) Postoperative views showing ↑interarch space by implant-supported FPD (Baeg et al., 2016).



Corticotomy: This method is an effective approach for gaining the intrusion of the supraerupted tooth/teeth without any side effects using skeletal anchorage. It is quick and provides highly predictable results when performed through ultrasonic piezoelectric surgery, which is safer and causes less bone trauma than other techniques for osteotomies (Grenga and Bovi., 2013) (Figs. 17A–17L).

Figure 17. (A–C) Preoperative view of tooth # 26. (D–F) During surgery and bone cuts adjacent to tooth. (G and H) Miniscrews inserted mesial and distal to vertical bone cuts with palatal ligature wire. (I and J) During intrusion. (K and L) Postoperative views (Grenga and Bovi 2013).



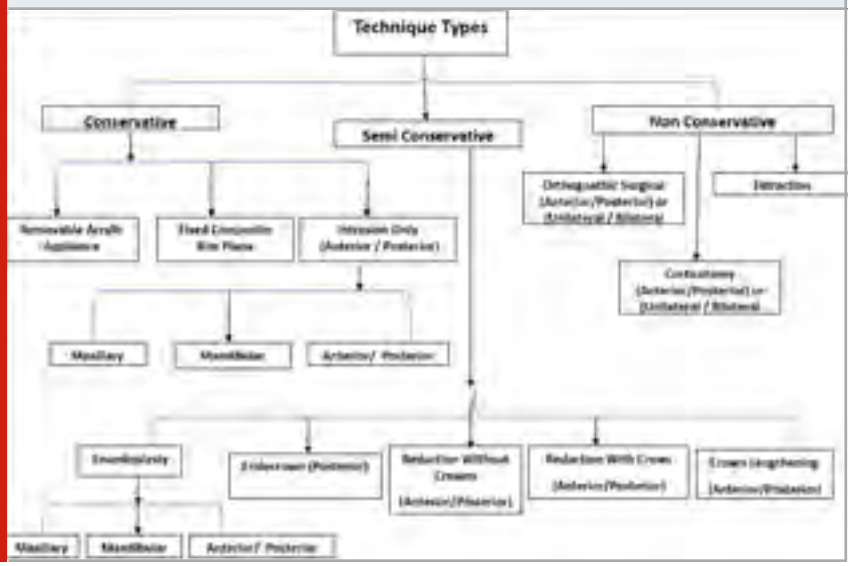
Tooth extraction: This method is applied for cases of resorption or the loss of the alveolar bone support around the supraerupted tooth/teeth, such as tri/bifurcation involvement, or for cases where supraeruption resulted in inadequate or improper crown/root ratio (Mahoorkar et al., 2010; Baeg et al., 2016; Mehta et al., 2018; Rosenstiel et al., 2015; Shillingburg et al., 2012). The removal of tooth/teeth is recommended if the tooth is not a key

tooth for future prosthetic option (Craddok & Youngson 2004).

Concluding Remarks from Previous Clinical Studies and Reviews: During routine examinations and treatment planning for the replacement of extracted teeth and its importance in preventing supraeruption, dentists should be aware and familiar with the occlusal changes following individual tooth/teeth loss. Moreover, they should advise patients on the importance of replacing missing teeth to maintain intact dentition throughout life (Craddock & Franklin 2005). A series of studies investigated the type and extent of supraeruption and tooth/teeth movements associated with posterior teeth without their antagonist and recorded any relationship between supraeruption and oral or patient factors. The relationship of tooth/teeth positional interferences following posterior tooth loss during different jaw movements were studied (Craddock et al., 2007a; Craddock et al., 2007b; Craddock 2008; Faggion et al., 2011). The following conclusions were obtained:

- The mean supraeruption for subjects without antagonists was between 0–3.99 mm and that for the control group was 0–1.46 mm (Craddock et al., 2007a).
- Supraeruption in the maxillary tooth/teeth was statistically higher than that in the mandibular tooth/teeth and was recorded in 92% of subjects with unopposed teeth.
- Supraeruption is classified on the basis of the form of attachment loss, periodontal growth, and palatal movement of the tooth distal to the extraction position. Three types of supraeruption, which may be recorded alone or in combination, have been identified. Attachment loss is associated with active eruption, whereas periodontal growth is inversely associated with attachment loss. Both conditions are common in the maxillary arch, among young female patients, and in premolar teeth. Wear is directly associated with increasing age and is highly common in unopposed mandibular teeth (Craddock et al., 2007a).
- A statistically significant difference was observed between the subjects and control groups in terms of the degree of tipping of maxillary tooth/teeth mesial and distal to the extraction site (nonvertical movements). Moreover, a significant difference was noted in the rotation of the tooth/teeth mesial to the site (Craddock et al., 2007b).
- Protrusive interferences are associated with the position and presence of tooth/teeth distal to the extraction area, whereas working side interferences are associated with the tipping of the tooth mesial to the extraction area, and many other jaw movements are related mainly to the other types of interferences (Craddock and Youngson 2004; Craddock 2008).
- An intraexaminer agreement using Kappa scores and Spearman's correlation revealed no statistically significant difference between supraeruption and occlusal interference even in the presence of supraerupted teeth (Craddock & Youngson 2004).
- A single study provided evidence for a statistically significant correlation between supraeruption resulting from the nonreplacement of mandibular first molars and recorded some gradual changes in the occlusal pattern; these interferences even extended to cause temporomandibular dysfunction (Gupta et al., 2014). In the same category, a study recommended that prosthodontic therapy in the presence of supraerupted teeth should be performed even in the presence of temporomandibular dysfunction if needed and indicated (de Carlsson & Klineberg 2000a; de Carlsson & Klineberg

Figure 18. Summary of techniques for correction of supraerupted teeth



2000b).

- An in vitro study measured the amount of the supraeruption of teeth following RPD treatment by comparing data collected from a surface-computer-aided design by dental casts at different time points (before and after RPD treatment). Supraeruption was exhibited by 38.1% of teeth antagonized with RPD and was considerably less than that in teeth that were unopposed by RPD (Matsuda et al., 2014).

CONCLUSION

Supraeruption of tooth/teeth is a serious problem faced by dental practitioners during their daily dental practice. The importance of the treatment of supraerupted teeth is to re-establish the proper occlusal plane and the relation of both arches during different jaw movements to minimize further trauma of the occlusion to the tooth/teeth and their surrounding structures. This problem can be managed through easy and straightforward approaches, such as a conservative technique. This technique can be performed without any reduction of the supraerupted tooth/teeth and can be applied with a removable appliance, composite bite plane, and tooth intrusion. The semiconservative technique can be performed with tooth reduction only or by enamloplasty, which is effective in reducing occlusal discrepancy in a moderately extruded tooth by reducing a single cusp. This approach improves the occlusal plane. The amount of reduction is controlled by the clinical crown length of the tooth or by the size of the dental pulp. In some cases, the reduction can be extended into intentional RCT followed by the crowning of the supraeruption tooth/teeth. Other treatment choices can be surgical orthodontics, corticotomy, or extraction of the severely supraerupted tooth/teeth. A summary of techniques for correction of the supraerupted tooth/teeth before prosthetic treatments is presented in Fig. 22.

Source of support: Nil

Conflict of interest: None

REFRENCES

Abduo J, Lyons K. (2012). Clinical considerations for increasing occlusal vertical dimension: a review. REVIEW. Austr Dent J; 57: 2–10.

Acar YB, Atek M. (2016). DIRECT USAGE OF MINISCREW ANCHORAGE TO INTRUDE OVERERUPTED MAXILLARY POSTERIOR TEETH BEFORE PROSTHODONTIC PREPARATION: A CASE REPORT. J Istanbul Univ Fac Dent 2016;50(1):43-50.

Al-Fraidi AA, Zawawi KH. (2010). Selective Intrusion of Overerupted Upper First Molars Using a Temporary Anchorage Device: Case Report. JCDA; 76(1): 25-30.

Arslan A, Ozdemir DN, Gursoy-Mert H, Malkondu O, Sencift K. (2010). Intrusion of an overerupted mandibular molar using mini-screws and mini-implants: a case report. Austr Dent J; 55: 457–461.

Ataoglu H, Kucukkolbasi H, Ataoglu T. (2002) Posterior segmental osteotomy of maxillary edentulous ridge: an alternative to vertical reduction. Int J Oral Maxillofac Surg; 31(5): 558–559.

Baeg SW, On SW, Lee JK, Song S. (2016). Posterior maxillary segmental osteotomy for management of insufficient intermaxillary vertical space and intermolar width discrepancy: a case report. Maxillof Plast Recon Surg; 38:28-33.

Basutkar N, Wali O, Ahmad SA-Q, Padala HK, Kumar RS, Sheetal A, Bahru H. (2018). "Management of Severely Supra-Erupted Teeth with Endocrown: Case Report". Acta Scie Dent Scie: 2(9): 21-24.

Bindl A and Mormann WH (1999). "Clinical evaluation of adhesively placed cerec endocrown after 2 years-preliminary results". J Adhe Dent; 1(3): 255-265.

Carranza F, Newman M, Glickman I. (2018). Clinical periodontology: WB Saunders Company; 18th Edition; page, 64, 70, 3174, 3208.

Choi BH, Zhu SJ, Han SG, Huh JY, Kim BY, Jung JH. (2005). The need for intermaxillary fixation in sagittal split osteotomy setbacks with bicortical screw fixation. Oral Surg Oral Med Oral Pathol Oral Radiol Endod;100: 292–295.

Compagnon D, Woda A. (1991). Supraeruption of the unopposed maxillary first molar. J Prosthet Dent; 66: 29-34.

Craddock HL, Youngson CC. (2004). A study of the incidence of overeruption and occlusal interferences in unopposed posterior teeth. Br Dent J; 196(6): 341-8.

Craddock HL (2008). Occlusal changes following posterior tooth loss in adults. Part 3. A study of clinical parameters associated with the presence of occlusal interferences following posterior tooth loss. J Prosthodont; 17(1): 25-30.

Craddock HL, Franklin P. (2005). Overeruption--another challenge? Dent Update; 32(10): 605-8, Dent Update; 33(1): 12.

Craddock HL, Youngson CC, Manogue M, Blance A. (2007a). Occlusal changes following posterior tooth loss in adults. Part 1: a study of clinical parameters associated with the extent and type of supraeruption in unopposed posterior teeth. J Prosthodont; 16(6): 485-94.

Craddock HL, Youngson CC, Manogue M, Blance A. (2007b). Occlusal changes following posterior tooth loss in adults. Part2. Clinical parameters associated with movement of teeth adjacent to the site of posterior tooth loss. J Prosthodont; 16(6): 495-501.

Dahl B L, Krogstad O, Karlsen K. An alternative treatment in cases with advanced localised attrition. J Oral Rehab 1975; 2: 209-214.

Davenport JC, Basker RM, Heath JR, Ralph JP. (1988).

A colour atlas of removable partial dentures. London: Wolfe Medical Publications: page, 11-13. de,
Carlsson GE, Klineberg IJ. (2000a). Need for occlusal therapy and prosthodontic treatment in the management of temporomandibular disorders. Part I: Occlusal interferences and occlusal adjustment. J Oral Rehabil; 27: 367-78. 15a.
de, Carlsson GE, Klineberg IJ. (2000 b). Need for occlusal therapy and prosthodontic treatment in the management of temporomandibular disorders. Part II: Tooth loss and prosthodontic treatment. J Oral Rehabil; 27(8): 647-59.
Djemal S, Bavisha K, GILMOUR G. (2004). Management of a Supra-Erupted Premolar: A Case Report. Dent Update 2004; 31: 220–222.
Erverdi N, Usumez S, Solak A. (2006). New generation open-bite treatment with zygomatic anchorage. Angle Orthod; 76(3):519-26.
Faggion CM Jr, Giannakopoulos NN, Listl S. (2011). How strong is the evidence for the need to restore posterior bounded edentulous spaces in adults? Grading the quality of evidence and the strength of recommendations. J Dent; 39:108-16.
Faot F, Ribeiro LF, Machado RM, Mello ACM, Del Bel Cury AA. (2015). Case Studies on the Use of Skeletal Anchorage Orthodontic Techniques Prior to Prosthetic Rehabilitation. J Oral Implantology; XLI (3): 332-6.
Gierie WV, Paterson RL, Proffit WR. (1999). Response of erupting human premolars to force application. Arch Oral Biol; 44: 423-428.
Grenga V, Bovi M. (2013). Corticotomy-Enhanced Intrusion of an Overerupted Molar Using Skeletal Anchorage and Ultrasonic Surgery. JCO; XLVII (1): 50-55.
Gupta SK, Pratibha PK, Bhat KM, Mutalik S, Guddattu V. (2014). Non-replaced Mandibular First Molars and Temporomandibular Joint Dysfunction. Nepal J Medi Scien; 3(1):57-62.
Hakami Z. (2016). Molar Intrusion Techniques in Orthodontics: A Review. J Inter Oral Health; 8(2):302-306.
Jepson NJA, Allen PF. (1999). Short and sticky options in the treatment of the partially dentate patient. Br Dent J; 187: 646-652.
Joshi U, Patil S K Siddiqua A, Thakur N. (2010). Posterior maxillary segmental osteotomy for management of supraerupted teeth. Int J Dent Clin; 2(3):64-67.
Kayser A. (1981). Shortened dental arches and oral function. J Oral Rehabil; 8: 457-462.
Kiliaridis S, Lyka I, Friede H, Carlsson GE, Ahlqwist M. (2000). Vertical position, rotation, and tipping of molars without antagonists. Int J Prosthodont; 13:480-6.
Mahoorkar S, Puranik SN, Moldi A, Chowdhary R, Majge B. (2010). Management of Supra-erupted Posterior Teeth- A Review. Inter J Dent Clins; 2(3)27-30.
Malathi K, Anand AG, Karthikeyan R, Garg S. (2014). Coronoplasty. IOSR-JDMS; 13(9): 64-67.
Marcus SE, Drury TF, Brown LJ, et al. (1996). Tooth retention and tooth loss in the permanent dentition of adults: United States, 1988–1991. J Dent Res;75(Spec): 684-695.
Matsuda K-I, Miyashita Y, Ikebe K, Enoki K, et al. (2014). Overeruption of Teeth Opposing Removable Partial Denture: A Preliminary Study. Int J Prosthodont; 27: 475-6.
McGuire MK, Scheyer ET, Gallerano RL. (2006). Temporary anchorage devices for tooth movement: a review and case reports. J Periodontol; 77:1613–1624.
Mehta S, Gupta K, Sonal S, Lodha S. (2018). "Multidisciplinary Management of Supra-Erupted Periodontally Compromised Maxillary Molar". Scie Arch Dent Sciences; 1(2): 10-13.
Meningaud J, Pitak-Arnnop P, Corcos L, Bertrand J. (2006). Posterior maxillary segmental osteotomy for mandibular implants placement: case report. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology; 102(5): e1-e3.
Meskin LH, Brown LJ. (1988). Prevalence and patterns of tooth loss in U.S. employed adult and senior populations, 1985– 86. J.Dent Educ; 52: 686-691.
Mizrahi E, Mizrahi B. (2007). Mini-screw implants (temporary anchorage devices): orthodontic and pre-prosthetic applications. J Orthod;34: 80– 94.
Patil SA, Kulkarni S, Thakur S, Naik B. (2016). Crown lengthening procedure following intentional endodontic therapy for correction of supra-erupted posterior teeth: Case series with long-term follow-up. J India Soc Periodontol; 20: 103-8.
Prakash P, Nishanth K, Jasani N, Katyal A, Nayak USK. (2014). Intrusion of overerupted molars using miniscrews and TMA spring: A case report. IJSS Case Reports & Reviews;1(1):4-8.
Punde PA. (2013). Gaining vertical interarch space for implant placement: The surgical protocol - Series of five cases. J Dent Implant; 3: 42-5.
Rosenstiel SF, Land MF, Fujimoto J. (2015). Contemporary Fixed Prosthodontics, 5th Ed. St. Louis (MO): Mosby Elsevier. pp. 40-66, 95-113, 161.
Rosenstiel S, Land M, Crispin B. (1998). Dental luting agents: a review of the current literature. J Prosthetic Dent; 80(3): 280-301.
Salazar G, Serrano AF, Mazzey GO. (2018). Intrusion of an overerupted maxillary molar with orthodontic mini implants for implant restorative purposes. J Int Oral Health; 10:44-6.
Sevimli G., et al. "Endocrowns: review". J Ista Univ Facu

Dent 2015; 49(2): 57-63.
Shafad M, Rahman S, Muddaiah S, Somaiah S, et al. (2017). Management of Supra Erupted Maxillary Molar by Using A Modified Removable Posterior Bite Plane –Two Case Reports. I J Adva Rese Development; 2(12): 1-6.
Shillingburg H, Hobo S, Whitsett LD, Richard J, Susan E. Brackett. (2012). Fundamentals of Fixed Prosthodontics. Chicago (4th Ed), Quintessence Publishing Co; page 21-23, 44-55, 88.
Solnit GS, Aquilino SA, Jordan RD. (1988). An etched metal splint to prevent the supereruption of unopposed teeth. J Prosthet Dent; 59: 381-382.
Stewart K, Rudd K, Kuebker W. (1983). Clinical removable partial prosthodontics. Impl Dent; 2(1): 94-112.
Sun J, Ruan W, He J, Lin X, et al. Clinical efficacy of different marginal forms of endocrowns: study protocol for a randomized controlled trial. BMC Trials 2019; 20: 454-60.
THE GLOSSARY OF PROSTHODONTIC TERMS, Ninth Edition (GPT-9). THE JOURNAL OF PROSTHETIC DENTISTRY 2017; 117(5S): e1- e105.
Tiago CM, Paulo LP, Nouer RA. (2016). Molar intrusion with orthodontic mini-implants: case reports. RGO, Rev Gaúch Odontol, Porto Alegre; 64(3): 327-332.
Tzimas K, Tsiafitsa M, Gerasimou P, Tsitrou E. (2018). Endocrown restorations for extensively damaged posterior teeth: clinical performance of three cases. Restor Dent Endod; 43(4):e38-e46.

Dental
Communication

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp-273-279

Effect of Finishing and Polishing Bulk-Fill Composites on Salivary and *Streptococcus mutans* Adhesion

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ABSTRACT

Bacterial adhesion over composite restorations may lead to secondary caries and periodontal inflammation. Post-curing finishing/polishing aim to reduce this exposure. This study investigated and compared the degree of single species *Streptococcus mutans (S. mutans)* and multi-species salivary biofilm adhesion on four restorative composites across three finishing/polishing systems. Standardized disc samples (2×10mm) were produced from each composite material. Ten discs of each material were subjected to three finishing and polishing systems. Half the samples (n=5) were incubated in human saliva and the other half were incubated in S. mutans for biofilm development for 48 and 24 h, respectively. Following dilution and bacterial growth, the mean number of colony forming units (CFU/mL×5; log10) was counted using a colony counter and analyzed using SPSS Statistics V22.0. Data were analyzed using three-way analysis of variance and the Tukey's post-hoc tests ($p<0.05$). There were no significant differences in biofilm adhesion in the saliva incubation group across the three polishing systems ($F=1.138$; $p=0.328$) or the four types of materials ($F=1.001$; $p=0.399$). There were significant differences in biofilm adhesion in the *S. mutans* group across the three polishing systems ($F=3.918$; $p=0.025$) and the four types of materials ($F=3.899$; $p=0.013$). Multiple comparisons revealed that biofilm adhesion was lowest in the Astropol® group. Filtek™ Bulk Fill had significantly lower biofilm adhesion than Filtek™ Z350 XT ($p=0.008$). Surface properties vary by composite materials and finishing and polishing techniques, which influences bacterial adhesion. The least bacterial adhesion was observed with Sof-LexTM finishing and polishing system and Filtek™ Bulk Fill composite material..

KEY WORDS: BULK-FILL COMPOSITES; RESIN COMPOSITE; SURFACE ROUGHNESS; BACTERIAL ADHESION; POLISHING SYSTEM; *STREPTOCOCCUS MUTANS*.

ARTICLE INFORMATION

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Received 13th Jan 2020 Accepted after revision 15th March 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/44

INTRODUCTION

Resin-based composites (RBCs) can provide maximum tooth-like esthetics with a compatible tooth bonding structure and minimal cavity preparation (Chesterman et al., 2017). Due to these features, RBC dental materials are now widely used in daily clinical practice. However, RBCs also suffer from various limitations, including technique sensitivity and the multi-step etching and bonding procedure that requires incremental application. To address these concerns, manufacturers introduced bulk-fill RBCs. Clinicians can place bulk-fill RBCs in large increments, ranging from 4 to 10 mm, with less polymerization shrinkage and postoperative sensitivity, but improved aesthetics, durability, and working time. SDR and Filtek bulk fills were introduced as flowable composites with enhanced features, including radio-opacity, visible light cured, fluoride-containing, and applicable in relatively large increments of 4 mm (Chan et al., 2010, Benetti et al., 2015 Benetti et al., 2015; Van Ende et al., 2017. Chesterman et al., 2017).

Although there is a diverse number of available bulk-fill RBCs and their physical and chemical properties keep advancing, a variable degree of oral microbiota still adheres to RBCs. These microbiota can exert unwanted complications, including development of secondary caries and risks of periodontal disease, which restoration longevity. Among the complex bacterial colonies that are present in the oral flora, *Streptococcus mutans* (*S. mutans*) species play a major role in dental caries. Further, these cariogenic species are increasingly prevalent on composite restorations compared to natural teeth. Jaberi et al., (2014) evaluated 1,339 posterior teeth restored with amalgam or RBCs for secondary caries. RBC restorations showed a higher prevalence of secondary caries compared to amalgam restorations, (Loesche 1986, Thomas et al., 2008, Jaberi et al., 2014, Denson et al., 2018 Soliman et al., 2019).

RBC restorations also presented with a higher percentage of replacement due to secondary caries compared to amalgam restorations (Mjör and Jokstad 1993; Bernardo et al., 2007). Growing evidence indicates that the surface geometry of RBCs, namely the surface roughness, chemical composition, and clinical manipulation, influences attraction and colonization of microbial flora (Derchi et al., 2017). It is well established that material surface roughness is a key factor that makes composites vulnerable to bacterial adhesion and biofilm development (Montonaro et al., 2004). A rough, unfinished, and poorly polished composite surface is more likely to accumulate plaque (Eick et al., 2004). Finishing and polishing RBC materials can minimize plaque accumulation, subsequent marginal tissue inflammation, and recurrence of caries while improving wear behavior and the marginal integrity of posterior fillings (Ferreira et al., 2004, 2017).

Recent developments in finishing and polishing systems have allowed for smoother composite surfaces that may impact microbial adhesion levels (Ferreira et al., 2017). Pereira et al.,(2011) evaluated S. mutans biofilm adhesion on the surface of three RBCs subjected to different finishing and polishing techniques. There was a significant increase in bacterial adherence on all three composite surfaces, regardless of the polishing treatment performed.A limited number of studies have reported how the surface characteristics of bulk-fill RBC's influence biofilm formation and bacterial adhesion (Montonaro et al., 2004). Of these, none have investigated the effect of different finishing and polishing systems with variable steps on biofilm formation on bulk-fill RBCs. Therefore, the aim of this *in-vitro* study was to investigate and compare the degree of single species *S. mutans* and multi-species salivary biofilm adhesion on the surface of four common restorative composites across three finishing and polishing systems.

MATERIAL AND METHODS

This study was approved by the institutional review board of King Saud University (Reg. # E-18-3347). One conventional (FiltekTM Z350 XT (FZ)) and three bulk-fill (SDR® flow+ (SBF), FiltekTM (FBF), SonicFillTM 3 (SF)) commercially available RBCs with variable types of filler particles were utilized in the present study (Table 1). Discs were created from the four materials using a stainless-steel mold with a diameter of 10 mm and thickness of 2 mm. Briefly, the RBC material were placed in the mold and a clear matrix strip was pressed to produce a bubble-free, smooth surface disc. The disc was then light cured for 80 s by applying the tip of a hand-held light curing unit (Spectrum 800; Dentsply Inc., York, PA, USA) directly on the matrix strip. Thirty discs were created from each material, subdivided into three groups (n = 10), and subjected to Sof-LexTM (SL) finishing and polishing wheels, Astropol® (AS), or Enhance® PoGo ® (EP) finishing and polishing systems according to the manufacturer instructions (Table 1). The materials were then cleaned for excess fragments, washed with distilled water, and air dried. They were then sterilized by ultraviolet light at a wavelength of 253.7 nm for 1 min. The specimens were stored in sterile plastic containers with distilled water prior to the experimental phase.

Experimental phase Saliva group: Unstimulated human saliva was collected from one healthy participant who volunteered. Briefly, the participant was instructed to stop her oral hygiene practice one day prior to collection as well as not to eat or drink for at least 1 h prior to collection. Then, saliva was collected between 9:00 and 10:00 a.m. post-fasting after their mouth was washed with water and a 5 min resting period. A 50 ml sterile polypropylene tube was provided for the volunteer to expectorate unstimulated (drooled) saliva to achieve a volume of 5–7 ml. The saliva was directly placed on ice and transferred to the laboratory for processing. Five samples per RBC type and finishing and polishing subgroup were placed in 24-well polystyrene tissue culture plates (Thermo Fisher Scientific Inc., USA) and incubated in 1 ml of thawed sterilized human saliva for 48 h at 37C° in a CO2 chamber.

Table 1. Description of materials used in the study.

	Material Trade Name	Abbreviation Used	Manufacturer	Description (% weight)	Inorganic filler
Composite Materials	Filtek™ Z350 XT	FZ	3M ESPE	Resin: Bis-GMA, UDMA, TEGDMA, and bis-EMA. Filler system: Combination of non-agglomerated /non-aggregated 20 nm silica filler, non-agglomerated/ non-aggregated 4 to 11 nm zirconia filler, and aggregated zirconia/silica cluster filler (comprised of 20 nm silica and 4 to 11 nm zirconia particles).	78.5%
	SDR® flow+ Bulk Fill Flowable	SBF	Dentsply	Resin: Modified UDMA, TEGDMA, and EBPDMA. Filler system: Barium and Strontium AluminoFluoroboro Silicate	68%
	Filtek™ Bulk Fill	FBF	3M ESPE	Resin: Bis-GMA, Bis-EMA, UDMA, and Procrylat. Filler System: Combination of Ytterbium trifluoride and zirconia/silica particles	64.5%
	SonicFill™ 3	SF	Kerr	Resin: Bis-GMA, TEGDMA, and EBPDMA. Filler system: SiO2, glass, oxides, and chemicals	84%
Finishing and Polishing Materials	Sof-LexTM® Spiral Finishing and Polishing Wheels	SL	3M ESPE	2-step finishing and polishing system composed of thermoplastic elastomer impregnated with aluminum oxide particles	-
	Astropol®	AS	Ivoclar Vivadent	3-step finishing and polishing silicon rubber system: Astropol F: Silicon carbide particles and color pigments Astropol P: Silicon carbide particles and color pigments Astropol HP: Diamond particles, aluminum oxide, titanium oxide, and iron oxide	-
	Enhance® PoGo®	EP	Dentsply	1-Step Diamond Micro Polishers composed of pre-mounted, diamond impregnated polishers	-

Bis-GMA: bisphenol A-glycidyl methacrylate; UDMA: Urethane dimethacrylate; TEGDMA: Tri-ethylene-glycol-dimethacrylate; bis-EMA: bisphenol A glycol dimethacrylate; EBPDMA: ethoxylated Bis-GMA

S. mutans group: The other half of the samples (n = 5) for each group were placed in 24-well plates then covered with 1.5 ml of brain heart infusion agar (BHI agar, Difco, Detroit, MI, USA). A standard suspension of S. mutans was then prepared by seeding *S. mutans* onto BHI agar and samples were incubated for 24 h at 37C° in a CO2 chamber.

Bacterial adhesion: Following the protocol described by Pereira et al (2011), the samples were removed and washed with sterile physiological buffered solution (Gibco®, ThermoFisher Scientific, MA, USA) to remove loosely bound material. Then, the samples were placed in tubes with 1 ml of sterile physiological solution and mixed on a mixer (Super Mixer® II, LAB-LINE Instruments, IL, USA) for 30 s to disperse the biofilms. The obtained suspension was diluted 10, 100, 1,000, and 10,000 times and 0.1 ml aliquots were seeded in duplicate onto BHI agar and incubated for 48 h at 37C° in a CO2 chamber. Following incubation, the plates with bacterial colonies were counted in a colony counter (Reichert Quebec® Darkfield Colony Counter, Cambridge Instruments. NY. USA)

Data analysis: Data was analyzed using SPSS version 21.0 (IBM Inc., Chicago, IL, USA) statistical software. The mean saliva and *S. mutans* values (CFU/ml x 5) were converted to log10 to attain a normal distribution for analysis. Descriptive statistics (mean ± standard deviations) were used to describe the quantitative outcome variables in saliva and *S. mutans* groups. One-way analysis of variance was used to compare the mean saliva and *S. mutans* values across the three polishing systems (SL, AS, and EP) and four composite materials (FZ, SBF, FBF, and SF). Tukey's multiple comparisons tests were used to compare the mean values of different pairs of polishing systems and composite materials. A p-value of <0.05 was used as the cut-off for statistical significance.

Table 2. Comparison of biofilm adhesion levels following saliva or *S. mutans* incubation across the three polishing systems.

Type of polishing system	Mean (SD) biofilm adhesion (log10) following saliva incubation	F-value	p-value	Mean (SD) biofilm adhesion (log10) following *S. mutans* incubation	F-value	p-value
SL	5.99 (0.52)	1.138	0.328	7.26 (0.39)1	3.918	0.025*
AS	6.11 (1.07)			6.89 (0.64)2		
EP	6.37 (0.74)			7.23 (0.24)3		
Overall Mean	6.15 (0.77)			7.12 (0.42)		

*Statistically significant; (SL vs AS: p = 0.040; SL vs EP: p = 0.985; AS vs EP: p = 0.059) (by Tukey's test) SL: Sof-LexTM finishing and polishing wheels; AS: Astropol®; EP: Enhance® PoGo ®

Table 3. Comparison of biofilm adhesion following saliva or *S. mutans* incubation across the four RBCs.

Type of material	Mean (SD) biofilm adhesion (log10) following Saliva incubation	F-value	p-value	Mean (SD) biofilm adhesion (log10) following *S. mutans* incubation	F-value	p-value
FZ	6.46 (0.41)	1.001	0.399	7.44 (0.42)1	3.899	0.013*
SBF	6.10 (0.77)			7.10 (0.61)2		
FBF	5.99 (1.32)			6.90 (0.37)3		
SF	6.06 (0.38)			7.07 (0.33)4		
Over all mean	6.15 (0.72)			7.12 (0.43)		

*Statistically significant; (FZ vs SBF: p-value = 0.157; FZ vs FBF: p-value = 0.008; FZ vs SF: p-value = 0.118; SBF vs FBF: p-value = 0.626; SBF vs SF: p-value = 0.999; FBF vs SF: p-value=0.712) (by Tukey's test). FZ: FiltekTM Z350 XT; SBF: SDR® flow+; FBF: FiltekTM; SF: SonicFillTM 3

RESULTS AND DISCUSSION

Finishing & Polishing systems: The mean biofilm adhesion level following saliva incubation (6.15 ± 0.77) across the three polishing systems was less than following *S. mutans* incubation (7.12 ± 0.42). The type of polishing system used did not significantly alter biofilm adhesion in the saliva incubation group (F = 1.138; p = 0.328). In comparison, there was a significant difference in the *S. mutans* incubation group (F = 3.918; p = 0.025), with AS having significantly less biofilm adhesion than SF (p = 0.040) and EP (p = 0.059) (Table 2).

Composite materials: The mean biofilm adhesion level following saliva incubation (6.15 ± 0.72) across the four RBCs was less than following *S. mutans* incubation (7.12 ± 0.43). The type of RBC did not significantly alter biofilm adhesion in the saliva incubation group (F = 1.001; p = 0.399). In comparison, there was a significant difference in the *S. mutans* incubation group (F = 3.899; p = 0.013), with FBF having significantly less biofilm adhesion than FZ (p = 0.008) and no other significant group differences (Table 3).

The performance and long-term service of RBC restorations is not only dependent on their intrinsic properties, but also is greatly influenced by their extrinsic properties (Barbosa et al., 2005; Jung M 2007). The intrinsic properties are closely related to the success or failure of the restoration in terms of its internal composition and resistance to fracture. However, the external surface properties following finishing and polishing directly affect the surrounding microflora and influence bacterial adhesion (Ikeda et al., 2007). Biofilm formation that results from poorly finished and polished RBCs increases the chances of periodontal disease, secondary caries, and esthetic complications like discoloration (Attar 2007; Koh et al., 2008). Surface roughness is one element that makes RBC materials susceptible to bacterial attachment and biofilm formation (Mei et al., 2011). Previous studies have proposed that a surface roughness value of 200 nm is the upper limit for bacterial retention. No reductions were seen in measures of bacterial retention below this value, whereas biofilm accumulation increased with higher roughness values (Ikeda et al., 2007). RBC surface roughness is influenced by resin matrix, filler type, size, shape, and distribution of the fillers in the matrix, as well as the finishing and polishing techniques used (Türkün and Türkün 2004).

Stoddard and Johnson (1991) suggested that the material itself, filler size, content, type of abrasive used, number of strokes, amount of pressure applied, time spent on each abrasion, direction of the abrading surfaces, and geometry of the abrasive instruments impact the effectiveness of finishing and polishing systems. These factors determine whether a surface is properly polished, which decreases the risk of initial bacterial adherence and subsequent colonization (Gedik et al., 2005; Yap et al., 1998). Previous studies have reported that lower *S. mutans* biofilm adhesion rates were observed in FZ RBCs due to their smaller particles and fillers size and their wide distribution in the resin matrix. These factors reduce surface roughness after finishing and polishing, consequently decreasing S. mutans adherence (Montonaro et al., 2004). However, the present study found that the highest bacterial adhesion rate was observed on the conventional FZ RBC regardless of incubation type, with the SF and AS polishing groups in the presence or the absence of human saliva, and it was higher compared to the FZ specimens finished and polished using EP finishing system.

This finding is in opposition to previous studies observing finishing and polishing techniques. Antonson et al. (2011), concluded that the SF finishing system provided the smoothest surface when compared to AS and EP (Pereira et al., 2011). SF disks were also found to provide a smoother RBC surface than carbide bur finishing, followed by the Astrobrush. This may be due to SF disk's inability to displace filler particles in RBCs, thereby providing a homogenous abrasion of the fillers and resin matrix which promotes less bacterial adhesion (Gyo et al., 2008). The results of the current study showed that SL disks in the presence of human saliva recorded the lowest bacterial adhesion over systems and composite materials (p=0.040), which also coincided with material surface roughness (data not shown). However, in the absence of human saliva, the SL group recorded the highest bacterial adhesion on SBF and FBF materials among all groups. In contrast, in the absence of saliva, in the AS group, the SBF and FBF composite materials recorded the lowest bacterial adhesion among all groups.

However, a study by Abuelenain et al. (2017) observed a surface roughness value greater than 200 nm was observed in SBF and SF RBCs, suggesting roughened surface beyond this threshold will lead to more bacterial retention due to presence of micro-retentive surface alterations, which increases surface area for pellicle formation as previously reported by Øilo et al., (2015). In the presence of saliva, SF composite in the EP group recorded the lowest bacterial adhesion among the four types of composites in the same group. In contrast, in the absence of saliva, SF recorded the highest bacterial adhesion in the EP group. Although we did not perform specific characterization and quantification of the salivary sample utilized in the experiment, it has been reported that human saliva contains and serves as a growth media for S. mutans species (Newman 1974). The specimens in the current study that were incubated in human saliva containing S. mutans exhibited a significant increase in biofilm growth using the conventional FZ composite. This demonstrates the powerful ability of salivary components to modulate biofilm adhesion because oral bacteria adhere to receptors of the host origin in saliva pellicle (Øilo et al., 2015).

Bacterial adhesion is not only influenced by the physical and chemical composition of composites, but also by the material type, polishing medium, finishing and polishing technique performed, and the presence of saliva pellicle. A study done by Nasoohi N et al. (2017) regarding polishing medium reported that dry finishing

and polishing of microhybrid and nanohybrid RBCs increased the micro hardness and surface roughness (Abzal et al., 2016). By comparing the RBCs with other dental materials, one previous study showed that glass ionomers with a rough surface and increased inorganic components harbors more bacteria than RBCs. Therefore, the increase in bacterial adhesion to RBCs after finishing and polishing may be due to the change in the surface roughness (Stoddard and Johnson 1991).

A study by Derchi et al. (2017) revealed that indirect dental restorative composite resins were less prone to biofilm adhesion than direct composite resins (Derchi et al., 2017). Another study that investigated surface roughness and *S. mutans* adhesion in the presence and absence of saliva on composites and ceramics found that enamel was the roughest, Leucite/feldspathic ceramics were rougher than the feldspathic ceramic, and indirect composites were similar to direct composites. The highest level of bacterial adherence was found on enamel in the presence and absence of saliva, whereas the leucite/feldspathic ceramic demonstrated greater adherence than the feldspathic ceramic, and the composites were all statistically similar (Ikeda et al., 2007). The present study used *S. mutans* to promote biofilm adhesion because *S. mutans* bacterium is known to be the main etiological factor in the initiation and progression of dental caries. Moreover, the bulk of the microorganisms present in dental plaque are S. mutans (Montonaro L et al., 2004), and its adherence to enamel surface and restorative materials is a preliminary condition for biofilm formation. These formations can eventually promote secondary caries and periodontal diseases (Jung et al., 2007; Sissons et al., 1991).

A possible limitation of the current research is that it only observes a few of many techniques that are available to investigate RBC performance within an experimental oral environment. Further, there is a lack of consistency across some experimental protocols, which means that the current study may not be directly comparable to some published results. Further, short term investigations concerning bacterial adhesion may not provide information that is representative of long-term intraoral RBC use, which could be gained by clinical studies. Nonetheless, an attempt was made to standardize and reproduce the conditions present in the oral environment. Use of the artificial mouth continuous culture, or systems as suggested by Sissons et al., (1991) and Sissons (1997) is recommended for future studies related to oral biofilms. These models reproduce biofilm-growing conditions similar to the in vivo environment.

CONCLUSION

Multiple conclusions can be made within the limitations of this study. Firstly, RBC surface properties differ across materials and finishing and polishing techniques, which influence bacterial adhesion. Due to differences in the size, shape, number of filler particles, and the type of resin, one finishing and polishing system was incapable of creating the smoothest surface for all the RBCs tested. Pairing RBCs with the polishing system recommended by the manufacturer is suggested for clinical use. Overall, the lowest levels of bacterial adhesion were observed with the SL system and FBK RBC. Understanding the relationships among surface roughness, saliva, and biofilm formation in environments containing *S. mutans* is important to preventing secondary caries around composite restorations.

ACKNOWLEDGEMENTS

Authors would like to thank the College of Dentistry Research Center http://cdrc-ksu.org/ and Deanship of Scientific Research at King Saud University, Saudi Arabia, for funding this research (Reg. # E-18-3347).

REFERENCES

Abuelenain DA, Neel EA, Al-Dharrab A.(2017) Surface characterization and mechanical behavior of bulk fill versus incremental dental composites. Tanta Dental Journal. 14:56–61. doi: 10.4103/tdj.tdj_56_16.

Abzal MS, Rathakrishnan M, Prakash V, Vivekanandhan P, Subbiya A, Sukumaran VG (2016) Evaluation of surface roughness of three different composite resins with three different polishing systems. J Conserv Dent.19:171–174.

Antonson SA, Yazici AR, Kilinc E, Antonson DE, Hardigan PC. (2011) Comparison of different finishing/polishing systems on surface roughness and gloss of resin composites. J Dent. 39:e9–e17.

Attar N. (2007)The effect of finishing and polishing procedures on the surface roughness of composite resin materials J Contemp Dent Pract. 8: 27-35.

Barbosa SH, Zanata RL, Navarro MF, Nunes OB. (2005) Effect of different finishing and polishing techniques on the surface roughness of micro filled, hybrid and packable composite resins. Braz Dent J. 16:39–44.

Benetti AR, Havndrup-Pedersen C, Honoré D, Pedersen MK, Pallesen U. (2015) Bulk-fill resin composites: polymerization contraction, depth of cure, and gap formation. Oper Dent. 40:190–200. doi: 10.2341/13-324-L.

Bernardo M, Luis H, Martin MD, Leroux BG, Rue T, Leitão J, et al. Survival and reasons for failure of amalgam versus composite posterior restorations placed in a randomized clinical trial. J Am Dent Assoc. 2007;138:775–783.

Cazzaniga G, Ottobelli M, Ionescu AC, Paolone G, Gherlone E, Ferracane JL, et al (2017) In vitro biofilm formation on resin-based composites after different finishing and polishing procedures. J Dent. 67:43–52. doi: 10.1016/j.jdent.2017.07.012.

Chan KHS, Mai Y, Kim H, Tong KCT, Ng D, Hsiao JCM. (2010) Review: resin composite filling. Materials (Basel). 3:1228–1243. doi: 10.3390/ma3021228.

Chesterman J, Jowett A, Gallacher A, Nixon P.(2017) Bulk-fill resin-based composite restorative materials: a review. Br Dent J. 222:337–344. doi: 10.1038/

sj.bdj.2017.214.
Denson N, Wells M, Tipton DA, Godoy FG, Babu JP. (2018) Bacterial adhesion and biofilm formation on direct, tooth-colored restorative materials: an in vitro study. Adv Dent Oral Health. 8:555736. doi: 10.19080/ADOH.2018.08.555736.
Derchi G, Vano M, Barone A, Covani U, Diaspro A, Salerno M.(2017) Bacterial adhesion on direct and indirect dental restorative composite resins: An in vitro study on a natural biofilm. J Prosthet Dent. 117:669–676.
Derchi G, Vano M, Barone A, Covani U, Diaspro A, Salerno M.(2017) Bacterial adhesion on direct and indirect dental restorative composite resins: An in vitro study on a natural biofilm. J Prosthet Dent. 117:669–676.
Eick S, Glockmann E, Brandl B, Pfister W.(2004) Adherence of *Streptococcus mutans* to various restorative materials in a continuous flow system. J Oral Rehab. 31:278–285.
Ferreira Rde S, Lopes GC, Baratieri LN.(2004) Direct posterior resin composite restorations: considerations on finishing/polishing. Clinical procedures. Quintessence Int. 35:359–66.
Gedik R, Hürmüzlü F, Coskun A, Bekta OO, Ozdemir AK.(2005) Surface roughness of new microhybrid resin-based composites. J Am Dent Assoc. 136:1106–1112.
Gyo M, Nikaido T, Okada K, Yamauchi J, Tagami J, Matin K. (2008) Surface response of fluorine polymer-incorporated resin composites to cariogenic biofilm adherence. Appl Envir Microbiol. 74:1428–1435.
Ikeda M, Matin K, Nikaido T, Foxton RM, Tagami J.(2007) Effect of surface characteristics on adherence of *S. mutans* biofilms to indirect resin composites. Dent Mater J. 26:915–923.
Jaberi AZ, Valizadeh HH. (2014)Secondary caries in the posterior teeth of patients presenting to the department of operative dentistry, Shahid Beheshti Dental School. J Dent Sch. 32:125–131.
Jung M, Eichelberger K, Klimek J. (2007) Surface geometry of four nanofiller and one hybrid composite after one-step and multiple-step polishing. Oper Dent. 32:347–355.
Koh R, Neiva G, Dennison J, Yaman P.(2008) Finishing systems on the final surface roughness of composites J Contemp Dent Pract. 9:138–145.
Loesche WJ. (1986) Role of *Streptococcus mutans* in human dental decay. Microbiol Rev. 50:353.
Mei L, Busscher HJ, van der Mei HC, Ren Y. (2011) Influence of surface roughness on streptococcal adhesion forces to composite resins. Dent Mater. 27:770–778. doi:10.1016/j.dental.2011.03.017.
Mjör IA, Jokstad A.(1993) Five-year study of Class II restorations in permanent teeth using amalgam, glass polyalkenoate (ionomer) cement and resin-based composite materials. J Dent. 21:338–343.
Montonaro L, Campoccia D, Rizzi S, Donati ME, Breschi L, Prati C.(2004) Evaluation of bacterial adhesion of *Streptococcus mutans* on dental restorative materials. Biomaterials. 25:4457–4463.
Nasoohi N, Hoorizad M, Tabatabaei SF. (2017) Effects of wet and dry finishing and polishing on surface roughness and microhardness of composite resins. J Dent (Tehran). 14: 69-75.
Newman HN.(1974) Diet, Attrition, Plaque and Dental Disease. Br Dent J. 136:491–497. doi: 10.1038/sj.bdj.4803220.
Oho T, Yamashita Y, Shimazaki Y, Kushiyama M, Koga T. Simple and rapid detection of *Streptococcus mutans* and *Streptococcus sobrinus* in human saliva by polymerase chain reaction. Oral microbiology and immunology. 2000;15:258–262.
Øilo M, Bakken V. (2015) Biofilm and dental biomaterials. Materials. 8:2887–2900.
Pereira CA, Eskelson E, Cavalli V, Liporoni PC, Jorge AO, do Rego MA.(2011) *Streptococcus mutans* biofilm adhesion on composite resin surfaces after different finishing and polishing techniques. Oper Dent. 36:311–317. doi: 10.2341/10-285-L.
Sissons CH, Cutress TW, Hoffman MP, Wakefield JS.(1991) A multi-station dental plaque microcosm (artificial mouth) for the study of plaque growth, metabolism, pH, and mineralization. J Dent Res. 70:1409–1416.
Sissons CH. (1997) Artificial dental plaque biofilm model systems. Adv Dent Res. 11:110–126.
Soliman WE, Ali AI, Elkhatib WF (2019) Evaluation of surface roughness and *Streptococcus mutans* adhesion to bulk-fill resin composites polished with different systems. Adv Microbiol. 9:87–101. 10.4236/aim.2019.91007.
Stoddard JW, Johnson GH.(1991) An evaluation of polishing agents for composite resins. J Prosthet Dent. 65:491–495.
Thomas RZ, van der Mei HC, van der Veen MH, de Soet JJ, Huysmans MC.(2008) Bacterial composition and red fluorescence of plaque in relation to primary and secondary caries next to composite: an in situ study. Oral Microbiol Immunol. 23:7–13.
Türkün LS, Türkün M.(2004) The effect of one-step polishing system on the surface roughness of three esthetic resin composite materials. Oper Dent. 29:203–211.
Van Ende A, De Munck J, Lise DP, Van Meerbeek B (2017) Bulk-fill composites: a review of the current literature. J Adhes Dent. 19:95–109. doi: 10.3290/j.jad.a38141.
Yap AU, Ang HQ, Chong KC (1998) Influence of finishing time on marginal sealing ability of new generation composite bonding systems. J Oral Rehabil. 25:871–876.

Biomedical
Communication

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp-280-283

BBRC
Bioscience Biotechnology
Research Communications

FMS-like Tyrosine Kinase 3 (FLT3) Gene as a Significant Biomarker for Acute Myeloid Leukemia

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ABSTRACT

Acute myeloid leukemia (AML), a cancer of myeloid cells involves the abnormal proliferation and differentiation of myeloid stem cells. AML accounts for approximately 30% of leukemia's but >40% of leukemia-related deaths. AML is highly diverse and cytogenetic analysis of metaphase cells reveals that approximately 40-50% of patients with de novo AML have a normal karyotype.. This study demonstrated activating mutations of FLT3 gene due to recent advances in cell and molecular biology have revolutionized our understanding of normal hematopoiesis. Mutations within the FMS-like tyrosine kinase 3 (FLT3) genes represent one of the most frequently identified genetic alterations that disturb intracellular signaling networks with a key role in leukemia pathogenesis. The present study has been designed to highlight and signify the importance of FLT3 and its related gene mutations involved in the onset and progression of Leukemias. Since mutations in FLT3 gene are one of the most common clinically relevant mutations which are expressed in 90% of leukemic blasts of patients with acute myeloid leukemia. Thus, there is an urgent need for the better understanding of the key genetic mutations involved in disease progression and prognosis. FLT3 testing should be done in parallel with cytogenetic testing and can open new horizons for better diagnosis and better treatment option.

KEY WORDS: ACUTE MYELOID LEUKEMIA, FMS-LIKE TYROSINE KINASE, GENETIC MUTATIONS.

INTRODUCTION

Leukemias are monoclonal diseases that originate from individual cells in the bone marrow. Leukemia like any other cancer also follows a multistep process which a normal cell must possess or pass through a number of distinct intermediate stages before attaining the status of malignancy. Based on the origin of the predominant cell type (myeloid or lymphoid) and the rate of disease progression (acute or chronic), leukemia is categorized into four major subtypes: acute myelogenous leukemia (AML), chronic myelogenous leukemia (CML), acute lymphocytic leukemia (ALL), and chronic lymphocytic leukemia (CLL). According to a recent report published by Taisen et al., (2019), the incidences of AML and CML has remained constant prior to 2011 but there has been a sudden increase in the incidence rates of CLL. Acute myeloid leukemia (AML), a cancer of myeloid cells involves the abnormal proliferation and differentiation of myeloid stem cells. It is generally accepted that survival, proliferation and differentiation are the three fundamental cellular processes that define normal hematopoietic cells. In acute myeloid leukemia

ARTICLE INFORMATION

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Received 6th Jan 2020 Accepted after revision 29th Feb 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/45

(AML), a heterogeneous disorder of the hematopoietic progenitor cells, abnormalities have been identified that affect the balance between cell proliferation, survival and differentiation. These abnormalities result in the expansion of an abnormal stem cell clone (Irons et al., 1996 Taisen et al., 2019 Nicholas et al 2020).

AML accounts for approximately 30% of leukemia's but >40% of leukemia-related deaths. It is the most common acute leukemia in adult population. The median age at diagnosis of AML is approximately 67 years and about 1/3rd of Leukemias among adults have been reported from developed countries. The therapeutic armamentarium of acute myeloid leukemia (AML) has rapidly expanded in the past few years, driven largely by translational research into its genomic landscape and an improved understanding of mechanisms of resistance to conventional therapies (Nicholas et al., 2020). Genetic alterations are the frequent features of all human cancers which include amplification, deletions, rearrangements and point mutations. Thus, genomic investigations of AML have also demonstrated the role of several genes which on recurrent mutations can lead to the new genomic classifications and predictive biomarkers, and new therapeutic targets (Daver et al., 2019 Nicholas et al 2020).

AML is highly diverse and cytogenetic analysis of metaphase cells reveals that approximately 40-50% of patients with de novo AML have a normal karyotype. These patients are classified with an intermediate clinical prognosis because clinically they do not have a reference marker and its biological origin is still unknown. The most frequent abnormalities are translocations t(15;17), t(8;21), inv(16) and a gain of number 8. Recently, with the development of methodologies of massive sequencing, new genetic mutations associated with acute myeloid leukemia have been identified. Some of the identified genes include KIT, FLT3, NPM1, CEBPA, RAS, WT1, BAALC, ERG, MN1, DNMT, TET2, IDH, ASXL1, PTPN11 and CBL. Of all these, WHO highlighting the related mutations in FLT3, NPM1 and CEBPα genes because they are associated with treatment response and progress of this disease (Swerdlow et al., 2008, Takahashi 2011, Martelli 2013 and Liesveld and Lichtman, 2016, Rubnitz et al., 2016 Nicholas et al 2020).

A number of genetic mutations, such as point mutations, gene rearrangements, and chromosomal translocations, which are involved in the pathogenesis of leukemia, have been documented. Recent advances in cell and molecular biology have revolutionized our understanding of normal hematopoiesis. Mutations within the FMS-like tyrosine kinase 3 (FLT3) genes represent one of the most frequently identified genetic alterations that disturb intracellular signaling networks with a key role in leukemia pathogenesis. FLT3, a receptor tyrosine kinase (RTK), is a membrane-bound receptor is primarily expressed on committed myeloid and lymphoid progenitors. FLT3 is composed of an immunoglobulin-like extracellular ligand-binding domain, a transmembrane domain, a juxtamembrane dimerization domain and a highly conserved intracellular kinase domain interrupted by a kinase insert. FLT3 belongs to the class III subfamily of RTKs, which include structurally similar members such as c-FMS, c-KIT, and PDGF receptor (Martelli et al, 1996 and Gabbianelli et al., 1995 Liesveld and Lichtman 2016).

FLT3 is expressed in 90% of leukemic blasts of patients with acute myeloid leukemia (Carow et al.,1996) . Approximately 20–30%ofAMLpatientsharbor a unique feature i.e. Internal Tandem Duplication (ITD) mutation in the FLT3 gene between exons 14 and 15 in the juxta membrane domain, which often results in high blast accounts, increased risk of relapse and decreased survival. FLT3-ITD is especially a frequent feature in patients with normal karyotype, t (15;17) (q22;q12) [PML-RARA] and t(6;9)(p23;q34) [DEK-NUP214]which leads to uncontrolled cellular proliferation, survival, and differentiation through constitutive activation of FLT3, (Meshinchi and Appelbaum (2009). Stirewalt and Radich 2003, Parcells et al, 2006) .FLT3-ITD occurs in the form of a replicated sequence in the juxta membrane domain and/or TKD1 of the FLT3 receptor and varies in location and length within these domains.

FLT3-ITD (high) is a driver mutation that presents with a high leukemic burden, confers a poor prognosis, and bears a significant negative impact on the management of patients with AML (Ding et al., 2012, Grimwade and Mrozek 2011). This ITD disrupts the auto inhibitory function of the juxta-membrane domain and results in ligand independent activation of the FLT3 receptor. This leads to a proliferative signal via activation of its downstream effectors (Kiyoi et al., 1998, Levis 2013) . Thus, the ITD leads to gain of function mainly by inducing hyper-responsivity of the FLT3 receptor to FL rather than through auto-activation of the receptor (Griffith et al., 2004), Therefore, the FLT3-ITD mutation directly or indirectly confers a selective advantage to a clone in its microenvironment. About 75% of patients with FLT3ITD–mutated AML at diagnosis continue to have the ITD mutation at relapse (Zheng et al., 2011) suggesting that FLT3-ITD may function as the driver mutation responsible for progressing the disease into overt leukemia.

The second common types of mutations in FLT3 are missense mutations in exon 20 of the activation loop (A-loop) in the tyrosine kinase domain (TKD). Almost all these mutations involve the substitution of an aspartate with a tyrosine at codon 835 (D835Y) by a point mutation (GAT→TAT). Aspartate in the 835 position belongs to the domain DFG (Aspartate-Phenylalanine-Glycine) in the A-loop, playing a critical role in preventing efficient binding of ATP. This type of mutation occurs in approximately 7% of patients with AML (Yomamoto et al., 2001 Kronke et al., 2013 Liesveld and Lichtman (2016).

In recent years, it has been shown that somatic activating mutations of the FLT3 gene are the most common genetic abnormalities in AML and have a significant impact on prognosis. Female patients are affected more

frequently and these mutations are also associated with hypercellularity and a higher incidence of recurrence (Abu- Duhier et al., 2001). Routine testing for FLT3 in patients with cytogenetically normal AML had been recommended since at least 2010 (Fenski et al., 2000), which corresponds to the time at which molecular testing was routinely performed in 100% of patients at academic centers but not at community sites. This suggests that there is a lack of awareness about the significance of molecular testing at community sites.

CONCLUSION

Identification of FLT3 mutations in AML has yielded novel approaches to the management of this disease. Over the last decade, the biology and the function of the wild-type and mutated FLT3 receptor have been well characterized. Whether it is through their utility as prognostic factors or their use as a target for directed therapies, FLT3 mutations have provided clinicians with novel therapeutic options for a large subset of AML patients. Identification of FLT3 mutations in AML has raised the potential for its utility as a molecular marker for risk-based therapy and as a target for directed therapy with novel small molecular inhibitors. Subsequent studies identified numerous other potential compounds (MLN518, PKC412, SU5416, SU5614, SU11248, CEP-701, CEP-5214) that also block FLT3 activation. Two compounds (CEP-701 and PKC-412) have shown some therapeutic promise for AML patients with FLT3 mutations. CEP-701 (Lestaurtinib) is an indolo carbazole compound that inhibits auto phosphorylation of the WT and mutant FLT3 receptors (Levis et al., 2005 and Levis et al., 2004, Nicholas et al 2020). Current clinical trials are combining FLT3 inhibitors with conventional chemotherapy in an attempt to increase the cytotoxic effect against leukemia cells and reverse the poor prognosis for AML patients with FLT3 mutations.

Funding Agency: Authors are extremely thankful to the Science and Engineering Research Board (SERB), DST, Govt. of India for providing financial assistance for carrying out the research work.

REFERENCES

Abu-Duhier F.M, Goodeve A.C, Wilson G.A (2011). Identification of novel FLT-3 Asp835 mutations in adult acute myeloid leukaemia. Br J Haematol, 113, 983-8.

Carow C.E., Levenstein M., Kaufmann S.H., et al (1996). Expression of the hematopoietic growth factor receptor FLT3 (STK-1/Flk2) in human leukemias. Blood., 87:1089–1096.

Daver N., Schlenk R.F., Russell N.H and Levis M.J. (2019). ¬¬¬Targeting FLT3 mutations in AML: review of current knowledge and evidence. Leukemia, 33, 299-312.

Ding L., Ley T.J., Larson D.E., Miller C.A., Koboldt D.C., Welch JS, et al. (2012). Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing. Nature, 481, 506– 10.

Dohner H., Estey E.H., Amadori S., et al. (2010). Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. Blood, 115, 453–474.

Fenski R., Flesch K., Serve S., et al. (2000). Constitutive activation of FLT3 in acute myeloid leukemia and its consequences for growth of 32D cells. Br J Haematol, 108, 322-30.

Gabbianelli M., Pelosi E., Montesoro E., et al. (1995). Multilevel effects of flt3 ligand on human hematopoiesis: expansion of putative stem cells and proliferation of granulomonocytic progenitors/monocyticprecursors. Blood, 86, 1661.

Griffith J., Black J., Faerman C., et al. (2004). The structural basis for autoinhibition of FLT3 by the juxtamembrane domain. Mol Cell ,13, 169-78.

Grimwade D., and Mrozek K. (2011). Diagnostic and prognostic value of cytogenetics in acute myeloid leukemia. Hematol Oncol Clin North Am. 25, 61.

Irons F G Burmit R.D, Stillman WS. (1996). The process of leukemogenesis. Environ Health Perspect,104, 1239-46.

Kiyoi H., Towatari M., Yokota S., et al. (1998). Internal tandem duplication of the FLT3 gene is a novel modality of elongation mutation which causes constitutive activation of the product. Leukemia, 12, 1333-7.

Kronke J., Bullinger L., Teleanu V., Tschurtz F., Gaidzik V.I., Kuhn M.W., et al. (2013). Clonal evolution in relapsed NPM1-mutated acute myeloid leukemia. Blood, 122,100–8.

Levis M., Smith B.D., Beran M., et al. (2005). A randomized, open-label study of lestaurtinib (CEP-701), an oral FLT3 inhibitor, administered in sequence with an oral chemotherapy in patients with relapsed AML harboring FLT3 activating mutations: clinical response correlates with successful FLT3 inhibition. Am Soc Hematol Annu Meet Abstracts, 106, 403.

Levis M. (2013). FLT3 mutations in acute myeloid leukemia: What is the best approach in 2013? Hematology Am Soc Hematol. Educ. Program, 220–6.

Levis M., Pham R., Smith B.D., Small D. (2004). In vitro studies of a FLT3 inhibitor combined with chemotherapy: sequence of administration is important in order to achieve synergistic cytotoxic effects. Blood, 104:1145.

Liesveld J.L., Lichtman M.A. (2016). Acute myelogenous leukemia. In: Kaushansky K, Lichtman MA, Prchal JT, et al., editors. Williams Hematology. 9th ed. . United States of America: McGraw-Hill Education.

Löwenberg B., Downing J.R., Burnett, A. (1999). Acute myeloid leukemia. N. Engl. J. Med., 341, 1051–1062.

Meshinchi S., Appelbaum F.R. (2009). Structural and functional alterations of FLT3 in acute myeloid leukemia. Clin Cancer Res., 15(13), 4263–9.

Nicholas J Short, Marina Konopleva, Courtney D. Di Nardo and Naval Daver (2020). Advances in the Treatment of Acute Myeloid Leukemia: New Drugs and New Challenges.

Martelli M.P., Sportoletti P., Tiacci E., et al. (2013). Mutational landscape of AML with normal cytogenetics: Biological and clinical implications. Blood Rev., 27(1), 13–22.)

Parcells B.W., Ikeda A.K., Simms-Waldrip T., et al. (2006). FMS-like tyrosine kinase 3 in normal hematopoiesis and acute myeloid leukemia. Stem Cells, 24(5), 174–84.

Rosnet O., Buhring H.J., Marchetto S., et al. (1996). Human FLT3/FLK2 receptor tyrosine kinase is expressed at the surface of normal and malignant hematopoietic cells. Leukemia, 10, 238

Rubnitz J.E., Gibson B., Smith F.O. (2010). Acute myeloid leukemia. Hematol. Oncol. Clin. North Am., 24(1), 35–63

Stirewalt D.L., Radich J.P. (2003). The role of FLT3 in haematopoietic malignancies. Nat. Rev. Cancer., 3(9), 650–65.

Swerdlow S.H., Campo E., Harris N.L., Jaffe E.S., Pileri S.A, Stein H., Thiele J., Vardiman J., (2008) editors. WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues. 4a ed. Lyon: International Agency for Research on Cancer (IARC).

Takahashi S. (2011). Current findings for recurring mutations in acute myeloid leukemia. J Hematol Oncol., 2011, 4, 36.

Taisen H., Min L.T., Alison B., & WenYong C. (2019). An emerging trend of rapid increase of leukemia but not all cancers in the aging population in the United States. Scientific reports, Nature.

Yamamoto Y., Kiyoi H., Nakano Y., et al. (2001) Activating mutation of D835 within the activation loop of FLT3 in human hematologic malignancies. Blood, 97, 2434-9.

Yamamoto, J.F.; Goodman, M.T. (2008). Patterns of leukemia incidence in the United States by subtype and demographic characteristics, 1997–2002. Cancer Causes Control, 379–390.

Zheng R., Bailey E., Nguyen B, et al. (2011). Further activation of FLT3 mutants by FLT3 ligand. Oncogene 2011, 30, 4004-14.

Ecological
Communication

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp-284-287

Macrophyte Diversity of a Tropical River from Nagpur India

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ABSTRACT

In present investigation, distribution and diversity of macrophytes in Kolar river in Nagpur region of Maharashtra state, India have been studied, to investigate overall health of the water body. Since the studies on macrophytes diversity are very less in Kolar river this paper is intended to report macrophytes diversity in present investigation, the present study was conducted on monthly basis for the period of two years from February 2010 to January 2012 by following standard protocols. The statistical analysis of the analytical data was computed and it reveals that submerged macrophytes are abundant followed by marginal and free floating. The species diversity is more at sampling Site-B in shallow water and less water current. In the present investigation, 25 species from three groups were recorded from Kolar lotic ecosystem under study which was categorized by free floating, submerged and marginal aquatic weeds. The data of the present investigation show that the enrichment of the shallow water with high bottom sediments provides an ideal habitat for luxuriant growth of macrophytes. It is also demonstrated that the diversity of macrophyte is less where water current is more and diversity increases as the water current decreases and organic contents increases.

KEY WORDS: MACROPHYTES, RIVER KOLAR, SPECIES DIVERSITY.

INTRODUCTION

In a natural ecosystem macrophytes have been shown to remove both toxic and nontoxic elements in the sediment and water, Narayan and Somshekhar, (1997). These are unchangeable biological filters and carry out purification of the water bodies by accumulating dissolved metals and toxins in their tissues, Shaha & Vyas (2015). The variation in water chemistry can be assessed by surveying the abundance of macrophytic communities. The trophic nature is mainly influenced the variety of communities and indicator species occur at the sources. Moreover, metabolic activities of macrophytic communities accelerate the metabolic and the physico-chemical conditions of stream Gregg and Rose, (1982). Some relevant and recent studies on aquatic macrophytes have been made by, Tenna Riis et al., (2019), Ester Vieira Noleto et al., (2019), Szymon Jusik and Staniszewski, (2019), Hanife Ozbay et al., (2019), Rameshkumar et al. (2019), Patel and Dubey (2019),), Prasad and Das (2018), Bhute and Harne (2017).

The macrophytes stimulate the growth of phytoplankton and help in the recycling of the organic matter. The submerged species of macrophytes at the margin also act as a green manure favorable the abundance of zooplankton and benthic fauna, supported by Bhute and Harne, (2017) from Nagrala Lake. Macrophytes serve as a substratum, manure and also provide food and

ARTICLE INFORMATION

*Corresponding Author: apsawane@gmail.com
Received 9th Jan 2020 Accepted after revision 17th March 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)
A Society of Science and Nature Publication, Bhopal India

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/46

shelter for many aquatic organism, Kudryavtsev and Yeshov, (1980) and Raut and Pejaver, (2005) Seasonal fluctuation in riverine water flow is responsible for limited macrophyte diversity, Rulikm et al., (2020). Macrophyte diversity significantly affects overall aquatic biodiversity, Prasad and Das, (2018). Since the studies on macrophytes diversity are very less in Kolar river this paper is intended to report macrophytes diversity in present investigation.

MATERIALS AND METHODS

The river Kolar is in the vicinity of Khaparkheda town, located at 21.3858107^0 north latitude and 78.9201379^0 east longitudes in Nagpur district of Maharashtra state. This river flowing besides the thermal plants (Khaparkheda TPS and Koradi TPS) and some villages in the downstream are located on the bank of this river and receiving effluents and domestic water. Therefore, the river was monitored by collecting samples from four locations covering the complete stretch of the river receiving discharges during the period of two years from February 2010 to January 2012 in winter, summer and monsoon seasons to know the seasonal variation. These sampling locations are Site - A (Dam Site), Situated at Nanda Dam, Site - B (Village Site), Situated near Kolar bridge on N.H.69 at Mahadula, Site - C (Village Site), Situated at Khaparkheda, Site - D (Confluence point Site), Situated at Confluence point of river Kolar and Kanhan at Waregoan. The macrophytes biodiversity of river Kolar was evaluated and assessment was made by analyzing parameters of interests.The sampling program was planned taking into account the objectives of the study and the parameters to be analyzed. Efforts were made to centralize the aim of sampling to achieve the representativeness and validity of the samples. Macrophytes were collected at monthly intervals during the period of investigation from shallow littoral zone by hand picking. After collection specimens were thoroughly washed with water, excess water was soaked with filter paper, kept in polythene bags, brought to laboratory in ice box and specimens identified up to species with the help of standard literature Edmondson (1959), APHA (1996), IAAB publication no.2, (1998) and Fassett (2000).

RESULTS AND DISCUSSION

In the present investigation, 25 species from three groups were recorded from Kolar lotic ecosystem under study which was categorized by free floating, submerged and Marginal aquatic weeds (Table-1 and Table-2). *Azolla species* were not recorded from Kolar river while *Eichhornia crassipes* was recorded. The *Azolla spp*. is considered as pollution free species and Eichhornia as pollution tolerant species Narayana and Somashekhar, (2002). The macrophytes also provide suitable breeding and sheltering place for macroinvertebrates and fishes Meshram, (2003). Macrophytes in fresh water play major ecological role and help in the regulation and stabilization of trophic state and mineral cycling in the aquatic ecosystem Melzer, (1981), Wielgleb, (1984). They serve as the bioindicators for the possible degree of damage in aquatic ecosystem Pieczynska and Ozimek, (1976). During investigation period of the total mcrophytes; free floating-20%, submerged-48% and Marginal aquatic weeds-32% were observed (Figure-1).

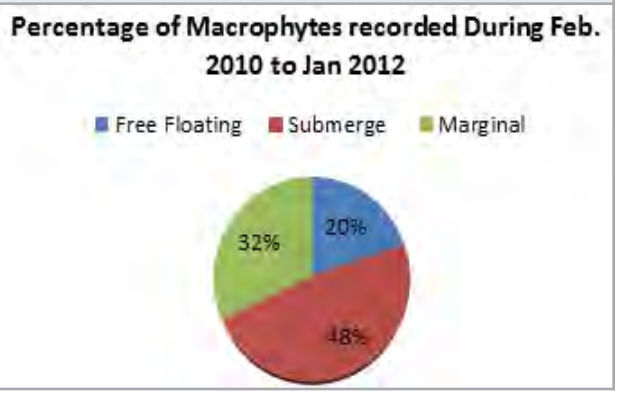
The free floating macrophytes were represented by, *Wolffia spp*, *Lemna spp, Spirodella spp*, *Pistia stratiotes* and *Eichhornia crassipes*.

The submerged macrophytes were represented by, *Hydrilla verticellata, Vallisneria spiralis, Potamogeton natans, P. crispus, P. pectinatus, P. richardsonii, Ceratophyllum demersum, Najas spp, Utricularia spp and Chara vulgaris. Myriophyllum, Hyperium spp*. The marginal aquatic weeds were represented by, *Rotala ramosior, Lythrum alatum, Penthorum spp, Cyperus diffuses, Typha angustata, Ludwigia spp. Marsilea quadrifolia* and *Ipomoea aquatic*, *Ludwigia spp*. Macrophyte diversity was found low in sampling site D which was confluence point of river Kolar and river Kanhan, where water current was fast. Maximum number of macrophyte species was recorded at sampling site-B (Village site near Kolar bridge where water current is slow, water is shallow and some anthropogenic activities were found Table-3 and Figure-3).

Table 1. Total numbers of Macrophytes recoded during Feb 2010 to Jan 2012

Type	Free Floating	Submerge	Marginal
Number of Species	05	12	08
Total	25		

Figure 1. Percentage of Macrophytes recorded During Feb. 2010 to Jan 2012



The data of the present investigation show that the enrichment of the shallow water with high bottom sediments provides an ideal habitat for luxuriant growth of macrophytes. It is also demonstrated that the diversity of macrophyte is less where water current is more and diversity increases as the water current decreases and organic contents increases, these data are well supported by the work of Tenna Riis et al., (2019) from River Gudena, Denmark, where shallow water was found to

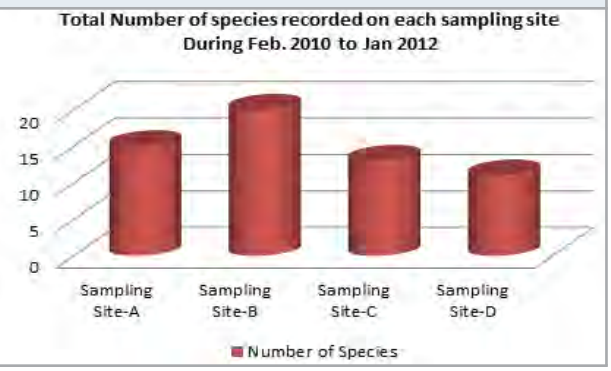
Table 2. The diversity of Macrophytes in the Kolar river During Feb. 2010 to Jan 2012

SN	Macrophytes	Family	Site A	Site B	Site C	Site D
A	Free Floating					
1	*Lemna spp,*	Salvanaceae	+	+	-	
2	*Pistia stratiotes*	Lemmaceae	+	+	-	
3	*Eichhornia crassipes.*	Lemmaceae	-	+	+	+
4	*Wolfia spp.*	Lemmaceae	+	+	-	
5	*Spirodella spp.*	Lemmaceae	-	+	+	+
B	*Submerged*					
1	*Najas spp.*	Najadaceae	+			+
2	*Potamogeton richardsonii*	Najadaceae		+	+	
3	*P. crispus*	Najadaceae	+	+	+	
4	*P. pectinatus*	Najadaceae		+	+	
5	*P. natans*	Najadaceae	+			+
6	*Ceratophyllum demersum*	Hydrocharitaccae	+	+	-	
7	*Hydrilla verticellata*	Hydrocharitaceae	+			+
8	*Valisnaria spiralis*	Hydrocharitaceae	+	+	-	
9	*Utricularia spp.*	Lentibulariaceae	+			+
10	*Hyperium spp,*	Hyparaceae		+	+	
11	*Chara vulgaris.*	Characeae	+			+
12	*Myriophyllum*	Hydrocharitaccae	+	+	-	
C	*Marginal*					
1	*Rotala ramosior*	Lythraceae		+	+	
2	*Lythrum alatum*	Lythraceae	+	+	+	+
3	*Typha angustata*	Iridaceae		+	+	+
4	*Ipomoea aquatica.*	Compositae	+	+	+	
5	*Penthorum spp.*	Crassulaceae		+	+	+
6	*Cyperus diffusus*	Cyperaceae		+	+	
7	*Ludwigia spp.*	Onagraceae	+	+	-	
8	*Marsilea quadrifolia*	Marsileaceae		+	+	+

Table 3. Total Number of species recorded on each sampling site During Feb. 2010 to Jan 2012

Sampling Site	A	B	C	D
Species Diversity	15	20	13	11

Figure 2. Total Number of species recorded on each sampling site During Feb. 2010 to Jan 2012



be enriched with high bottom sediments for luxuriant growth of aquatic plants. This has also has been recently reported by Patel and Dubey, (2019). Environmental factors such as topography, season, rain fall expected to create numerous ecological niches, also leads to high diversity of aquatic plants as shown by Prasad and Das (2018). The findings of the present investigation that the shallow water when enriched with high bottom sediments provides an ideal habitat for luxuriant growth of macrophytes has also been reported by the recent findings of Rulik et al., (2020).

REFERENCES

APHA (1996); Standard Methods for the examination of water and waste water, 20th Edition American Public Health Association, Washington DC.

Bhute KB and Harney NV (2017); Macrophytes biodiversity of Nagrala lake of Bhadrawati, district-Chandrapur (M.S.), India. Int. Res. J. of Science & Engineering, 2017; Vol. 6 (1): 17-19 http://www.irjse.in ISSN: 2322-0015

Edmondson, W. T. (1959); Freshwater Biology. II Ed.

John Willey & Sons, New York.
Ester Vieira Noleto, Marcus Vinícius Moreira Barbosa, Fernando Mayer Pelicice, (2019); Distribution of aquatic macrophytes along depth gradients in Lajeado Reservoir, Tocantins River, Brazil. Acta Limnologica Brasiliensia Print version ISSN 0102-6712 On-line version ISSN 2179-975X
Fasset, C. Norman (2000); A Manual of aquatic plants, Agrobios (India), Jodhpur.
Gregg, W. W. and Rose F. L. (1982); The effects of aquatic macrophytes on the stream micro-environment., Aquat. Bot.14 : 309-324.
Hanife Ozbay, Ahmet Emre Yaprak & Nesibe Turan (2019); Assessing water quality in the Ceyhan River basin (Turkey) with the use of aquatic macrophytes. Journal Chemistry and Ecology Volume 35, 2019 - Issue 10
IAAB Pub. No. 2 (1998); Methodology for water analysis, Hyderabad (A. P.). Ibid (1993b); Assessment of water quality of river Bhagirathi at Uttarakashi., J. Env. and Zoology. 7(2): 118-123.
Kudryavtsev VM, Yeshov YV (1980); Abundance dynamics of microorganisms in the decomposition of macrophytes. Ins. of Bio. of Inland waters, USSR, Academy of Sci. Brok, 1980, 14-18.
Melzer, A. (1981); Veranderungen der Macrophyten Vegetation der starn berger Sees and ihre dikatarische Bedeutung. Limnologica. 13: 449¬458.
Meshram, C. B. (2003); Macro invertebrate fauna of lake Wadali, Amaravati, Maharashtra., J.Aqua.Biol.Vol.18(2): 47- 50.
Muslim Ahmad Shan and Vipin Vyas (2015); Assessment of Macrophyte diversity in selected reaches of river Narmada at Hoshangabad District of Madhy Pradesh. International Journal of Science Engineering and Technology Research (IJSETR) Volum 4 Issue 10: 3338-3344
Narayana, J. and Somshekhar, R. K. (1997); Heavy metal composition in the sediment and plants of the river Cauvery., J. Environ. Pollut., 4(4); 325-328.
Narayana, J. and Somashekhar R. K. (2002); Distribution and species diversity of Macroinvertebrates in river Cauvery., Ecology and Conservation of Lakes, Reservoirs and Rivers, ABD publishers, Rajasthan, India.1-43.
Patel Karuna, Dubey Sanjeev, (2019), Diversity and distribution of macrophytes in Govindgarh Lake of Rewa district (M.P.). International Journal of Advanced Science and Research ISSN: 2455-4227, www.allsciencejournal.com, Volume 4; Issue 2; March 2019; Page No. 20-22
Pieczynska, E. and Ozimek T. (1976); Ecological significance of macrophytes., Int. J. Ecol. Environ. Sci. 2: 155-158.
Prasad Nami and Das Tapati (2018); Diversity and distribution of aquatic macrophytes with special reference to invasive species in Barak Valley, Assam, Northeast India. NeBIO An international journal of environment and biodiversity Vol. 9, No. 1, March 2018, 102-108 ISSN 2278-2281(Online Version) www.nebio.in
Rameshkumar, S., Radhakrishnan, K., Aanand, S.(2019); Influence of physicochemical water quality on aquatic macrophyte diversity in seasonal wetlands. Appl Water Sci 9, 12 (2019) doi:10.1007/s13201-018-0888-2
Raut Nayana S, Madhuri Pejaver, (2005); Survey of diversity of plankton attached to macrophytes from weed infested lakes. J Aqua Biol. 2005; 20(1):1-7.
Rulik M., Opatrilova L., Jurajda P., Spacek J., Grulich V., (2020); Rivers in the Czech Republic: In: Zelenakova M., Fialov J. Negm A (eds), Assessment and protection of Czech Republic, Springer water, Springer Cham online ISBN 978-3-030-18363-9
Szymon Jusik, Ryszard Staniszewski (2019), Shading of River Channels as an Important Factor Reducing Macrophyte Biodiversity. Pol. J. Environ. Stud. Vol. 28, No. 3 (2019), 1215-1222
Tenna Riis, Jennifer L. Tank, Alexander J. Reisinger, Antoine Aubenau, Kevin R. Roche, Peter S. Levi, Annette Baattrup-Pedersen, Anette B. Alnoee, Diogo Bolster, (2019): Riverine macrophytes control seasonal nutrient uptake via both physical and biological pathways. Freshwater Biology. 2019;00:1–15. vileyonlinelibrary.com/journal/ fwb, 2019 John Wiley & Sons Ltd.
Wielgleb, G. (1984); A study of habitat conditions of the macrophytic vegetation in selected river systems in Western Lower Sexony (Federal Republic of Germany)., Aquatic Botany. 18 :313-352

Psychological Communication

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp-288-295

BBRC Bioscience Biotechnology Research Communications

Role of Parents Education and Occupation in Parental Pressure and Adolescents Test Anxiety

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ABSTRACT

Failure in academics is a leading cause of suicides in India. Among students appearing for Board examinations, 82 percent reported academic pressure and 74 percent experienced test anxiety. Parental pressure over academics is specific to South Asian cultures and related to parents' socioeconomic status. The present study aimed to explore role of parents' education and occupation in parental pressure and test anxiety of students appearing for Board examinations. It adopted sequential explanatory mixed method. 123 students appearing for Board exams of grade 10 from Pune participated in the first (quantitative) phase. Parental pressure was found to be a significant predictor of test anxiety (B= .022, p=.000, adjusted R2= .095). The mean scores of test anxiety and parental pressure did not vary based on education and occupation of parents. Four participants who had reported high parental pressure and test anxiety were interviewed in the second (qualitative) phase. Themes about antecedents and effects of test anxiety and parental pressure, role of education/occupation of parents in these, solutions for mitigation got revealed. The findings would aid to device specific interventions and stress free evaluation systems.

KEY WORDS: PARENTAL PRESSURE, TEST ANXIETY, BOARD EXAMINATION, PARENTS' EDUCATION, PARENTS' OCCUPATION.

INTRODUCTION

India would become the youngest country in the world by 2020 with 64 percent of her population in the working age group (The Hindu, 2013). Contrastingly, India has the highest rate of suicides among people ranging from 15 to 29 (Patel et.al, 2012). Failure in examination is among the top ten causes of suicide in India accounting for 2 percent of total number of suicides (National Crimes Records Bureau, 2016). The literature identifies academics as a stressor for students. Frequency and performance in examinations was one among the stressors (Swaminathan et al., 2016). Competition in Indian educational system makes academics a stressor. Grades obtained in 10th and 12th standard influence the choice of subjects, stream, college and career (Deb et al., 2015). 82 percent of students appearing for board examinations reported academic pressure and 74 percent experienced test anxiety (Deb et al., 2014) irrespective of the curriculum and type of school (Sasikala and Karunanidhi, 2015). The pressure may reach the extent of verbal and physical abuse pushing students to suffer depression or take extreme steps like committing suicide (Times of India, 2011). It adversely affects students' overall well being by interfering with academic, interpersonal and intrapersonal domains (Desai & Sathiyaseelan, 2017, Nair and Sathiyaseelan, 2018).

The biopsychosocial model highlights the role of social systems like family, school and community in shaping academic self efficacy of the child which is related to test anxiety (Lowe et al, 2008). South Asian countries observe a trend of parental pressure because education is considered a way to move up higher in the socioeconomic hierarchy (Gulf News, 2015). Putwain, Woods and Symes (2010) found parental pressure to be a predictor of test

ARTICLE INFORMATION

*Corresponding Author: tanmayidesai98@gmail.com
Received 12th Jan 2020 Accepted after revision 28th Feb 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)
A Society of Science and Nature Publication, Bhopal India

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/47

anxiety. Comparison and acceptance based on grade was prominent among traditional Asian families (Putwain, 2009). In the Indian context, test anxiety was observed to be a mediator between parental pressure and test anxiety (Nagpal & Sinha, 2016). Social derogation was salient in experiences of test anxiety Indian students they perceived failure as bringing shame to the family. They feared examinations due to disapproval, punishment and shame which might follow test evaluations (Bodas, Ollendick & Sovani, 2008). Socioeconomic status of parents influenced parental pressure and test anxiety (Chen, 2012). In India, education of parents and occupation of mother were related to parental pressure. Parental pressure was related to academic stress but not test anxiety (Deb, Strodl & Sun, 2015). However, Deb, Strodl and Sun (2014) did not find any relationship in these variables. Considering the large scale extent and debilitating consequences of test anxiety and parental pressure, detailed study is warranted.

MATERIAL AND METHODS

The present study was aimed to investigate the role of Parents' Education and Occupation in Parental Pressure and Test Anxiety among students appearing for Board examinations of grade 10. It adopted sequential explanatory mixed method. Results obtained in the initial quantitative phase are explained with the aid of later qualitative phase (Creswell, 2014).

First (Quantitative Phase): Two schools following the state syllabus from Pune, India were chosen based on the convenience. School principals signed the informed consent as guardians. 123 students of grade 10 signed the assent form to participate in the study. The exclusion criteria were repeaters, physically challenged and staying away from either/both parents. Participants responded to the Demographic Data Sheet which included columns to indicate education and occupation of each parent based on revised Kuppuswamy Socioeconomic Status Scale (Oberoi, 2014), Parental Pressure Subscale (PPS) of the Inventory of Parental Influence (Campbell, 1994) and Westside Test Anxiety Scale (WTAS) (Driscoll, 2007). Participants meeting the predetermined criteria (Cut off score of 39 on PPS, 3 on WTAS) were shortlisted for the next phase. Second (Qualitative) Phase. To maintain the homogeneity, education (both parents were graduates) and occupation (either parent is employed) were checked. Participants and their parents' consents were obtained for audio recordings. Adopting Interpretative Phenomenological Approach, face to face interviews were conducted based on the interview guide which had been validated by three experts. Memo interviews were conducted based on transcripts. Member check was carried out to ensure validity. Thematic analysis revealed basic, organizing and global themes.

RESULTS AND DISCUSSION

Phase One: Quantitative Phase: The characteristics of the sample are mentioned in table 1.

$$\text{Test anxiety} = 1.685 + .022 \times \text{Parental pressure}$$

Several studies conducted worldwide (Putwain et al., 2010; Chen, 2012) and in India (Nagpal & Sinha, 2016) identified parental pressure as a predictor of test anxiety. Deb, Strodl & Sun (2014) presented evidence indicating no relationship between parental pressure and test anxiety. However, the models tested in America (Pang, 1994 as cited in Chen, 2012) and China (Chen, 2012) could explain 20 and 70 percent variance in test anxiety respectively. Bodas et al., (2008) suggested that the unique cultural setting in India allows for several influences on test anxiety which may not be applicable to other countries. This could account for the lower beta coefficients.

Hypothesis 2. H1 Education and occupation of parents play significant roles in parental pressure and test anxiety of the student: The education and occupation of a parent was measured using seven categories each described in revised Kuppuswamy Socioeconomic Status Scale (Oberoi, 2014). The data was regrouped into two categories each for education and occupation of each parent (table 2).

As per the results of T test and Mann Whitney U test, the mean scores on test anxiety and parental pressure did not differ when the education and occupation of each parent was considered ($p > .05$). Thus, hypothesis 2 is rejected. The findings are similar to a study done Kolkata which found no relation between parents' education and exam related anxiety (Deb, Strodl & Sun, 2014). But, studies conducted in China (Chen, 2012) and India (Deb, Strodl & Sun, 2015) observed that education and occupation of parents' influence parental pressure and test anxiety. Wards of non graduate parents and self employed mothers were likely to experience more academic and parental pressure (Deb, Strodl & Sun, 2015). In the light of the mixed evidence, qualitative analysis would help us understand and explain the results obtained in the first phase.

Phase Two: Qualitative Analysis: Profiles of four participants interviewed for phase two are mentioned in table 3.

[PC: Participant's code, WTAS: Westside Test Anxiety Scale (Discroll, 2007); PPS: Parental Pressure Subscale

Table 1: Demographic Details of the Participants

Descriptions	Frequency	Percentage	Mean	SD	N
School					123
School 1	70	56.9			
School 2	53	43.1			
Sex					123
Male	77	62.6			
Female	46	37.4			
Age			14.72	.563	123

(Campbell, 1994); Education and Occupation based on revised Kuppuswamy Socioeconomic Status Scale (Oberoi, 2014).Thematic analysis of transcripts reveled three global themes which are presented below with its organizing and basic themes.

Theme One. Antecedents, experiences and consequences of exam fear: It deals with students' experience of test anxiety.

1.1. Perception of Exam Fear: It deals with subjective meaning of the phenomenon.

1.1.1 Meaning of Test Anxiety: Participants noted test anxiety to be fear of the outcome as low grades have potential consequences in future. The views are similar to definition given by Zeidner (1998), "test anxiety is emotional, physiological, and behavioural responses surrounding the potential consequences of negative evaluation on an upcoming test or exam" (as cited in Von Der Embse, Barterian & Segool, 2013).

1.1.2 Expression of Test Anxiety: Test anxiety is a multidimensional phenomenon having affective, cognitive and behavioural facets (Zeidner, 1998). Participants perceived test anxiety as combinations of affective (fear), cognitive (going blank) and behavioural (not writing) indicators.

1.2 Manifestation of Exam Fear: Three components of exam fear were explored.

1.2.1 Affective Manifestation: Students reported to be 'tensed', 'scared, 'nervous' before exam but relaxed after the same. Academics related activities in India are associated with experience of negative states (Verma, Sharma & Larson, 2002).

1.2.2 Cognitive Manifestation: Participants mentioned apprehensions about other's reactions to their grades, especially parents. Going blank, getting confused and performing lower than desired were experienced frequently during exams. Bodas*, Ollendick & Sovani (2008) concluded that Physiological symptoms dominate the presentation of test anxiety among Indian students. The current study falls short to conclude it.

1.2.3 Behavioural Manifestation: Participants experienced sweating, inability to write and hence kept on studying till the last moment. Bodas, Ollendick and Sovani (2008) found that 59 percent of Indian students preferred studying harder to deal with test anxiety and stress. Academic procrastination, a way to escape anxiety was seen as a self harming behaviour (Nair and Sathiyaseelan, 2018).

1.3 Antecedents/ Contributing Factors: Perception and preparation of academics, parental pressure and board examinations were found to contribute to test anxiety.

1.3.1 Perception and preparation of academics: The dislike of students towards studies or a subject was seen as a reason of fear by them. Putwain, Woods & Symes (2010) noted personal beliefs as a contributor to test anxiety.

1.3.2 Parental Pressure. Parental pressure was found to be a predictor of test anxiety (B= .022, p=.000): In students opinion, parental expectations, repetitive nagging and exaggeration of Board examinations made them fear exams. Hill and Wigfield (1984) noted that unreasonable expectations and derogation after failure pressurizes one to perform beyond one's capacity. Test anxiety and poor performance form a vicious cycle by influencing motivational dynamics.

1.3.3. Board Examinations: Performance in Board exams is crucial for admission to colleges and streams of career. The scarcity of good colleges leads to cut throat competition (Deb, Strodl & Sun, 2015). Students cited the same reasons.

Table 2. Frequencies of Regrouped data regarding Parent's Education and Occupation

		Description of Categories	N	Percentage (%)
Mother's Education	1	Graduates and above	85	69.1
	2	Intermediate/high school diploma and below	38	30.9
Father's Education	1	Graduates and above	86	69.9
	2	Intermediate/high school diploma and below	37	30.1
Mother's Occupation	1	Profession, semiprofession	22	17.9
	2	Clerical, workers and unemployed	101	82. 1
Father's Occupation	1	Profession, semiprofession	90	73.2
	2	Clerical, workers and unemployed	33	26.8

Table 3: Profile of participants in qualitative analysis phase (phase two)

PC	Age	Gender	Scores in Phase one	Education	Occupation			
			WTAS	PPS	Mother	Father	Mother	Father
P1	15	Female	3.4	39	Graduate or Post Graduate	Graduate or Post Graduate	Unemployed	Skilled Worker
P2	15	Female	3.7	51	Graduate or Post Graduate	Graduate or Post Graduate	Unemployed	Semi Profession
P3	15	Male	3.0	42	Profession or Honours	Profession or Honours	Unemployed	Profession
P4	14	Male	3.4	40	Graduate or Post Graduate	Graduate or Post Graduate	Profession	Skilled Worker

1.4 Consequences of Exam Fear: Exam fear had implications for the individual, her surroundings and academics.

1.4.1. On self and surroundings: Participants highlighted that exam fear affects their health, routine and surroundings like family environment marked with stress and tension.

1.4.2. On preparation. Exam fear interferes with exam preparation and studies: Due to exam fear participants faced non completion of syllabi, intruding thoughts and longer time to learn. It corroborates with finding that students with high test anxiety experience encoding difficulty (Hembree, 1988).

1.4.3 On performance in exams: Hembree (1988) stated that test anxiety could have facilitating as well as debilitating consequences. Same opinion was expressed by participants in the present study. Participants experience confusion, uncertainty of answers and increased chances of silly mistakes. These tend to lower their performance in exams.

Theme Two. Parental pressure and relevance of parent's education, occupation to it: Campbell (1994, 1996) defined parental pressure as the amount of pressure parents exert on their children to achieve high levels of academic performance (as cited in Wei, 2008). The theme attempts to explain role of parents' education and occupation in parental pressure.

2.1 Changes in parents after commencement of child's 10th grade: The year of board examination brought changes in parents' behaviour.

2.1.1 Dynamics within parents: Three participants mentioned that the changes were more dramatic with their fathers and one opined for mother.

2.1.2 Emphasis on studies: Often, parents expressed dissatisfaction over child's study and repetitively instructed to study harder. The pressure exerted by parents over academics could go to extremes making them get glued to study tables (Times of India, 2011).

2.1.3 Token offered: Parents offered reinforcements like buying a phone, vehicle or threats like cutting of television connection. Bodas, Ollendick and Sovani (2008) found fear of punishment and disapproval as causes of test anxiety.

2.2 Expectations of parents from students: The theme allows inferring about implicit assumptions of parents noted from parents' expectations regarding academic.

2.2.1 Overemphasis on grades: Parents had stringent criteria about grades and considered that to be the sole indicator of academic progress. Parents were not satisfied thought students scored the said grades.

2.2.2. Expression of parental pressure: Parents were reported to make comparison of their wards with toppers, classmates and insisted on following the same schedule to study. Traditional Asian families tend to compare their wards with other high achieving individuals (Putwain, 2009).

2.2.3 Implicit assumptions and discrepancies: Parents equated grades in exams as an indicator of successful life. Securing higher grades was associated with number of hours of study. While insisting on grades, the learning aspect was ignored. But, the emphasis on academic achievements did not translate to active involvement of parents. A participant complained that parents valued academic performance more than any other achievements. This could be attributed to cultural factors in South Asia where education in considered to be a way to move up in socioeconomic hierarchy (Gulf News, 2015).

2.3 Involvement of parents in academics: This theme explores parents' involvement in academics.

2.3.1 Extent and Manner: Parents got involved in academics only during the exam time. Direct involvement (aiding in academics) and indirect involvement (maintaining conducive environment) were reported. However, a participant complained about the involvement being not authentic indicating that parents did not share the responsibility over academics with their children.

2.3.2 Responsibility of studies: Parents attributed the responsibility of academics to the child and his/her teachers, guides etc.

2.3.3 Students' reactions to involvement of parents: Students explained positive as well as negative sides of academic involvement of parents.

2.4 Approaching disagreements or failures of students: The theme elaborate how parents handle failure of children.

2.4.1 Reaction of parents to disagreements or failure of child: Parents would express their disappointment by cutting rewards, not talking or shouting at them etc. The observations corroborate with the finding that academic performance of the child is a condition of acceptance (Putwain ,2009). However, participants clarified that their parents would also encourage them to study better.

2.4.2 Role of other parent: In cases of disappointment over failure, parent and child are at conflict. The other parent could exacerbate or minimize the severity of conflicts.

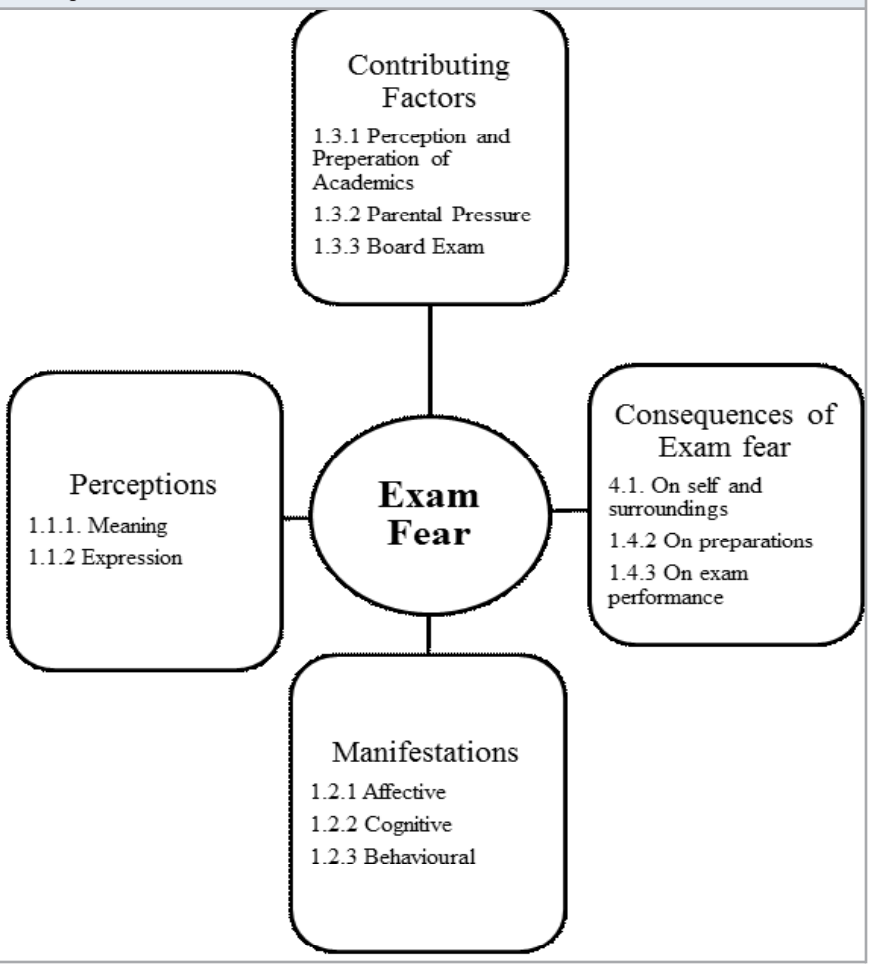
2.5 Influence of parental pressure: Parental pressure impacts physical, psychological health (Times of India, 2011) and academic performance (Nagpal & Sinha, 2016). Findings here corroborate with the same.

2.5.1 On students. Participants perceived parental pressure to be a usual phenomenon: It influenced students' schedules i.e having no time to play. The Social Cognitive theory emphasizes the role of environment in shaping of an individual (Bandura, 1977). Hence, it is vital to note that parental pressure has potential to influence students' affect, behaviours and cognitions.

2.5.2 On parent child relationship: Students' reaction to parental pressure had potential to influence parent child relationship as well as family environment. Desai and Sathiyaseelan (2017) have summarized influences of parental pressure and test anxiety on overall wellbeing.

2.6 Role of Parents' education and occupation in parental pressure: Students noted several influences of parents' education and occupation on academics.

Figure 1. Global theme one. Antecedents, experiences and consequences of exam fear



2.6.1 Effect on students' lives: According to the participants, advanced education of parents contributed to better understanding and respect.

2.6.2 Influence on academics: Occupation of parents influenced the time they could involve in their child's academics. To the contrary, homemaker mothers were said to monitor student's schedule. Besides the allotment of time, education and occupation acted as a resource in academics. Deb, Strodl and Sun (2015) found non graduate fathers and mothers in business as more pressurizing. It can be argued that parents with higher education (graduation in this case) were seen as a resource rather than pressure. Homemaker mothers were seen as support due to their availability and involvement in studies.

2.6.3 Influence on expectations: Nair (2014) asserted that parents see children as a tool to fulfil their dreams. Here, parents would insisting on selecting careers they had chosen and expressed negative attitude towards other disciplines. Cultures where academics is seen as matter of family glory, pride experience trend of parental pressure (Chen, 2012).

Theme Three: Proposed Solutions to Exam fear and Parental Pressure. Participants explained how they manage their exam fear and the desired changes in parents' related to academics that would help reduce parental pressure (figure 3).

3.1 Coping with exam fear: Participants tried to manage anxiety on their own and occasionally sough support.

3.1.1 Managing on own: Participants resorted to changing study strategies or taking time off studies. In order to avoid poor results, they try to write the little they know. But, failure to do so may increase their anxiety. It is important to note that students did not try to deal with the fear directly. Rather, tried to minimize its impact on the exam performance.

3.1.2 Support seeking from parents and other sources: Bodas*, Ollendick and Sovani (2008) discovered that only eight percent Indian students with high test anxiety sought support from parents or professionals. All participants mentioned of discussing it with parents and some sought help of friends or counselors. But, negative reactions from parents like blaming discouraged students. The nature of support was limited to sharing only.

Figure 2. Global theme two. Parental pressure and relevance of parent's education, occupation to it.

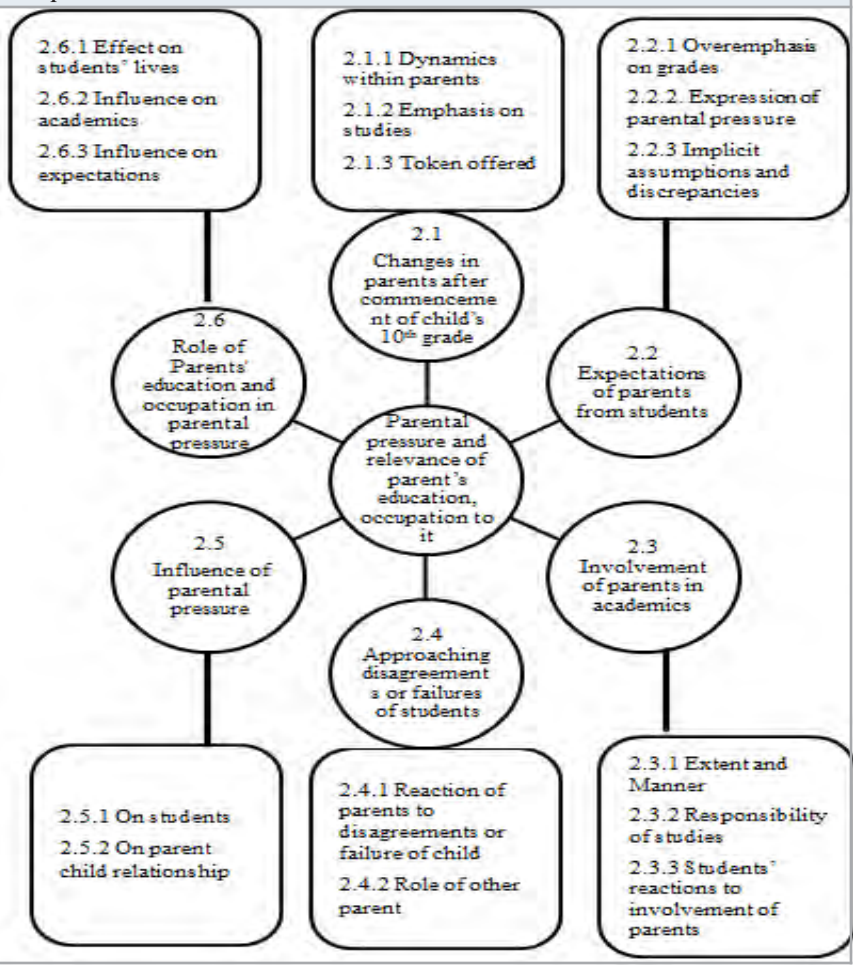
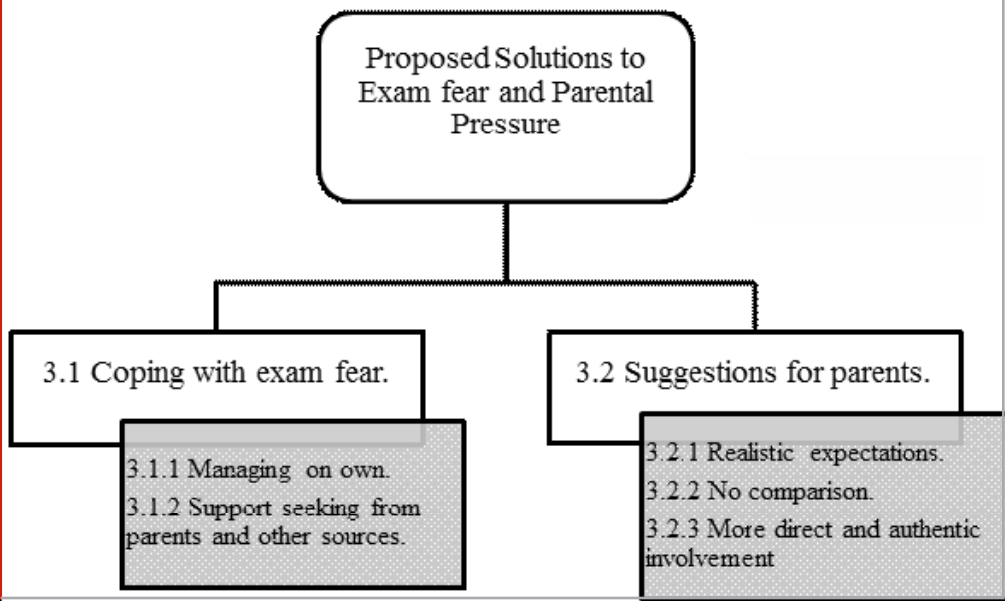


Figure 3. Global theme three. Proposed solutions to exam fear and parental pressure



3.2 Suggestions for parents: Students wanted parents to be a support in academics.

3.2.1 Realistic expectations: Highlighting the need for mutual goal setting, participants wanted parents to consider their perspective and abilities to set goals.

3.2.2 No comparison: Participants expressed dislike towards parents comparing them with toppers, relatives or classmates.

3.2.3 More direct and authentic involvement: Participants wanted direct and authentic involvement in studies. A participant wanted them to monitor her progress, enquire about her studies and allocate time to solve her queries. Nagpal and Sinha (2016) opined that parents together with teachers need to create environment fostering child's learning. Thus psychologists, school counsellors and school authorities need to conduct awareness creating workshops regarding parental pressure and test anxiety (Deb, Strodl & Sun, 2014). Imbibing the concept of 'mindful living' would help both students and parents to excel beyond the daily stressors (Sathiyaseelan & Sathiyaseelan, 2014).

CONCLUSION

The study threw lights on role of socioeconomic factors in test anxiety and parental pressure. Parental pressure was found to be a predictor of test anxiety. Quantitative analysis did not favor for influence of education and occupation of parents on parental pressure and test anxiety experienced by students. However, the qualitative phase provided insights about links among parents' education, occupation, test anxiety and parental pressure. Parents could not give time for studies because of their busy schedules. Children of homemaker mothers reported active involvement, support and monitoring of studies. Parents were reported to compare academic performance of child with peers, relatives. Insistence on selecting a particular career and rigid standards about scores contributed to parental pressure and test anxiety. But, the present study found contradictory evidences in two phases. The findings need to be interpreted by keeping the limitations of the study in mind.

The study has important theoretical as well as applied implications. The findings are relevant to the fields of education, parenting and counselling. To treat the root cause of the issues, all stakeholders of students' wellbeing like students, parents , teachers, psychologists, administrators and policy makers must device an effective examination system. Efforts by academia along with professionals are required to address the issues of education and parenting in India holistically. Trealease (2006) reminds us of our responsibility of children's well being saying, "The child spends 900 hours a year in school and 7,800 hours outside school. Which teacher has the bigger influence?"

REFERENCES

Bandura, A. (1977). Self-efficacy: toward a unifying theory of behavioral change. Psychological review, 84(2), 191.

Bodas J., Ollendick, T. H., & Sovani, A. V. (2008). Test anxiety in Indian children: A cross-cultural perspective. Anxiety, Stress, & Coping, 21(4), 387-404.

Campbell, J. R. (1994). Inventory of Parental Influence. Developing cross-cultural/cross-national instruments: Using cross-national methods and procedures. International Journal of Educational Research, 21, 675-684.

Chen, H. (2012). Impact of parent's socioeconomic status on perceived parental pressure and test anxiety among

Chinese high school students. International Journal of Psychological Studies, 4(2), 235.
Creswell, J. W. (2014) Research Design. Qualitative, Quantitative, and Mixed Methods Approaches. USA: Sage
Deb, S., Strodl, E., & Sun, J. (2015). Academic stress, parental pressure, anxiety and mental health among Indian high school students. International Journal of Psychology and Behavioral Sciences, 5(1), 26-34.
Deb, S., Strodl, E., & Sun, J. (2014). Academic-related stress among private secondary school students in India. Asian Education and Development Studies, 3(2), 118-134.
Desai & Sathiyaseelan. (2017). Parental Pressure and Test Anxiety: Threats to Students' Well Being. Recent Advances in Psychology: An International Journal, 4(1).
Driscoll, R. (2007). Westside Test Anxiety Scale Validation. Online Submission.
Experts take aim at South Asia parents (2015, March 1).Gulf News India. Retrieved from http://gulfnews.com/news/asia/india/experts-take-aim-at-south-asia-parents-1.1464587
Hembree, R. (1988). Correlates, causes, effects, and treatment of test anxiety. Review of educational research, 58(1), 47-77.
Hill, K. T., & Wigfield, A. (1984). Test anxiety: A major educational problem and what can be done about it. The Elementary School Journal, 85(1), 105-126.
India is set to become the youngest country by 2020 (2013, April 17). The Hindu. Retrieved from https://www.thehindu.com/news/national/india-is-set-to-become-the-youngest-country-by-2020/article4624347.ece
Lowe, P. A., Lee, S. W., Witteborg, K. M., Prichard, K. W., Luhr, M. E., Cullinan, C. M., ... & Janik, M. (2008). The test anxiety inventory for children and adolescents (TAICA) examination of the psychometric properties of a new multidimensional measure of test anxiety among elementary and secondary school students. Journal of Psychoeducational Assessment, 26(3), 215-230.
Nagpal, M., & Sinha, C. (2016). Perceived parental pressure and academic achievement among Students: Exploring the mediating effect of test anxiety among school students, Bangalore. Open Journal of Educational Psychology.
Nair, M.(2014, November 27). Is Exam the sole factor causing stress among students in India?. Maps of India. Retrieved from http://www.mapsofindia.com/my-india/education/is-exam-the-sole-factor-causing-stress-among-students-in-india
Nair, Ganesh & Sathiyaseelan, A. (2018). Academic procrastination: adaptive or self harming behaviour. Research journal of social science and management. 7. 8 - 13.
National Crime Records Bureau (2016), Accidental Deaths & Suicides in India, Ministry of Home Affairs, Government of India, New Delhi. Retrieved from http://ncrb.nic.in/StatPublications/ADSI/ADSI2015/adsi-2015-full-report.pdf
Oberoi, S. (2015). Updating income ranges for Kuppuswamy's socio-economic status scale for the year 2014. Indian journal of public health, 59(2), 156-156.
Patel, V., Ramasundarahettige, C., Vijayakumar, L., Thakur, J. S., Gajalakshmi, V., Gururaj, G., ... & Million Death Study Collaborators. (2012). Suicide mortality in India: a nationally representative survey. The lancet, 379(9834), 2343-2351.
Putwain, D. W., Woods, K. A., & Symes, W. (2010). Personal and situational predictors of test anxiety of students in post-compulsory education. British Journal of Educational Psychology, 80(1), 137-160.
Putwain, D. W. (2009). Assessment and examination stress in Key Stage 4. British Educational Research Journal, 35(3), 391-411.
Sasikala, S., & Karunanidhi, S. (2010). Test anxiety in relation to behavioural problems of school students. J. Ind. Heal. Psy, 5(1), 109-120.
Sathiyaseelan, A., & Sathiyaseelan, B. (2014). Integrating 'mindful living'for a peaceful life. Research journal of social science and management, 4, 187-192.
Swaminathan, A., Viswanathan, S., Gnanadurai, T., Ayyavoo, S., & Manickam, T. (2016). Perceived stress and sources of stress among first-year medical undergraduate students in a private medical college–Tamil Nadu. National Journal of Physiology, Pharmacy, and Pharmacology, 6(1), 9-14.
Trelease, J. (2013). The Read-Aloud Handbook. Penguin.
Tender minds crushed by parental pressure (2011, November 1). The Times of India. Retrieved from http://timesofindia.indiatimes.com/city/kolkata/Tender-minds-crushed-by-parental-pressure/articleshow/10563010.cms
Verma, S., Sharma, D., & Larson, R. W. (2002). School stress in India: Effects on time and daily emotions. International Journal of Behavioral Development, 26(6), 500-508.
Von Der Embse, N., Barterian, J., & Segool, N. (2013). Test anxiety interventions for children and adolescents: A systematic review of treatment studies from 2000–2010. Psychology in the Schools, 50(1), 57-71.
Wei, D. (2008). Perceptions of family processes and the effects on middle school students' achievement. ProQuest.
Zeidner, M. (1998). Test anxiety: The state of the art. Springer Science & Business Media.

Biotechnological Communication

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp-296-306

Isolation, Characterization and Evaluation of Probiotic Potential of Lactic Acid Bacteria Isolated from Human Colostrum

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ABSTRACT

The first thick milk produced immediately after the delivery is called human colostrum (HC). Its composition and functions are quite different than mature milk. It contains high levels of proteins, vitamins, immunoglobulins, carbohydrates, amino acids and many other nutrients. Apart from its nutritional aspects, HC also contains large number of Lactic Acid Bacteria (LAB) with huge probiotic potential. These LAB helps in nourishment, proper growth and development of infants in the early stages of life. The main objective of the study was to characterize and evaluate the probiotic potential of LAB from HC. The study showed several LAB with probiotic potential. The isolated LAB fulfilled all the necessary criteria of a standard probiotics such as growth at low pH, different temperatures, tolerance against bile salts, resistance against antibiotics and antimicrobial activities against common human pathogens. Four isolates of the study were found to be very promising in showing resistance against antibiotics and antimicrobial response against common pathogens such as *Escheria coli* ATCC 25922, Proteus vulgaris ATCC 33420, *Staphylococcus aureus* ATCC 25922, *Salmonella typhi* ATCC 733 and *Pseudomonas aeruginosa* ATCC 27853. On the basis of biochemical characterization, the isolates were identified as *Lactobacillus brevis*, *L. acetotolerans, L. casei* and *Pediococcus acidilactici*. The present paper deals with the isolation, characterization and evaluation of probiotic potentials of LAB isolated from HC.

KEY WORDS: ANTAGONISTIC ACTIVITY, HUMAN COLOSTRUM, INFANT GUT, LACTIC ACID BACTERIA, PROBIOTICS.

ARTICLE INFORMATION

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Received 10th Feb 2020 Accepted after revision 20th March 2020

Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)

A Society of Science and Nature Publication, Bhopal India

Online Contents Available at: http//www.bbrc.in/

DOI: 10.21786/bbrc/13.1/48

INTRODUCTION

For many years, HC was considered to be a sterile fluid, but recent studies have revised this dogma (Fernandez et al., 2013). The period of flow of HC is from 1st to 6th day of lactation. The milk produced after the 6th day is mature milk (Castellote et al., 2011). HC is a thick fluid rich in nutrients and contains vitamins, proteins, amino acids, carbohydrates and lipids along with several immune cells which provide immunity to infants in early stages of growth and development (Ballard et al., 2013). Recent studies reveals that apart from all the nutritional aspects of HC, it also contains large number of probiotic bacteria which helps in digestion and protection against infections (Marchesi et al., 2016). The study on milk of Rheses monkey (*Mucaca mulatta*) first showed that milk contains 19 different species of bacteria belonging to 8 genera in its constituents (Jin et al., 2011). HC also contains large number of other bacteria (Hunt et al., 2011). These bacteria also play a very important role in the development of immune system of infant (Wang et al., 2018). The number of bacteria in HC are about thrice more than mature milk. The number of bacteria in mature milk lower downs with the continuous regular flow of milk (McGuire et al., 2015). From the studies carried out in the past, majority of the bacteria isolated from human milk were generally Lactic Acid Bacteria (LAB) (Jost et al., 2015). LAB is a large group of bacteria used worldwide as a probiotic.

This group of bacteria involves the microorganisms of genera *Lactobacillus, Lactococcus, Aerococcus, Enterococcus, Pediococcus, Leuconostoc, Streptococcus, Sporolactobacillus, Vagococcus* and *Carnobacterium* (Pavli et al., 2018 & Neha 2019). The bacteria of these genera have high probiotic potential and have been proven safe for human consumption (Guesh et al., 2019). The first bacteria that enters the infant gut is from HC. These bacteria enters infant gut through HC and remains in the gut for entire life (Pang et al., 2007). The gut microflora get involves in various biochemical processes and serves several functions in the welfare of human gut (Dunlop et al., 2015). LAB have innumerable health benefits such as blood pressure lowering (Robles-Vera et al., 2017), prevention of colon cancer (Rafter 2003), reduction of allergic symptoms (Cuello-Garcia et al., 2017), reduction of cholesterol (Agerholm-Larsen et al., 2002), boosting of immune system (King et al., 2014), prevention of urinogenital infections (Shortliffe et al., 2013), reduction of *Helicobacter pylori* infections (Hamilton 2003), Intestinal Inflammation (Jin-Sil et al., 2018), antimicrobial effects on pathogens (Tankoano et al., 2019) and many more. Therefore, it can also be said that LAB are boon to infant gut. The present study deals with isolation, characterization and evaluation of probiotic potential of HC.

MATERIAL AND METHODS

Sample Collection: Total 60 different HC samples were collected from lactating mothers who voluntarily gave their consent for our study. All the samples were collected immediately after the delivery from the maternity ward of Jaipur National University Hospital, Jaipur (India). The tubes used for sample collection were autoclaved using standard protocols. The nipples of lactating mothers were cleaned properly with cotton dipped in alcohol to avoid any contaminations of breast skin microflora. The mid flow of HC was carefully aseptically collected in the tubes with the help of experts.

Isolation of LAB: The isolation of LAB from HC was quickly processed after completion of sample collection. The HC samples were serially diluted upto 10-6 using sterile peptone water. The last three dilutions were inoculated on MRS agar plates using Spread Plate Technique. The inoculated plates were incubated at 3 for 48 h under anaerobic conditions using anaerobic gas jar.

Enumeration of LAB: After incubation, the bacterial colonies were counted using digital colony counter and Colony Forming Unit (CFU) per mL of HC were calculated using standard method.

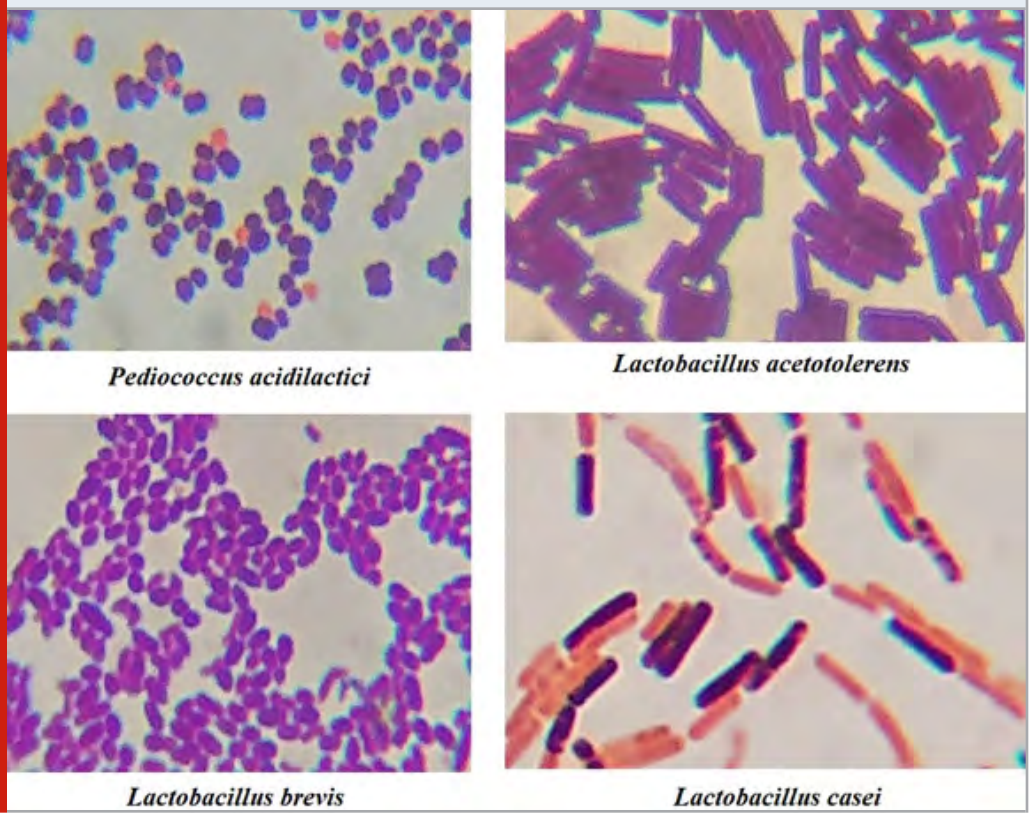
Biochemical Characterization of Isolates: Isolated bacteria were sub-cultured to get pure form of colony. The colony characteristics of each isolate were recorded carefully. The pure colonies were further chosen for biochemical characterization. Gram's staining, Catalase test, Oxidase Test, Arginine Hydrolysis Test and Sugar Fermentation tests were performed to characterize the isolates to be LAB as per recommendation of Bergey's Manual of Determination Bacteriology.

Gram's Staining: A single drop of sterile water was dropped on a clean glass slide and a pure colony of isolate was picked from the plate and was mixed gently to prepare a smear. The smear was heat fixed carefully. The standard procedure of Gram's staining was followed and the slide was observed under oil emulsion lens (10x X 100x) of compound microscope. As LAB are Gram positive in nature, all the isolates which showed Gram positive nature were further processed for other biochemical tests.

Catalase Test: Catalase test was performed for all the isolates which were Gram's positive. Catalase is a type of enzyme which is produced by several microorganisms that breaks down hydrogen peroxide into water and oxygen and forms bubbles of gas. The 3% hydrogen peroxide solution was mixed gently on the surface of clean glass slide and was observed for bubble formation. As LAB are catalase negative, all the isolates that showed negative results of catalase test were further tested for oxidase test.

Oxidase Test: All the isolates which showed catalase activity negative were further tested for oxidase test. Cytochrome c oxidase is an enzyme found in several bacterial electron transport chain. Presence of cytochrome c oxidase oxidizes the reagent called tetramethyl-phenylenediamine into indophenols (purple color) end product. As LAB are oxidase negative, all the

Figure 1. Microscopic observation of Gram's Staining



isolates which showed negative results were further tested for its arginine hydrolysis.

Arginine Hydrolysis Test: Nessler's reagent and arginine MRS medium were used to check the production of ammonia from arginine. 5 mL of MRS broth was transferred to empty test tube and 100 µl of test culture (O. D 1.0 at 600 nm) was inoculated and the tubes were incubated at 37±1°C for 24h. After incubation, an equal volume of Nessler's reagent was added to each tube. The immediate appearance of dark orange color was interpreted as positive (presence of ammonia) while indication of yellowish color was interpreted as negative reaction (absence of ammonia) (Kavitha and Devasena 2013).

Sugar Fermentation Test: Carbohydrate when fermented by microorganisms form an acid or acid with gas at the end. Depending on the microorganisms involved, the end products may vary. All the isolates which were Gram positive and catalase and oxidase negative were tested for their sugar fermentation activity. Sugars were prepared using standard protocol (HiMedia) and each tube of sugar contained Durham's tube in inverted position. Each isolate was inoculated in all different sugars (Glucose, Lactose, Maltose, Fructose, Mannitol, Galactose and Sucrose) to note down the breakdown of sugars into acid and/or acid + gas. Incubation for 48 h at 37 were given to all the sugars. Results were recorded after completion of incubation period. On the basis of Sugar fermentation activity, the isolates were identified using Bergey's Manual of Systematic Bacteriology (Hammes P et al., 2009). Determination of Probiotic Potential.After biochemical characterization, all the isolates were tested for their probiotic potential by testing their growth at low pH, different temperatures, tolerance against bile salts, resistance against common human pathogens and resistance to antibiotics.

Growth at low pH: The pH of human stomach ranges between 2 to 3. It is also believed that food eaten by us stays in stomach for at least 4 h (Bistha N et al., 2019). Therefore, it is the necessary for the isolate to survive at low pH for more than 4 h. To check the growth of isolates at low pH, all the isolates were inoculated in peptone water prepared with different pH (6, 5, 4, 3, 2) for a period of 6 h. After incubation period, the isolates were inoculated on MRS agar plates and were incubated under anaerobic conditions to check their survival at different pH. All the isolates were further checked for their tolerance against bile salts.

Tolerance against Bile Salts: The concentration of bile salts in the intestine is believed to be 0.3% (w/v) and the food eaten stays in small intestine is suggested to be 4 h (Kumari A et al., 2019). Therefore, all the isolates were examined for their growth at different bile salts concentrations. Peptone water with different bile salts concentration was prepared using Oxoid and active cultures of isolates were inoculated in the medium for 6 h. After incubation, the isolates were inoculated on MRS agar plates for its viable count.

Table 1. Fermentation of different sugars for identification of isolates as per the recommendations of Bergey's Manual

Sample No.	Isolate No.	Glucose A	Glucose G	Maltose A	Maltose G	Lactose A	Lactose G	Mannitol A	Mannitol G	Galactose A	Galactose G	Fructose A	Fructose G	Sucrose A	Sucrose G	Identification Based on Bergey's Manual
HC1	I1001	+	+	+	+	+	+	-	-	+	+	+	-	+	-	*Lactobacillus acidophilus*
	I1002	+	+	+	+	+	-	-	-	+	-	+	-	+	-	*Lactobacillus sakei*
HC2	I2001	+	-	+	+	+	-	+	-	+	-	+	-	+`	-	*Lactobacillus paracasei*
	I2002	+	+	+	-	+	-	-	-	+	-	+	+	+	+	*Lactobacillus fermentum*
	I2003	+	+	+	+	+	-	+	-	+	-	+	-	+	-	*Streptococcus oralis*
HC3	I3001	+	+	+	+	+	+	-	-	+	+	+	-	+	-	*Lactobacillus gasseri*
	I3002	+	+	+	-	+	-	+	-	+	-	+	-	-	-	*Lactobacillus agilis*
HC4	I4001	+	+	+	-	+	-	+	-	+	-	+	-	+	-	*Bifidobacterium longum*
	I4002	+	-	+	+	+	-	-	-	+	-	+	-	+`	-	*Lactobacillus rhamnosus*
HC5	I5001	+	+	+	+	+	+	+	-	+	+	+	-	+	-	*Bifidobacterium breve*
	I5002	+	+	+	+	+	-	+	-	+	+	+	+	-	-	*Pediococcus demnosus*
HC6	I6001	+	+	+	+	-	-	+	-	+	-	+	+	+	-	*Lactobacillus oris*
	I6002	+	+	+	-	+	-	+	-	-	-	-	-	+	-	*Lactobacillus curtus*
HC7	I7001	+	+	+	-	+	+	+	-	+	+	+	+	+	-	*Bifidobacterium magnum*
	I7002	+	+	+	+	+	-	-	-	+	+	+	+	+	-	*Lactococcus garvieae*
HC8	I8001	+	+	+	+	-	-	+	+	+	+	+	-	-	-	*Bifidobacterium dentium*
	I8002	+	+	+	+	+	+	-	-	+	+	-	-	+	-	*Lactobacillus johnsonii*
HC9	I9001	+	+	+	+	+	+	+	-	-	-	+	-	+	+	*Lactobacillus gasseri*
	I9002	-	-	+	+	+	-	+	-	+	-	-	-	-	-	*Lactobacillus helviticus*
	I9003	+	-	+	+	+	-	+	-	+	-	+	-	+`	-	*Lactobacillus backii*
HC10	I0101	+	+	+	+	+	-	+	-	+	-	+	-	+	-	*Lactobacillus parakefiri*
	I0102	+	-	+	-	+	+	-	-	+	-	+	-	+	-	*Stretococcus bovis*
HC11	I1101	+	+	+	+	-	-	+	-	+	-	+	-	-	-	*Lactobacillus silage*
	I1102	+	+	+	+	+	-	+	-	+	-	-	-	+	-	*Lactobacillus rennini*
HC12	I1201	+	+	+	+	+	-	+	-	+	-	+	-	+	-	*Bifidobacterium bifidum*
	I1202	+	-	+	-	+	-	+	+	+	+	+	-	+	-	*Lactobacillus rapi*
HC13	I1301	+	+	+	+	+	+	+	+	+	+	+	+	+	-	*Pediococcus cellicola*
	I1302	+	+	+	+	+	-	-	-	-	-	+	-	-	-	*Lactobacillus ozensis*
HC14	I1401	+	+	-	-	-	-	+	-	+	-	+	-	+	+	*Lactobacillus helviticus*
	I1402	+	-	+	+	+	+	-	-	+	-	+	-	-	-	*Bifidobacterium hapal*
HC15	I1501	-	-	+	+	+	-	+	-	-	-	+	-	+	-	*Bifidobacterium merycicum*
	I1502	+	+	+	+	+	+	-	-	+	+	+	-	+	-	*Lactobacillus acidipiscis*
	I1503	+	+	+	+	+	-	+	-	+	-	+	-	+	-	*Lactococcus piscium*
HC16	I1601	+	+	+	+	+	+	+	+	+	-	+	+	+	-	*Lactobacillus acetotolerens*
	I1602	+	+	+	+	+	+	+	-	+	-	-	-		+	*Lactobacillus florum*
HC17	I1701	+	+	+	+	+	+	+	-	+	+	+	-	+	-	*Bifidobacterium breve*
	I1702	+	+	+	+	+	-	+	-	+	-	+	+	-	-	*Lactococcus plantarum*
HC18	I1801	+	+	+	+	+	-	+	-	+	-	+	-	+	-	*Bifidobacterium reuteri*
	I1802	+	+	+	-	+	+	+	-	+	-	+	+	-	-	*Bifidobacterium bifidum*
HC19	I1901	+	+	+	-	+	-	-	-	+	-	+	+	+	+	*Lactobacillus plantarum*
	I1902	+	+	+	+	+	+	+	-	+	+	+	-	+	-	*Lactobacillus acidophilus*
HC20	I0201	+	+	+	+	+	-	+	-	+	-	+	-	-	-	*Lactobacillus agalis*
	I0202	+	+	+	+	-	-	+	+	+	+	+	-	-	-	*Bifidobacterium dentium*
HC21	I2101	-	-	+	-	+	+	+	-	+	+	+	-	+	-	*Pediococcus inopinatus*
	I2102	+	+	+	-	+	+	+	+	-	-	+	-	+	-	*Lactobacillus casei*
	I2103	+	+	+	+	+	+	-	-	+	-	+	+	+	-	*Lactobacillus larvae*
HC22	I2201	+	-	+	+	-	-	+	-	+	-	-	-	+	-	*Lactobacillus nagelii*
	I2202	+	+	+	+	+	+	+	+	-	-	+	-	+	-	*Lactococcus formosensis*
HC23	I2301	+	+	+	-	+	-	+	-	-	-	-	-	+	-	*Pediococcus stilesii*
	I2302	-	-	+	+	+	-	+	-	+	-	-	-	-	-	*Lactobacillus helviticus*
HC24	I2401	+	+	+	+	+	-	-	-	+	+	+	+	+	-	*Lactobacillus brevis*

	I2402	+	-	+	+	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
HC25	I2501	+	+	+	+	+	+	-	-	+	+	-	-	+	-	+	+	-	-	+	-
	I2502	+	+	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
	I2503	-	-	+	+	+	-	+	-	+	-	-	-	-	-	-	-	+	-	+	-
HC26	I2601	+	+	-	-	+	+	+	-	+	+	+	+	+	-	+	-	+	-	+	-
	I2602	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
HC27	I2701	+	-	+	-	+	+	-	-	+	-	+	-	+	-	+	-	+	-	+	-
	I2702	+	+	+	+	-	-	+	-	+	-	+	-	+	-	-	-	+	-	+	-
HC28	I2801	+	+	+	+	+	-	+	-	+	-	-	-	+	-	+	-	+	-	+	-
	I2802	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
HC29	I2901	-	-	+	+	+	-	+	-	+	-	-	-	-	-	-	-	+	-	+	-
	I2902	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	-
HC30	I0301	+	+	+	+	+	-	-	-	-	-	+	-	-	-	-	-	+	-	+	-
	I0302	+	-	+	+	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
HC31	I3101	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
	I3102	-	-	+	+	+	-	+	-	-	-	+	-	+	-	+	-	+	-	+	-
	I3103	+	+	+	+	+	-	+	-	+	-	+	-	+	-	-	-	+	-	+	-
HC32	I3201	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
	I3202	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
HC33	I3301	+	+	+	-	+	+	+	-	+	-	+	+	-	-	-	-	+	-	+	-
	I3302	+	+	-	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
HC34	I3401	+	+	+	+	+	-	+	-	+	-	+	+	-	-	-	-	+	-	+	-
	I3402	+	+	+	-	-	-	+	-	+	-	+	+	+	-	+	-	+	-	+	-
HC35	I3501	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
	I3502	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
HC36	I3601	+	+	+	-	+	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+
	I3602	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
HC37	I3701	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
	I3702	+	+	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
HC38	I3801	+	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	+	-	+	-
	I3802	+	-	+	+	+	-	-	-	+	-	+	-	+	-	+	-	+	-	+	-
	I3803	+	+	+	+	+	-	+	-	+	-	+	+	-	-	-	-	+	-	+	-
HC39	I3901	+	+	+	+	+	+	+	-	+	-	-	-		+	+	+	+	+	+	+
	I3902	+	-	+	+	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
HC40	I0401	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
	I0402	+	+	+	+	+	+	-	-	+	+	+	-	+	-	+	-	+	-	+	-
HC41	I4101	+	+	+	-	+	+	+	+	-	-	+	-	+	-	+	-	+	-	+	-
	I4102	+	+	+	+	+	+	+	-	+	+	+	-	+	-	+	-	+	-	+	-
HC42	I4201	+	+	+	-	+	+	+	-	+	-	+	+	-	-	-	-	+	-	+	-
	I4202	+	-	+	+	-	-	+	-	+	-	-	-	+	-	+	-	+	-	+	-
HC43	I4301	+	+	+	+	+	-	+	-	+	-	-	-	+	-	+	-	+	-	+	-
	I4302	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
HC44	I4401	+	+	+	-	+	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+
	I4402	+	+	+	-	+	+	+	+	-	-	+	-	+	-	+	-	+	-	+	-
HC45	I4501	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	-	+	-
	I4502	+	+	+	+	+	+	+	-	+	+	+	-	+	-	+	-	+	-	+	-
	I4503	+	+	+	-	+	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+
HC46	I4601	+	+	+	+	+	-	+	-	+	-	+	+	-	-	-	-	+	-	+	-
	I4602	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
HC47	I4701	-	-	+	+	+	-	+	-	-	-	+	-	+	-	+	-	+	-	+	-
	I4702	+	+	+	+	+	-	+	-	+	+	+	+	-	-	-	-	+	-	+	-
HC48	I4801	+	+	+	+	-	-	+	-	+	-	+	+	-	-	-	-	+	-	+	-
	I4802	+	-	+	+	-	-	+	-	+	-	-	-	+	-	+	-	+	-	+	-
HC49	I4901	+	+	+	+	+	+	-	-	+	+	+	-	+	-	+	-	+	-	+	-
	I4902	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
HC50	I0501	+	+	+	+	+	-	-	-	+	+	+	+	+	-	+	-	+	-	+	-
	I0502	+	+	+	+	-	-	+	-	+	-	+	-	-	-	-	-	+	-	+	-

HC51	I5101	+	+	+	+	+	-	+	-	+	-	*Lactobacillus pasteurii*
	I5102	+	+	+	+	+	-	+	-	+	-	*Bifidobacterium bifidum*
HC52	I5201	+	+	+	+	-	-	+	-	+	-	-	-	*Lactobacillus perolens*
	I5202	+	+	+	-	+	+	+	-	+	+	-	-	*Aerococcus sanguinicola*
	I5203	+	+	+	-	+	-	-	-	+	-	+	+	*Lactobacillus plantarum*
HC53	I5301	+	+	+	+	+	+	+	-	+	+	+	-	*Lactobacillus gasseri*
	I5302	+	+	+	+	+	-	+	-	+	-	-	-	*Lactobacillus vini*
HC54	I5401	+	+	+	+	+	+	-	-	+	-	+	+	+	-	*Lactobacillus larvae*
	I5402	+	-	+	+	+	-	+	-	+	-	+`	-	*Lactobacillus paracasei*
HC55	I5501	-	-	+	+	+	-	+	-	-	-	+	-	+	-	*Lactococcus hircilactis*
	I5502	+	+	+	+	+	+	+	-	-	-	+	-	+	+	*Lactobacillus gasseri*
HC56	I5601	+	-	+	+	-	-	+	-	+	-	-	-	+	-	*Lactobacillus acetotoleren*
	I5602	+	+	+	+	+	+	+	+	+	+	+	+	+	-	*Lactobacillus mobilis*
HC57	I5701	+	+	+	+	+	-	+	-	+	-	+	-	-	-	*Lactobacillus agalis*
	I5702	+	+	+	+	+	-	+	-	+	-	+	-	+	-	*Lactococcus lactis*
	I5703	+	+	+	-	+	+	+	-	+	-	+	+	-	-	*Bifidobacterium minimum*
HC58	I5801	+	+	+	+	-	-	+	-	+	-	+	+	+	-	*Lactobacillus oris*
	I5802	+	+	+	+	+	-	+	-	+	-	+	-	+	-	*Lactobacillus casei*
HC59	I5901	+	+	+	-	+	-	+	-	-	-	-	-	+	-	*Pediococcus stilesii*
	I5902	+	+	+	-	+	-	+	-	+	-	+	-	+	-	*Bifidobacterium longum*
HC60	I0601	+	+	+	+	+	+	+	+	+	+	+	+	+	-	*Lactobacillus mobilis*
	I0602	+	+	+	+	+	-	-	-	+	+	+	+	+	-	*Lactococcus garvieae*

The isolates which showed good growth on plates were further tested for their growth at different temperatures.

Growth at Different Temperatures: To examine the growth of isolates at different temperature, the active cultures of isolates were inoculated on MRS agar plates and were incubated at different temperatures (25, 30, 35, 40) in anaerobic conditions. The isolates which showed best growth at both high as well as low temperatures were further screened for their resistance against antibiotics. Resistance to Antibiotics:The isolates which gave best growth at high as well as low temperature were tested for their resistance against common antibiotics using Kirby Bauer method. The isolates were spreaded on the entire surface of MH agar plates and the discs of antibiotics with different concentrations were placed on the surface of agar and gently pressed. The plates were allowed to incubate at room temperature for 24-48 h. The isolates which did not give appropriate zone of inhibition around the discs of antibiotics according to standard chart were further examined for their antimicrobial activities against common human pathogens.

Antimicrobial activity: All the isolates which fulfilled the above mentioned criteria were further tested for their antimicrobial activities against common human pathogens using agar well diffusion method. The indicator pathogenic microorganisms were spreaded on the entire surface of Muller Hilton (MH) agar plates and using a sterile core borer of 7 mm diameter. 5 different wells of same size were made by puncturing the MH agar plates. Using micropipettes, 80 µL of overnight grown culture of isolate were inoculated carefully in the wells. The plates were incubated for 24 h in upright position. Thereafter, the zone of inhibition were measured. The isolates which showed greater zones of inhibition were considered having good probiotic potential.

RESULTS AND DISCUSSION

A total of 130 LAB were isolated from the HC of 60 different lactating mothers. The isolates were identified on the basis of physiological and biochemical characteristics. On the basis of Bergey's Manual of Systematic Bacteriology, 72 different species of LAB were identified. Of these, 4 isolates of LAB were found to be very promising with the potential of probiotics. These isolates were selected on the basis of their antimicrobial activities and their resistance against antibiotics. The average number of LAB count per ml of HC of a health lactating mothers were found to be 108 to 109. The LAB count was measured on the basis Standard Plate Count (Total Viable Count). The isolates were initially confirmed by using biochemical test such as Catalase, Oxidase, Grams Staining, Arginine Hydrolysis test and Sugar Fermentation test. All the isolates in the present study were found Gram's positive, Catalase and Oxidase negative and also had the capacity to breakdown sugars into acids and gas [Table 1]. On the basis of their Sugar Fermentation activity and Gram's morphology [Figure 1], the isolates were identified using Bergey's Manual of Systematic Bacteriology (Hammes et al., 2009).

Determination of LAB to be potentially probiotic: All the isolates identified as LAB through biochemical tests were further screened for determining their probiotic potential. Firstly, the growth of isolates were checked at low pH. Out of 130 isolates, 79 showed its positive growth at pH 2 which were further screened for their tolerance against different bile salts concentrations.

Table 2. Determination of probiotic potential based on growth at low pH, bile salt tolerance and growth at variable temperatures

Sample No.	Isolate No.	Growth at different pH				Bile Salt Tolerance (%)					Growth at different Temperatures ([])			
		pH6	pH5	pH4	pH3	pH2	0.2	0.3	0.4	0.5	25	30	35	40
HC1	I1001	+	+	+	-	-	+	+	+	-	+	+	+	+
	I1002	+	+	+	+	+	+	+	+	+	-	+	+	+
HC2	I2001	+	+	+	+	+	+	-	-	-	+	+	+	+
	I2002	+	+	+	+	+	+	+	+	-	-	-	+	+
	I2003	+	+	+	-	-	+	+	+	+	+	+	+	-
HC3	I3001	+	+	+	+	+	-	-	-	-	+	+	+	+
	I3002	+	+	-	-	-	+	+	+	+	-	+	+	+
HC4	I4001	+	+	+	+	+	+	-	-	-	+	+	+	+
	I4002	+	+	+	+	-	+	+	+	+	+	+	+	-
HC5	I5001	+	+	+	+	-	+	+	+	+	+	+	+	+
	I5002	+	+	+	+	+	-	-	-	-	-	-	+	+
HC6	I6001	+	+	-	-	-	+	+	+	+	+	+	+	-
	I6002	+	+	+	+	+	+	-	-	-	+	+	+	+
HC7	I7001	+	+	+	-	-	+	+	+	+	+	+	+	+
	I7002	+	+	+	+	+	+	+	+	+	+	+	+	+
HC8	I8001	+	+	+	+	+	+	-	-	-	+	+	+	+
	I8002	+	+	+	+	+	+	+	+	+	-	+	+	+
HC9	I9001	+	+	+	+	+	+	+	+	+	+	+	+	+
	I9002	+	+	+	-	-	+	+	+	+	+	+	+	-
	I9003	+	+	+	+	-	-	-	-	-	+	+	+	+
HC10	I0101	+	+	+	+	+	+	+	+	+	-	-	+	+
	I0102	+	+	+	+	+	+	+	+	+	+	+	+	+
HC11	I1101	+	+	+	+	-	+	+	+	+	+	+	+	+
	I1102	+	+	+	+	+	+	+	-	-	-	+	+	+
HC12	I1201	+	+	+	-	-	+	+	+	+	+	+	+	+
	I1202	+	+	+	+	+	-	-	-	-	+	+	+	+
HC13	I1301	+	+	+	+	+	+	+	+	+	+	+	+	+
	I1302	+	+	+	+	+	+	+	+	+	-	+	+	+
HC14	I1401	+	+	-	-	-	+	+	+	+	+	+	+	-
	I1402	+	+	+	+	-	-	-	-	-	+	+	+	+
HC15	I1501	+	+	+	+	+	+	+	+	+	+	+	+	+
	I1502	+	+	+	+	-	+	+	+	+	+	+	+	-
	I1503	+	+	+	+	+	+	+	+	+	-	-	+	+
HC16	I1601	+	+	+	+	+	+	+	+	+	+	+	+	+
	I1602	+	+	+	-	-	+	-	-	-	+	+	+	+
HC17	I1701	+	+	+	+	+	+	+	+	+	+	+	+	+
	I1702	+	+	+	+	+	+	+	+	+	-	+	+	+
HC18	I1801	+	+	-	-	-	+	+	+	+	+	+	+	+
	I1802	+	+	+	+	+	-	-	-	-	+	+	+	+
HC19	I1901	+	+	+	+	-	+	+	+	+	+	+	+	+
	I1902	+	+	+	+	+	+	+	+	+	-	+	+	+
HC20	I0201	+	+	+	+	+	+	-	-	-	+	+	+	+
	I0202	+	+	+	+	-	+	+	+	+	+	+	+	-
HC21	I2101	+	+	+	+	+	-	-	-	-	+	+	+	+
	I2102	+	+	+	-	-	+	+	+	+	+	+	+	+
	I2103	+	+	+	+	+	+	+	+	+	-	-	+	+
HC22	I2201	+	+	+	+	+	+	+	+	+	+	+	+	+
	I2202	+	+	+	+	+	+	+	-	-	+	+	+	+
HC23	I2301	+	+	+	-	-	+	+	+	+	+	+	+	+
	I2302	+	+	+	+	+	+	+	+	+	+	+	+	+

HC24	I2401	+	+	+	+	+	+	+	+	+	-	-	+	+
	I2402	+	+	+	+	+	-	-	-	-	+	+	+	+
HC25	I2501	+	+	+	+	+	+	+	+	+	+	+	+	+
	I2502	+	+	+	-	-	+	+	+	+	+	+	+	-
	I2503	+	+	+	+	+	+	-	-	-	+	+	+	+
HC26	I2601	+	+	+	+	-	+	+	+	+	+	+	+	+
	I2602	+	+	+	+	+	+	+	+	+	+	+	+	+
HC27	I2701	+	+	-	-	-	+	+	+	+	-	-	+	+
	I2702	+	+	+	+	+	+	+	+	+	+	+	+	+
HC28	I2801	+	+	+	+	+	+	+	+	+	+	+	+	+
	I2802	+	+	+	-	-	+	+	+	+	-	+	+	-
HC29	I2901	+	+	+	+	+	+	+	-	-	+	+	+	+
	I2902	+	+	+	+	-	+	+	+	+	+	+	+	+
HC30	I0301	+	+	-	-	-	+	+	+	+	+	+	+	+
	I0302	+	+	+	+	-	+	+	+	+	+	+	+	-
HC31	I3101	+	+	+	+	+	-	-	-	-	+	+	+	+
	I3102	+	+	+	+	+	+	+	-	-	+	+	+	+
	I3103	+	+	+	-	-	+	+	+	+	-	-	+	+
HC32	I3201	+	+	+	+	+	+	+	+	+	+	+	+	+
	I3202	+	+	+	+	+	+	-	-	-	+	+	+	+
HC33	I3301	+	+	+	+	+	+	+	+	+	+	+	+	+
	I3302	+	+	+	-	-	+	+	+	+	+	+	+	-
HC34	I3401	+	+	+	+	+	-	-	-	-	+	+	+	+
	I3402	+	+	-	-	-	+	+	+	+	+	+	+	+
HC35	I3501	+	+	+	+	+	+	+	+	+	-	+	+	+
	I3502	+	+	+	+	+	+	+	+	+	+	+	+	+
HC36	I3601	+	+	+	+	+	+	+	+	+	+	+	+	+
	I3602	+	+	+	-	-	+	+	+	+	+	+	+	+
HC37	I3701	+	+	+	+	+	+	+	+	+	+	+	+	+
	I3702	+	+	+	+	+	+	-	-	-	+	+	+	+
HC38	I3801	+	+	+	+	+	+	+	+	+	-	-	+	+
	I3802	+	+	-	-	-	+	+	+	+	+	+	+	-
	I3803	+	+	+	+	-	-	-	-	-	+	+	+	+
HC39	I3901	+	+	+	+	+	+	+	+	+	+	+	+	+
	I3902	+	+	+	+	+	+	+	-	-	+	+	+	+
HC40	I0401	+	+	+	+	-	+	+	+	+	-	-	+	+
	I0402	+	+	+	+	-	+	+	+	+	+	+	+	+
HC41	I4101	+	+	+	+	+	+	+	+	+	+	+	+	+
	I4102	+	+	-	-	-	+	+	+	+	+	+	+	-
HC42	I4201	+	+	+	+	+	+	-	-	-	+	+	+	+
	I4202	+	+	+	+	+	+	+	+	+	+	+	+	+
HC43	I4301	+	+	+	+	+	+	+	+	+	+	+	+	+
	I4302	+	+	+	+	-	-	-	-	-	+	+	+	+
HC44	I4401	+	+	+	+	-	+	+	+	+	-	-	+	+
	I4402	+	+	+	+	+	+	+	-	-	+	+	+	+
HC45	I4501	+	+	+	+	+	-	-	-	-	+	+	+	+
	I4502	+	+	+	-	-	+	+	+	+	+	+	+	-
	I4503	+	+	+	+	+	+	+	+	+	+	+	+	+
HC46	I4601	+	+	+	+	+	+	+	+	+	+	+	+	+
	I4602	+	+	+	+	-	-	-	-	-	+	+	+	+
HC47	I4701	+	+	+	+	+	+	+	+	+	-	-	+	+
	I4702	+	+	-	-	-	+	+	+	+	+	+	+	-
HC48	I4801	+	+	+	+	+	+	+	+	+	+	+	+	+
	I4802	+	+	+	+	+	+	+	+	+	+	+	+	+
HC49	I4901	+	+	+	+	+	-	-	-	-	+	+	+	+
	I4902	+	+	+	+	+	+	-	-	-	+	+	+	+
HC50	I0501	+	+	-	-	-	+	+	+	+	+	+	+	-

	I0502	+	+	+	+	+	+	+	+	+	+	+	+	+
HC51	I5101	+	+	+	+	+	+	+	+	+	-	-	+	+
	I5102	+	+	+	+	-	-	-	-	-	-	+	+	-
HC52	I5201	+	+	+	+	+	+	+	+	+	+	+	+	+
	I5202	+	+	+	+	+	+	+	+	+	+	+	+	+
	I5203	+	+	+	+	+	+	+	-	-	+	+	+	+
HC53	I5301	+	+	+	+	+	+	+	+	+	-	+	+	+
	I5302	+	+	+	-	-	+	+	+	+	-	+	+	-
HC54	I5401	+	+	+	+	-	+	+	+	+	+	+	+	+
	I5402	+	+	+	+	+	+	+	+	+	+	+	+	+
HC55	I5501	+	+	+	+	-	-	-	-	-	+	+	+	+
	I5502	+	+	+	+	+	+	+	+	+	+	+	+	+
HC56	I5601	+	+	+	+	+	+	+	+	+	+	+	+	+
	I5602	+	+	+	+	+	+	+	+	+	-	+	+	+
HC57	I5701	+	+	+	+	+	+	+	+	+	-	-	+	+
	I5702	+	+	+	+	-	+	+	+	+	+	+	+	+

Table 3. Evaluation of Resistance of isolates against common antibiotics using disc diffusion method

Isolate No.	Names of Antibiotics													
	Erythromicin		Tetracycline		Pencillin		Gentamicin		Streptomicin		Amoxicillin		Ciprofloxacin	
Measurement on Zone of Inhibition in (mm) and its Resistance (R) or Sensitivity (S) against Antibiotics														
L. casei	13	R	9	R	10	R	11	R	9	R	12	R	13	R
L. brevis	12	R	10	R	12	R	12	R	8	R	9	R	14	R
P. acidilactici	9	R	11	R	8	R	9	R	10	R	10	R	10	R
L. acetotoleren	14	R	9	R	9	R	13	R	9	R	11	R	12	R

Table 4. Antimicrobial activity of isolated LAB against common pathogens

Isolate No.	Names of Pathogens				
	E.coli ATCC-25922	*P. vulgaris* ATCC-33420	*S. aureus* ATCC -25922	*S. typhi* ATCC-733	*P. aeruginosa* ATCC-27853
L. casei	18 mm	17 mm	18 mm	17 mm	18 mm
L. brevis	21 mm	14 mm	16 mm	19 mm	20 mm
P. acidilactici	19 mm	18 mm	18 mm	20 mm	18 mm
L. acetotoleren	20 mm	16 mm	15 mm	16 mm	16 mm

Out of 130 total isolates, 96 were found to be prominent against tolerating the 0.3% (w/v) bile salts concentrations. These isolates were further examined for their growth at different temperatures. Out of 130 isolates, 77 showed a good growth at 40 and even at 25 . On basis of these three criteria's, 34 best isolates were selected for checking their resistance against antibiotics from which 16 best isolates were screened for testing their antimicrobial activity against common human pathogens. Out of 16 isolates, only 4 showed very high degree of zone of inhibition against pathogenic bacteria. The details of these for isolates are mentioned below in Table I, II, III and IV.

4 best species of LAB were screened out of 130 isolates. Identification of LAB was made on the basis of colony morphology, physiological and biochemical tests as per the guidelines mentioned in Bergey's Manual of Systematic Bacteriology (Hammes et al., 2009). Similar tests performed by earlier researchers found 8 species of LAB that were Gram positive, catalase and oxidase negative and also showed active hydrolysis of arginine (Kang et al., 2019). The acidic pH of stomach and antimicrobial actions of pepsin provide an effective barrier for LAB to survive in gastrointestinal tract (Kang et al, 2019). For exerting beneficial effects on host, probiotic should be able to maintain its viability along the gastrointestinal transit by surviving under harsh conditions (Tongwa et al., 2019). The survival rate of the isolates of our study were found to be best even at the pH of 2. Traditional techniques of microbiology were used in the study rather than modern molecular techniques because it is more reliable. Modern techniques have some limitations such as the viability of milk microbes cannot be analyzed, total

bacteria counts may be over- or underestimated because of cell-wall composition, DNA extraction methods and the number of microbial 16S gene copies which may lead to the over- or underestimation of bacteria counts. Contamination in DNA extraction kit and reagents was also reported in the past studies (Mc Guire, 2015).

CONCLUSION

Human Colostrum contains of large number of bacteria with probiotic potential which greatly helps the infant in boosting up its immunity and in maintaining the gut microbiome. The number of LAB below this count can be a cause of worry for infant. We also found that LAB have the great potentials of fighting against common human pathogens. In our study, we have found that some LAB have great efficiency to resist against antibiotics. Such species of LAB should be commercialized and marketed at a global stage so that problems related to imbalance in gut microbiome can be solved. Through our studies, we also came to know that unnecessary consumption of antibiotics during the time of pregnancy may reduce the LAB count in HC. Therefore, use of antibiotics used be minimized. There are several other facts which are still not known till date such as existence of LAB in HC is still a mystery. LAB in HC is a wide area of research and still needs lots of genuine studies to be carried out to solve the unknown.

ACKNOWLEDGMENTS

Our team is greatly thankful to the entire staff members of Institute for Medical Sciences and Research Center, Jaipur National University (JNU) for directly and indirectly contributing to our studies. A special thanks to the members of Institutional Ethics Committee of JNU for approving our work. We are also thankful to the nursing staff of JNU Hospital especially Mrs. Mamta for helping us in sample collection throughout our entire study.

REFERENCES

Agerholm-Larsen L, Bell ML, Grunwald GK, Astrup A (2002). The effect of a probiotic milk product on plasma cholesterol: a meta-analysis of short term intervention studies, European Journal of Clinical Nutrition. Vol 54 (11), Pages: 856–860.

Ballard O and Morrow AL (2013). Human milk composition: nutrients and bioactive factors. Pediatr. Clin. North Am. Vol 60, Pages: 49–74.

Bishtha, Neha and A.P. Garg (2019). Diversity of Probiotic Bacteria in milk and milk products. In: National Seminar Proceedings on Biodiversity. (In press)

Castellote C, Casillas R, Ramírez-Santana C, Pérez-Cano J, et al., (2011). Premature delivery influences the immunological composition of colostrum and transitional and mature human milk. Journal of Nutrition. Vol 141, Pages: 1181–1187.

Cuello-Garcia C, Fiocchi A, Pawankar R, Yepes-Nuñez J, Morgano GP, et al., (2017). Prebiotics for the prevention of allergies: A systematic review and meta-analysis of randomized controlled trials. Clin. Exp. Allergy (Systematic review). Vol 47 (11), Pages: 1468–1477.

Dunlop AL, Mulle JG, Ferranti EP, Edwards S, Dunn AB, Corwin EJ (2015). Maternal microbiome and pregnancy outcomes that impact infant health: A Review. Adv Neonatal Care. Vol 15(6), Pages: 377-385.

Fern_andez L, Langa S, Martín V, Maldonado A, Jimenez E, Martín R, et al. (2013). The human milk microbiota: origin and potential roles in health and disease. Pharmacol Res. Vol 69 (1), Pages: 1-10.

Guesh Mulaw ,1,2 Tesfaye Sisay Tessema,3 Diriba Muleta ,3 and Anteneh Tesfaye3 (2019). In Vitro Evaluation of Probiotic Properties of Lactic Acid Bacteria Isolated from Some Traditionally Fermented Ethiopian Food Products. Int. J. Micro.(Hindawi). Vol 2019, Pages: 1-11.

Hammes P H and Hertel C, In: Vos P.D., Garrity, G.M., Jones, D, et al. (2009). Bergey's Manual of Systematic Bacteriology, Springer, New York, Edi 3rd , Pages: 456-479.

Hamilton-Miller JM (2003). The role of probiotics in the treatment and prevention of Helicobacter pylori infection. International Journal of Antimicrobial Agents. Vol 22 (4), Pages: 360–366.

Hunt KM, Foster JA, Forney LJ, Schütte UM, Beck DL, Abdo Z, et al., (2011). Characterization of the diversity and temporal stability of bacterial communities in human milk. PLoS One. Vol 6, Pages: 213-233.

Jin-Sil P, Jeong W, JooYeon J, et a. (2019). Lactobacillus acidophilus Improves Intestinal Inflammation in an Acute Colitis Mouse Model by Regulation of Th17 and Treg Cell Balance and Fibrosis Development. J. Medicinal Food. Vol 21(3), Pages: 1-11.

Jin L, Hinde K, Tao L (2011). Species diversity and relative abundance of lactic acid bacteria in the milk of rhesus monkeys (Macaca mulatta). J Med Primatol. Vol 40(1), Pages: 52-58.

Jost T, Lacroix C, Braegger C, Chassard C (2015). Impact of human milk bacteria and oligosaccharides on neonatal gut microbiota establishment and gut health. Nutr Rev. Vol 73(7), Pages: 426-437.

Kavitha R J and Devasena T (2013). Isolation, Characterization, Determination of probiotic properties of Lactic Acid Bacteria from Human Milk. ISOR J. Phar. and Biolo. Sci. Vol 7(3). Pages: 01-07.

King S, Glanville J, Sanders ME, Fitzgerald A, Varley D (2014). Effectiveness of probiotics on the duration of illness in healthy children and adults who develop common acute respiratory infectious conditions: a systematic review and meta-analysis ". Br. J. Nutr. Vol 112 (1), Pages: 41–54.

Kumari A, Garg PA, Makeen K and Lal M (2008). A bacteriocin production on soya nutri nuggets extract medium by Lactococcus lactis CCSUB202. Int. J. Dairy. Sci. Vol 3(1), Pages: 49-54.

Marchesi JR, Adams DH, Fava F, Hermes GD, Hirschfield GM, Hold G, et al. (2016). The gut microbiota and host health: a Review. Clinical frontier. Gut. Vol 65(2), Pages:

3309-3319.
McGuire MK and McGuire MA (2015). Human milk: mother nature's prototypical probiotic food? Adv Nutr. Vol 6(1), Pages: 112-123.
Neha B and Garg AP (2019). Antagonistic Activity of Lactic Acid Bacteria Against Common Enteric Pathogens Isolated from Milk and Milk Products and Evaluation of their Probiotic Attributes. Bioscience Biotechnology Research Communications. Vol 12 No. (4) 1173-1184
Pang W and Hartmann P (2007). Initiation of human lactation: secretory differentiation and secretory activation. J. Mammary Gland Biol. Neoplasia. Vol 12, Pages: 211–221.
Pavli F, Tassou C, Nychas E J G and Chorianopoulos N (2018). Probiotic Incorporation in Edible Films and Coatings: Bioactive Solution for Functional Foods. Int J Mol Sci. Vol 19, Pages: 150-168.
Rafter, J (2003). Probiotics and colon cancer. Best Practice and Research Clinical Gastroenterology. Vol 17(5), Pages: 849-859.
Robles-Vera I, Toral M, Romero M, Jiménez R, Sánchez M, Pérez-Vizcaíno F, Duarte J (2017). Antihypertensive Effects of Probiotics. Curr. Hypertens. Rep. (Review). Vol 19 (4), Pages: 26-37.
Shortliffe L and Wein AJ (2013). Infection and Inflammation of the Pediatric Genitourinary Tract. Urology. Saunders Elsevier. Vol 4 (10), Pages: 3121-3143.
Tankoano A, Diop B M, Lingani S H, Mbengue M, Kaboré D, Traoré Y and Savadogo A (2019). Isolation and Characterization of Lactic Acid Bacteria Producing Bacteriocin like Inhibitory Substance (BLIS) from "Gappal", a Dairy Product from Burkina Faso. Adva. Microbi. Vol 9, Pages: 343-358.
Wang H, Wei XC, Min L and Zhu L (2018). Good and bad: gut bacteria in human health and disease. Reviews. Medi. Biotech. Vol 32 (5), Pages: 1075-1080.

Biotechnological Communication

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp-307-312

Synthesis and Characterization of Biogenic Gold Nanoparticles Using *Aegle marmelos* Extracts: Antibacterial Assay

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ABSTRACT

In the present paper we discuss about the biogenic synthesis of gold nanoparticles using *Aegle marmelos* extract. The gold nanoparticles were synthesized via eco-friendly and low cost effective method. Preparation of the aqueous leaf extracts of *Aegle marmelos* was carried out deionized water and the extracts acted as reducing agent as well as capping agent. The synthesized gold nanoparticles were characterized by UV-Visible spectroscopy, Fourier transform infrared spectroscopy (FTIR), X-ray Diffraction (XRD), Field emission gun Scanning electron microscopy (FEG-SEM) with EDS and High Resolution Transmission electron microscopy (HR-TEM). The elemental composition and purity of gold nanoparticles was analysed by EDS. XRD patterns showed average particle size of 22 nm and also UV absorption peak showed around 534 nm. The antibacterial activity of gold nanoparticles was studied against micro organisms like against *Bacillus subtilis* (MTCC 121) and Escherichia coli (MTCC 119).

KEY WORDS: GOLD NANOPARTICLES, AEGLE MARMELOS, BIOGENIC SYNTHESIS, ANTIMICROBIAL ACTIVITY.

INTRODUCTION

Nanostructures with various properties like physical, chemical and electrical empower broad applications such as an antibacterial, catalysis, optical, electrical, as an energy transformation as well as reservoir devices and production of biomedicals. In the past, research has shown that antioxidant compounds present in *Aegle marmelos* inactivate the free radicals or make them less reactive and thus protect against reactive oxygen species. Health-promoting compounds present in Bael include Terpenoids, Flavonoids, Steroids, Phenolic Compounds, Alkaloids, Tannins, and Saponins. Out of all these, tannins have a very strong free radical scavenging property and act as a primary antioxidant, (Iqbal et al.,2015 Ashar et al.,2016 Cao et al.,2016 Iqbal et al.,2017).

The Bael plant or *Aegle marmelos* grows in the soil with a pH range between 5 – 10. The *Aegle marmelos* tree tolerates all kinds of soil situation and stands water logging situation. This tree has a wide tolerance for temperature and grows well in the temperature range 7° C to 48°C. It grows best on rich, well-drained soil. it grows very well in summer and its growth declines during winter. The preliminary step in the synthesis of AuNPs is the reduction of gold ions (Au1+) to neutral gold atoms (Au0). It occurs by reduction of chloroauric acid (H [AuCl4]) in a solution in the presence of suitable reducing agent. The gold nanoparticles synthesized using plant extracts do not require reducing agent as

ARTICLE INFORMATION

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Received 14th Jan 2020 Accepted after revision 20th March 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/49

the compounds which readily exists in plants acts as reducing agents as well as stabilizing agents. The synthesis of gold nanoparticles using plant extracts has been tried and it has become successful. Biosynthesis of gold nanoparticles from plants like *Hibiscus rosa-sinensis* (Daizy et al., 2010), *Cinnamomum camphora, Azadirachta indica* (Shankar et al., 2004), *Geranium*, (Shankar et al., 2003), *lemon grass* (Grunwald et al., 2004), *Aloe vera* (Chandran et al., 2006), *Moringa pterygosperma* and *Boerhavia diffusa* (Vaghela et al., 2018), Crateva Religiosa (Parmar et al., 2016), and *Bauhinia variegate* (Vaghela et al. (2017,2018).

Recently, synthesized AgNPs using fresh fruit extract of *Phyllanthus emblica* have been and evaluate for their antibacterial efficacy against pathogen *Acidovorax oryzae* strain RS-2 of rice bacterial brown stripe (Masum et al., 2019). Balasubramanian et al., (2020) have reported biogenic synthesis of gold nanoparticles using *Jasminum auriculatum* leaf extract and have reported their catalytic, antimicrobial and anticancer activities. Vo et al. (2020) have reported biosynthesized silver and gold nanoparticles from *Lactuca indica* leaf extract and their applications in catalytic degradation of toxic compounds. Uzma et al. (2020) have reported biogenic synthesis of gold nanoparticles using Commiphora wightii and their cytotoxic effects on breast cancer cell line (MCF-7). Similarly, Aisida et al. (2020) have reported biogenic synthesis of iron oxide nanorods using *Moringa oleifera* leaf extract which showed good antibacterial activity. Siddiquee et al. (2020) have reported green synthesis of silver nanoparticles from *Delonix regia* leaf extract and its application in cytotoxicity interaction studies with bovine serum albumin.

In this study, we report the plant-mediated synthesis of gold nanoparticles using the aqueous leaf extracts of *Aegle marmelos* an evergreen shrub which is found in many parts of India. We prepared metallic gold nanoparticles via green biogenic synthesis. The reduction of aqueous Au+ ions with the thallus broth of marine algae, *P. pavonica* its characterization and tested its anti-microbial activity tested against microorganisms namely *Escherichia coli* and *Bacillus subtilis*. The gold nanoparticles (AuNPs) synthesized were characterized by UV-Vis spectroscopy, SEM, TEM, XRD, EDAX, and FTIR. The Antibacterial/Antimicrobial Activity effects of gold nanoparticles.

MATERIALS AND METHODS

All the chemical reagents used in this experiment were of analytical grade purchased from s.d. fine chemicals. The fresh and healthy leaves were collected around patan (N.G.) and washed with distilled water to remove dust particles.All the chemical reagents used in this experiment were of analytical grade purchased from s.d. fine chemicals. The fresh and healthy leaves were collected around patan (N.G.) and washed with distilled water to remove dust particles.After washing the leaves were spread evenly in a clean paper and the leaves were allowed to dry in shade for about 3-4 days. When the leaves were dried completely they were finely powdered using mixer and was used for extract preparation. First we cut all the leaf and then Initially 10 gm of leaf boiled with 100 ml double distilled water for 60 min. in heating on Soxhlet at temperature 600C. The mixture was brought to the room temperature and the aqueous leaf extract was collected using Whatman filter paper. The resulting product was filtered and stored in refrigerator for further use. All solutions were prepared using double distilled water. Initially 10 gm of leaf Aegle Marmelos were boiled with double distilled water and heated on Soxhlet for 60 min. than it was filtered using Whatman filter paper No.1 after filtration, 20 ml extract was added in 30 ml gold (AuNO3) which was put on magnetic stirrer with hot plate for around 8 hours. The resultant solution was dark black color which turned to dark brownish color within one hour. The solid products were obtained from it. Subsequently the solution was then centrifuged at 12000 rpm for 20 min. The supernated centrifugation was used as plant extract. Gold nanoparticles were synthesized by adding 20 ml of plant extract to 1x10-3M AuNo3 (ACS extra pure).

RESULTS AND DISCUSSION

After completion of reaction, reaction mass was observed for the colour changes from dark black to dark brownish in comparison to the control solution. The colour change is the visual method of detection of synthesis of gold nanoparticles. The gold nanoparticles were characterized by using UV-Vis spectroscopy (Shimadzu UV 1800 UV-Visible spectrophotometer) reduction of gold ions was monitored by measuring the UV-Vis range of the reaction mixture at 8 hour (Martínez et al., 2012). FTIR spectroscopy analysis was carried out to identify the biomolecules responsible for the reduction of Au+ ions (Elia ET AL., 2014). FTIR spectroscopy analysis was carried out to find the biomolecules that were bound specifically on the gold oxide nanoparticles surface. The morphology of the obtained nanoparticles was characterized by using a high-resolution transmission electron microscope (HR-TEM) (Krishnaswamy et al., 2014). Chemical composition of the obtained nanoparticles were analysed by EDAX technique using scanning electron microscope (FEG-SEM) (Hezard et al., 2012). The crystallographic structure of Gold nanoparticles and the phase properties was examined by XRD measurements using Rigaku D/max 40 kV diffractometer (Yan et al., 2005).

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 Gold oxide nanoparticles. The mono dispersed Gold oxide nanoparticles are shown in synthesis figure-1. The room temperature spectra exhibited strong excitonic absorption peaks at 534 nm for samples respectively, which is in good agreement with previous work (Islam et al., 2015).

Figure 1: Uv-visible spectra of Biogenic synthesized Gold nanoparticles

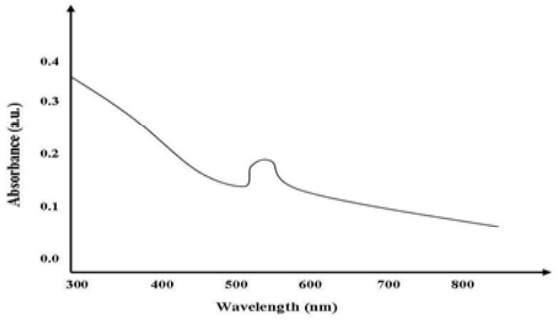


Figure 2: FTIR spectra of Biogenic synthesized Gold nanoparticles

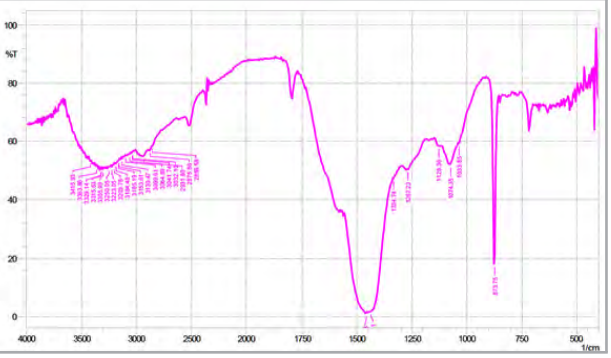


Table 1. XRD spectral data of gold nanoparticles

2θ	Particle size (nm)	(h k l)
38	19.94	(111)
44	16.34	(200)
64	20.68	(222)
77	34.24	(311)
Average Particle size D = 22 nm		

FTIR spectroscopy analysis was carried out to identify the biomolecules of extract responsible for the reduction of Au+ ions. FTIR spectroscopy analysis was carried out to find the biomolecules that were bound specifically on the Gold nanoparticles surface. Fig. 2 shows the FTIR spectra of Biogenic synthesized Gold nanoparticles. The spectrum showed sharp bands at 873 cm-1 corresponding to metal–oxygen (M–O). Strong bands were observed at 1033, 1074 and 1450 cm-1 and have been referred to as alcohols and phenolic groups, C–N stretching vibrations of aliphatic and aromatic amines, respectively. The bands obtained at 3000 cm-1 have been representing to stretching vibrations of primary alkanes, amines and water molecules.

X-ray diffraction (XRD) measurement of the Biogenic synthesis of Gold nanoparticles carried out on a Rigaku D/max 40 kV diffractometer equipped with the graphite mono chromator and Au target. Fig. 3 shows the XRD analysis of Biogenic synthesized Gold oxide nanoparticles. This is used for further confirmation of Gold oxide phase of nanoparticles. The observed intense peaks are 380, 440, 640 and 770respectively representing the (111), (200), (222) and (311)reflections indicating the face centered cubic (fcc) structure of AuNPs XRD pattern reveals the face centered cubic structure indicating the crystalline nature of AuNPs and the particle size calculated using Debye-Scherrer equation,

$$D = \frac{0.9\lambda}{\beta \cos\theta} \quad (1)$$

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 22 nm (Dubey et al., 2010).

Figure 3: XRD spectra of Biogenic synthesized Gold nanoparticles.

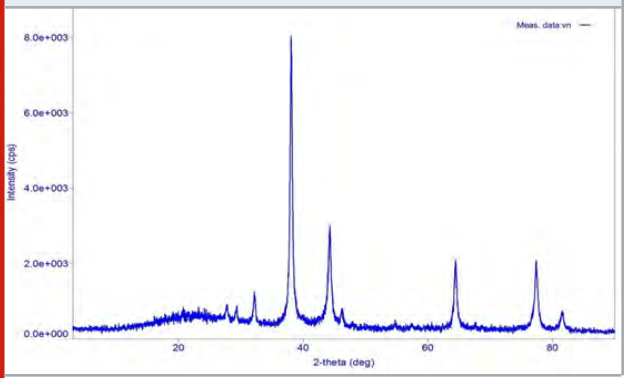
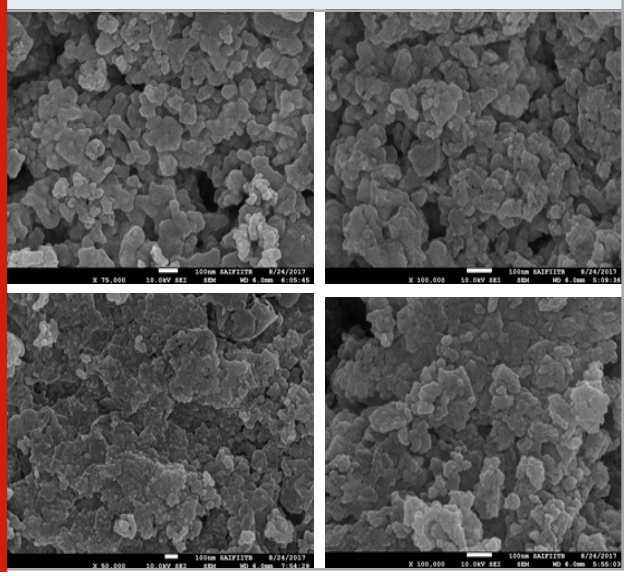


Figure 4: FEG-SEM spectra of Biogenic synthesis of Gold nanoparticles



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 Gold nanoparticles are shown in Fig.4. Gold nanoparticles by this method show nearly mono dispersed distribution of particle sizes. The average particle size of the Au nanoparticles is around 15-30 nm. The composition of Gold nanoparticles was further probed by energy-dispersive X-ray (EDS) analysis. Fig. 5 shows the EDS pattern of AuNPs prepared using Gold sulphate, which indicates the presence of Au and small amount of oxygen. EDS spectrum of Gold nanoparticles shows the peaks for Gold and respective elements indicating the formation of Gold nanoparticles. Peak indexing of the elements is oxygen 0.5 keV and Gold 1.5 & 2.2 keV. The compositions in the mass percentage of the elements are oxygen 45.12% and Gold 41.21 %. The experimental composition matches with the theoretically calculated composition.

High-resolution Transmission electron microscope (HR-TEM) images were recorded on a Tecnai G2-F30 electron microscope. Fig. 6 shows the HR-TEM images of Au NPs prepared using Mixtures. 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 Gold nanoparticles. The antibacterial screening test of the synthesized Gold nanoparticles was performed by both agar well diffusion method against Bacillus subtilis (MTCC 121) and Escherichia coli (MTCC 119). The antibacterial activity of Gold nanoparticles was carried out by agar cup plate method. The antibacterial activity of Gold nanoparticles was evaluated against *Bacillus subtilis* and *Escherichia coli*. Fresh overnight Culture of each strain swabbed uniformly by cotton on plates containing Mueller Hinton agar and 4 wells (diameter size- 6 mm) were prepared using cup borer. Different concentration (20, 40, 60 µL) samples of nanoparticles pour into each well and incubated it for 24 hr at 370C, after that around the well diameter of inhibition zone was observed in millimeter (Figure 7)(Table 2). Inhibition zone of bacterial growth is due to inhibitory compounds from the tested sample. We concluded that agar well diffusion method exhibited good antibacterial activity in which *Escherichia coli* showed excellent zone of inhibition and Bacillus subtilis showed weak results compare to *Escherichia coli* bacteria. Plant extract didn't give any results (Bhawasar et al., 1965, Behl and Srivastava, 2002, Shah and Qadry, 1971, Zafar, 1994). Experiments with each strain performed three times for good results.

Figure 5: EDS spectra of Biogenic synthesis of Gold nanoparticles

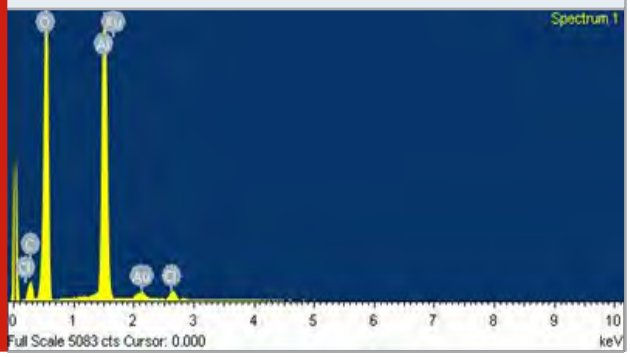


Figure 6: HR-SEM spectra of Gold nanoparticles

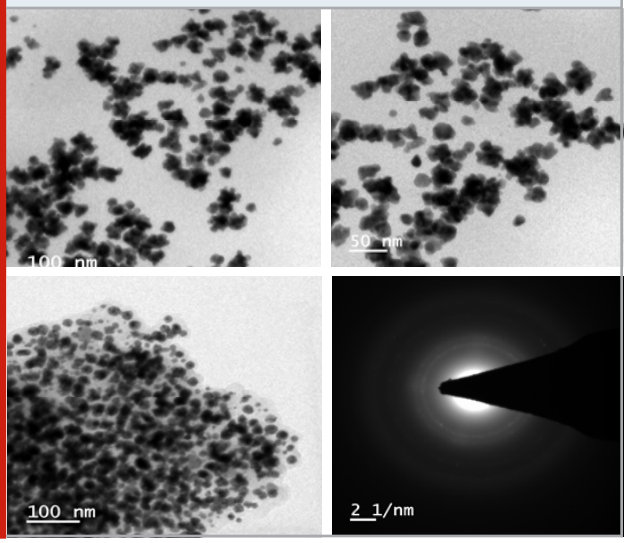
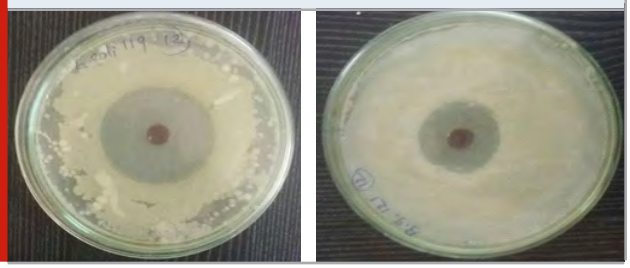


Table 2. Antimicrobial activity data for synthesized gold nanoparticles.

Bacteria Inhibition	Zone of Inhibition of Biosynthesized Gold Nanoparticles with different concentrations			Average zone of
	20 µL	40 µL	60 µL	
B. subtilis	4mm	8mm	13mm	8.3 mm
E. Coli	7mm	12mm	17mm	12mm

Figure 7: Antimicrobial activity of Au nanoparticles



In summary, the biogenic synthesis of gold nanoparticles was performed using *Aegle marmelos* extracts without involving any toxic chemicals. In this reduction reaction metal ions were reduced (Au+2 to Au+1) very rapidly and reaction was finally completed within 8 hours to produce Gold nanoparticles. Synthesized gold nanoparticles mediated from Aegle marmelos leaves exhibited excellent antibacterial activity against gram-

positive (*Bacitililus subs*) and gram negative (*Escherichia coli*) bacteria which exhibited considerable zone of inhibition. Increase in the dose of AuNPs resulted in the rise in antibacterial activity. So it can concluded that biologically synthesized gold nanoparticles using Aegle marmelos extracts can be used as environment-friendly low cost formulation with excellent antibacterial activity against Escherichia coli microorganisms.

REFERENCES

Ashar, A., Iqbal, M., Bhatti, I. A., Ahmad, M. Z., Qureshi, K., Nisar, J., & Bukhari, I. H. (2016). Synthesis, characterization and photocatalytic activity of ZnO flower and pseudo-sphere: Nonylphenol ethoxylate degradation under UV and solar irradiation. Journal of Alloys and Compounds, 678, 126-136.

Aisida, S. O., Madubuonu, N., Alnasir, M. H., Ahmad, I., Botha, S., Maaza, M., & Ezema, F. I. (2020). Biogenic synthesis of iron oxide nanorods using *Moringa oleifera* leaf extract for antibacterial applications. Applied Nanoscience, 10(1), 305-315.

Balasubramanian, S., Kala, S. M. J., & Pushparaj, T. L. (2020). Biogenic synthesis of gold nanoparticles using *Jasminum auriculatum* leaf extract and their catalytic, antimicrobial and anticancer activities. Journal of Drug Delivery Science and Technology, 101620.

Behl, P. N., & Srivastava, G. (2002). Herbs useful in dermatological therapy (Vol. 11). CBS Publishers & Distributors.

Bhawasar, G. C., Guru, L. V., & Chadda, A. K. (1965). Antibacterial activity of some indigenous medicinal plants. Medical-Surgical Nursing, 5, 11-14.

Cao, J., Sun, X., Zhang, X., & Lu, C. (2016). Homogeneous synthesis of Ag nanoparticles-doped water-soluble cellulose acetate for versatile applications. International journal of biological macromolecules, 92, 167-173.

Chandran, S. P., Chaudhary, M., Pasricha, R., Ahmad, A., & Sastry, M. (2006). Synthesis of gold nanotriangles and silver nanoparticles using *Aloe vera* plant extract. Biotechnology progress, 22(2), 577-58

Dubey, S. P., Lahtinen, M., & Sillanpää, M. (2010). Tansy fruit mediated greener synthesis of silver and gold nanoparticles. Process Biochemistry, 45(7), 1065-1071.

Elia, P., Zach, R., Hazan, S., Kolusheva, S., Porat, Z. E., & Zeiri, Y. (2014). Green synthesis of gold nanoparticles using plant extracts as reducing agents. International journal of nanomedicine, 9, 4007.

Grunwald, A. (2004). The case of nanobiotechnology. EMBO reports, 5(S1), S32-S36.

Hezard, T., Fajerwerg, K., Evrard, D., Collière, V., Behra, P., & Gros, P. (2012). Gold nanoparticles electrodeposited on glassy carbon using cyclic voltammetry: Application to Hg (II) trace analysis. Journal of Electroanalytical Chemistry, 664, 46-52.

Iqbal, M., & Bhatti, I. A. (2015). Gamma radiation/H2O2 treatment of a nonylphenol ethoxylates: degradation, cytotoxicity, and mutagenicity evaluation. Journal of hazardous materials, 299, 351-360.

Iqbal, M., Nisar, J., Adil, M., Abbas, M., Riaz, M., Tahir, M. A.& Shahid, M. (2017). Mutagenicity and cytotoxicity evaluation of photo-catalytically treated petroleum refinery wastewater using an array of bioassays. Chemosphere, 168, 590-598.

Islam, N. U., Jalil, K., Shahid, M., Rauf, A., Muhammad, N., Khan, A. & Khan, M. A. (2015). Green synthesis and biological activities of gold nanoparticles functionalized with *Salix alba*. Arabian Journal of Chemistry.

Krishnaswamy, K., Vali, H., & Orsat, V. (2014). Value-adding to grape waste: Green synthesis of gold nanoparticles. Journal of Food Engineering, 142, 210-220.

Martínez, J. C., Chequer, N. A., González, J. L., & Cordova, T. (2012). Alternative methodology for gold nanoparticles diameter characterization using PCA technique and UV-VIS spectrophotometry. Nanosci. Nanotechnol, 2(6), 184-189.

Masum, M. M. I., Siddiqa, M. M., Ali, K. A., Zhang, Y., Abdallah, Y., Ibrahim, E. & Li, B. (2019). Biogenic synthesis of silver nanoparticles using *Phyllanthus emblica* fruit extract and its inhibitory action against the pathogen *Acidovorax oryzae* Strain RS-2 of rice bacterial brown stripe. Frontiers in microbiology, 10.

Parmar, Kokila A., A. K. Pathan, Kavita R. Desai, J. R. Jadhav and Rahul Shah. (2016). Synthesis of Biogenic Silver Nanoparticles from Medicinal Plant and its Antibacterial Activity. IOSR Journal of Applied Chemistry, 9(8), 29-33.

Philip, D. (2010). Green synthesis of gold and silver nanoparticles using *Hibiscus rosa sinensis*. Physica E: Low-Dimensional Systems and Nanostructures, 42(5), 1417-1424.

Shah, C. S., & Qadry, J. S. (1971). A textbook of pharmacognosy. Messrs BS Shah.

Shankar, S. S., Ahmad, A., & Sastry, M. (2003). Geranium leaf assisted biosynthesis of silver nanoparticles. Biotechnology progress, 19(6), 1627-1631.

Shankar, S. S., Rai, A., Ahmad, A., & Sastry, M. (2004). Rapid synthesis of Au, Ag, and bimetallic Au core–Ag shell nanoparticles using Neem (*Azadirachta indica*) leaf broth. Journal of colloid and interface science, 275(2), 496-502.

Siddiquee, M. A., ud din Parray, M., Mehdi, S. H., Alzahrani, K. A., Alshehri, A. A., Malik, M. A., & Patel, R. (2020). Green synthesis of silver nanoparticles from *Delonix regia* leaf extracts: In-vitro cytotoxicity and interaction studies with bovine serum albumin. Materials Chemistry and Physics, 242, 122493.

Vaghela, H., Shah, R., & Pathan, A. (2018). Palladium nanoparticles mediated through Bauhinia variegata: potent in vitro anticancer activity against mcf-7 cell lines and antimicrobial assay. Current Nanomaterials, 3(3), 168-177.

Vaghela, Hiral, Rahul Shah, and Kokila A. Parmar. (2018). Plant Mixture Mediated Biogenic Copper Nanoparticles: Antibacterial Assay. Current Nanomaterials 3(2), 86-94.

Vo, T. T., Dang, C. H., Doan, V. D., Dang, V. S., & Nguyen, T. D. (2020). Biogenic synthesis of silver and gold nanoparticles from Lactuca indica leaf extract and their application in catalytic degradation of toxic compounds. Journal of Inorganic and Organometallic Polymers and Materials, 30(2), 388-399.

Uzma, M., Sunayana, N., Raghavendra, V. B., Madhu, C. S., Shanmuganathan, R., & Brindhadevi, K. (2020). Biogenic synthesis of gold nanoparticles using *Commiphora wightii* and their cytotoxic effects on breast cancer cell line (MCF-7). Process Biochemistry.

Shah, C. S., & Qadry, J. S. (1971). A textbook of pharmacognosy. Messrs BS Shah.

Yan, W., Petkov, V., Mahurin, S. M., Overbury, S. H., & Dai, S. (2005). Powder XRD analysis and catalysis characterization of ultra-small gold nanoparticles deposited on titania-modified SBA-15. Catalysis Communications, 6(6), 404-408.

Zafar, R. (1994). Medicinal plants of India CBS Publications and Distributors. New Delhi, 105.

Biotechnological Communication

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp-313-319

Efficiency of *Bacillus subtilis* and *Bacillus cereus* to Abate Salinity Stress and Augment Plant Growth

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ABSTRACT

PGPR are potential tools to alleviate plant growth and augment tolerance to abiotic stress tolerance, with reduced level of agro-chemical application, as excessive use of fertilizers poses threat to soil fertility, soil ecology and fertilizer run-off leads to water contamination, water eutrophication. PGPR also elicit 'induced systemic tolerance' to salt and drought. The present study indicated that soil inoculation with rhizobacterial strains of *Bacillus subtilis* and *Bacillus cereus* (NCBI accession numbers: LC480918 and LC481470 respectively) promotes growth of *Capsicum annuum* under both non-saline and saline conditions by directly or indirectly regulating plant chlorophyll content, leaf osmotic potential. The potential of the two rhizobacterial strain to produce exopolysaccharide, indole acetic acid, gibberelic acid production confirmed its ability as plant growth promoting isolates. Present study recorded maximum root length of 22.9 cm and total chlorophyll content of 182 µg/g in *B.subtilis* inoculated plants compared to root length of 22.6 cm and total chlorophyll content of 160 µg/g in control plants, under non saline condition. *B. subtilis* inoculated plants under salt stress showed root length of 17.2 cm total chlorophyll content of 69 µg/g compared to root length of 16.0 cm total chlorophyll content of 64 µg/g in control plants at 200mM salt concentration. Different paradigms of applicability of the PGPR have been displayed comprehensively under both normal and stress conditions to highlight the recent trends with the aim to develop future insight into the role of PGPRs inoculum as biofertilizers for sustainable agriculture productivity and reclaiming soil fertility unlike chemical fertilizers.

KEY WORDS: PGPR, BACILLUS SUBTILIS, BACILLUS CEREUS, SALT STRESS TOLERANCE, CAPSICUM ANNUUM.

INTRODUCTION

Soil fertilization is required for agricultural production but can also cause nitrate and phosphate accumulation that eventually contaminates surface and ground waters. Fertilizer run-off leads to water contamination and phosphate run-off augments eutrophication of surface waters, resulting in fish mortality (Srivastava et al., 2017). These environmental impacts of fertilization can be attributed, in part, to low uptake nutrient efficiency of crops. Phosphorous is highly reactive with iron, aluminum and calcium in soils, which can result in precipitation of up to 90% of the soil phosphorous, making it largely unavailable to plants (Meller et al., 2019).

Owing to these environmental concerns and the increasing prices of fertilizers, there is an urge from farmers worldwide to reduce use of fertilizers below the recommended amount for optimum yields; however, such reductions would exert abiotic stress on plants. Over the last few decades, the agriculture policy in

ARTICLE INFORMATION

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Received 5th Jan 2020 Accepted after revision 25th March 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)

*Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/50*

India has undergone a major change influenced by the root system, referred to as the rhizosphere is a versatile environment of intensive plant microbe interaction for extracting essential micronutrients from a confident nutrient pool (Singh et al., 2012). The plant roots exude a huge diversity of organic nutrients and signals that attracts the microbial populations, especially those able to metabolize plant excluded compounds and proliferate in this habitat (Lareen et al., 2016). PGPR allevate plant growth by various mechanisms that involve soil structure formation, decomposition of organic matter, recycling of essential elements, solubilization of mineral nutrients, producing numerous plant growth regulators, modulating phytohormone level, degrading organic pollutants, stimulation of root growth, vital for soil fertility, biocontrol of soil and seed borne plant pathogens based on their ability to produce antimicrobial or hydrolytic enzymes (Kamilova et al., 2009). *Bacillus species* with potent plant growth promoting traits such as essential phytohormone production and biocontrol attributes are considered as safe microorganism that holds remarkable abilities for synthesizing vast array of beneficial substances (Hashem et al., 2019).

Recent work by several groups shows that PGPR also elicit so-called 'induced systemic tolerance' to salt and drought. Salinity in arid regions is frequently a crucial limiting factor for the productivity of agricultural crops, with adverse effects on germination, plant vigour and crop yield (Singh and Jha, 2016). Soil salinity promotes osmotic stress, water deficit, stomatal closure and reduced leaf expansion (James et al. 2011); moreover, soil salinity causes deficiency of essential nutrients such as K+. Elevated Na+ inside plants can decrease plant photosynthetic rates and biomass accumulation (Zhang and Shi, 2013). Therefore, use of PGPR is a new ways to cope with the threat of global soil salinization to agriculture. A wide range of PGPR produces 1-aminocyclopropane-1-carboxylate (ACC) deaminase, conferred induced systemic tolerance (IST) to salt and drought stress in pepper (*Capsicum annuum L.*) and tomato (*Solanum lycopersicum L.*) plants (Mayak et al., 2004). Many current studies are underway that will further define the utility of PGPR in nutrient management strategies aimed at reducing fertilizer application rates and nutrient runoff from agricultural sources. To maintain the growth and development of *Capsicum annuum* (chilli) in saline condition, the current work was to evaluate the efficiency of Bacillus subtilis and *Bacillus cereus* for growth promotion and salt tolerance in *Capsicum annuum*.

MATERIALS AND METHODS

Present study was conducted at Department of Molecular and Cellular Engineering, SHUATS University, Prayagraj whereas Capsicum annuum seeds (Kashi Surkh) variety was obtained from Indian Institute of Vegetable Research, Varanasi, Uttar Pradesh, India.

Isolation of Rhizobacterial Strains: After selecting Dhatura plant a 6"X 2.5"X 5" rhizospheric soil sample was serially diluted and appropriate dilutions (10-3, 10-5, 10-7) were spread plated on nutrient agar plates. The plates were incubated at 37°C and developed colonies were identified on the basis of cultural, morphological characteristics as described in the Bergey's Manual of Determinative Bacteriology (Holt et al. 1994). Selective colonies were taken and streaked on selective media for individual strains and kept in incubator at 37°C for 24 h to obtain colonies.

Screening of Rhizobacteria for plant growth promoting activities: Production of Indole Acetic Acid (IAA):Nutrient broth amended with 5-mmol tryptophan were inoculated with overnight raised selected bacterial cultures (0.5 OD at 600 nm) and incubated at 37°C for 48 h. One ml of culture was centrifuged at 3,000 rpm for 20 min and supernatant separated. To the supernatant, 4 ml of Salkowasky reagent were added followed by incubation for 1 h at room temperature under dark conditions. Absorbance of the pink colour developed was read at 530 nm (Pandey et al., 2013). Concentration of the proteins in the pellet was determined by Bradford method and the amount of IAA produced was expressed in µg/ml.

Production of Gibberellic Acid (GBA): Extraction of Gibberellic Acid: For gibberellic acid production bacterial isolate was grown in 100 ml nutrient broth medium at 30°C for 72 h. Following the incubation period, cultures were centrifuged at 3,000 rpm for 10 min and the pH of supernatant was adjusted to 2.5 using 1 N HCl. It was extracted with equal volume of ethyl acetate in a separating funnel, shaked vigorously and excess of ethyl acetate fraction was discarded from funnel and allowed the ethyl acetate to by exposing in air. The extract was transferred to a separating funnel and retreated with equal volume of ethyl acetate and then solvent fraction was separated and allowed to by exposing in air. The process was repeated 2 to 3 times to get a large amount of fine quality of gibberellic acid. The concentrated solution was re-evaporated and the residues were dissolved in water containing 0.5% Tween20 (Holbrook et al., 1961).

Spectrophotometric method for gibberellic acid: In this method, one ml of gibberellic extract was pipetted into 15 ml of phyosphomolybdic acid reagent. The content was mixed thoroughly and kept in boiling water bath for 1 h. Flask was removed and rapidly immersed in ice cold, After cooling till room temperature, volume was made till 25 ml with double distilled water and optical density was taken at 780 nm.

Ninhydrin assay for ACC deaminase concentration determination: The DF-ACC medium (with an ACC concentration of 3.0 mmol $l^{-1}$was diluted with the DF medium to respective ACC working concentrations of 0.005, 0.01, 0.015, 0.02, 0.03, 0.04, 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.40 and 0.50 mmol l^{-1}. After the addition of 1 ml of ACC working solution and 2 ml of ninhydrin reagent, glass test tubes were capped and shaken and placed in a boiling water bath. After 15 min, the tubes were moved into a water bath at room temperature for

2 min and then shaken for 30 sec according to Li et al. (2011). After standing at room temperature for 10 min solution turns to purple (Ruhemann's purple), the solution was transferred into a cuvette and absorbance was measured at 570 nm with spectrophotometer. The DF medium was used as a blank. Each working solution was run in triplicate. In addition, 1 ml of a tenfold diluted supernatant of a bacterial culture was used to determine ACC in bacterial cultures with the standard ninhydrin assay.

Characterization of PGPR strains: After confirming their potential for plant growth promoting efficacy, selected culture(s) were characterized by several biochemical analysis viz., indole, methyl red, Voges-Proskauer, citrate utilization, carbohydrate fermentation, amylase, uerease and nitrate reduction according to Bergey's Manual of Determinative Bacteriology (Holt et al., 1994) and then molecular characterization using 16s rRNA gene amplification under standard conditions (initial denaturation 94°C for 3 min, 35 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30s, extension at 72°C for 60s, and final extension at 72°C for 7 min). The amplified product was sequenced and found the size of 237 and 1247 bp for Bacillus cereus and *Bacillus subtillis* respectively. The sequence of 16S rRNA genes of both isolates were compared with the existing database using BLAST and submitted to GenBank of NCBI.

In-vivo test: Screening for salt (NaCl) tolerance on Capsicum annuum plant by isolated plant growth promoting B. cereus and B. subtilis rhizobacteria: Pots were filled with of air dried sieved soil and single surface sterilized seeds were placed in each pot. After germination, five ml of inoculum (*B. cereus/B. subtilis*) with population density of 107-108 cfu/ml were applied into the rhizosphere through a syringe. *Capsicum annuum* plants were irrigated with 800 ml of sodium chloride (0, 50, 100, and 200 mM) according to water requirement. Each treatment was performed in triplicates. Plants were harvested after 30 days and data regarding root length, shoot length, root and shoot fresh and dry weights were recorded according to Shahzadi et al. (2013).The three replicates taken for each treatment were used to calculate the mean of each measurement. Several measurements like length of the shoot system, primary root legth, number of plant leaves as well as plant's fresh (immediately) and dry weights (keeping in oven at 70oC until the weight were stable).

Estimation of total chlorophyll: The conc. of chlorophyll was determined by measuring in a 1.0 ml cell suspension by taking 1 gm of fresh leaves. Few drops of liquid nitrogen was added and grounded with mini pestles. 1.0 ml DMSO was added to each eppendorf containing grounded leaf and mix well followed by centrifugation at 4000 rpm for 5 min. Supernatant was removed and 1.0 ml DMSO to pellet and re-extract followed by centrifugation. Supernatant was discarded and 1.0 ml DMSO was added and absorbance was recorded at 645 (Ali et al., 2014).

Statistical analysis: Basic descriptive statistics was calculated for all the growth parameters of all samples. Standard deviation was obtained by using mean values in order to have treatments comparison at P≤0.05 significance level. All of the statistical procedures were performed by using Statistics software i.e., Web Agri Stat Package 1.0 (an ICAR Web based package).

RESULTS AND DISCUSSION

Many colonies of *Bacillus spp*. were isolated from the soil sample as plates were incubated at 37+2°C and developed colonies selective isolation of bacillus species. Many Colonies of *Bacillus spp*. were identified on the basis of cultural, morphological (Table 1) as described in the Bergey's Manual of Determinative Bacteriology and then screening for plant growth promotion was done. Spectrophotometric quantification of isolated genomic DNA was done and found 1.04 µg/ml for B. cereus and 1.04 µg/mL for *B. subtilis*. Genomic DNA samples were amplified and found size of 237, 1247 bp for *B. cereus* and *B. subtilis* respectively and were then sequenced for 16S r RNA gene and submitted to NCBI under accession number of LC481470 and LC480918.

Table 1. Culture, Morphological and biochemical characteristics

Culture Characteristics	Results	
	B. subtilis	*B.cereus*
Colony shape	Irregular	Round
Margin	Undulate or lobate	Lobate
Colony elevation	Umbonate	Slightly convex
Color	White or dull	Opaque
Texture	Dry	Dry
Gram staining	Positive	Positive
Indole	Positive	Negative
Methyl red	Positive	Negative
Voges-Proskauer	Positive	Positive
Citrate utilization	Positive	Positive
Amylase production	Positive	Positive
Gelatin hydrolysis	Positive	Positive
Urease	Positive	Negative
Ammonia production	Positive	Positive
Nitrate reduction	Positive	Positive
Carbohydrate fermentation (galactose, fructose, Inositol, rafinose, riboflavin, manitol)	(+,+,-,+, +,+,+)	(+,+,-,+, +,+,+)

The results obtained from both qualitative and quantitative assays of Indole Acetic Acid (IAA) reflected the ability of tested bacteria to produce indole compounds. The bacillus sp. exhibited a purple colour with a little variation in intensity. In the quantitative measurements, the highest value of IAA production was 0.398 and 0.26 µg/ml by

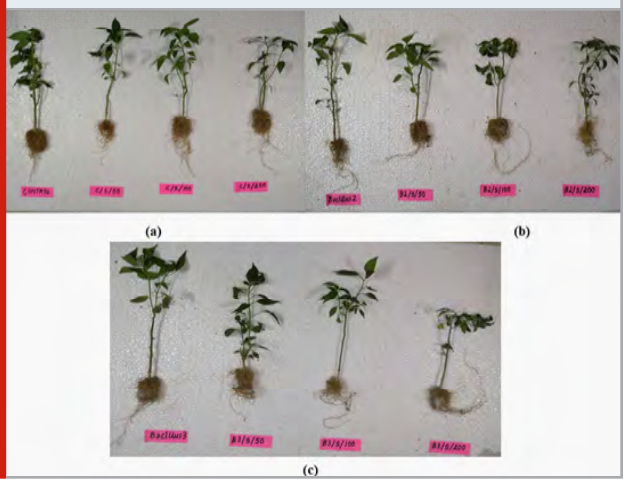
B. subtilis by *B.cereus* species.Isolate screened for EPS production under both no stressed conditions as well as under minimum water potential (–0.30 MPa). The strain *B. subtilis* produced maximum amount of EPS (2.9mg/mg) whereas *B. cereus* could produce 2.1 mg/mg protein. Ali et al. (2014) conducted study on Pseudomonas isolate and found maximum 3.22 mg/mg protein EPS production (from Rdgp10 strain) while another strain (BriP15). Razack et al. (2013) conducted their study to understand the influence of various parameters on elevation of EPS yield by *B. subtilis* in agro waste containing medium and observed positive results.

In Ninhydrin assay, selected isolate were found to positive for ACC deaminase activity a as sample colour turned from yellow to red. The ACC deaminase activity of rhizobacteria was quantified. The ACC deaminase activity of *B. subtilis* and *B. cereus* was 0.13 and 0.05 µg/ml respectively. ACC deaminase-containing rhizobacteria were evaluated for their potential to promote growth (Maxton et al., 2018). Studies of Zhang et al. (2008) showed that ethylene content in tomato seedlings when exposed to high salt was reduced due to Achromobacter piechaudii (PGPR), indicating that bacterial ACC deaminase was functional and its significant role under stress conditions. A. piechaudii, which produces ACC, increased the growth of tomato seedlings by as much as 66% in the presence of high salt contents. *Bacillus subtilis* was tested for gibberellic acid production. Gibberellic acid concentration by *B. subtilis* was recorded as 0.42µg/ml whereas *B. cereus* showed 0.32µg/ml gibberellin production. Our results are in accordance with another study which states that the growth promotion in plants induced by *Azospirillum* infection, may occur by a combination of both gibberellin production and gibberellin glucoside or glucosyl ester de-conjugation by the bacterium (Piccoli et al., 1997) .

Similar concentration of gibberellins was recorded in cultures of Bacillus subtilis; *A. brasiliense* (Janzen et al., 1992). Plants of *Capsicum annuum* were treated with *B. cereus/B. subtilis* inoculum under varying salt concentration (0,50,100 and 200mM) and physical parameters like shoo length, primary root length, number of leaves, plant fresh, dry weight (fig. 1) and chlorophyll content was recorded (fig. 2,3). Inoculation *of B. cereus/B. subtilis* significantly increased the plant growth compare to control plant (fig. 2,3). However plant growth started to decrease as salinity stress increases but significant increase compares to control plant was noticed almost in all plant growth parameters (fig. 1). Compared to uninoculated plant, plant with *Bacillus cereus* inoculum shows maximum shoot length 23cm without any salt stress and maximum primary root length 37.2 cm at 50mM salt conc. (fig. 2a) but decreased with further increases in salinity levels.

Our results were confirmed by Ghorbani et al. (2014) with *Nitraria schoberi* and Panahi et al. (2015) with *Salsola orientalis*, showing moderate salinity levels may improve several growth parameters and the plant will be injured as increasing salinity level increases. Inoculated plant at 50mM salt conc. shows maximum number of leaves (32) in comparison to uninoculated plant which is 26 in number (fig. 3a). Studies of Zhang et al. (2008) showed that in *Arabidopsis, B. subtilis* strain regulates cell expansion and auxin homeostasis, augments photosynthesis by lowering glucose sensing levels that utlimately promotes salt tolerance as well as reduces total Na+ by regulating tissue specific expression.

Figure 1: Effect of *B. cereus/ B. subtilis* on growth measurements, for the plant exposed to saline treatments (a) and (b) without rhizobacteria (c) and (d) *Bacillus cereus* treatment.



Whereas 24.7 cm maximum shoot length was recorded without salt stress compare to 21.2 cm without inoculated plant and sustained till 16.1 cm even at 200mM salt conc. while *B. subtilis* inoculum was applied (fig. 2a). Primary root length was also showed sustainable improvement in the presence of inoculated plant and recorded 17.2cm compare to 16 cm (fig. 2b) in uninoculated plant even at 200mM salinity stress whereas study conducted by Hussein and Joo (2014) on raddish using *Azotobacter chrococcum* showed 9cm root length at 150mM NaCl conc. However in some treatments elongated root development was noticed compare to noticeable increase in shoot/number of leaves present (fig. 1) as some PGPR promote root development (Kloepper et al., 2007) and alter root architecture by the production of phytohormones such as indole acetic acid (IAA), resulting in increased root surface area and numbers of root tips. Such stimulation of roots can aid plant defense against pathogens and can also relate to induced systemic resistance (IST). Similar increase pattern was noticed in plant fresh (3.1 gm with B.cereus while 2.9 gm with *B. subtilis* at 200mM compare to 2.4 gm in contol) and dry weight as well (fig. 2c,d).

The notable differences recorded in all plant growth parameters may be due to the bacterial inoculation efficiency and their quorum sensing ability with another microbes present in soil. When plant growth suppression is the result of ethylene stress, PGPR with ACCD can be exploited (Saleem et al., 2007). ACCD has ability to metabolize ACC, a precursor of ethylene in biosynthesis

pathway and thus controlling total amount of ethylene stress that may be produced (Arora et al., 2012).

During our study significant development in number of leaves at almost inoculum stage was notices (fig. 3a) that is in accordance with studies of Han et al. (2014) using *B. subtilis* strain where around 80% more leaves were observed PGPR have been demonstrated to activate the synthesis of antioxidants and indole acetic acid, which can stimulate root growth (Jha and Subramanian, 2014). Total chlorophyll content (µg/g) was also recorded (fig. 3b) in both *B. cereus/B. subtilis* inoculam and recorded as 160/172 µg/g in uninoculated whereas 182µg/g in inoculated plant under zero salinity stress. However, 71 µg/g was recorded with *B. cereus* inoculated and 69 µg/g with *B. subtilis* inoculated plant at 200mM salinity stress compare to 64 µg/g (fig. 3b) in uninoculated plant that showed another significant effect of these inoculums to as plant growth promotion. Studies of Han et al. (2014) using *B. subtilis* also observed promotion of leaf growth, as well as leaf chlorophyll content under both non-saline and salinity stress.

Figure 2: Comparison of (a) Shoot length (b) Root length (c) Fresh plant weight and (d) Dry plant weight for *B. cereus/B.subtilis* in treated/untreated *Capsicum annuum* plant under salt stress where error bars indicate standard deviation and represents significance at p≤0.05 level.

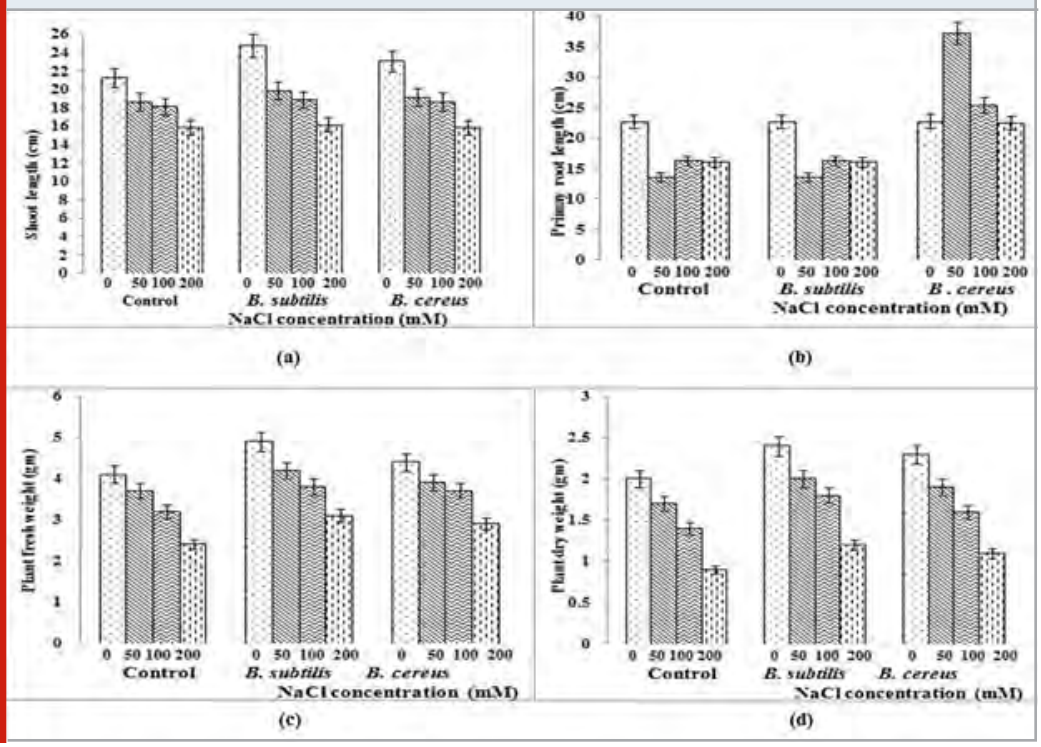
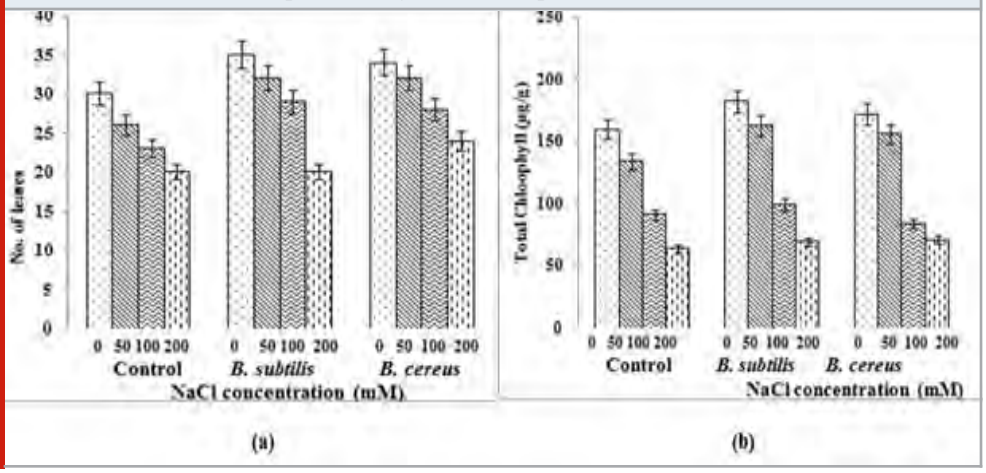


Figure 3: Comparison of (a) Number of leaves (b) Chlorophyll content for *B. cereus/B.subtilis* in treated/untreated *Capsicum annuum* plant under salt stress where error bars indicate standard deviation and represents significance at p≤0.05 level.



Several polyamines secreted through PGPR have also been exposed to mitigate stress ethylene levels as well as alleviate osmotic stress (Xie et al., 2014). Recently, PGPR were shown to alter mineral uptake, which results in a favorable increase in the cellular ratio of K+/Na+; and elevated generation of quorum-sensing molecules, which can lead to modifications.

CONCLUSION

Different paradigms of the PGPRs have been displayed comprehensively under both normal and salinity stress conditions, with the aim to develop future insight into the role of PGPR inoculum as biofertilizers for sustainable agriculture productivity and reclaiming soil fertility. The present study established that the inoculation of the rhizobacteria *Bacillus subtilis* and *Bacillus cereus* are capable of significantly increasing plant growth and biomass of *Capsicum annuum* under both non-saline and saline conditions, by the production of specific rhizobacterial determinants like IAA, GBA, exopolysaccharides, ACC deaminase. Plant root and shoot length, fresh and dry weight, number of leaves, chlorophyll content was recorded to be higher in inoculated plants as compared to control plants, under both non-saline and saline conditions. Therefore PGPR elicited stress tolerance can aid the growth of crops in environmentally unfavourable conditions.

Conflict of interest: There is no conflict of interest in the present study

ACKNOWLEDGMENT

Authors are thankful to SHUATS University, Prayagraj for granting In-house project (DR/Cir/JIBB/IRPs/2018/23) and providing required lab facility.

REFERENCES

Ali S, Charles TC and Glick BR (2014) Amelioration of high salinity stress damage by plant growthpromoting bacterial endophytes that contain ACC deaminase. Plant Physiol. Biochem. 80:160-67.

Arora NK, Tewari S, Singh S, Lal N and Maheshwari DK (2012) PGPR for protection of plant health under saline conditions, pp. 239-258. In Maheshwari DK (ed.). Bacteria in Agrobiology: Stress Management. Springer, Berlin.

Ghorbani M, Ranjbar FA, Panahi F, Attarha J, Marzbani N and Moases M (2014) Salinity and *Nitraria schoberi*: growth parameters, chlorophyll content and ion accumulation. Int. J. Agri. Crop Sci. 11:853-62.

Han QQ, Lü PX, Bai JP, Qiao Y, Pare PW, Wang SM, Zhang JL, Wu YN, Pang XP , Xu WB and Wang ZL (2014) Beneficial soil bacterium *Bacillus subtilis* (GB03) augments salt tolerance of white clover. Front. Plant Sci. 5:1-8.

Hashem A, Tabassum B. and Allah E. (2019). *Bacillus subtilis*: A plant-growth promoting rhizobacterium that also impacts biotic stress. Saudi J. Biol. Sci. 26: 1291-1297.

Holbrook A, Edge W and Bailey F (1961) Spectrophotometric method for determination of gibberellin acid. Adv. Chem. Serv. 28: 159-167.

Holt JG, Krieng N, Sneath P, Staley P and Williams J (1994) S.T. Bergey's Manual of Determinative Bacteriology. (9nd ed.). Baltimore.

Hussein KA and Joo JH (2018) Plant Growth-Promoting Rhizobacteria Improved Salinity Tolerance of *Lactuca sativa* and *Raphanus sativus*. J. Microbiol. Biotechnol. 28:938–45.

James RA, Blake C, Byrt CS and Munns R (2011) Major genes for Na+ exclusion, Nax1 and Nax2 (wheat HKT1;4 and HKT1;5), decrease Na+ accu- mulation in bread wheat leaves under saline and water logged conditions. J. Exp. Bot. 62: 2939-47.

Janzen RA, Rood SB, Dormaar JF and McGill WB (1992) *Azospirillum brasilense* produces gibberellin in pure culture on chemically-defined medium and in co-culture on straw. Soil Biol. Biochem. 24:1061-64.

Jha Y and Subramanian RB (2014) PGPR regulate caspase-like activity, programmed cell death, and antioxidant enzyme activity in paddy under salinity. Physiol. Mol. Biol. Plant. 20: 201-7.

Kloepper JW, Gutiérrez EA and McInroy JA (2007) Photoperiod regulates elicitation of growth promotion but not induced resistance by plant growth-promoting rhizobacteria. Can. J. Microbiol. 53: 159-67.

Lareen A, Burton F, Schäfer P (2016) Plant root-microbe communication in shaping root microbiomes. Plant Mol Biol. 90(6):575–587.

Li Z, Chang C, Lin L, Li Y and An Q (2011) A colorimetric assay of 1-aminocyclopropane-1-carboxylate (ACC) based on ninhydrin reaction for rapid screening of bacteria containing ACC deaminase. Lett. Appl. Microbiol. 53: 178-85.

Lugtenberg B and Kamilova F (2009) Plant-Growth-Promoting Rhizobacteria. Ann. Rev. Microbiol. 63 :541-56.

Maxton A, Singh P, Aruna A, Prasad SM and Masih SA (2018) PGPR: A Boon in Stress Tolerance and Biocontrol. Res. J. Biotechnol. 13: 105-11.

Mayak S, Tirosh T and Glick BR (2004) Plant growth-promoting bacteria that confer resistance to water stress in tomatoes and peppers. Plant Sci. 166: 525-30.

Meller S, Frossard E and Luster J (2019) Phosphorus Allocation to Leaves of Beech Saplings Reacts to Soil Phosphorus Availability. Front. Plant Sci. 10:744.

Panahi F, Asareh MH, Jafari M, Givar A, Tavili A, Arzani H and Ghorbani M (2015) The responses of *Salsola*

orientalis to salt stress. Int. J. Advanced Biol. Biomed. Res. 3(2):163-71.

Pandey S, Ghosh PK, Ghosh S, De TK and Maiti TK (2013) Role of heavy metal resistant *Ochrobactrum spp*. and *Bacillus Spp*. Strains in bioremediation of a rice cultivar and their PGPR like activities. J. Microbiol. 51: 11-17.

Piccoli P, Masciarelli O and Bottini R (1997) Metabolism of 17,17-[2H2]-gibberellin A4, A9 and A20 by *Azospirillum lipoferum* in chemically defined culture medium. Symbiosis. 21: 263–274.

Razack AS, Velayutham V and Thangavelu V (2013) Medium optimization for production of Exopolysaccharide by *Bacillus subtilis* using synthetic sources and agro wastes. Turkish J. Biol. 37: 280-288.

Saleem M, Arshad M, Hussain S and Bhatti AS (2007) Perspective of plant growth promoting rhizobacteria (PGPR) containing ACC deaminase in stress agriculture. J. Indust. Microbiol. Biotechnol. 34: 635-48.

Shahzadi IA, Khalid S, Mahmood MA, Mahmood T and Aziz I (2013) Effect of bacteria containing ACC deaminase on growth of wheat seedlings grown with chromium contaminated water. Pak. J. Bot. 45: 487–94.

Singh RP and Jha PN (2016) A Halotolerant *Bacterium Bacillus* licheniformis HSW-16 Augments Induced Systemic Tolerance to Salt Stress in Wheat Plant (*Triticum aestivum*). Front. Plant Sci. 7:1890

Singh, S., Najar, G., and Singh, U. (2012). Phosphorus management in field pea (*Pisum sativum*)-rice (*Oryza sativa*) cropping system under temperate conditions. Ind. J. Agric. Sci. 82:494.

Srivastava V, Sarkar A, Singh S, Singh P, Araujo A and Singh R (2017) *Agroecological* Responses of Heavy Metal Pollution with Special Emphasis on Soil Health and Plant Performances. Front. Environ. Sci. 5:64.

Xie SS, Wu HJ, Zang HY, Wu LM, Zhu QQ and Gao XW (2014) Plant growth promotion by spermidine-producing *Bacillus subtilis* OKB105. Mol. Plant Microbe Int. 27:655-63.

Zhang H, Kim MS, Sun Y, Dowd SE, Shi H and Paré PW (2008) Soil bacteria confer plant salt tolerance by tissue-specific regulation of the sodium transporter HKT1. Mol. Plant Microbe. Int. 21: 737-44.

Zhang JL and Shi H (2013) Physiological and molecular mechanisms of plant salt tolerance. Photosynthesis Research 115:1-22.

Technological Communication

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp-320-325

A Study on Some Network Attacks and Their Preventive Measures

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ABSTRACT

Spoofing and hijacking arer a major threat in network security. Spoofing involves attacks which are associated with the impersonation of third party to steal the credential information's from a network. Major IP spoofing attacks include ARP spoofing attacks and DNS spoofing attacks, which target the server. These are also called as IP address forging which try to take away the major information from the organizations network systems. There are several tools available to prevent this intrusion prevention system. Some of them are snort, suricata, firewall, netfilter and IPfilter.Penetrating into the network can be prevented using be found using some testing tools like Nmap, Netcat and Hping. Certain attacks denial of service attack and man in the middle attack are more prone to these penetrating malicious threats. Therefore, i is mandatory to take necessary actions to prevent the network from these attacks. Defensive strategies like filtering the packets, using an upper layer, using access control list and using a router that is encrypted in nature are encouraged to make the network secure. In this paper, various hijacking spoofing attacks are analyzed and their preventive methods are mentioned to enable the network to be well secured. Certain specific protocols are encouraged to do this security measure to prevent the network attacks.

KEY WORDS: HIJACKING,SPOOFING,BLINDSPOOFING,ZOMBIE,COOKIE,FERRET,WIRESHARK.

INTRODUCTION

Session hijacking is a major threat in network security where the ongoing connection with a server will lose its connectivity for a period of time (Ali,et al., 2014).The credentials like username, password and other secret information's will be steeled away by the attacker for that particular period of time. To be precise an unauthorised access is been given to a third party who acts as though he is serving from the system (Prabhu et al., 2017).

ARTICLE INFORMATION

**Corresponding Author: alibaggar@hotmail.com*
Received 7th Feb 2020 Accepted after revision 19th March 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/51

Spoofing is an illegal trick to steel the security of the network. Current internet packet delivery relies on packets destination IP address and forwarding techniques. IP source address spoofing or IP spoofing attack refers to all the attackers where the packets with forged IP source addresses so that they can conceal on their identities (Zhan et al., 2019).

IP spoofing threat is derived from the design that internet packet forwarding in routers only relies on packets destination. Despite anti IP-Spoofing has been studied extensively in the past decade feasible and integrated solutions to cover both of intra domain and inter domain scopes till exists on the weaker side. Intra domain solutions like IP source address filtering, IP source address encryption, protocol and host stack redesign and SDN based source address validation are validated for the required manner by the attacker. Security with IP address assignment and spoofing for Smart IOT devices

are carried out using special techniques. Since cyber security is of main importance the smart IOT device to the cloud storage is carried with the IoT Gateway present in-between each other. Methods to implement the sensors on the smart IOT devices were carried out and implemented in both the IPv4 and IPv6 (Rajshree et al., 2019). IP spoofing prevention using reverse path forwarding as applied to software defined networks are described. Light weight approach to IP spoofing assumes IP filtering rules which are on the gateway of the devices. Key generation is also done separately for IPv4 and IPv6.This makes the network stronger and less prone to network attacks.

Using certain strategies like ferret, hamster and Wireshark this session hijacking can be prevented. Ferret is a software tool that which checks the host to find out the vulnerabilities present in the system. It was originally designed to work on UNIX but later it was developed for windows (Anand et al., 2004). Hamster is an external tool used by the networking devices to prevent the forgery of false and fake fingerprint readers. Wireshark is an analyser application, which uses the network protocol to analyse the traffic over the network. Major advantage of Wireshark is that this is a multi-platform supporting tool (Shaoqiang Wang et al., 2010). Comparative analysis of Tcpdump and Wireshark was carried out by Goyal et al., (2019) which compares all the features between Wireshark and Tcpdump. The first feature compared was power consumption and got the result that Wireshark takes power hundred times as tcpdump.The next factor analysed is the memory and Wireshark is several times that of the Tcpdump. Speed is the next factor analysed and observed that Wireshark is speedier than Tcpdump. Assessment of website security is done by penetration testing using Wireshark tool (Sandhya et al., 2019).

The breakdowns in the security systems which has caused the tool to be used in a wiser material for a more prolonged usage of security testing. Some common methods like metasploit, web application audit and attack framework, nipper studio, OWASP zed attack proxy, backtrack and skipfish.There is a step by step procedure to crack any website and load into the server. Selecting interface and starting the live capture of the data packets is the first step. The second step involves logging into the vulnerable website and looking for a post request. The final stage has the analyser mode where the traffic is analysed for further processing.

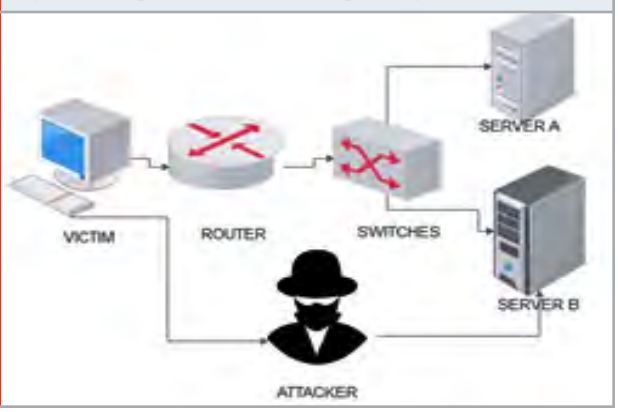
The main purpose of the contribution of this paper is that to prevent all the session hijackings like blind hijacking, hybrid hijacking, and a zombie attack on a network, cookie spoofing techniques and prevention mechanisms.Certain other strategies like fingerprint validation, session verification, innet strategy, outnet strategy are taken into consideration. On the conclusion part, we conclude by saying what steps we have taken to reduce the risk of server and other Dynamic Host Configuration Protocol (DHCP) being attacked (Tripathi, et al., 2015). In this research paper a detailed view of the hijacking and spoofing mechanisms are termed in detail. The corresponding defense strategy to provide security to the network protocol has also been suggested for a secured transmission of data packets. Hijacking can be termed as different types such as session hijacking, blind hijacking and hybrid hijacking.

Various Spoofing and Hijacking Attacks: There are several types of network attacks among them few common attacks are IP address spoofing attack, fingerprint attack, zombie attack, and session hijacking attack.

IP Address spoofing attack: Spoofing in general happens at a different level more specifically in ARP Spoofing, MAC Spoofing, Blind Spoofing and Non blind spoofing. Blind spoofing is a method where the attacker can inject data into the stream of packets without having a proper authentication itself when the connection is established at first stage. In this, the data packets are injected where the target is aware of the packets, which are got as sequence. A proper IDS (Intrusion Detection System) has to be used for security system of computer networks. This can be of Host-Based IDS and Network-Based IDS (Tamizi and Weinstein 2017). Honey spots are identified at the first stage and soft spot which means the weak spot in the network has to be checked. IP Spoofing detection for preventing the DDoS Attack in fog computing is carried using proper design works. Fingerprinting is carried out to prevent the network from getting attacked by the third party. This is further divided into active fingerprinting and passive fingerprinting which results in actively detected Os and passively detected Os.

After checking the weak soft spot, the IP address spoofing process starts. A prime example of IP address spoofing attack is given in the diagram below. Consider an organization with the following configuration with following specifications.Intra domain solutions like IP source address filtering,IP source address encryption, Protocol and host stack redesign and SDN based source address validation. The Inter domain solutions involves end based source address filtering, path based source address filtering, end to end based source address filtering are been carried out to ensure the proper data packet travel on the specified network path. The system to be attacked is been passed to a router and to a switch.

Figure 1: Representation of IP Spoofing Attack



Then it is been transmitted to two servers server A and server B.The IP address that is transmitted through the network is been attacked and spoofed by the attacker. Now the original IP address of the victims system that is the system been attacked is changed to another IP by the attacker (Pomsathit et al.,2012). Now the fake IP address, which is transmitted from the attacker, is been sent to servers and for users. This shows the IP been spoofed by the third party attacker.

Session Hijacking Attack: Hijacking a particular session of a network taking away all the credential information's from the network between client and server is termed as session hijacking attack. Denial of Service is more common to this type of attack. During session, hijacking attack an adversary sets up a fake point to access the hijackers. This attack is a mixture of DOS and man in the middle attack. A network sniffer is present in this attack. This attack leads the user to go to the fake website leading to take away all the information's like password and usernames. Cross-site scripting is used in hijacking the session of a particular network path. This stolen information's can lead to loss in integrity and confidentiality of the network systems (Cashion et al 2017). This attack is more often in wireless network than in wired network. The session ID of victim and the fake masquerade authorized user, is the computer session been exploited. This is also termed as SHA (Session Hijacking Attack).A more detailed view of the organizations network is been given in the below system where the information of the network is been stolen and taken away by the attacker. Session hijacking leads to a major headache for networks that which are connected without a proper network throughout the organization. Consider the following criteria happening in the below given system architecture.

In the above system, the victim transmits a session ID from its end to web server. There is an attacker present illegally between two systems sniffing the session ID.This ID after been sniffed the attacker takes control of the entire system. The attacker will terminate the endpoint connection between client and the server (Enos and Sunday,2017). Therefore this session is hacked and a fake session ID is been transmitted to web server which leads to sensitive information been taken away from the web server.

Zombie Attack: Zombies can be generally referred as spam or junk messages which are been transmitted to a particular system to make it a malicious thing. Spam zombie detection scheme blocks the emails and mail server that which transmits the junk of data. SPOT is a lightweight spam zombie detection system, which detects the intrusion detection system as required by the bothunter.Mails which are designated from various sources comes into the zombie and they all together form an attacked system, which can cause heavy damage to the system of the organization. Spam zombie algorithm is available to find out the zombie-attacked system (Duan et al., 2009). Emails servers provide the junk of the zombie system where enormous amount of information is been involved for the system to developed. An organization with different zombie attacked system is been given below and their functionalities are termed below. Zombies plays a vital role in network security by sending junks of data an mails throughout the system network enabling the organization to cause damage to the system and to define the problems. Consider an organization with the following configuration with following specifications.

Figure 2: Representation of Session Hijacking Attack.

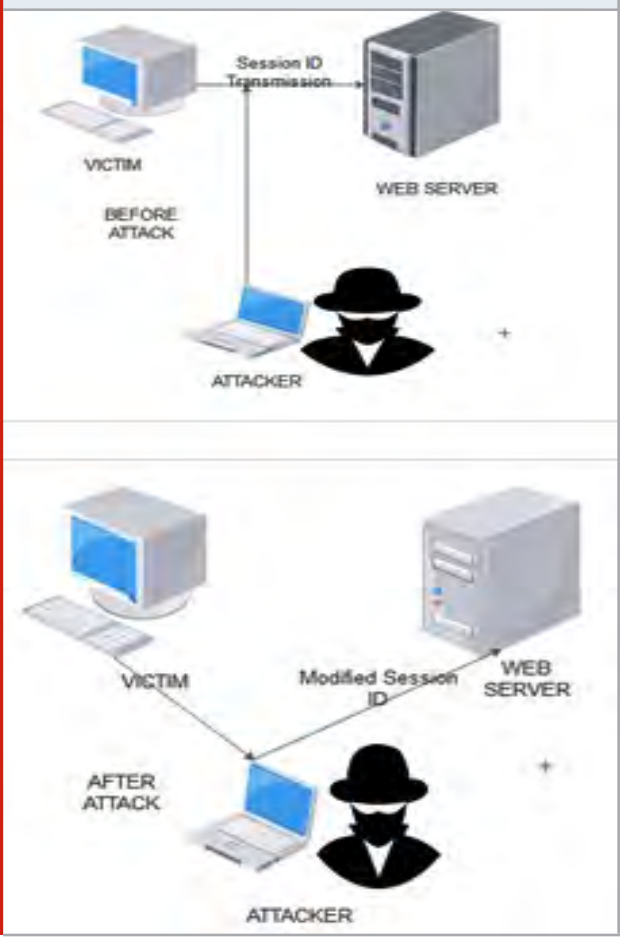
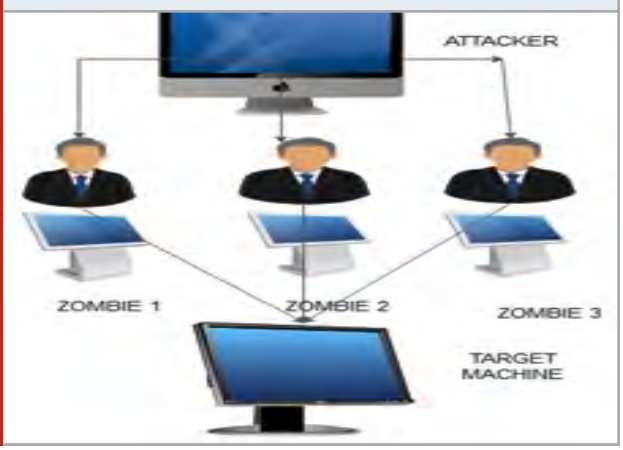


Figure 3: Representation of Zombie Attacked System



The attacker has assigned three systems to act as zombie to attack the main system. After getting all the junk messages and emails from across all the sections the zombies just passes the information's from one system to another. The attacking machine on receiving information to be theft from the system gives the zombie system with each task. The targeted machine will then be attacked by the various junk messages to jam the main system. This is working of zombie attack.

RESULTS AND DISCUSSION

Strategies to prevent network attacks: There are plenty of methods and techniques to prevent network attacks. Some of the network attacks are mentioned in this paper with their diagrammatic representation. Some common methods are using Wireshark tool, in network strategy, out network strategy been implemented.

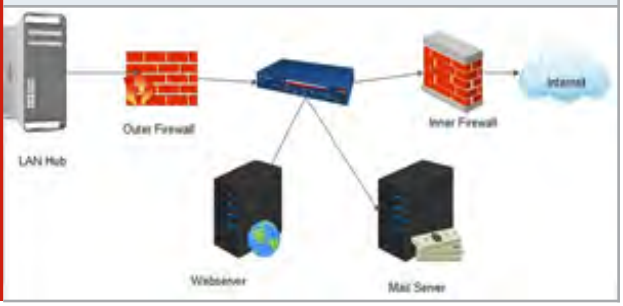
Wireshark Tool: Wireshark is a network protocol application to analyse the data packets been transferred from one section to the other. This is a multi-platform supportive tool, which supports Linux, Mac OS and BSD platforms as well. This tool can be used to prevent the system from being been affected by the external internet traffic. When huge traffic comes to a webserver intented to attack a network Wireshark will provide as a supportive tool to the operating system with the traffic been not affected by the network. Wireshark can capture the data and include PROFINET by using a capability to discovering the topology of network to work on the required spots. Network experts use Wireshark as a troubleshooting tool and for analysing the security issues. Protocols such as TCP/IP, MAC, IP datagram and the data transmission of PDU can be seen and analysed using the Wireshark tool. Hence, this tool is a good analyser and security tool for making the network connections more secure.

Figure 4: Representation of a system that is protected by Wireshark

In this system architecture a system with heavy internet traffic is been sent to jam a network system. A Wireshark is present in-between the network. Snort and an alarm is kept for analysing and sending alarm to inform the incoming internet traffic. It analysis and divides the traffic according to the organisations needs and splits up into various parts. The snort will be then divided into different PCs.Hence Wireshark provides, as a block between the networks been not being damaged. It analysis the traffic and sends the needed data and blocks the unwanted data as an integral part of a network.

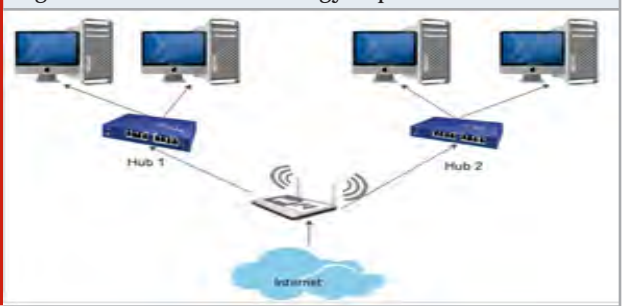
In network Strategy: This is a unique method of preventing network security where there will be two firewalls one on the outer and one on the inner firewall. The organisation is double protected with the firewalls on both the sides before exposing the network to the internet exposure. This serves as the double-blinded system. In the above system, the Router is present in-between two firewalls thus providing which access to be given to the required system. The router sends the data to web server and to mail server. Hence it is provided between two walls whereas the data integrity is been protected by having the double protection. This strategy is followed in many of the companies and organization, which handles very confidential data like bank and government websites.

Figure 5: In network Strategy Representation



Out network Strategy: Out network strategy is a method of giving out the internet sources to outer systems that which are present in the environment in an open account to be accessed by the individual users controlled system. The below picture shows the representation of out network strategy of networking system. A massive amount of internet is been provided by the internet provider to the router at initial stage. After it gets give, the internet from the provider the router divides the network into for hubs. The hubs transfer's data for the systems present in the organisation. In extend to this a wireless router is present which provides access to the Wi-Fi enabled zone such as mobile users and office providers. This method is followed in organisations where the architecture of the system is well formed without leaking any confidential information's from the source to destination. This is the overview of the out network strategy of the system.

Figure 6: Out Network Strategy Representation



Other Techniques to provide defence strategy for network protocols: Apart from the above-mentioned techniques, there are other strategies to prevent the network attacks by using a proper filter to avoid the proxy, using ferret and hamster strategies. Using proper filter is a good technique that which can be applied in order to provide a good security for networks. Filtering the data packets based on the nature of the source whether it contains any harmful contents or malicious natured codes to threaten the network system to be collapsed. Ferret is another technique, which can be analyzed to choose what type of attacks to be caused by individual networks. Hamster is another network security-providing tool, which helps in finding the network faultering.This tool uniquely provides the entire network from to be being accessed by an illegal third party attacker.

The main features and findings of this paper includes

1. Intimating the Hijacking, which includes whether the incident happened, is a hijack or not. More common
   Problem first faced is the suspicious activity is an attack or any ordinary network cracks (Tamizi and Weinsten 2017).
2. Identifying what has happened in the network is the next factor of the work that which has been carried out. It is necessary to analyse what sort of attack has happened in the network and the need is must to be fulfilled.
3. Find out whether the thing happened is an attack is another foremost thing, which has been carried out because not every single activity can be termed as attack.
4. Amount of detection been found is another major role because the security has to be preserved well enough in order to give the attackers a solution.
5. Respected solution for attacks, which has happened, is the next thing, which has been studied in this paper. This has resulted in providing multiclient with the feature of providing a well-supervised material to execute the attacks, which has happened. We do not want to use big firewalls to get a secured network authentication. Just these preventive actions will do a great doing for the given organisation. Almost 65*/* of detection is increased because of the system that we have proposed (Sharma et al., 2016).
   This also provides and supports multiclients, which prevents zombie attack. Detection rate can be improved by using the server broadcasts that which we can use some secured data mechanism such as, DHCP (Dynamic Host Configuration Protocol) where the management of network usually gets to use to assign dynamically the Internet protocol address to any device or any node or any particular network for which the security needs to be provided.

CONCLUSION AND FUTURE SCOPE

Future scope of the networking includes DHCP. Dora (Discovery, offer, request, acknowledgement) process, which exists in this paper for a future work to be discovered. Dora is a process of handling the servers that which distributes dynamically the network configuration parameters such as IP addresses interface methods and their services. This is been standardized by following network protocols used in the network communication. Discovery includes the network sub netting that is followed by destination address.DHCP offer provides a DHCPDISCOVER message from a client where it receives the IP address that is to lease the request from the server, (Veysel et al.,2017).DHCP request is followed up with a client server connection thus leading to a well secured authentication scheme. Acknowledgement includes the receipts to be involved in the system to enable the data packets to be included in the duration and other configurations. The final IP configuration can be done in this system.

REFERENCES

Abdullah H. Alqahtani, Mohsin Iftikhar (2013) Hijacking and spoofing attacks,International Journal of Science and Modern Engineering, Volume-1, Issue-10

Anand N, Anil Sharma (2004) Ferret: A Host Vulnerability Checking Tool, Proceedings of the 10th IEEE Pacific Rim International Symposium on Dependable Computing

Enos Letsoalo, Sunday OJO (2017) Session Hijacking Attacks in Wireless Networks: A Review of Existing Mitigation Techniques,IIMC International Information Management Corporation

Feng Luo (2010) Method and Implementation of Building ForCES Protocol Dissector Based on Wireshark, IEEE

Goyal P (2019) Comparitive study of two most popular packet sniffing tools-Tcpdump and Wireshark,International conference on computational intelligence and communication Networks

Jeffrey Cashion and Mostafa Bassiouni (2017) Protocol for Mitigating the Risk of Hijacking Social Networking Sites,International Conference on Collaborative Computing: Networking, Applications and Worksharing (CollaborateCom), Orlando, Florida, USA

Nikhil Tripathi, Neminath Hubballi (2015) Exploiting DHCP Server-side IP Address Conflict Detection: A DHCP Starvation Attack

Pedro R. M. In´acio, Paulo P. Monteiro (2010) Zombie Identification Port, The Third International Conference on Internet Monitoring and Protection, 67-73

Pomsathit A (2012) Effective of Unicast And Multicast IP Address Attack Over Intrusion Detection System with Honeypot, U.S. Government work not protected by U.S. copyright

Purkayastha SS (2019) Assessment of website Security by penetration testing using wireshark,International

conference on Advanced computing and communication systems

Raguvaran S (2014) Spoofing Attack: Preventing in Wireless Networks, International Conference on Communication and Signal Processing,117-121

Resul Das, Gurkan Tuna (2017) Packet Tracing and Analysis of Network Cameras with Wireshark,IEEE

Saurabh Jha, Shabir Ali(2014) Mobile Agent Based Architecture to Prevent Session Hijacking Attacks in IEEE 802.11 WLAN, International Conference on Computer and Communication Technology

Shaoqiang Wang, DongSheng Xu (2010) Analysis and Application of Wireshark in TCP/IP Protocol Teaching ,International Conference on E-Health Networking, Digital Ecosystems and Technologies, IEEE

Sharma P , Sanjeev Kumar (2016) BotMAD: Botnet Malicious Activity Detector Based on DNS Traffic Analysis, 2nd International Conference on Next Generation Computing Technologies

Soman R (2019)Security with IP Address Assignment and Spoofing for Smart IOT Devices,IEEE

Tamizi M and Matt Weinstein (2017) Automated Checking for Windows Host Vulnerabilities, Proceedings of the 16th IEEE International Symposium on Software Reliability Engineering

Thawatchai Chomsiri (2008) Sniffing Packets on LAN without ARP Spoofing,Third International Conference on Convergence and Hybrid Information Technology,472-477

Vanajakshi, Srikanth Prabhu (2017) An Effective Method for Preventing SQL Injection Attack and Session Hijacking, IEEE International Conference On Recent Trends in Electronics Information & Communication Technology

Veysel Harun (2017) Topology discovery of profinet networks using wiresharK,2013 IEEE

Yongle Wang, Jun Zhang Chens, (2013) Hijacking spoofing attack and defense strategy based on Internet TCP sessions, IEEE 2nd International Symposium on Instrumentation and Measurement, Sensor Network and Automation

Zhang C ,Guangwu Hu (2019) Towards a SDN based Integrated Architecture for mitigating IP Spoofing Attack,IEEE

Zhenhai Duan, Peng Chen, Fernando Sanchez (2009) , Detecting Spam Zombies by Monitoring Outgoing Messages, IEEE INFOCOM proceedings

Zhenhai Duan, Peng Chen, Fernando Sanchez (2013) Detecting Spam Zombies by Monitoring Outgoing Messages,IEEE

Biotechnological Communication

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp-326-334

Design and Prediction of Absorption, Distribution, Metabolism, Excretion and Toxicity Properties of Drugs for H1N1 Flu (Swine Flu) Using In-Silico Approaches

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ABSTRACT

H1N1 Flu (Swine Flu) protein NA (neuraminidase), assists newly prepared viruses. It is released from infected cells by cutting up sialic acids. This study was aimed at in-silico drug design and prediction of Absorption, Distribution, Metabolism, Excretion and Toxicity (ADMET) properties of potential hits against H1N1 Flu (Swine Flu) protein NA (neuraminidase). It contains ligand named Glycerol, (propane-1,2,3-triol). It was identified as a drug target protein, whose structure (PDB format) was downloaded from the Protein Data Bank. Ligand Glycerol, (propane-1,2,3-triol) was obtained for further analysis. Compounds were selected from DrugBank, PubChem, ChemSpider and they were screened along with Glycerol using PyRx-Virtual Screening software for Structure based drug design (SBDD) and compared using similarity score. Absorption, Distribution, Metabolism, Excretion and Toxicity (ADMET) properties were determined by using online tool Danish Quantitative Structure-Activity Relationship (QSAR) database. The Absorption, Distribution, Metabolism, Excretion and Toxicity (ADMET) analysis and docking results revealed 18 compounds as potential leads.

KEY WORDS: ADMET, GLYCEROL (PROPANE-1,2,3-TRIOL), NEURAMINIDASE, SWINE FLU.

INTRODUCTION

In April 2009, a new strain of influenza virus A, H1N1, generally known as “swine flu,” commenced to extend in numerous countries all over the world. This new strain could pass from human to human guided the World Health Organization (WHO) to promptly elevate its pandemic awareness (Rubin, Amlôt, Page, & Wessely, 2009). Influenza A (H1N1) virus has caused severe respiratory infection and death over the years. The first confirmed case was acknowledged in May 2009 in India. H1N1 occurrence in India had directed to significant morbidity and mortality (Kshatriya1 et al., 2018). Gujarat has its highest case fatality rate with 1674 cases and 144 deaths as on February 2015 (Baria, Solanky, Shah, & Patel, 2017). Influenza A, influenza B, and influenza C are the three major groups of Influenza viruses. Out of these influenza A viruses are main cause of infection in Human, which is further categorized into HA (hemagglutinin) and NA(neuraminidase) (Jilani and Jamil 2019). Neuraminidase (NA) is the most significant in the pathogenesis of infection. Hence, it is the most important target for agents used in the treatment and prophylaxis of influenza (Young, Fowler, & Bush, 2001). Influenza viruses are typically round however they can also be amorphous or even form long filaments. Each virus has a membrane taken from the inflamed cellular

ARTICLE INFORMATION

*Corresponding Author: arpit3290@gmail.com
Received 05th Jan 2020 Accepted after revision 21th Feb 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)
A Society of Science and Nature Publication, Bhopal India

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/52

that produced it. There are several crucial viral proteins on this outer layer giving the virus a studded appearance. Hemagglutinin allows the virus to bind to cells within the respiratory tract with binding to sialic acid on the cell surface (Jilani and Jamil, 2019).

Neuraminidase enables newly made viruses launch from infected cells by slicing up sialic acids. Matrix protein 2 (M2) is the alternative protein found inside the viral membrane. It is an ion channel. The inner of the viral membrane is lined with matrix protein 1(M1). Influenza viruses consist of a single-stranded RNA genome which encodes all the other proteins of Virus. This genome is divided into eight segments. Each and every section is covered with nucleoprotein(NP) to defend the fragile RNA (Jilani and Jamil 2019). The viral RNA polymerase catalyzes its replication and transcription (Stubbs & Te Velthuis, 2014). The RNA is replicated by means of a polymerase composed of 3 diverse kinds of proteins: Polymerase basic protein 1 (PB1), Polymerase basic protein 2 (PB2), Polymerase Acidic Protein A (PA). Altogether, the RNA, NP, and polymerase are known as a ribonucleoprotein particle (RNP). Other two viral proteins are non-structural protein 1 (NS1) and non-structural protein 2 (NS2) also present inside the virus in limited amounts. NS1 allows preventing the cell from shutting down crucial cellular pathways and detecting the virus. NS2 plays an important role in assembly of new born viruses (Jilani and Jamil 2019).

Neither presently available vaccines nor natural immunity from earlier strains of influenza A suggest protection against swine flu (Coker, 2009).To design the drug with the help of the computer is a specialized branch that uses computational approaches to create drug-receptor interactions (Bissantz, Folkers, & Rognan, 2000).These types of methods are greatly dependent on the tools of bioinformatics, applications and databases. Many of the promising drugs fail in clinical trials after many years of research. The failuret is due to toxicity or problems with metabolism. The ADMET properties of drugs are Absorption, Distribution, Metabolism, Excretion and Toxicity in other words bioavailability and bioactivity. However these properties are typically measured in the wet lab, they can also be assumed with help of bioinformatics software and tools. The wide range of bioinformatics tools and web indexes (search engines) are available for this work (Bissantz et al., 2000; Iskar, Zeller, Zhao, Noort, & Bork, 2011). Once potential lead molecules have been identified the next step is to find their structural and ADMET properties. This commonly consists of the arrangement of changes in accordance with the essential and secondary structure of the compound. The protocol can be enhanced by using software that explores related compounds to the lead candidate (Song, Lim, & Tong, 2009).

MATERIALS AND METHODS

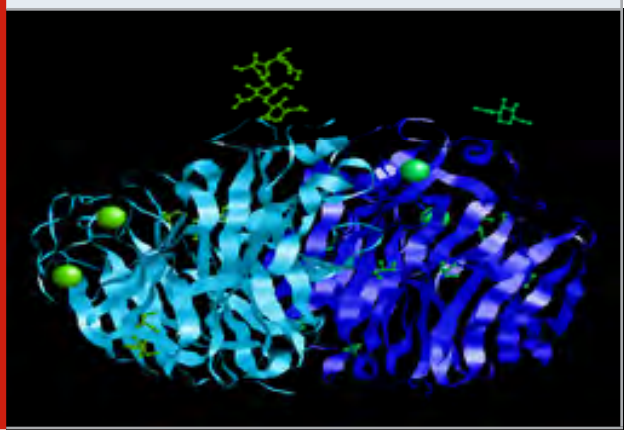
Target Identification: H1N1 Flu (Swine Flu) contains certain proteins. Among all these proteins Neuraminidase of Influenza A virus, H1N1 strain (figure 1) was identified as target. The structure of the protein molecule was downloaded in from Protein Data Bank (PDB) with PDB ID 3BEQ. The experimental detail shows, Resolution[Å]: 1.64, R-Value: 0.180 (obs.) and R-Free:0.210.

Drug Library: Online chemical databases throughout the web were employed to obtain compounds. This mixes were screened which had related structures and properties with Glycerol, (propane-1,2,3-triol) using similarity score. Such properties like Common and IUPAC name of Compounds, Melting and Boiling Point of Compounds, pKa Value, Polarizability, Molecular Weight have been reported (Table 1) (Dias, Filgueira, & Jr, 2008; Fernando et al., 2008).

Docking: Structure files such as .sdf/.mol/.pdb of those chemical compounds were downloaded from the chemical compound database like PubChem, DrugBank, KEGG, ChemSpider. Docking was performed on the structure files of the compounds by using docking programming named PyRx. Binding energy was obtained as a result of the docking process. These chemical compounds were used for the ADMET analysis.

ADMET: ADMET properties of docked chemical substance were scrutinized in dry work experiment. ADMET properties of chemical compounds were examined by online database named Danish QSAR database. Multicase Acute Aquatic Toxicity, Carcinogenicity, Arylhydroxylase Activity, Lethal Body Burden, Bioconcentration, Mutagenicity, Biodegradation, Environmental Partitioning and General Properties etcetera are included in ADMET properties. Forty eight compounds have affirmed ADMET properties using software but out of these forty eight compounds eighteen compounds lastly fulfill maximum of the characteristics of drug target hopefuls. Thus Physical and Chemical Properties, Binding Energy and ADMET of those eighteen compounds have been shown in the outcome tables (Table:1, Table:2 and Table 3 respectively).

Figure 1. Neuraminidase of Influenza A virus, H1N1 strain



RESULTS AND DISCUSSION

In this work total 75 chemical compounds were screened

Table 1. Physical and Chemical Properties of Chemical Compounds

Drug Accession Number	State	pKa	Polarizability	Molecular Weight (g/mol)	Boiling Point	Melting Point	Log P
CID 1030	Liquid	14.9	NA	76.095	187.6 °C	-60 °C	-0.92
CID 259994	NA	NA	NA	76.095	NA	NA	NA
CID 439846	Solid	NA	NA	76.095	NA	NA	NA
DB00302	Solid	(Acidic)4.56, (Basic) 10.22	17.28 Å3	157.2102	NA	>300 °C	0.3
DB00548	Solid	4.55 (at 25 °C)	20.5 Å3	188.2209	286.5 °C	106.5 °C	1.57
DB00858	Solid	(Acidic)19.39, (Basic) -0.88	36.79 Å3	304.4669	NA	151 °C	3.99
DB01561	Solid	(Acidic)19.78, (Basic)	33.84 Å3	288.4244	NA	NA	3.97
DB01856	Solid	-7.1 4.51 (at 25 °C)	4.51 (at 25 °C)	160.1678	342 °C	106 °C	0.51
DB02399	Solid	(Acidic)12.7, (Basic)-3	16.25 Å3	166.1724	NA	NA	-2.7
DB02817	Solid	(Acidic)14.88, (Basic)	18.62 Å3	168.2328	NA	NA	1.24
DB02938	Solid	-2.9 5.15	15.33 Å3	130.1849	NA	NA	2.41
DB03193	Solid	4.95	38.64 Å3	284.4772	383 °C	68.8 °C	8.23
DB03600	Solid	4.9	21.61 Å3	172.2646	268.7 °C	31.9 °C	4.09
DB03703	Solid	(Acidic)18.18,	-0.44	100.1589	160.8 °C	25.4 °C	1.23
DB03741	Solid	(Basic) -1.4 4.97	10.99 Å3	102.1317	NA	NA	1.47
DB01637	Solid	(Acidic)17.11,	41.01 Å3	298.5469	NA	NA	8.18
DB02145	Solid	(Basic) -1.9 16.1 (at 25 °C)	9.21 Å3	74.1216	117.7 °C	-89.8 °C	0.88
DB03900	Solid	(Acidic) 18.03, (Basic) -1.4	8.94 Å3	74.1216	NA	NA	0.54

Table 2. Showing Binding Energy of Chemical Compounds

Drug Accession Number	Binding energy
CID1030	-3.1
CID259994	-3.1
CID439846	-3.2
DB00302	-5.9
DB00548	-5.2
DB00858	-8.7
DB01561	-7.8
DB01856	-8.5
DB02399	-4.9
DB02817	-6.2
DB02938	-4.3
DB03193	-5.3
DB03600	-4.8
DB03703	-4.1
DB03741	-4.2
DB01637	-5.7
DB02145	-.3.5
DB03900	-3.2

as drug target candidate for Neuraminidase of Influenza A virus, H1N1 strain. Chemical and physical properties of these compounds were collected from chemical database. These properties such as pKa value, Molecular weight(MW), its state, Polarizibility, Melting and Boiling Point play essential role in the drug molecule (Dias & de Azevedo Jr., 2008). A weak acid incorporates a pKa worth within the scope between -2 to 12 and pKa values over twelve area unit are said to be alkaline drug. Acid with a pKa worth of less concerning -2 area units are said to be a strong acid. Similarity score denotes relevance of the new chemical with the chemical which may fit the drug target pocket.Polarizability shows the ability of a molecule to be polar; it is dependent upon the charge distribution and effects the bond formation in a chemical compound.Docking results denote that lower binding energy show the higher affinity of the drug target candidate (Modis, Ogata, Clements, & Harrison, 2003). Every drug must comply with its ADMET properties. Amongst all their properties Mutagenicity, Toxicity, In-vitro test and Carcinogenicity are important properties of drug candidates to be examined. Preferably these properties are examined by tests like Ames test, Food and Drug Administration's Center for Drug Evaluation and Research (FDA- CDER) properties on RAT and Mouse, Lethal Concentration, Lethal Dose in human, and Hypoxanthine-guannine phosphoribosyltransferase (HGPRT) test. These properties can also be predicted using ADMET software (Yang & Chen, 2004). Table 3 enlists skin irritation, skin sensitization, Respiratory sensitization and other properties of these molecules. Most of the molecules show negative skin sensitization, skin irritation, respiratory irritation and negative Ames

and Hypoxanthine-guannine phosphoribosyltransferase (HGPRT) test indicates that these molecules can be considered as suitable hits for further drug design. Ames test can be utilized to check mutagenicity of the drug. Positive result indicates that the drug will be the mutagenic (Wadood et al., 2013; Williamson, 2014). CID439846, DB02938 compounds demonstrate positive Ames test, thereupon they are mutagenic and can only be used as drug target candidates after bringing changes in their molecular structure. Remaining compounds show negative Ames test; thus they are not mutagenic and can be considered as drug target candidates.

Certain tests such as-CDER Proprietary Male Rat, FDA Cancer Male Rat, FDA Cancer Female Rat, FDA Cancer Male Mouse, CDER Proprietary Male Mouse, FDA Cancer Female Mouse, -CDER Proprietary Female Rat, -CDER Proprietary Female Mouse are used to determine carcinogenicity. Positive result denotes that the drug will be the carcinogenic(Wadood et al., 2013; Williamson, 2014) DB02817, DB01637 compounds indicate positive carcinogenicity thence they can only be considered as drug candidates after modification in the structure. Remaining compounds show negative carcinogenicity thus they are not carcinogenic and can be considered as drug target candidates. Lethal dose is a way to determine the short term poisoning potential of the material. Lethal concentration values solicit the concentration of the chemical. This toxicity is established on lethal concentration of the drug. The range of lethal concentration 100 – 1000mg/L indicates moderately toxicity of compound and based on that concentration lethal dose is set as 0.5-5 gm/kg. If lethal concentration range is 10 -100 mg/L then it indicates highly toxicity of the substance and hence lethal dose has to be 5-50 mg /kg. If lethal concentration range is 1000 – 10000 mg/L then it indicates slightly toxicity of the compounds and lethal dose is set as 5 – 15 gm/ kg (Wadood et al., 2013; Williamson, 2014). Here screened each of the eighteen compounds have lethal concentration range 100 – 1000 mg/L thus they are sensibly toxic and their lethal dose should be 0.5 – 5 gm/Kg. After evaluating physical and chemical properties, docking and ADMET analysis of all the screened chemical compounds we validate below chemical compounds as potential lead molecules for Neuraminidase of Influenza A virus, H1N1 strain. PROPYLENE GLYCOL - (CID1030), (R)-PROPANE-1,2-DIOL – (CID259994), S-1,2-PROPANEDIOL-(CID439846), TRANEXAMIC ACID – (DB00302), AZELAIC ACID – (DB00548), DROSTANOLONE – (DB00858), ANDROSTANEDIONE – (DB01561), PIMELIC ACID – (DB01856), L-RHAMNITOL – (DB02399), 5-EXO-HYDROXYCAMPHOR – (DB02817), HEPTANOIC ACID – (DB02938), STEARIC ACID – (DB03193), CAPRIC ACID – (DB03600), CYCLOHEXANOL – (DB03703), 2-METHYLBUTANOIC ACID – (DB03741), 3,7,11,15-TETRAMETHYL-HEXADECAN-1-OL – (DB01637), BUTYL ALCOHOL – (DB02145), 2-METHYL-2-PROPANOL.

Table 3. Absorption, Distribution, Metabolism, Excretion and Toxicity Properties

Drug Accession Number		Health End Point		Mutagenicity	In Vitro Tests	Carcinogenicity
	Severe skin irritation	Skin sensitization	Respiratory sensitization	Ames test (Salmonella)	HGPRT	
CID1030	NEG	NEG	NEG	NEG	NEG	NEG
CID259994	NEG	NEG	NEG	NEG	NEG	NEG
CID439846	NEG	NEG	NEG	POS	NEG	NEG
DB00302	POS	NEG	POS	NEG	POS	NEG
DB00548	NEG	NEG	POS	NEG	NEG	NEG
DB00858	NEG	NEG	NEG	NEG	NEG	NEG
DB01561	NEG	NEG	NEG	NEG	NEG	NEG
DB01856	NEG	NEG	POS	NEG	NEG	NEG
DB02399	NEG	POS	POS	NEG	NEG	NEG
DB02817	NEG	NEG	NEG	NEG	NEG	POS
DB02938	NEG	NEG	NEG	POS	NEG	NEG
DB03193	NEG	NEG	NEG	NEG	NEG	NEG
DB03600	POS	NEG	NEG	NEG	NEG	NEG
DB03703	NEG	NEG	POS	NEG	POS	NEG
DB03741	NEG	POS	NEG	NEG	NEG	NEG
DB01637	NEG	NEG	NEG	NEG	NEG	POS
DB02145	NEG	NEG	POS	NEG	NEG	NEG
DB03900	NEG	NEG	NEG	NEG	NEG	NEG

(* POS = POSITIVE) (* NEG =NEGATIVE)

Figure 2. Showing the Docking Pose of PROPYLENE GLYCOL (CID1030) with Neuraminidase of Influenza A virus, H1N1 strain

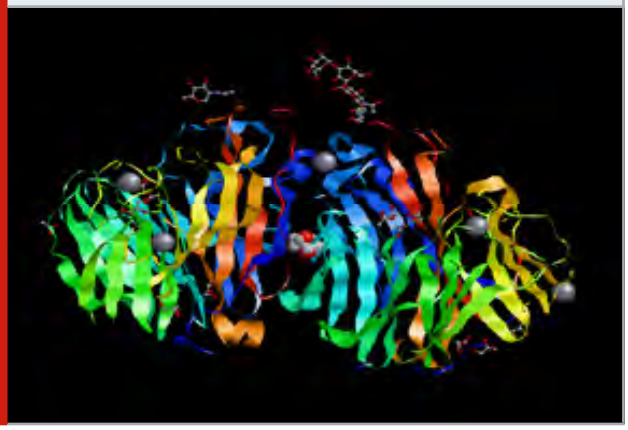


Figure 3. Showing the Docking Pose of (R)-PROPANE-1,2-DIOL (CID259994) with Neuraminidase of Influenza A virus, H1N1 strain

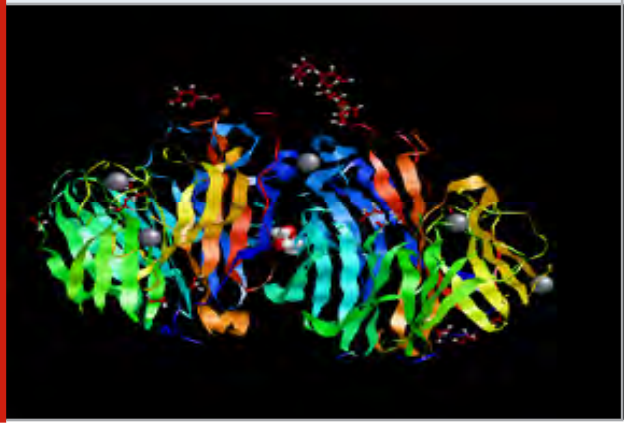


Figure 4. Showing the Docking Pose of S-1,2-PROPANEDIOL (CID439846) with Neuraminidase of Influenza A virus, H1N1 strain

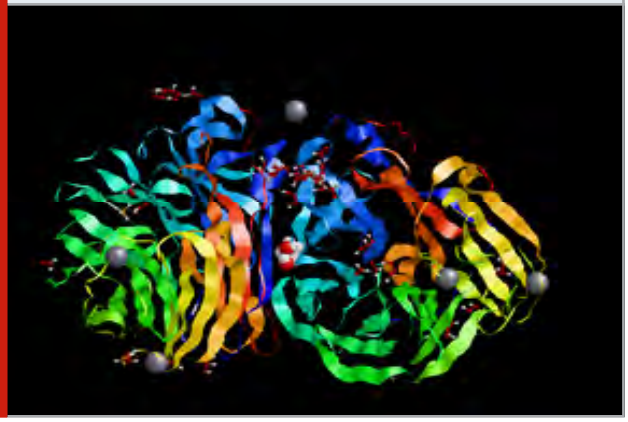


Figure 5. Showing the Docking Pose of TRANEXAMIC ACID (DB00302) with Neuraminidase of Influenza A virus, H1N1 strain

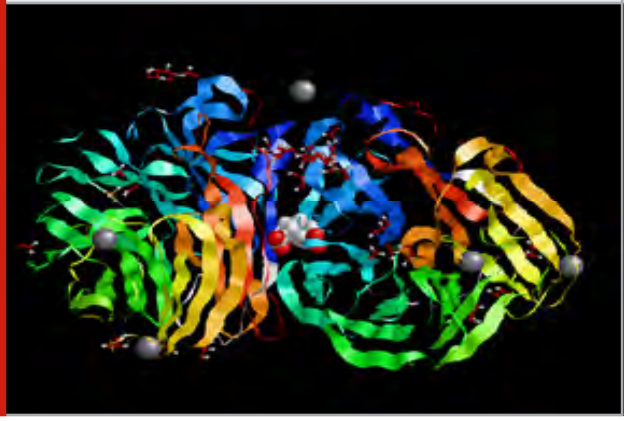


Figure 6. Showing the Docking Pose of AZELAIC ACID (DB00548) with Neuraminidase of Influenza A virus, H1N1 strain

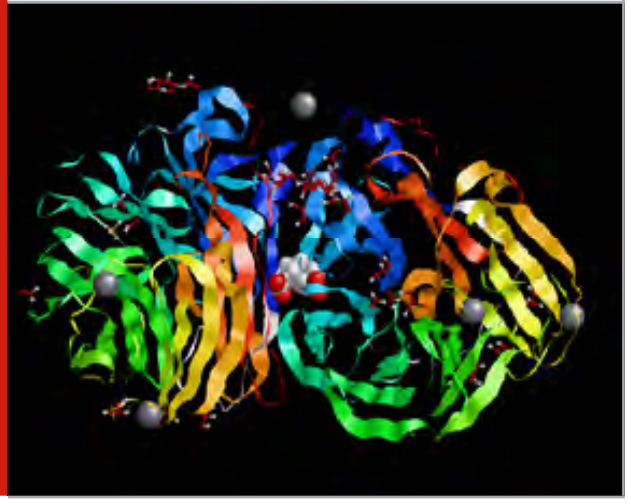


Figure 7. Showing the Docking Pose of DROSTANOLONE (DB00858) with Neuraminidase of Influenza A virus, H1N1 strain

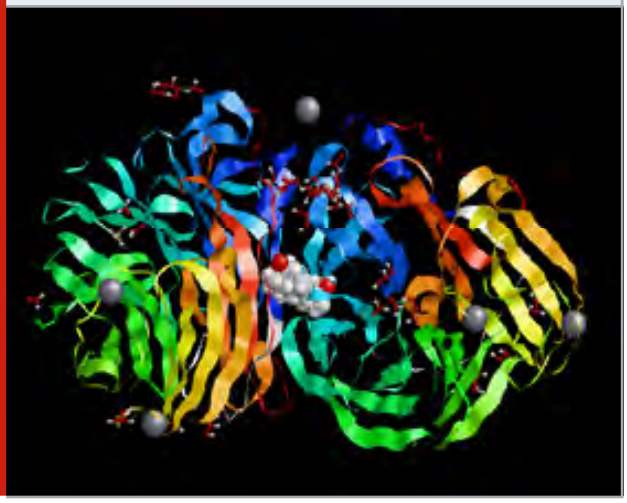


Figure 8. Showing the Docking Pose of ANDROSTANEDIONE (DB01561) with Neuraminidase of Influenza A virus, H1N1 strain

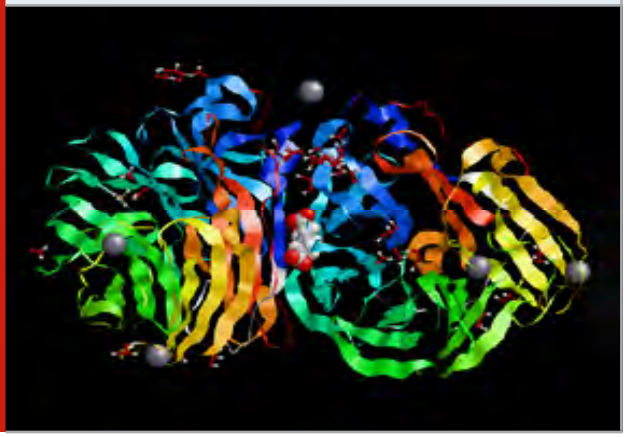


Figure 9. Showing the Docking Pose of PIMELIC ACID (DB01856) with Neuraminidase of Influenza A virus, H1N1 strain

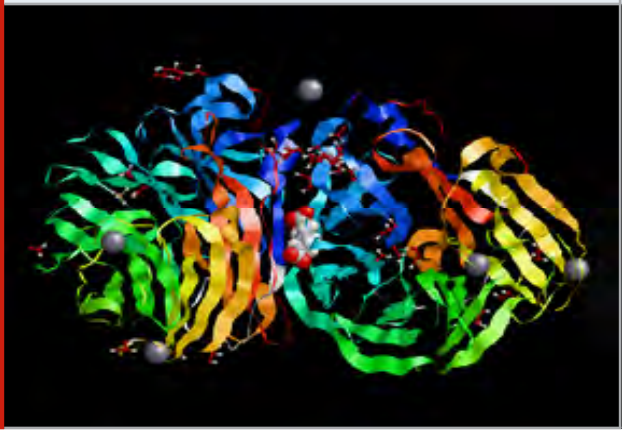


Figure 10. Showing the Docking Pose of L-RHAMNITOL (DB02399) with Neuraminidase of Influenza A virus, H1N1 strain

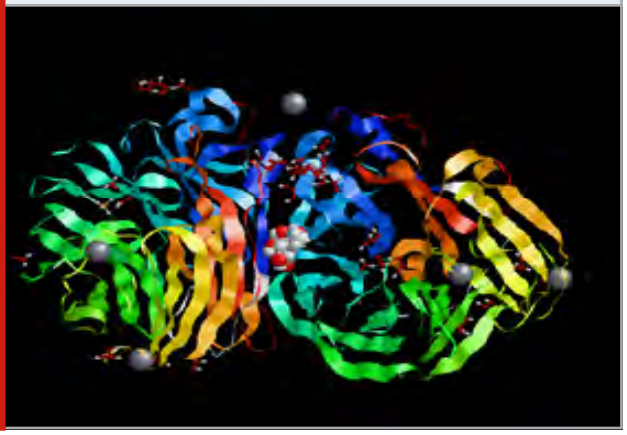


Figure 11. Showing the Docking Pose of (R)-PROPANE-1,2-DIOL (CID259994) with Neuraminidase of Influenza A virus, H1N1 strain

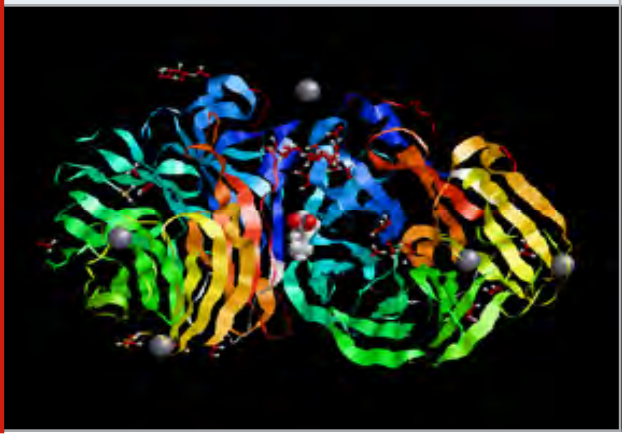


Figure 12. Showing the Docking Pose of (R)-PROPANE-1,2-DIOL (CID259994) with Neuraminidase of Influenza A virus, H1N1 strain

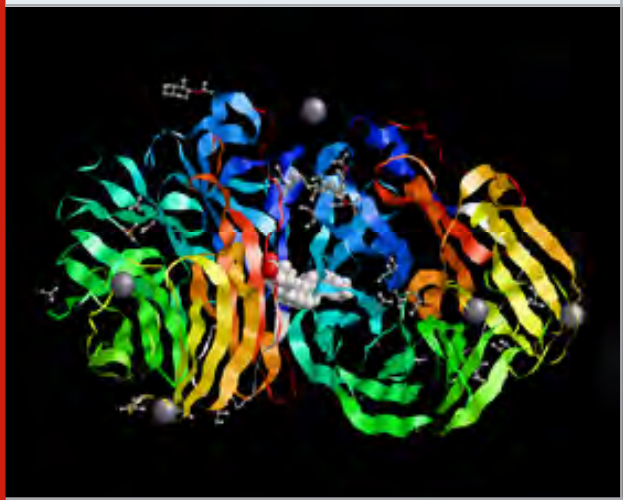


Figure 13. Showing the Docking Pose of (R)-PROPANE-1,2-DIOL (CID259994) with Neuraminidase of Influenza A virus, H1N1 strain

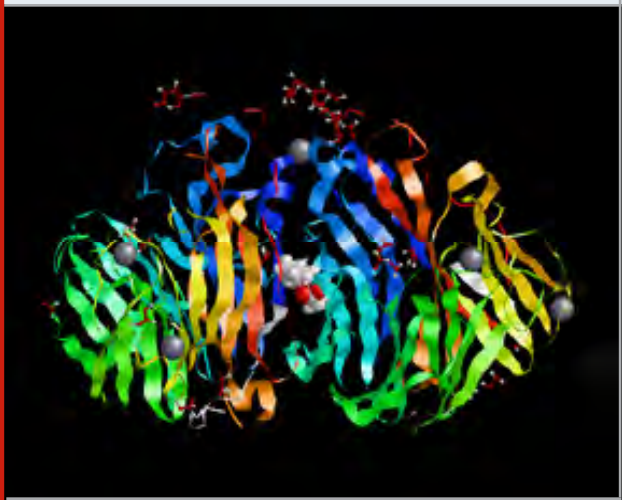


Figure 14. Showing the Docking Pose of CAPRIC ACID (DB03600) with Neuraminidase of Influenza A virus, H1N1 strain

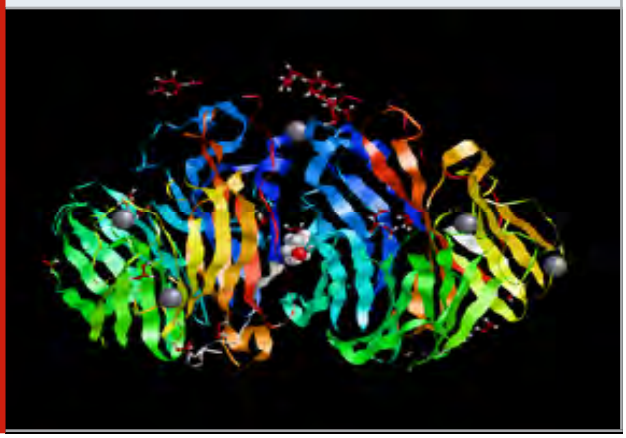


Figure 15. Showing the Docking Pose of (R)-PROPANE-1,2-DIOL (CID259994) with Neuraminidase of Influenza A virus, H1N1 strain

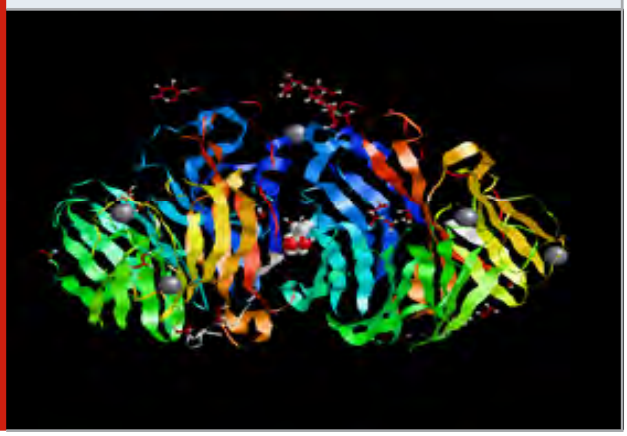


Figure 16. Showing the Docking Pose of 2-METHYLBUTANOIC ACID (DB03741) with Neuraminidase of Influenza A virus, H1N1 strain

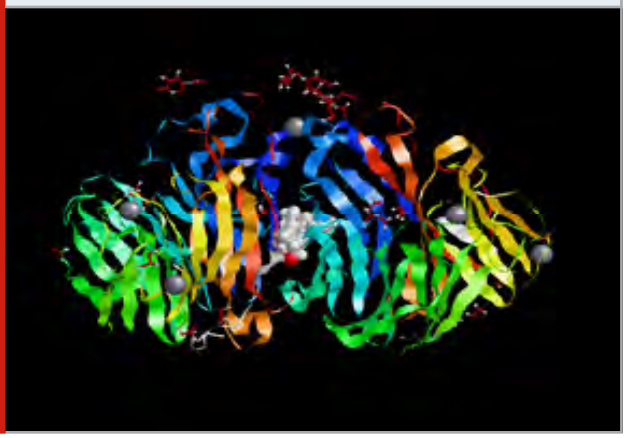


Figure 17. Showing the Docking Pose of 3,7,11,15-TETRAMETHYL-HEXADECAN-1-OL (DB01637) with Neuraminidase of Influenza A virus, H1N1 strain

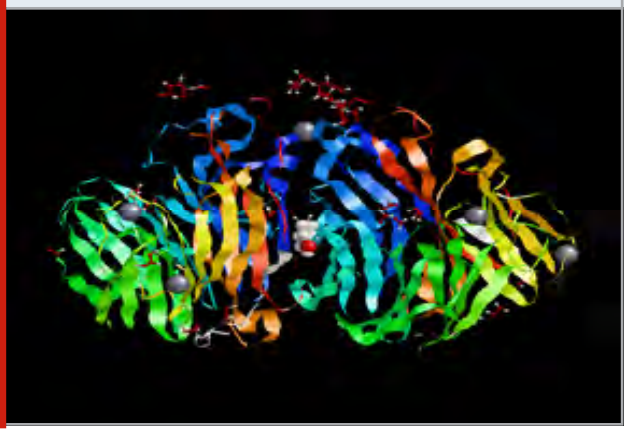


Figure 18. Showing the Docking Pose of BUTYL ALCOHOL (DB02145) with Neuraminidase of Influenza A virus, H1N1 strain

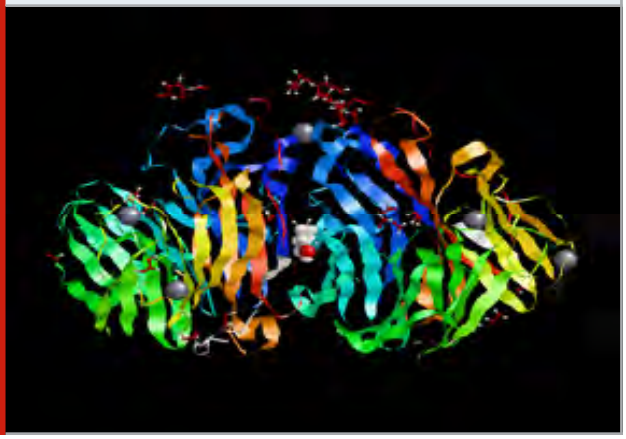
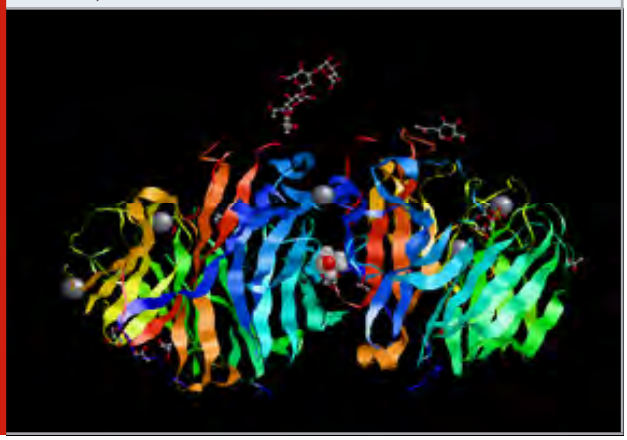


Figure 19. Showing the Docking Pose of 2-METHYL-2-PROPANOL (DB03900) with Neuraminidase of Influenza A virus, H1N1 strain



– (DB03900) Chemical Compounds apart from these are missing in above criteria. Thus, those chemical compounds can't be employed in present form as drug candidate for the same target.

SUMMARY AND CONCLUSION

Swine flu protein NA (neuraminidase), assists newly prepared viruses. It release from infected cells by cutting up sialic acids. It contains ligand named Glycerol, (propane-1,2,3-triol). It was identified as a Drug Target protein. The structure of protein (PDB format) was downloaded from the Protein Data Bank. Ligand Glycerol, (propane-1,2,3-triol) was obtained for further analysis. Similar structure search of different chemical compounds was done which were related to Glycerol, (propane-1,2,3-triol). Lastly, 75 structures were obtained with their physical and chemical properties. Further, Docking and Screening were performed. ADMET study was done. At the end of ADMET analysis forty eight Chemical compounds were found to affect Protein Target.

Further, all parameters of those forty eight chemical compounds were analyzed and following eighteen chemical compounds confirm their potential as drug target candidates. PROPYLENE GLYCOL - (CID1030), (R)-PROPANE-1,2-DIOL – (CID259994), S-1,2-PROPANEDIOL- (CID439846), TRANEXAMIC ACID – (DB00302), AZELAIC ACID – (DB00548), DROSTANOLONE – (DB00858), ANDROSTANEDIONE – (DB01561), PIMELIC ACID – (DB01856), L-RHAMNITOL – (DB02399), 5-EXO-HYDROXYCAMPHOR – (DB02817), HEPTANOIC ACID – (DB02938), STEARIC ACID – (DB03193), CAPRIC ACID – (DB03600), CYCLOHEXANOL – (DB03703), 2-METHYLBUTANOIC ACID – (DB03741), 3,7,11,15-TETRAMETHYL-HEXADECAN-1-OL – (DB01637), BUTYL ALCOHOL – (DB02145), 2-METHYL-2-PROPANOL – (DB03900). From the above eighteen compounds these two are hormone derivatives : DROSTANOLONE – (DB00858), ANDROSTANEDIONE – (DB01561). Thus it can be concluded that above eighteen chemical compounds show great affinity against H1N1 Flu (Swine Flu). Hence, these eighteen chemical compounds can be further analyzed as drug target candidates for H1N1 Flu (Swine Flu) in wet lab for the development of new drug.

ACKNOWLEDGEMENTS

This study was supported by Dr. Indu Dayal Meshri College of Science and Technology and the department of Biotechnology, Hemchandracharya North Gujarat University, Patan, Gujarat, India for Ph.D scholar.

Conflict of Interest statement: We, the authors of the submitted manuscript declare that the work and data present in the manuscript entitled - Design and prediction of Absorption, Distribution, Metabolism, Excretion and Toxicity properties of drugs for H1N1 Flu (Swine Flu) using in-silico approaches is genuine research carried out by us. The work finally belongs to the institutes. We have not misused the data previously published and also have not manipulated the original work.

REFERENCES

Baria, H. G., Solanky, P., Shah, H., & Patel, R. (2017). A study on swine flu (H1N1) awareness among college students of Valsad city. International Journal Of Community Medicine And Public Health, 4(10), 3668–3672. https://doi.org/10.18203/2394-6040.ijcmph20174231

Bissantz, C., Folkers, G., & Rognan, D. (2000). Protein-Based Virtual Screening of Chemical Databases . 1 . Evaluation of Different Docking / Scoring Combinations. J. Med. Chem., 43, 4759–4767.

Coker, R. (2009). Swine flu. BMJ, 10(2), 338–340. https://doi.org/10.3329/jom.v10i2.2810

Dias, R., & de Azevedo Jr., W. (2008). Molecular Docking Algorithms. Current Drug Targets, 9(12), 1040–1047. https://doi.org/10.2174/138945008786949432

Iskar, M., Zeller, G., Zhao, X., Noort, V. Van, & Bork, P. (2011). Drug discovery in the age of systems biology : the rise of computational approaches for data integration. Current Opinion in Biotechnology, 23, 1–8. https://doi.org/10.1016/j.copbio.2011.11.010

Jilani TN, Jamil RT, S. A. (2019). H1N1 Influenza (Swine Flu). Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK513241/

Modis, Y., Ogata, S., Clements, D., & Harrison, S. C. (2003). A ligand-binding pocket in the dengue virus envelope glycoprotein. Proceedings of the National Academy of Sciences of the United States of America, 100(12), 6986–6991. https://doi.org/10.1073/pnas.0832193100

R. M. Kshatriya1, N. V. K., Ganjiwale, J., & , S. D. Lote, S. N. Patel1, R. P. P. (2018). Lessons learnt from the Indian H1N1 (swine flu) epidemic: Predictors of outcome based on epidemiological and clinical profile. Journal of Family Medicine and Primary Care, 7(6), 1506–1509. https://doi.org/10.4103/jfmpc.jfmpc

Rubin, G. J., Amlôt, R., Page, L., & Wessely, S. (2009). Public perceptions, anxiety, and behaviour change in relation to the swine flu outbreak: Cross sectional telephone survey. BMJ (Online), 339(7713), 156. https://doi.org/10.1136/bmj.b2651

Song, C. M., Lim, S. J., & Tong, J. C. (2009). Recent advances in computer-aided drug design. Briefings in Bioinformatics, 10(5), 579–591. https://doi.org/10.1093/bib/bbp023

Stubbs, T. M., & Te Velthuis, A. J. W. (2014). The RNA-dependent RNA polymerase of the influenza A virus. Future Virology, 9(9), 863–876. https://doi.org/10.2217/fvl.14.66

Wadood, A., Ahmed, N., Shah, L., Ahmad, A., Hassan, H., & Shams, S. (2013). In-silico drug design : An approach

which revolutionarised the drug discovery process In-silico drug design : An approach which revolutionarised the drug discovery process. OA Drug Design & Delivery, 1(1), 1–4. https://doi.org/10.13172/2054-4057-1-1-1119

Williamson, E. M. (2014). Synergy : Interactions within Herbal Medicines Synergy and other interactions in phytomedicines. Phytomedicine, 8(5), 401–409.

Yang, J., & Chen, C. (2004). GEMDOCK : A Generic Evolutionary Method for Molecular Docking. Proteins: Structure, Function, and Bioinformatics, 55, 288–304. https://doi.org/10.1002/prot.20035

Young, D., Fowler, C., & Bush, K. (2001). RWJ-270201 (BCX-1812): A novel neuraminidase inhibitor for influenza. Philosophical Transactions of the Royal Society B: Biological Sciences, 356(1416), 1905–1913. https://doi.org/10.1098/rstb.2001.1004

Agricultural Communication

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp-335-339

BBRC Bioscience Biotechnology Research Communications

Genetic Variability, Heritability and Genetic Advances in the Garlic, *Allium sativum* Genotypes

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ABSTRACT

The present investigation was carried out to find out the Genetic variability, heritability and genetic advance in garlic in Central Uttar Pradesh during Rabi season. The experiment was laid out in randomized block design. All the treatments were randomly distributed among the plots and replicate three times. Most of the characters under study exhibited high estimates of heritability viz., plant height, and number of cloves per bulb, bulb weight, dry weight of bulb, weight of 10 uniform cloves and fresh weight of bulb. These characters would be effective in selection for garlic improvement.

KEY WORDS: GARLIC, GENETIC VARIABILITY, HERITABILITY AND GENETIC ADVANCE.

INTRODUCTION

Garlic (*Allium sativum L.*) is an important spice and condiment crop grown throughout the country as well as world. It is one of the most important bulb vegetable crop which have been used since ancient for its culinary, medicinal and health benefits (Narayan et al., 2019). Garlic is the second most important bulb crop after onion. It is an important spice crop belonging to family Alliaceae and botanically known as (*Allium sativum L.*). Growth of garlic mainly depends on the time of planting as the vegetative growth is stimulated under a short photoperiod and low temperature and bulb production is enhanced by a long photoperiod and high temperature (Atif et al,. 2020). The economic yield is obtained from its underground bulb, which is consisted of bulblets, popularly called as cloves. Garlic is used in flavoring foods, preparing chutneys, pickles, curry powder, tomato ketchup etc. It contains protein (6.3%), phosphorus (0.31%), potash (0.40%), calcium (0.03%), magnesium (0.025%), carbohydrates (29%) and a colourless as well as odourless water soluble amino acid called allicin. On crushing the blub clove, an enzyme *allinase* acts upon *allicin* and breaks down to produce *allicin*. Garlic contains volatile oil known as *diallel - disulphide* which is the major flavouring component in garlic. Beneficial use of garlic extract has been found against many fungi and bacteria (Pandey, 1997). Besides the nutritive value of garlic and its use in various forms, it is included in Indian system of medicines (Ayurvedic, Unani and Siddha) as carminative and gastric stimulant to help indigestion and absorption of food. Garlic is a scapigerous foeti perennial medicinal herb with underground compound bulbs covered by outer white thin scales with simple smooth round stem surrounded by the bottom by tublar leaf sheath (Yadav et al, 2018).

Allicin present in aqueous extract of garlic reduce blood cholesterol concentration in human blood (Shankaracharya, 1974). Garlic oil or its juice is

ARTICLE INFORMATION

*Corresponding Author: drmeena1977@gmail.com
Received 9th Feb 2020 Accepted after revision 20th March 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)
A Society of Science and Nature Publication, Bhopal India

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/53

recommended to inhale in cases of pulmonary tuberculosis, rheumatism, sterility, impotency, cough and redness of eyes (Pruthi, 1979). India ranks second after China in area (171.45 thousand hectare) and second in production (923.250 thousand tonnes) of garlic with an average productivity of 4.38 tonnes per hectare (Anonymous, 2009). The major garlic producing states of India are Maharashtra, Madhya Pradesh, Orissa, Rajasthan, Karnataka, Uttar Pradesh and Gujarat. India is one of the most garlic exporting countries in the world. The export was 13008.78 tonnes (worth Rs 1962.66 lakh) in 2010- 11 In Uttar Pradesh, garlic is grown extensively in the districts, Kanpur, Bundel Khand, Mirjapur, Fathepur, Varanasi, Raebareli, Agra, Mathura, Lucknow, Jaunpur and Azamgarh. Despite the importance of crop, so far very limited breeding work has been done. The adequacy of germplasm collection is determined by the amount of genetic variability present in the germplasm. Assessment of variability present in these genotypes is helpful in selection of suitable genotype. Hence, to boost the economy of garlic growing farmers in Uttar Pradesh, there is urgent need to select/develop superior varieties for our zone i.e. Agro climatic zone nine.

MATERIALS AND METHODS

The twenty five genotypes of garlic used for the present investigation were collected from the different parts of India. The experiment was laid down in a randomized block design with three replications. Randomization of the genotype was done. The row to row spacing 15 cm and plant to plant 10 cm in double row of 5 meter length of each genotype were planted. All the standard package of practices and plant protection measures were timely adopted to raise the crop successfully. The Department of Applied Plant Science (Horticulture), BBAU, Lucknow. The twenty five germplasm/ genotypes lines of garlic were obtained from the "Department of Plant Breeding and Genetics, S.K.N. College Of Agriculture, Jobner, Sri Karan Narendra Agriculture University Jobner. Geographically, Lucknow is situated at an elevation of 111 meter above Mean Sea Level in the subtropical climate of Central Uttar Pradesh at 260 56" North latitude and 800 52' East longitudes. The climate of region is subtropical with maximum temperature ranging from 22 0 C to 45 0C in summer, minimum temperature ranging from 3.5 0C to 15 0C in winter and relative humidity ranging from 60-80% in different season of the year.

Ten randomly selected plants from each replication were utilized for recording observations and drawing sample for estimating variability, heritability and genetic advance in garlic. The observations were recorded on plant height (cm), number of leaves per plant, bulb weight (g), number of cloves per bulb, weight of ten uniform cloves (g), bulb yield (q/h-1), fresh weight of bulbs (g), dry weight of bulb (g), fresh weight of leaves (g), dry weight of leaves (g), neck thickness (cm), circumference of bulb (cm), volume of bulb, total soluble solids (%) (0 brix) and Vitamin 'C' (mg/100g). The critical differences for the treatments comparison were worked out, wherever the "F" test was found significant at 5 per cent level of significance. The mean values obtained from the data were used for estimating the analysis of variance. The data were analyzed to work out various components coefficient of variation and heritability in broad sense and expected genetic advance as percent of Mean were estimated as suggested by (Johnson et al, 1955 and Burton and Devane (1952). The data was statistically analyzed for variance and test the significance of variance using the standard procedure by (Panse and Sukhatme, 1961).

RESULTS AND DISCUSSION

The analysis of variance for the design of experiment indicated that the mean squares due to genotypes were highly significant for most of the characters indicating a wide genetic variability among the genotypes (Table 1). The analysis of variance revealed significant differences among the genotypes for different traits viz., plant height, number of leaves per plant, fresh weight of leaves, dry weight of leaves, number cloves per bulb, weight of 10 uniform cloves, fresh weight of bulb, dry weight of bulb, neck thickness, circumference of bulb, volume of bulb, total soluble solids, vitamin C, bulbs weight and bulb yield. The result of the present investigation revealed that there exists significant variations were observed for different characters. The range of 25 genotypes for all the twelve characters is presented in the (Table 2). The highest genotypic variance was observed for bulb yield (288.15) whereas moderate genotypic variance was obtained for number of cloves per bulb (29.63), plant height at 90 DAS (28.63) and bulb weight (27.55) and low genetic variance was obtained for volume of bulb (9.59), weight of 10 uniform cloves (9.38), dry weight of bulb (5.89), fresh weight of leaves (2.81), TSS (2.40), vitamin C (0.46), number of leaves per plant (0.09), neck thickness (0.003) and. Highest phenotypic variance was also observed for bulb yield (421.26) followed by number of cloves per bulb (31.88), bulb weight (29.88), plant height at 90 DAS (29.14) and fresh weight of bulb (26.38).

The highest genotypic coefficient of variation was recorded for weight of 10 uniform cloves (23.03) followed by dry weight of bulb (22.52), number of cloves per bulb (22.02) and bulb weight (21.52) whereas moderate genotypic coefficient of variance was obtained for fresh weight of bulb (19.44), dry weight of leaves (18.36), fresh weight of leaves (17.73), volume of bulb (17.10), bulb yield (14.28) and plant height at 90 days after sowing (10.11) and low genotypic coefficient of variation was recorded for circumference of bulb (8.01), vitamin C (6.38), TSS (3.80), neck thickness (3.48) and number of leaves per plant (3.12). The phenotypic coefficient of variation was also highest for weight of 10 uniform cloves (24.79) followed by dry weight of bulb (24.39), number of cloves per bulb (22.84), bulb weight (22.42), dry weight of leaves (21.98), fresh weight of bulb (21.41) and fresh weight of leaves (20.59) whereas moderate genotypic coefficient of variance was obtained for volume of bulb (19.53), bulb yield (17.27), circumference of bulb (10.49) and plant height at 90 days after sowing (10.20).

Table 1: Analysis of variance for fifteen characters in garlic germplasm

Source of variation	D.f	Plant height (cm)	No. of leaves /plant	Fresh weight of leaves (g)	Dry weight of leaves (g)	No. Of cloves/ bulb	Weight of 10 uniform cloves (g)	Fresh weight of bulb (g)	Dry weight of bulb (g)	Neck thickness (cm.)	Circumfe-rence of bulb (cm)	Volume of bulb (cc)	TSS (%)	Vitamin C (mg/ 100g)	Bulb weight (g)	Bulb yield (q/ha)
Replication	2	0.15	0.91	0.47	0.19	4.32	0.14	3.60	1.18	0.000004	0.52	0.38	4.42	0.02	6.35	110.79
Genotypes	24	86.40**	0.56*	9.40**	1.39**	91.13**	29.62**	69.90**	18.68**	0.0055**	3.63**	31.69**	10.17**	1.90**	84.98**	997.56**
Error	48	0.51	0.31	0.98	0.18	2.25	1.49	4.63	1.02	0.0026	0.70	2.92	2.98	0.52	2.33	133.11

*Significant at 5%
**Significant at 1%

Similar to genotypic coefficient of variation low genotypic coefficient of variation was recorded for vitamin C (9.32), TSS (5.70), neck thickness (6.69), number of leaves per plant (6.68). Heritability a wide range of heritability (21.81% to 98.23%) was observed for the characters under study. High values of heritability were observed for plant height (98.23%), number of cloves per bulb (92.94%), bulb weight (92.19), dry weight of bulb (85.22%), weight of 10 uniform cloves (86.29) and fresh weight of bulb (82.47%), whereas moderate heritability was obtained for volume of bulb (76.67%), fresh weight of leaves (74.17%), dry weight of leaves (69.78%), bulb yield (68.40%) and circumference of bulb (58.38%) and low value of heritability was recorded for vitamin C (46.44%), TSS (44.58), neck thickness (27.14%) and number of leaves per plant (21.81%).

The highest genetic advance was recorded for the character bulb yield (28.92) followed by plant height (10.92), number of cloves per bulb (10.81) and bulb weight (10.38) whereas, fresh weight of bulb (8.73), weight of 10 uniform cloves (5.86), volume of bulb (5.59), dry weight of bulb (4.61), fresh weight of leaves (2.97), TSS (2.13), circumference of bulb (1.56), dry weight of leaves (1.10), vitamin c (0.96 number of leaves per plant (0.28), neck thickness (0.03) and exhibited the low genetic advance. Genetic Advance as per cent of mean the high genetic gain was recorded for dry weight of leaves (30.17%), and dry weight of bulb (19.94%), neck thickness (20.46%) and fresh weight of leaves (18.25%). The genetic advance as per cent of mean were low for number of cloves per bulb (13.30%), bulb weight (13.21%), volume of bulb (13.05%), fresh weight of bulb (12.31%), circumference of bulb (10.11%), vitamin C (9.19%), plant height (6.25%), number of Experimental Results 56 leaves per plant (5.66%) and bulb yield (4.52%). The character TSS recorded the lowest (3.59%) genetic advance.

The range in mean values an indicator of variability revealed high variation for bulb yield, plant height at 90 days after sowing, bulb weight, number of cloves per bulb and fresh weight of bulb and low for other characters. Comparison of coefficient of variation indicated that the phenotypic coefficient of variation was higher than genotypic coefficients of variation for all the characters which indicated effect of environment on the character expression. Among all the characters high GCV and PCV were observed for weight of 10 uniform cloves (23.03) followed by dry weight of bulb (22.52), number of cloves per bulb (22.02) and bulb weight (21.52) in comparison of other characters, indicating the presence of high amount of genetic variability for these traits and selection for these characters would be effectives because the response to selection is directly proportional to the variability present in the experimental material. These results are in broad conformity to earlier researchers (Mohanty and Prusti, 2001) and (Gurjar and Singhania, 2006) in onion.

Estimates of PCV and GCV observed for vitamin C, TSS, neck thickness and number of leaves per plant indicated that the genotypes used had less genetic variability

for these characters. The heritability estimate of a quantitative character is very important as phenotypic expression of a genotype may be altered by environment at various stages of its development. Heritability indicates the effectiveness with which selection for genotypes can be done on the basis of its phenotypic variation. It expresses the extent to which individual phenotypes are determined by their genotypes. The heritability estimates serve as a useful guide to the breeder because selection would be fairly easy for the characters with high heritability. Thus, there would be a close correspondence between the genotypes and phenotype will be attributed to a relatively smaller contribution of the environment to phenotype. But for a character with low heritability, selection may not be effective due to the masking effect of the environment on genotypic effect. The response to selection depends upon the relative magnitude of heritable variation present in relation to the phenotypic variation. Therefore, it is desirable to partition observed variability into its heritable and non-heritable components. (Burton, 1952) suggested that genotypic coefficient of variation along with heritability would give a better idea about the efficiency of selection.

Thus, a character with high genotypic coefficient of variation and high heritability will be more valuable in selection programme. In present investigation, high heritability along with high genotypic coefficient of variance was recorded for number of cloves per bulb, bulb weight and dry weight of bulb, weight of 10 uniform cloves and fresh weight of bulb. This indicates good correspondence between genotypic and phenotypic values and thereby low environmental effect on the expression of these characters.

Thus phenotypic selection might be effective for number of cloves per bulb, bulb weight, and dry weight of bulb, weight of 10 uniform cloves and fresh weight of bulb. Moderate heritability was obtained for volume of bulb, fresh weight of leaves, dry weight of leaves, bulb yield and circumference of bulb and low value of heritability was recorded for vitamin C, TSS, neck thickness and number of leaves per plant.Similarly, high heritability for bulb weight in onion (Mohanty, 2001 and Atif et al., 2020), number of cloves per bulb in garlic (Singh and Chand, 2004), weight of 10 uniform cloves in garlic and fresh weight of bulb in onion (Hossain et al., 2008) and (Singh et al., 2004) in garlic have been reported by earlier researchers. Low heritability in number of leaves in garlic (Agarwal and Tiwari, 2004). Moderate heritability in onion is reported by (Mohanty, 2001 and Yadav et al., 2018). Heritability estimates alone do not provide reliable information about the gene action governing the expression of a particular character and also this does not provide the information of the amount of genetic progress that would result from the selection of best individuals.

Table 2. Estimates of range, general mean, genotypic and phenotypic coefficient of variation in percent of mean for fifteen characters in garlic.

Characters	Mean± S.Em	Range	Genotypic variance	Phenotypic variance	Coefficient of variance		Herita bility (%)	Genetic Advance	G.A. as % of mean
					Genotypic	Phenotypic			
Plant height (cm)	52.91±0.24	36.33-60.70	28.63	29.14	10.11	10.20	98.23	10.92	6.25
No. of leaves per plant	9.38±0.18	8.57-10.13	0.09	0.39	3.12	6.68	21.81	0.28	5.66
Fresh weight of leaves (g)	9.45±0.33	6.00-13.63	2.81	3.79	17.73	20.59	74.17	2.97	18.25
Dry Weight of leaves (g)	3.47±0.14	2.37-4.83	0.41	0.58	18.36	21.98	69.78	1.10	30.17
No. of cloves per bulb	24.72±0.50	16.03-38.27	29.63	31.88	22.02	22.84	92.94	10.81	13.30
Weight of 10 uniform cloves (g)	13.30±0.41	7.90-17.87	9.38	10.87	23.03	24.79	86.29	5.86	18.20
Fresh weight of bulb(g)	23.99±0.72	14.97-31.60	21.76	26.38	19.44	21.41	82.47	8.73	12.31
Dry weight of bulb(g)	10.77±0.34	6.05-16.50	5.89	6.91	22.52	24.39	85.22	4.61	19.94
Neck thickness (cm.)	0.89±0.02	0.81-0.95	0.002	0.004	3.48	6.69	27.14	0.03	20.46
Circumference of bulb (cm)	12.34±0.28	8.76-14.35	0.98	1.67	8.01	10.49	58.38	1.56	10.11
Volume of bulb (cc)	18.11±0.57	12.37-24.00	9.59	12.51	17.10	19.53	76.67	5.59	13.05
TSS (%)	40.69±0.58	36.50-43.17	2.40	5.38	3.80	5.70	44.58	2.13	3.59
Vitamin C (mg/100g)	10.64±0.24	9.24-11.56	0.46	0.98	6.38	9.32	46.84	0.96	9.19
Bulb weight (g)	24.39±0.51	12.50-32.13	27.55	29.88	21.52	22.42	92.19	10.38	13.21
Bulb yield (q/ha)	118.85±3.85	82.60-151.40	288.15	421.26	14.28	17.27	68.40	28.92	4.52

(Johanson et al., 1955) had pointed out that the heritability estimates along with genetic advance were more useful than heritability estimates alone in predicting the response to selection. In the present investigation, genetic advance as per cent of mean was also estimated in order to determine the relative merits of different characters that can be further utilized in the selection programme.

Relative comparison of heritability along with genetic advance as per cent of mean over the characters indicated that characters viz. weight of 10 uniform cloves and dry weight of bulb had high heritability estimates along with high genetic advance as per cent of mean. Earlier, Panse, (1961) had suggested that the genotypic variations for such characters is probably due to high additive gene effects and are least influenced by the environment. The phenotypic selection based on such a character is likely to be more effective for improvement and hence these characters offer good promise for further breeding programme using simple breeding methods. Similar results were also reported by (Singh and Chand, 2004 and Narayan et al., 2019) in garlic. Thus, high heritability along with high genetic advance as a percentage of mean was observed for other characters also like weight of 10 uniform cloves and dry weight of bulb. In this condition selection will be more effective for these characters. However, low heritability and low genetic gain was reported for vitamin C, TSS, neck thickness and number of leaves per plant, therefore, selection in these characters would not be much effective.

CONCLUSION

Based on the present results, it can be concluded that Analysis of variance revealed highly significant differences among the genotypes for all the characters showing thereby considerable amount of genetic variability for all the characters and were amenable to improvement. The estimates of phenotypic coefficient of variation (PCV) were higher than the genotypic co-efficient of variation (GCV) for all the characters. Most of the characters under study exhibited high estimates of heritability viz., plant height, and number of cloves per bulb, bulb weight, dry weight of bulb, weight of 10 uniform cloves and fresh weight of bulb. These characters would be effective in selection for garlic improvement. The number of cloves had positive and desirable association with bulb yield and selection of these traits would be effective for yield improvement in garlic.

REFERENCES

Atif, M. Jawaad, Bakht Amin, Muhammad I. Ghani, Muhammad Ali and Zhihui Cheng (2020). Variation in morphological and quality parameters in garlic (*Allium sativum L.*) bulb influenced by different photoperiod, temperature, sowing and harvesting time. Plants (9): 155

Agrawal A. and Tiwari, R. S. (2004) Genetic variability in garlic. Indian Journal of Agriculture Science, 74: 164-165.

Anonymous. (2009) Vital Agriculture Statistics, Directorate of Agriculture, Jaipur.

Baiday, A. C. and Tiwari, R. S. (1995) Character association and evaluation of garlic germplasm. Recent Horticulture, 2: 117-123.

Burton G. M. and Devane E. H. (1952) Estimating heritability in tall Fescue (*Festuca arundinancea*) from replicated clonal material. Agronomy Journal.; 45: 478-481.

Gurjar R. S. S. and Singhania D. L. (2006) Genetic variability, correlation and path analysis of yield and yield components in onion. Indian Journal of Horticulture, 63: 1.

Hossain, M. S., Khalkuzzaman, M., Rashid, M. H. and Rahman, M. S. (2008) Variability and interrelationship among yield and yield contributing characters in onion (*Allium cepa L.*). Journal of Biological Science, 16: 85-88.

Johnson H. W., Robinson H. F. and Comstock R. E. (1955) Estimates of genetic and environmental variability in soybeans. Agronomy Journal, 47: 314-318.

Mohanty, B. K. (2001) Analysis of genetic divergence in kharif onion. Indian Journal of Horticulture, 58: 260-263.

Mohanty B. K. and Prusti A. M. (2001) Multivariate analysis for genetic divergence in onion seed crop. Progressive Horticulture, 33: 134-137.

Panday, U. B. (1997) Garlic cultivation in India, NHRDF. Nasik, Technical Bulletin No. 7, 8-9.

Panse V. G. and Sukhatme P. V. (1961) Statistical methods for Agricultural workers. ICAR, New Delhi.

Pruthi, J. S. (1979) Spices and Condiments, National Book Trust of India, New Delhi.

Raj Narayan, Arun Kishor, Mukesh S. Mer, Ravindra Kumar Singh and Vivek Kumar Tiwari (2019). Evaluation of Long Day Garlic (*Allium sativum L.*) Ecotypes For Growth, Yield and Quality Performance. Chem Sci Rev Lett., 2019, 8 (32), 247-251

Shankarachaya N. B. (1974) Symposium on Spice Industry in India, AFST, CFTRI, Mysore, pp. 24-36.

Singh, Yudhvir and Chand, Ramesh. (2004) Genetic variability in garlic (*Allium sativum L.*). Haryana Journal of Horticulture Science, 33: 146-147.

Singh, R. K., Dubey, B. K. and Gupta R. P. (2012) studies on variability and genetic divergence in elite lines of garlic (Allium satium L.). Journal of Spices and Aromatic Crops; 21: 136-144.

Shubham Yadav, VP Pandey, Rohit Maurya, Sriom and Akshay Jain (2018). Studies of genetic variability and correlation among the characters of different genotypes of garlic (*Allium sativum L.*) International Journal of Chemical Studies 2018; 6(4): 128-130

Biotechnological Communication

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp-340-344

Biological Software for Recognition of Specific Regions in Organisms

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ABSTRACT

The identification of specific regions in an organism of a plant, animal or a single-celled life form, or something that has interdependent parts and that is being compared to a living creature. User can choose and select any type of organism's image such as plant or animal. After successful selection, the proposed tool automatically analyses and finds the name of the organisms and identifies the specific region with some other related information in an effective manner.In comparative and evolutionary genomics, a detailed comparison of common features between organisms is essential to evaluate genetic distance. However, identifying differences in matched and mismatched genes among multiple genomes is difficult using current comparative genomic approaches due to complicated methodologies or the generation of meager information from obtained results. This research reduces the manual activity to analyze the details. Using this software tool, it easily helps us to know all the related organisms information of specific region and also can make report effectively. This makes the system user-friendly consequently reducing the manual work. The system has been developed with advanced features. The objective of this work is to establish an identification of specific region organisms and related information.The system developed with the main intension to progress an effective and user friendly tool for identification of the specific region in organisms. From the experimental results, the proposed system grasps and more and more identification accuracy.

KEY WORDS: CLASSIFICATION, ORGANISM, ORGANISM SEARCH, REGION DETECTION, SPECIFIC REGION.

INTRODUCTION

This communication describes the main event, ie to identify organisms using protein sorting process, which provides an overview of the computational contributions made to this field, and finally gives a few guiding words to potential users. Gene amplification and sequencing of broad-range gene targets for bacteria and fungi have emerged as important tools to diagnose infections. During the past decade, clinical laboratories have applied PCR amplification and gene sequencing to characterize organisms from culture, and occasionally, to directly detect pathogens from patient samples. Gene sequencing is a more accurate and reproducible method to identify organisms and has increased our ability to capture the diversity of microbial taxa. This is a new technology that has resulted in the identification of unusual organisms and the detection of novel, difficult-to-cultivate organisms, such as Tropherymawhipplei, (.Jung et al., (2019).

However, clinicians are now faced with interpreting microbiological reports that include taxa that are unfamiliar and cannot be assimilated in a meaningful clinical setting. Also, application of broad-range bacterial and fungal PCR directly from clinical material is now more widely available, shifting from research to the clinical setting. This review defines sequence-based

ARTICLE INFORMATION

*Corresponding Author: gripsyjebs@gmail.com
Received 5th Jan 2020 Accepted after revision 26th Feb 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)

*Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/54*

identification of bacteria and fungi, with particular emphasis on improving the understanding of increasingly complex world of microbial taxonomy as it relates to the clinical context. Additionally, this review discusses a rational approach to broad-range bacterial PCR and gene sequencing when applied directly to clinical samples, (Guha et al. 2018).

Cheng and Prayogo, (2014) have presented the Symbiotic Organism Search Algorithm, which is an optimization metaheuristic inspired by the symbiotic relationships that occur along with the organisms in nature. In the last few years, the SOS algorithm attracted increasing attention due to its good performance on various real-world problems, despite the fact that no specific parameter adjustment is required. In this paper, we propose an improved version of Symbiotic Organism Search by modifying the organism's selection strategy. In the offered version of the algorithm, three organisms are selected from the population without having a predefined symbiotic relationship. Once the organisms are selected, an assignment step is conducted to assign each organism to a symbiotic relationship. The testing process was done to analyze the performance of the proposed algorithm using twenty benchmark instances of the flow shop scheduling problem.

The proposed modification improved the performance of the SOS algorithm in the search for the global optimum value in most of the instances. Guha et al. (2018) have shown that user data are aligned, gene information is recognized, and genome structures are compared based on user-defined GenBank files. Information regarding inversion, gain, loss, duplication and gene rearrangement among multiple organisms being compared is provided by geneCo, which uses a web-based interface that users can easily access without any need to consider the computational environment. Recently, Jung et al., (2019) demonstrated the density of marine organisms, size and direction of the current, an early warning model of marine organism invasion based on BP neural network was established. A quantitative evaluation of the intensity of marine organism invasion can be obtained through the designed early warning model. BPNN is used to estimate the relationship between marine organisms density and invasion intensity in different current velocity (Kanimozhi et al., 2016, Guha et al 2018).

Rodrigues et al (2018) proposed by the author, shows an attempt has been made to incorporate a nature inspired optimization algorithm namely symbiotic organism search (SOS) for optimistic results of load frequency control problem. SOS mimics the symbiotic relations received by an organism to stay in the ecosystem. Similarly Yang et al., (2018) proposed control technique while they evaluated a two-area multi-unit multi-source power plant equipped with classical controllers. The power system nonlinearities such as generation rate constraint, governor dead band and time delay of transmission system are considered in the study to appraise feasibility of SOS algorithm. The controller settings are concurrently optimized using SOS algorithm via minimization of integral time square error based fitness function. The tuning ability of SOS algorithm is demonstrated by relative study with other optimization techniques reported before. Furthermore, a frequency stabilizer is included in the LFC loop to provide faster damping to the system oscillations. Simulation results shows that proposed coordinated damping controller exhibits greater dynamic performance in terms of overshoot, peak time, settling time (Prakash and Rajathy 2015).

Salwa (2019), presented DNA polymorphisms in DNA sequences among individuals, groups, or populations. Polymorphism at the DNA level includes a wide range of variations from single base pair change, many base pairs, and repeated sequences. Genomic variability can be present in many forms, including single nucleotide polymorphisms (SNPs), variable number of tandem repeats (VNTRs, e.g., mini- and microsatellites), transposable elements (e.g., Alu repeats), structural alterations, and copy number variations. Different forms of DNA polymorphisms can be tracked using a variety of techniques; some of these techniques include restriction fragment length polymorphisms (RFLPs) with Southern blots, polymerase chain reactions (PCRs), hybridization techniques using DNA microarray chips, and genome sequencing. During the last years, the recent advance of molecular technologies revealed new discoveries of DNA polymorphisms, (Guha etal 2018). Taraswinee et al., (2015) have proposed an efficient and reliable Symbiotic Organism Search algorithm for solving Economic Load Dispatch problem. The superiority of Symbiotic Organism Search is revealed for 6 bus system including the transmission limitation. It has been gathered that Symbiotic Organism Search method gives considerable results for Security Constrained Economic Dispatch problem.

Problem Definition: Most commonly existing method is to handle large numbers of biological elements in high-throughput data like microarray data, various gene set-based methods have been proposed with successful applications, and so on. A key idea of the gene set based methods is to evaluate enrichment of the significant genes in the prescribed gene set; this leads to the results biologically more interpretable. All details are maintained manually. The existing systems don't have the ability to identify these organism facilities. We can only identify several regions.

- Identify several regions only performed.
- Not suitable for all regions.

Proposed System: In the proposed system, user can choose and select any type of organism's image such asplant, animal, etc.After successful selection the proposed tool automatically analysis the training data set and it finds the name of the organisms along with this, it identifies the specific region with some other related information in an effective manner. This project reduces the manual activity analyzing the details. Using this software tool easily know all the related organisms specific region

information also can make report effectively. This makes the system user-friendly thus reducing the manual work.

- Easily identify plant or animal region.
- Easy identification.

Process Involved In Proposed Work Authentication: This module is mainly based on admin. System will check the admin user name and password for authentication. After the verification for authorization the admin can be able to precede the process. All works are done under his control.

Organism and region upload: In this module the admin have to login this application and using their username and password. After successful login admin can upload the Organism and region details in this application. The collection details can be uploaded in this module.

User registration and Login: This module is based on the user control. The user can register their details in the module. After registration, the user can login to the application and view the organism and region details.

Upload image process: In this module used to the analyze region. In this module user upload organism details such as a plant, animal or a single-celled life form, or something that has interdependent parts and that is being compared to a living creature. An example of an organism is a dog, person or bacteria.

RESULTS AND DISCUSSION

This section describes the implementation result and report process. Implementation is the realization of an application, or execution of plan, idea, model, design of a research. This section clarifies the software, datasets and process which are used to develop the research. The proposed system has been successfully implemented which has the effective searching properties and functions. Software Specifications:The system has used Visual Studio.Net framework. And C#.Net has been used for developing the front end and SQL Server for the back end. The reason for using C#.Net is its flexibility. For the experiment, An Intel I3 2.2 GHz processor with 2 Gb RAM is used to measure the execution time and detection speed. The table 4.1 shows the proposed software specification of the proposed application development. The main reason of using .Net framework is, it's a complete GUI and have many unique features to deploy a high featured windows application.

Table 4.1 software specification

Operating System	Windows 10
Front End	ASP.Net , C#
Back End	MS SQL Server

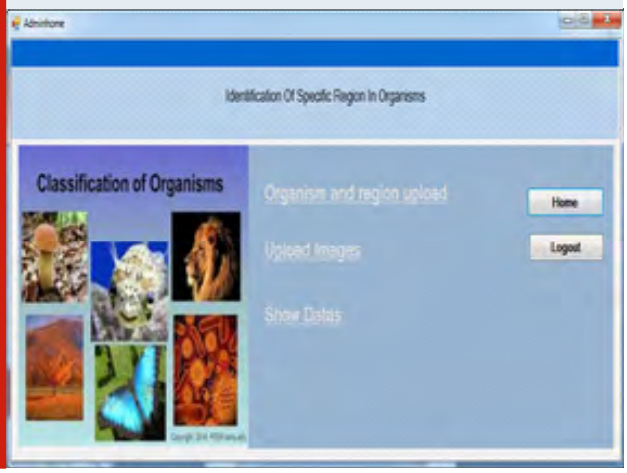
4.2 Screen Shot: It is the initial form for identification of specific region in organism tool. This tool consists of two login, one is Admin login and another one is user login. Which has shown below:

Home Page:

Figure 1

Admin Home: This is the admin home page. The admin process is shown in this form. This page comprises the details like organism and region upload, Upload images, show datas.

Figure 2



Upload Data: This is the page where admin clicks the upload data tab and upload the organism dataset in the page. This page shows the uploaded dataset details like organism name and organism description.

Search Organism: This form refers the detailed search for the organism, if the admin clicks the organism tab; this tool displays the detailed description of an organism. This page isused to search organism. The user can enter organism name and search the organism details.

Find Organism: This page shows the region detection details of organism. The Selected organism name and the region descriptions are displayed. If the user enters sequence id and search the details, it will show the organism name and its descriptions.

Figure 3

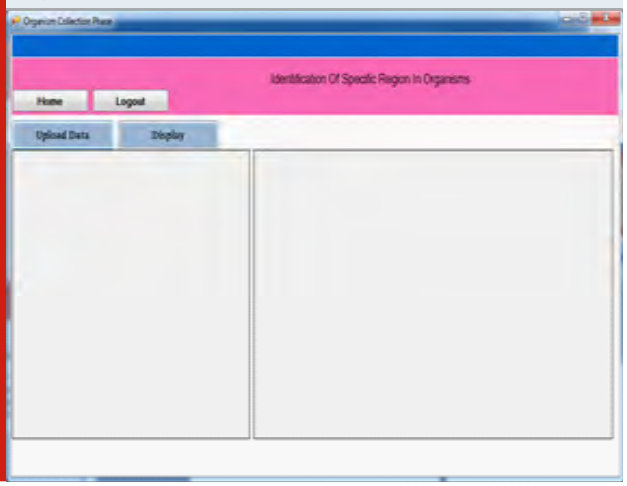


Figure 4

Figure 5

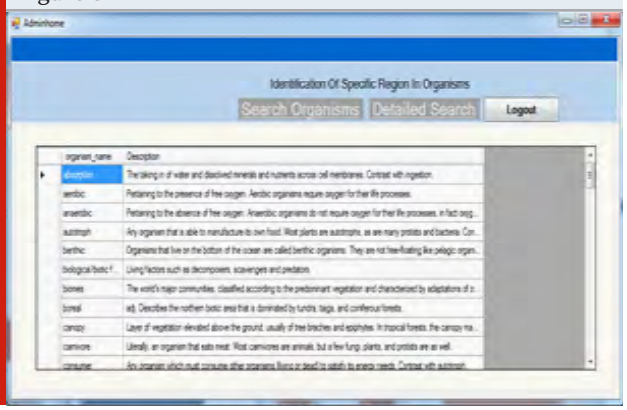


Figure 6

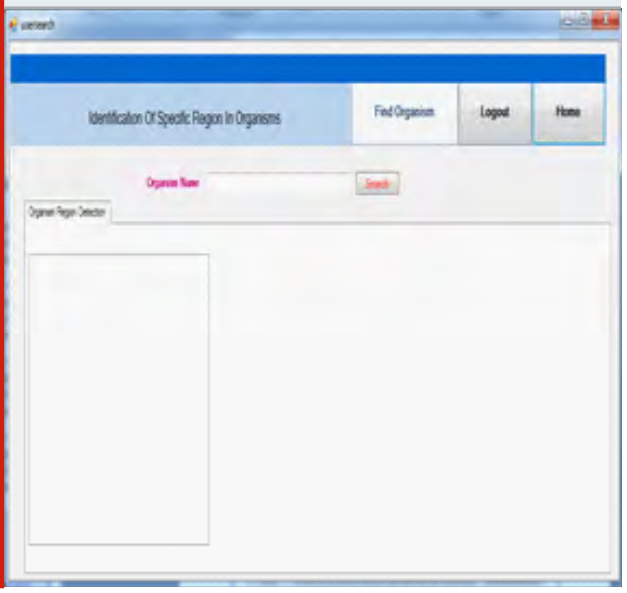


Figure 7

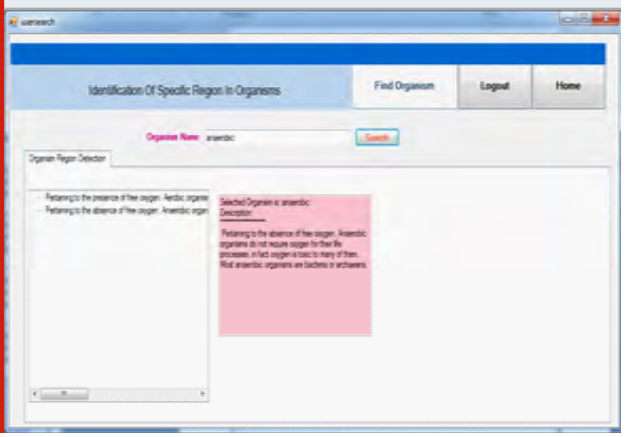
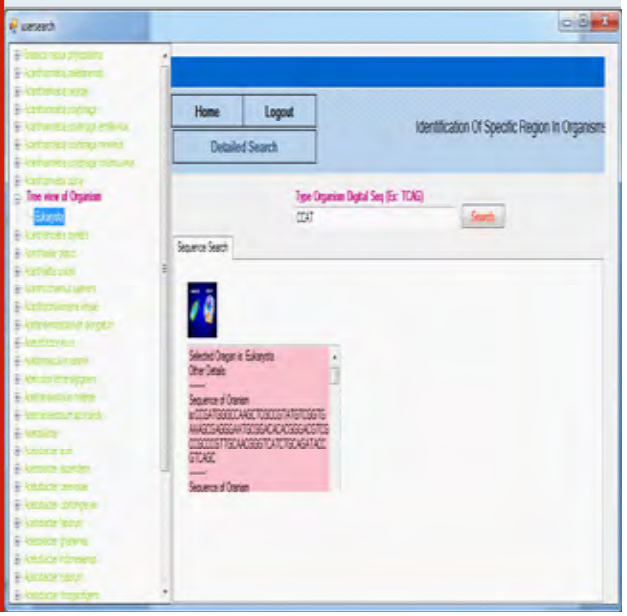


Figure 8



CONCLUSION

It is concluded that the application works well and satisfies both the admin and user. The application is tested very well and errors are properly debugged.System can solve the problems from the literature review and it additionally improves the identification of specific regions in the organism. The experimental results are evaluated by using the DotNet environmental area. In this experiment, it shows that a proposed software tool indicates better identification quality assessment compared to the existing models.

REFERENCES

Cheng, M. Y., &tPrayogo, D. (2014). Symbiotic organisms search: a new metaheuristic optimization algorithm. Computers & Structures, 139, 98-112.http://dx.doi.org/10.1016/j.compstruc.2014.03.007

Guha D, Provas Kumar Roy, Subrata Banerjee,(2018) Symbiotic Organism Search Based Load Frequency Control with TCSC", IEEE, 978-1-5386-3039-6

Jung, J., Kim, J. I., & Yi, G. (2019).geneCo: a visualized comparative genomic method to analyze multiple genome structures. Bioinformatics.https://doi.org/10.1093/bioinformatics/btz596

Kanimozhi, G., Rajathy, R., & Kumar, H. (2016). Minimizing energy of point charges on a sphere using symbiotic organisms search algorithm. International Journal on Electrical Engineering and Informatics, 8(1), 29-44.

Prakash, S, &tRajathy, R. (2015). Implementation of symbiotic organism search algorithm for extracting maximum power from the pv system under partially shaded condition. International Journal of Control Theory and Application, 8(5), 1871-1880.

Rajathy, R., Taraswinee, B., & Suganya, S. (2015, March). A novel method of using symbiotic organism search algorithm in solving security-constrained economic dispatch.In 2015 International Conference on Circuits, Power and Computing Technologies [ICCPCT-2015] (pp. 1-8).IEEE.

Rodrigues, L. R., Gomes, J. P. P., Neto, A. R. R., & Souza, A. H. (2018, July). A Modified Symbiotic Organisms Search Algorithm Applied to Flow Shop Scheduling Problems. In 2018 IEEE Congress on Evolutionary Computation (CEC) (pp. 1-7).IEEE.

Salwa Teama (2019), DNA Polymorphisms: DNA-Based Molecular Markers and Their Application in Medicine Genetic Diversity and Disease Susceptibility.

Yang, L., Humin, Z., & Wei, M. (2018). Early warning model for marine organism detection based on BP neural network. In 2018 37th Chinese Control Conference (CCC) (pp. 1909-1914).IEEE.

Biotechnological
Communication

BBRC
Bioscience Biotechnology
Research Communications

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp-345-352

Identification and Molecular Characterization of Tumour-Associated antigen Expressed on Hepatocytes in Mice Exposed to Diethyl Nitrosamine

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ABSTRACT

The study of antigens on tumor cells and their role in immune response against the tumor is an important aspect of tumor immunology, and the recognition and characterization of novel tumor associated antigens (TAA) is fundamental to the advancement of cancer immunotherapy. In the present investigation an attempt has been made to purify a highly over-expressed membrane surface glycoprotein of approximately 58 kDa molecular size on liver cell of Swiss albino mice exposed to diethylnitrosamine (DEN), an established hepato carcinogen in rodents. Carcinogenesis induction was monitored by assays of gamma glutamyl transpeptidase (GGT), acetylcholine esterase (AChE), glutathione S-transferase (GST) activities and the glutathione level (GSH) in liver. Animals exposed to DEN showed cell distortion and extensive necrosis as observed in the histological examination and transmission electron microscopy (TEM) study of the liver tissues. An over-expressed glycoprotein on the membrane surface of hepatocytes was purified by ion-exchange chromatography, further characterized by SDS-PAGE and identified as TAA. The glycoprotein contains significantly high carbohydrate moieties and targeted for specific active immunotherapy in mice. The preliminary results of our studies suggest that an effective immune response could be achieved against TAA formulated with either alhydrogel or CFA and could eventually be used to counter tumor regression. The results suggest that specific proteins are uniquely susceptible to alterations in expression and carry implications for the further investigation of their potential as therapeutic and prognostic markers.

KEY WORDS: CARCINOGENESIS RESPONSE MODULATION, CANCER IMMUNOTHERAPY DIETHYLNITROSAMINE, HEPATOCARCINOGEN, TUMOR ASSOCIATED ANTIGEN.

ARTICLE INFORMATION

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Received 9th Jan 2020 Accepted after revision 29th Feb 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

Clarivate Analytics crossref

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/55

INTRODUCTION

Hepatocellular carcinoma (HCC) is being reported as the third most frequent cause of cancer-related death worldwide and it accounts for around 90% of primary liver cancers .In the early 1970s, there were outbreaks of liver disorders, including cancer, in various farm animals in Norway. Intensive investigation revealed that all the affected animals had consumed rations containing herring meal, which had been preserved by the addition of relatively large amounts of sodium nitrite. Further investigation showed that the herring meal contained dimethylnitrosamine (DMN), a chemical class of N-nitroso compounds (NOCs). Later it was found that DMN was formed in the fish meal as a result of chemical reaction between dimethylamine, a commonly occurring amine in fish meal, and a nitrosating agent that formed from the sodium nitrate (Liao et al., 2001; Brown, 1999). DEN is known to induce damage in many enzymes involved in DNA repair and is normally used to induce liver cancer in experimental animal models (Heindryckx et al., 2009 Wong et al., 2016, Santos et al., 2017; Sklavos et al., 2018 Kaltenecker et al., 2019).

Diethyl nitrosamine (DEN) is a well-established hepatocarcinogen over a wide range of doses with or without promotors in experimental animals (Matuoka et al., 1993; Pascale et al., 1993; Williams et al., 1993; Montesano, 1981; Pariat and Sharan, 1995). This derivative of NOCs has very high degree of cell and tissue specificity depending on its systemic distribution (Montesano, 1981). DEN in its parent form is not active carcinogen. It requires metabolic activation induced by cytochrome P450s and Flavin dependent-oxidases that yield electrophiles which readily alkylate nucleophilic sites in DNA. Exposure to DEN has also been associated with hepatocellular accumulation of reactive oxygen species (Hanahan and Weinberg, 2000), which may lead to oxidative damage to DNA and other nucleophiles, that may further enhance DEN-induced hepatocarcinogenesis (Arboatti et al., 2018). During nitrosamine-induced hepatocarcinogenesis in mice, processes such as gene expression, replication and differentiation are altered in the transformed cells, which might lead either to the expression of a novel antigen unique to that tumor or the differential expression of some membrane surface proteins usually called as tumor-associated antigens (TAA) (Raghupati, 1996).

Many studies have suggested that TAA may be used as a potential candidate for active immunotherapy against cancer (Raghupati, 1996; Goydos et al., 1996; Rodolf et al., 1996; Xing et al., 1995). The present investigation was an attempt with an objective to identify tumor-associated antigen (TAA) on liver cells of DEN-exposed mice, its subsequent purification and to study its immunogenicity upon immunization in mice. The interest in the study of these carcinogens stemmed from the finding that N-nitroso compounds are present in industrial occupational hazards. In recent years these compounds are of great concern, not only to industrial workers, but also to the population at large and have emerged as one of the most important classes of environmental carcinogens.

MATERIALS AND METHODS

Acetylcholine chloride, Acrylamide, Albumin bovine, Ammonium per sulphate(APS), Brilliant Blue G, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), Ethylenediamine tetra acetic acid (EDTA), L-γ-Glutamyl-p-nitroanilide, Glycylglycine, 2-Mercaptoethanol, N,N'-Methylene bisacrylamide, N-Nitrosodiethylamine (DEN), Tween 20, Sephacryl S-200 HR, Q-Sepharose Fast Flow, Sodium Lauryl Sulphate, N,N,N',N'-Tetramethylethylene , Triton-X-100, Trizma base. Trypsin,Protein Molecular weight markers, Rabbit anti-mouse IgG-HRP, TMB/H2O2, Freund's Complete Adjuvant (CFA), Freund's Incomplete adjuvant (IFA). Cancer induction- Healthy Swiss albino balb/c mice 6-8 weeks old weighing approximately 25g from inbred colony, maintained at controlled temperature (20 ± 2 o C), provided with standard mouse pellets and drinking water ad libitum (National Research Council, 1996) were administered an aqueous solution of DEN at a dose of 10 mg kg-1 body weight by intravenous route at weekly intervals for a period of 16 weeks. Sham treated age-matched normal Swiss albino balb/c mice served as control.

Tissue preparation for enzyme assays- After completion of DEN treatment, mice were killed by cervical dislocation. The liver was then quickly excised and removed, rinsed in chilled normal saline (0.9 % NaCl), blotted dry and weighed. A 10 % homogenate of the liver was prepared in chilled 0.25 M sucrose and centrifuged at 20,000 x g for 30 min at 4°C. The resulting supernatant was used for the enzyme assays as described below. The total protein content in the supernatant was estimated by Bradford's method (Bradford, 1976). γ-Glutamyl transpeptidase assay- The GGT activity was assayed using the method described by Meister (Meister et al., 1975) with slight modification. Acetylcholine esterase assay- AChE activity was assayed according to the method described by Ellman (Ellman et al., 1961) with slight modificationGlutathione-S-transferase assay- GST activity in the liver was determined by the method described by Habig and Jacoby (Habig and Jacoby, 1981) with slight modificationGlutathione assay-GSH levels were determined using the method described by Ellman (Ellman,1959) with slight modifications. The concentration of GSH in each test sample was read from a standard GSH curve.

Histological study- Microtomy technique described by Ratcliffe (Ratcliffe, 1983) was employed for the histological examination of liver tissues of DEN-treated and age-matched normal control mice. Glycoprotein extraction- Glycoproteins extraction from liver tissues of DEN-exposed and normal control mice was based on the method described Liao (Liao et al., 1985). Gel electrophoresis- The final 1-Butanol extracts containing membrane surface glycoprotein were analysed by SDS-PAGE. The sample were run on acrylamide gel (10%) at a current of 60 mA and constant voltage of 200 V

using Mini-PROTEINR Electrophoresis Cell. The gels were stained with Coomassie brilliant blue.

Ion-exchange chromatography- The crude 1-butanol extract was dialysed in phosphate buffer (0.05 M, pH 6.5) overnight. The dialysed sample was then loaded on Q-Sepharose (Anion-exchanger) column pre-equilibrated with Tris HCl buffer (0.01 M, pH 7.5). The column was washed with the tris buffer for 60 min and the bound proteins from the column were then eluted with a salt gradient of 0 – 0.1 M NaCl for another 60 min. Fractions of 2 ml were collected and read at 280 nm. Preparative SDS-PAGE analysis of bound proteins- The fractions corresponding to different peaks in the bound region obtained from anion exchanger were subjected to run on preparative SDS-PAGE for the final purification of the desired protein. A longitudinal section of the gel corresponding to the crude extract lane was cut and stained. This was used as a reference to locate the exact position of the TAA in the rest of the gel. The portion of the gel corresponding to the TAA was cut out of the unstained gel. It was cut into small pieces and homogenized in 3% 1-butanol. The homogenate was centrifuged at 8000 x g for 30 min at 4 ^{0}C. The supernatant was collected and the pellet was re-suspended in 3% 1-butanol and centrifuged. The supernatant collected was pooled, dialysed against distilled water overnight and lyophilised. It was then run on a SDS-PAGE gel for homogeneity of TAA.

Molecular weight determination- A fresh, filtered solution of Blue Dextran was prepared. This was applied to the column to determine the void volume (Vo), and to check the column packing. The selected calibration proteins were dissolved in the running buffer and applied to the column. The elution volumes (Ve) for the standards were determined by measuring the volume of the eluent from the point of application to the centre of the elution peak. A calibration curve of logarithm of their molecular weights versus Ve/Vo was prepared. The sample is applied in a volume < 2 % of the total column volume (Vt) and the elution volume (Ve) of the molecule of interest is determined. The corresponding molecular weight of the protein was read from the calibration curve after determining its elution volume. Estimation of neutral hexoses- The total neutral hexoses in purified TAA was determined using the method described by Spiro (Spiro, 1966a). Estimation of total sialic acid- Amount of total sialic acid in TAA was determined by Resorcinol-HCl assay (Spiro, 1966b). Immunization of animals- Three groups of mice consisting of at least six each were immunized separately with three different antigen formulations which are (a) TAA in normal saline, (b) TAA adsorbed on Alhydrogel (1:1) and (c) TAA emulsified with Freund's adjuvant (1:1) by intra-muscular route. Formulations (a) & (b) were administered thrice at weekly intervals as primary immunization.

However animals immunized with formulation (c) received only one injection. The booster injection was given on 30th day after the primary immunization. Mice that were administered only saline served as control. Blood was collected from the animals on 3rd and 7th day after the booster injection. The serum obtained was pooled and stored at 20oC. ELISA- Microtitre plate wells were coated with 50 µl of 1 µg/ml TAA in coating buffer. The plate was covered and incubated overnight at 4°C. The wells were then washed with washing buffer for 2-3 times. This was followed by blocking the wells with 100 µl of blocking buffer and incubated overnight at 4°C. After washing the wells again, 50 µl of serially diluted serum (in blocking buffer) was applied to each well of the plate and was incubated for 2 hr at 37°C followed by washing with washing buffer. The commercially supplied anti-mouse-HRP conjugate was diluted 1000 times (in blocking buffer) and 50µl of it was added to each well and incubated for 2hr at 37°C followed by washing step. 50µl of the substrate (20-fold diluted in distilled water) was added and incubated in the dark for 10 min at room temperature. The reaction was stopped by adding 50µl of 1M H2SO4. The absorbance of resulting yellow coloured product was read at 450 nm.

RESULT AND DISCUSSION

Cancer induction- Upon complete treatment, the mice were sacrificed, and the liver was examined for any visible morphological changes. The liver of DEN-treated mice showed hardening and swelling in some portions of the liver as shown in Fig. I-b & c respectively. However, in some cases nodule formation were also seen in liver (Fig. I-d & e). No such changes observed in the liver of any sham-treated normal control mice. Marker enzyme activities- The marker enzyme activities viz GGT, AChE, GST and the GSH level in the liver tissues of DEN-treated and that of untreated mice were monitored separately in the supernatant fractions. The GGT and AChE activities were markedly elevated upon DEN exposure in comparison to that of normal control. How7ever, enzyme GST activity in DEN-exposed animals was found significantly low in comparion to normal control. The GSH level was also found elevated significantly in DEN-exposed animals as compared to the normal control (Table-I). Liver histology- The liver tissues of DEN-exposed mice and age-matched normal control mice were examined microscopically for any morphological differences in liver cells. When compared with the control, DEN-exposed liver micro section showed many changes in liver cells such as loss of regular arrangement, variation in the shape and size, multi-nucleated cells, and loss of contact with the neighbouring cells. In contrast, the liver cells of the untreated normal control showed mono- and bi-nucleated cells only with a regular morphology and well defined outlines (Fig.II).

SDS-PAGE analysis of membrane glycoprotein- The membrane glycoproteins 1-butanol extracts obtained from normal control and DEN-exposed mice liver tissues were analysed on SDS-PAGE (Fig. III). A glycoprotein of approximately 58 kDa molecular weight was prominently over-expressed in the treated animals as compared to normal control and was identified as TAA. Ion-exchange chromatography- An anion-exchanger (Q-Sepharose Fast Flow) was employed for the purification of the desired

glycoprotein. The elution profile of anion-exchanger is shown in Fig. IV. The desired specific glycoprotein eluted out in the bound fraction and present in in all the three peaks 2, 3 and 4 that was confirmed when run on SDS-PAGE. All the peaks were pooled together and run on preparative SDS-PAGE for final purification as discussed in methods and materials section. The glycoprotein was analysed for its recovery from the gel by running it on SDS-PAGE to insure its homogeneity and molecular weight (Fig. V). The purified TAA obtained from gel was subsequently used for molecular weight determination, carbohydrate analysis and immunization.

Molecular weight determination- The molecular weight of the purified protein was determined from the plot of log molecular weight versus Ve/Vo of several known calibration standards. The logarithm of their respective molecular weights (Log Mr) was plotted against the ratio of their elution volume to void volume (Ve/Vo). From the calibration curve the molecular weight of the purified glycoprotein sample was calculated to be 57.95 kDa (Fig.VI). Estimation of neutral hexoses, sialic acid and protein- Neutral hexoses, sialic acid and the polypeptide contents of the glycoprotein were estimated, and the results are shown in Fig. VII. The protein content in the sample was estimated by Bradford's method, while the amount of sialic acid and neutral hexoses in the sample was calculated as described in method section. The total carbohydrate to peptide ratio was found to be >2:1, which signifies TAA as highly glycosylated protein. ELISA- Presence of anti-TAA antibody in the immune sera were determined using ELISA. The assay carried out showed significantly very high titer of anti-TAA antibodies in the immune serum obtained from of all the three antigenic formulations. The presence of antibodies in test serum were carried out using ELISA and high titer was observed even up to a dilution of 1:256 as shown in Fig. VIII.

Diethynitrosamine (DEN), an important environmental and food carcinogen was employed as the chemical carcinogen to induce carcinogenesis in Swiss albino mice. DEN is a genotoxic, carcinogenic nitrosamine having its place among N-nitrosodialkylamines (Fausto et al., 2010). DEN was administered intravenously at a weekly dose of 10 mg/kg body weight for a period of 16 weeks. DEN is well known for contributing to

Figure 1: Liver Photographs: (a) Normal Control Mice; (b), (c), (d) And (e) DEN-Treated Mice. Arrows Indicate The Hardening, Swelling And Nodule Formations In The Liver Of DEN-Exposed Mice

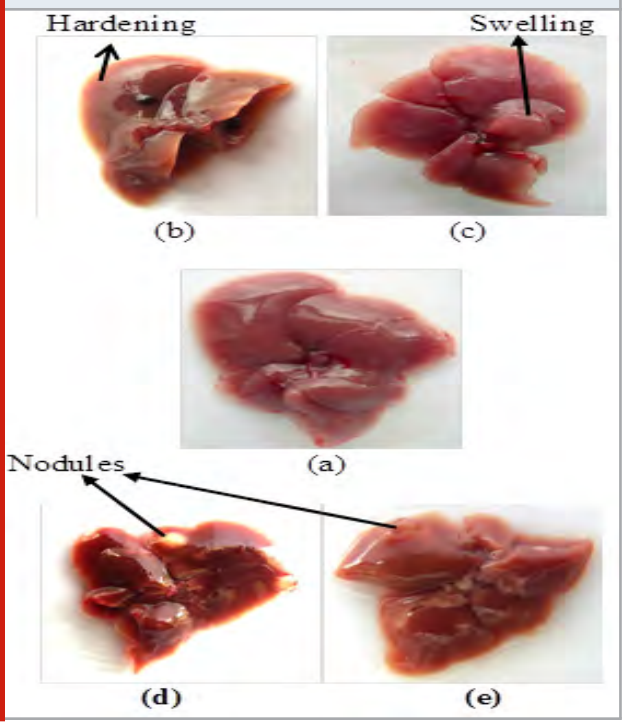
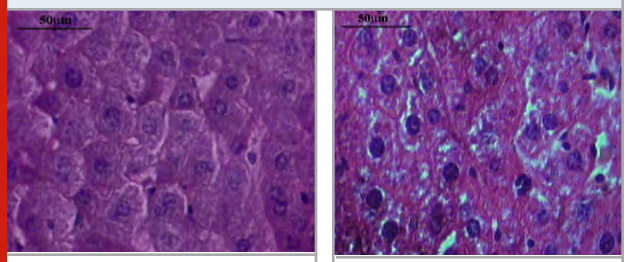


Figure 2: Microphotographs Of Histological Section Of Liver From Normal Control Mice (A) And DEN-Treated Mice (B). The Slides Were Stained By Haematoxylin And Eosin. Slides Were Examined Microscopically After Drying. Magnification X 40.



(A) (B)

Table I. Marker Enzyme Activities And GSH Level In The Liver Tissue Of DEN-Treated And Sham-Treated Normal Control Mice. Enzyme Activities Were Measured In The Supernatant Fractions Of Liver Tissue

Marker Enzymes	Normal control Mean ± SEM (n=12)	DEN-treated Mean ± SEM (n=12)	Fold increase / decrease	Test of significance
GGT activity (U/mg protein)	0.0107 ± 0.001	0.0179 ± 0.0005	~1.7	P < 0.0003
AChE activity (U/mg protein)	0.0168 ± 0.002	0.0558 ± 0.002	~3.3	P < 0.0001
GST activity (U/mg protein)	0.5894 ± 0.019	0.3705 ± 0.011	~1.6	P < 0.0001
GSH level (mg/gm tissue)	0.5894 ± 0.003	1.7480 ± 0.014	~3.0	P < 0.001

the pathogenesis in hepatocarcinogenesis (Chen et al., 2008). Reports available state that the initiation of carcinogenesis especially in the liver is induced only upon coupling it with proliferative stimuli (Ying et.al.,1982), however, this protocol successfully triggered initiation of carcinogenesis in the experimental mice without coupling any proliferative stimulus with DEN. Morphological changes such as swelling, hardening of the tissue and nodule formation seen in the liver of the treated animals signify carcinogenesis induction upon DEN-exposure (Fig.II). Carcinogenesis induction in liver of DEN-exposed mice was followed by enzyme marker assays viz. GGT, AChE, GST and the level of GSH and compared with age-matched normal control animals. These marker enzymes activities in liver have been recognised as a positive marker for hepatocytes which have undergone malignant transformation (Boelsterli, 1979). Increased generation of ROS and abnormal production of antioxidant enzymes in liver tissues have been reported in many models of DEN-induced hepatocellular carcinoma (Sivaramakrishnan et al., 2008).

DEN-treated mice liver showed marked elevation in GGT and AChE activities as compared to the normal control (Table-I). The hyper-activation of these enzymes in DEN-exposed mice signifies hepatocellular transformation. Glutathione-S-transferase (GST) was another marker enzyme studied to monitor cancer induction. It plays a protective role for the cell towards cytotoxic and mutagenic effects of electrophiles and perhaps it evolved to protect cells against reactive oxygen metabolites. Upon DEN-exposure the GST activity in liver drastically decreased as compared to normal control mice (Table-I). The decrease in GST activity in mice exposed to DEN signifies that it leaves the cell vulnerable to these agents. Such alterations have been shown earlier in mice exposed to DBN (Alam et al., 2005). Intracellular GSH plays an important role in a series of physiological functions in the plasma membrane, particularly in tumor cells. Because of its reducing properties of GSH, it can inactivate some carcinogens, protect DNA against free radicals that are damaging, protect the integrity of different tissues, and prevent lipid peroxidation (Traverso et al., 2013). Reduced glutathione acts as a nucleophile that protects the DNA and other components from attack by the reactive forms of these carcinogens (Moldeus and Jernstrom, 1983). High intracellular levels of glutathione would also prevent oxidative damage. An increase in GSH level was observed in the liver tissue of DEN-exposed mice (Table-I).

Figure 3: SDS-PAGE Analysis Of 1-Butanol Extract Of Membrane Surface Glycoproteins Obtained From Liver Of DEN-Treated And Normal Control Mice. A Gel Consisting Of 10% (W/V) Acrylamide Was Used. Sample Of 25 µg Each Were Run Under The Reduced Condition. The Gel Was Fixed In Methanol/Acetic Acid And Stained With Commassie Brilliant Blue. Lane (A)-Molecular Weight Markers; Lane (B)- DEN-Treated And Lane (C)- Normal Control.

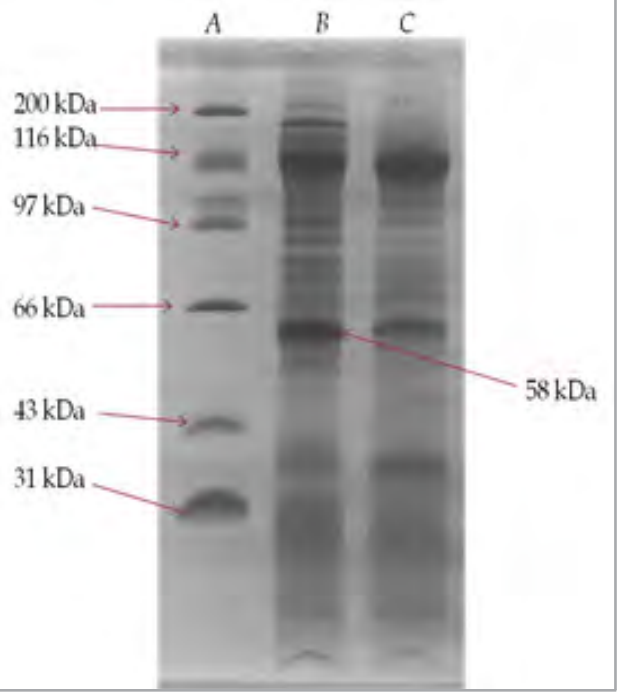
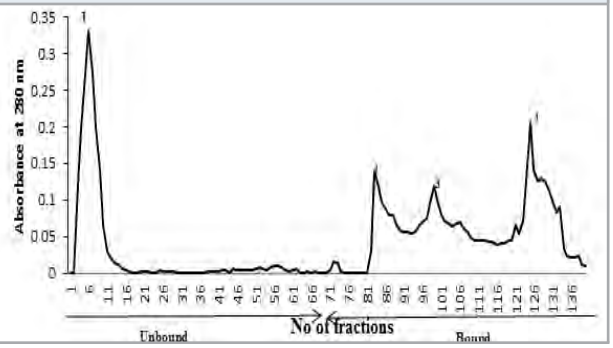


Figure 4: Q-Sepharose An Anion-Exchange Protein Profile Of Crude Membrane Glycoprotein Extract Of Liver From DEN-Treated Mice. Unbound Glycoprotein Were Eluted With 10 Mm Tris-Hcl Buffer, Ph 7.0 And Bound Glycoprotein Were Eluted With An Increasing Gradient Of 0-0.1M Nacl In The Same Buffer. Fractions Of 2 Ml Were Collected And Read At 280 Nm. The Desired Glycoprotein Was Eluted Out In All Three Peaks Of Bound Fraction Confirmed When Run Of SDS-PAGE



This increase in GSH level may also be likely due to a rise in the GGT activity, as modulation in cellular GSH levels has been correlated with hyper-activation of GGT. Overproduction of GGT results in increased intracellular GSH synthesis, it plays an important role in the development of resistance to certain chemotherapeutics, such as alkylating agents (Bansal et al., 2018). The histological examination of liver tissues of DEN-treated mice exhibited that the hepatocytes in liver section were in a neoplastic state. Many changes in hepatocytes such as, irregular arrangement, variation in the shape and size, multi-nucleated cells, and loss of contact with the neighbouring cells were noticed. In contrast, the liver cells of the untreated normal control mice did not show any such changes and were found with a regular morphology and well-defined outlines (Fig.II). Thus, the

morphological changes seen in the liver, alterations in the marker enzyme activities, the increase in GSH level and changes in histological section of liver tissue, all these observations support the development of cancer induction in liver of mice upon DEN-exposure.

Figure 5: Preparative SDS-PAGE Purified TAA. The Portion Of The Gel Corresponding To The TAA Was Cut Out Of The Unstained Gel. It Was Cut Into Small Pieces And Homogenized In 3 % 1-Butanol. The Homogenate Was Centrifuged At 8000 X G For 30 Mi At 4 O C. The Supernatant Collected Was Pooled, Dialysed Against Distilled Water Overnight And Lyophilised. Lane (A)- Molecular Weight Markers; Lane (B)- Purified TAA

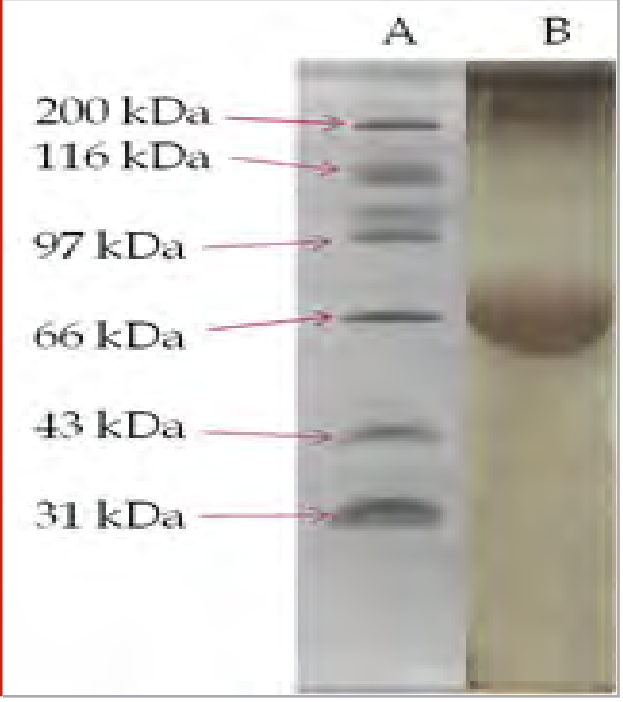
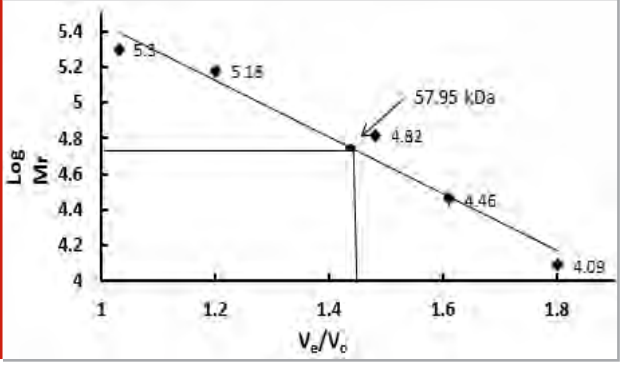


Figure 5: Sephacryl S200 HR Gel Filtration Profile To Determine Molecular Weight Of Purified TAA. Different Calibration Standards Were Used. The Logarithm Of Their Respective Molecular Weights (Log Mr) Was Plotted Against The Ratio Of Their Elution Volume To Void Volume (Ve / Vo). From The Calibration Curve The Molecular Weight Of The Purified Glycoprotein Sample Was Calculated And Found To Be 57.95 Kda.



After having established cellular transformation in mice upon exposure of DEN, identification of TAA was carried out. The SDS-PAGE analysis of the 1-butanol liver extract showed that a membrane surface glycoprotein of ~58 kDa molecular weight identified as TAA, was found to be significantly over-expressed in DEN-treated mice liver cells (Fig. III). Observed alteration in the expression of above glycoprotein clearly indicate that DEN has caused major changes in the membrane of liver cells, which may involve in distortions and alterations of cell membrane during treatment. Attempt was made to purify the above membrane glycoprotein TAA using ion-exchange chromatography followed by gel filtration. The desired glycoprotein was found to be an anionic protein as evident from the elution profile shown in Fig. IV. Further purification of the glycoprotein was carried out by gel filtration chromatography using sephadex G-100 but could not be achieved due to small differences in the molecular weight of other anionic protein eluted with it. Hence was finally purified by preparative SDS-PAGE (Fig. V).

Figure 7: Total Amount Of Neutral Hexoses, Sialic Acid And Protein In Purified TAA Sample Determined As Per The Method Described In Method Section.

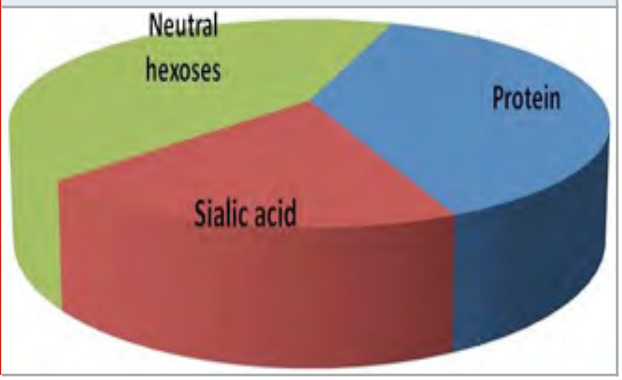
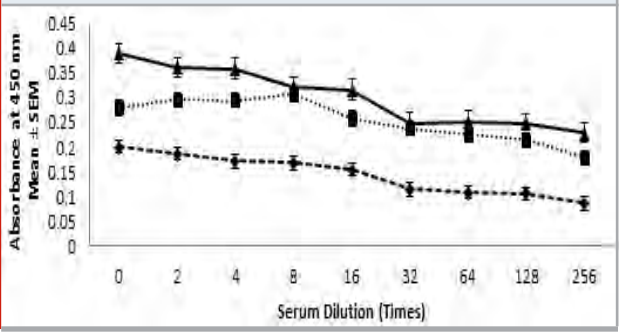


Figure 8: Anti-TAA Antibody Titres Were Determined Using ELISA In Serum Samples Obtained From Mice Immunized With Various Antigenic Formulation As Described In Material And Method Section. Mice Injected With Saline Alone Served As Normal Control. The Absorbance Measured In Serum Sample Of Normal Control Were Subtracted From The Experimental Sample Readings. Antibody Levels At Various Dilutions In Mice Immunized With TAA-CFA , TAA-Alhydrogel And TAA-Saline Formulations.



Molecular weight of TAA was determined by gel filtration chromatography. Membrane glycoprotein TAA was analysed for the carbohydrate moities i.e. neutral hexoses and sialic acid and for its protein content. The results are shown in Fig. VII. The total carbohydrate to protein ratio was calculated to be >2:1. Thus, the carbohydrate moieties seem to make up a large portion of the TAA, as is evident from its high content as compared to the protein. The substantially high concentration of sialic acid observed during carbohydrate analysis of TAA also reveal about the anionic nature of TAA. Sialic acid content on membrane surface varies from cancer to cancer and has been associated with malignancy, if not with every stage of carcinogenic process, but starting with initiation, progression and finally metastasis. The purified TAA was tested for its immunoreactivity in allogenic normal control Swiss albino mice through active immunization using three different TAA-formulations as discussed in method section. The anti-TAA antibody concentrations in immune sera were determined by ELISA as has been shown in Fig. VIII. These observations clearly indicate that the purified TAA is an anionic glycoprotein with very high content of carbohydrate moieties, highly immunogenic, may elicit a significantly high antibody response against it in mice upon immunization and thus could suitably be used as an effective target for active immunotherapy against cancer. The results suggest that specific proteins are uniquely susceptible to alterations in expression and carry implications for the further investigation of their potential as therapeutic and prognostic markers.

ACKNOWLEDGEMENT

This investigation was supported by a project grant from Department of Science and Technology (DST), New Delhi sanctioned to Prof. A. Alam.

REFERENCES

Alam A Singha LI and Singh V (2005) Molecular characterization of tumor associated antigen in mice exposed to a hepatocarcinogen Molecular and Cellular Biochemistry Vol 271 Pages 177-188.

Arboatti AS Lambertucci F Sedlmeier MG Pisani G Monti J Álvarez ML Francés DEA Ronco MT and Carnovale CE (2018) Diethylnitrosamine Increases Proliferation in Early Stages of Hepatic Carcinogenesis in Insulin-Treated Type 1 Diabetic Mice BioMed Research International Vol 2018: 9472939 Pages 1-10.

Bansal A and Simon MC (2018) Glutathione metabolism in cancer progression and treatment resistance Journal of Cell Biology Vol 217 No 7 Pages 2291-2298.

Boelsterli U (1979) Gamma-glutamyl Transpeptidase (GGT) – an early marker of hepatocarcinogenesis in rats Trends in Pharmacological Sciences Vol 1 No 1 Pages 47-49.

Bradford MA (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilising the principle of dye binding. Analytical Biochemistry Vol 72 No7 Page 245.

Brown JL (1999) N-Nitrosamines Occupational Medicine Vol 14 No 4 Pages 839-848.

Chen Qi Y Chan X and Cy (2008) Two-dimensional differential gel electrophoresis/analysis of diethylnitrosamine induced rat hepatocellular carcinoma,International Journal of Cancer Vol 122 Page 2382-2688.

Ellman GL (1959) Tissue sulfhydryl group Archives of Biochemistry and Biophysics Vol 82 No 1 Pages 70–77.

Ellman GL Courtney DK Andres V and Featherstone RM (1961) A new and rapid colorimetric determination of acetylcholine esterase activity Biochemical Pharmacology Vol 7 No 2 Pages 88-95.

Fausto N Campbell J (2010) Mouse Models of Hepatocellular Carcinoma Seminars in Liver Disease Vol 30 No 1 Page 87–98.

Goydos JS Elder E Whitiside TL Finn OJ and Lotze MT (1996) A phase I trial of a synthetic mucine peptide vaccine Journal of Surgical Research Vol 63 No 1 Pages 298-304.

Habig WH and Jakoby WB (1981) Glutathione S-transferases (Rat and Human) Methods in Enzymology Vol 77 Pages 218–237.

Hanahan D and Weinberg RA (2000) The hallmarks of cancer Cell Vol 100 No 1 Pages 57–70.

Heindryckx F Colle I and Vlierberghe HV (2009) Experimental mouse models for hepatocellular carcinoma research International Journal of Clinical and Experimental Pathology Vol 90 No 4 Pages 367–386.

Kaltenecker D Themanns M Mueller KM Spirk K Schwarzl NG Friedbichler K Kenner L Haybaeck J Moriggl R (2019) STAT5 deficiency in hepatocytes reduces diethylnitrosamine-induced liver tumorigenesis in mice Cytokine Vol 124 Pages 154573.

Liao DJ Blanck A Eneroth P Gustafsson JA and Hällström IP (2001) Diethylnitrosamine causes pituitary damage, disturbs hormone levels and reduces sexual dimorphism of certain liver functions in the rat Environmental Health Perspective Vol 109 No 9 Pages 943–947.

Liao S Smith JW and Kwong PC (1984) Selective extraction by 1-butanol of surface glycoprotein antigens from human melanoma cells Cancer Immunology Immunotherapy Vol 17 No 2 Pages 95–99.

Matuoka K Marus I Wong A and Smith GJ (1993) Diethylnitrosamine and partial hepatectomy induced in alpha 2μ-globulin mRNA level in the rat liver Journal of Cancer Research and Clinical Oncology Vol 119 No 10 Pages 572-275.

Meister A Tate SS and Griffith OW (1975) γ-Glutamyl transpeptidase Methods in Enzymology Vol 77 Pages 237–251.

Moldeus P and Jernstrom B (1983) In: Interaction of glutathione with reactive intermediates Pp 99-108

(Edited by) Larsson A Orrenivs S Holmgren A and Mannervik B Functions of Glutathione: Biochemical, Physiological, and Clinical Aspects Raven Press New York.

Montesano R (1981) Alkylation of DNA and tissue specificity in nitrosamine carcinogenesis Journal of supramolecular structure and cellular biochemistry Vol 17 No 3 Pages 259-73.

National Research Council (2011) Guide for the care and use of laboratory animals. National Academy Press 39.

Pariat T and Sharan RN (1995) Low dose exposure of diethylnitrosamine affects mice liver thymidine kinase Life Science Vol 57 Pages No 26 Pages 2431-2431.

Pascale RM Simile MM Gaspa L Diano L Seddain MA Pinna G Carta M Zolo P and Feo F (1993) Alterations of ornithine decarboxylase gene during the progression of rat liver carcinogenesis Carcinogenesis Vol 14 No 5 Pages 1077-1080.

Raghupati G (1996) Carbohydrate antigens as targets for active specific immunotherapy Cancer Immunology Immunotherapy Vol 43 No 3 Pages 152-157.

Ratcliffe NA (1983) Practical illustrated histology. The Macmillan Press London and Basingstoke 24-23.

Rodolf M Zilocchi C Melani C Cappetti B Arioli I Parmiani G and Colombo PM (1996) Immunotherapy of experimental metasis by vaccination with interleukin gene-transduced adenocarcinoma cells sharing tumor-associated antigens. Journal of Immunology Vol 157 No12 Pages 5536-5542.

Santos NP Colaço AA and Oliveira PA (2017) Animal models as a tool in hepatocellular carcinoma research: A Review Tumor Biology Vol 39 No 3.

Sivaramakrishnan V Shilpa PNM Praveen Kumar VR and Niranjali DS (2008) Attenuation of N-nitrosodiethylamine-induced hepatocellular carcinogenesis by a novel flavonol-Morin. Chemico-Biological Interactions Vol 171 No 1 Page 79–88.

Sklavos A Poutahidis T and Giakoustidis A (2018) Effects of wnt-1 blockade in DEN-induced hepatocellular adenomas of mice Oncology Letters, Vol 15 No 1 Pages 1211–1219.

Spiro RG (1966a) In: Determination of neutral hexoses Vol 8 Pp 4-5 (Edited by) Neufeld EF and Ginsburg V. Methods in Enzymology Academic Press New York.

Spiro RG (1966b) In: Resorcinol reaction Vol 8 Pp 15-16 (Edited by) Neufeld EF and Ginsburg V. Methods in Enzymology Academic Press New York.

Traverso N Ricciarelli R Nitti M Marengo B Furfaro AL Pronzato MA Marinari UM and Domenicotti C (2013) Role of Glutathione in Cancer Progression and Chemoresistance Oxidative Medicine and Cell Longevity Vol 2013:972913.

Williams GM Gebhardt R Sirma H and Stenback F (1993) Non-linearity of neoplastic conversion induced in rat liver by low exposure to Diethylnitrosamine. Carcinogenesis Vol 14 No 10 Pages 2149-2156.

Wong CR Nguyen MH and Lim JK (2016) Hepatocellular carcinoma in patients with non-alcoholic fatty liver disease World Journal of Gastroenterology Vol 22 No 37 Pages 8294-8303.

Xing P Michael M Apostopoulos V Prenzoska J Marshall C Bishop J and Mckenzie (1995) Phase I study of synthetic MUC1 peptides in breast cancer International Journal of Oncology Vol 6 No 6 Pages 12883-1287.

Ying TS Enomoto K Sharma DSR and Farber E (1982) Effects of delays in the cell cycle on the initiation of preneoplastic lesions in rat liver by 1,2-dimethylhydrazine Cancer Research Vol 42 No 3 Pages 876-880.

Biotechnological Communication

Biosc.Biotech.Res.Comm. Vol 13 Number (1) Jan-March 2020 Pp-353-361

Optimization of Cultural Conditions, Temperature and pH for Production of Pectinases by Two Species of *Aspergillus*

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ABSTRACT

Pectinases are major constituents in many fundamental life processes and have copious relevance in biotechnology and industry. Pectinase have immense perspective in food, textile and pharmaceuticals industries. With this regard, there has been a great increase in industrial applications of pectinase owing to their significant biotechnological uses. This study was undertaken with main objectives of meeting the growing industrial demands of pectinase, by improving the yield without increasing the cost of production. The present investigations were aimed at to study the occurrence and distribution and later on to isolate and characterize pectinolytic fungi from different habitats in and around the Warangal district of Telangana state, India. Further the study includes the factors influencing pectinase production by selected isolates, optimization of parameters for over production of pectinase. In the present investigation thirty soils known to harbor the pectinolytic fungi were selected for sampling. About 30 isolates of fungi showing pectinase production were isolated. Colonies exhibiting more than 2.0 mm pectinolytic zone was picked and further screened for pectinolyic activity on pectin screening agar medium. Out of these studies 2 efficient strains producing pectinolytic zone, were selected and an attempt was made to characterize and identify them tentatively by following the guidelines of Bergeys manual. The selected strains were identified as *Aspergillus niger* and *Aspergillus flavus* based on analysis profile of 18S rRNA sequence. These 2 isolates were showing promising pectinolysis were chosen for further studies. The production of pectinase was improved in submerged fermentation. Both the organisms produced all the pectic enzymes (Exo-pectinase, Exo-PGase, Endo-PG, Endo-PL and PME). However, the optimal pH and temperature varied with the species. Maximum enzyme production by *A. niger* and *A. flavus* was recorded at 8th and 12th days of incubation with optimum temperature 30°C and 35°C, optimum pH 6.0 and 5.0 respectively at agitation rate 140 rpm.

KEY WORDS: *A. NIGER, A. FLAVUS*, PECTINASES, OPTIMIZATION, SUBMERGED FERMENTATION.

ARTICLE INFORMATION

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Received 24th Dec 2019 Accepted after revision 29th March 2020
Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal

NAAS Journal Score 2020 (4.31) SJIF: 2019 (4.196)

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/13.1/56

INTRODUCTION

Pectinases are enzymes that catalyze break down of the glycosidic bonds in the galacturonic acid chains of the pectin rich materials. Because of their wide range of applications in food and feed industry, pectinases make up almost 25% of the global food enzyme market. Pectinases are major enzymes involved in pectin degradation process in the cell wall of plants and also act as major cell wall components in plants. Microbes are well known source for the production of extracellular enzymes and industrially important secondary metabolites. Pectic substances are a class of complex glycosidic polysaccharide compounds with a high molecular weight. Pectin an important component of plant cell wall is a polymeric material having carbohydrate group esterifies with methanol. It is present in high concentrations in the middle lamella, where it acts as a cementing material between adjacent cells, (El Enshasy et al. 2018, Kamalambigeswari et al. 2018 and Abd El Tawab et al. 2019).

Three major pectic polyssacharide groups viz, HG, RGI ansd RGII are recognized. Homogalacturonan (HG) is a linear polymer formed by D-galacturonic acid which can be acetylated and/or methyl esterified. It can also be called as smooth regions of pectin, (Jayani et al. 2005). Rhamnogalacturonan I (RGI) is composed of the repeating disaccharide rhamnose galacturonic acids and Rhamnogalacturonan II (RGII) is a homogalacturonan chain with complex side chains attached to the galacturonic residues, (Willats et al. 2001). The occurrence of pectinolytic enzymes has been reported in a large number of bacteria and fungi. However, most commercial preparations of pectic enzymes are obtained from fungal sources. This is due to a wide range of pH optima of enzymes produced by fungal strains. These enzymes not only provide an economically viable alternative, but are also ecofriendly, (Vikari et al. 2001). The microbial pectinase accounts approximately for 25% of the total worldwide enzyme sale, (Voragen et al. 2004 El Garhy, et al. 2020).

These groups of enzymes harbor a very huge commercial potential as their biotechnological applications span broad spectra in diverse industries such as biofuels, pulp-paper, food, animal feed, textile, fiber, etc. Out of these, biofuel industries demand these xylanolytic and pectinolytic enzymes play their major role to the enzymes for improving plant biomass saccharification. Whereas, animal feed industries require combination of cellulase, xylanase and pectinase for improving the nutrition quality of grain and feed (Thite et al. 2020). The important constraint for commercialization of new sources of enzymes is higher cost of the production. It defines the optimal microbial cultivation conditions for capable microbial strains and cheap raw substrate may reduce the cost of enzymes production. A large number of bacteria, yeasts and many filamentous fungi are potential pectinase producers. Fungi like *T. viride, A. flavus, A. niger, F. oxysporum, A. terreus,* and *P. chrysogenum* have attracted the most attention as enzymes producers because of the prolific yield and their long history in fermentation industries (El Garhy, et al. 2020).

Enzyme breakdown of the biomolecules depends up on the type of enzyme, application, temperature, incubation time, agitation, concentration, pH and use of different enzyme preparations (Dominguez et al. 1994 and Chadha et al. 2003). Owing to the vast potential applications of pectinase in various sectors of industries it is pertinent to undertake research on screening of microorganisms for pectinases and determine optimal conditions for production of microbial pectinase. Generally for the production of high-priced materials and for the study of biochemical and physiological aspects of the microbial metabolites, submerged fermentation system is very useful (Pereira et al. 1993). The usage of submerged fermentation is technically easier than solid state fermentation (Pedrolli et al. 2009). The purpose of this research was to evaluate pectinase production by the selected fungi using various vegetable waste dump yard soils as substitutes of pectin to make its production cost effective under submerged state fermentation.

MATERIAL AND METHODS

Isolation and Screening of pectinolytic fungi: Soil samples were collected from the site where the vegetable wastes were dumped and laced in sterile polythene bags and transferred to the laboratory. One gram of soil sample from each collection site was mixed in 100 ml of sterilized distilled water and 10-fold serial dilutions were prepared. One ml of each dilution was spread on potato dextrose agar (PDA) plates and incubated in an inverted position at 28˚C for 7 days (Kaur et al. 2004). Fungal colonies developing from the plates were picked up and purified and sub cultured onto slants and maintained for identification and enzyme studies. Promising producers of pectinase enzymes were screened by plating on modified pectin agar medium. (Pectin-10 g, $K2HPO_4$-0.05%, $MgSO_4$-0.05% KCl-0.05%, $FeSO_4$–0.01% Sucrose-1%, $ZnSO_4$-0.001%, $CuSO_4$–0.001%, Agar-20g, Distilled water-1000ml, pH-5.5) supplemented with streptomycin.

Screening and Identification of Fungal Isolates for Pectinolytic Activity: Pectin agar medium was used for the screening of isolated fungal strains. They were incubated for a week at room temperature. After incubation, the plates were flooded with iodine-potassium iodide solution (Iodine-1.0g, potassium-iodide-5.0g in 330ml distilled water) observed for zone of hydrolysis around the colonies. Positive cultures were selected from these isolates after screening and identified as *A. niger* and *A. flavus* by 18S rRNA sequencing.

Production of Pectinase Enzyme Submerged fermentation: Cultures were grown in 250 ml Erlenmeyer flask containing 100 ml of broth [pH 7.0, contains 1% of rice bran 0.2%, NaNO3, 0.1%, K2HPO4; 0.05%, $MgSO4.7H_2O$; 0.05%, KCl; 0.01%, $FeSO_4.7H_2O$; 0.001%, $ZnSO_4$; 0.001%, $CuSO_4$] for production of pectinases and exo-

polygalacturonases. After sterilization of the Erlenmeyer flasks containing fermentation medium, young fungal mycelium of 3 days old cultures at the growing edges were used to inoculate aseptically. Inoculated culture flasks were incubated in the incubator shaker operating at 120-180 rpm at 28±1°C for 16 days. 10 ml of culture broth was withdrawn from the flasks at different time intervals of incubation. The supernatants obtained from the centrifugations of the same were used as enzyme sources for enzyme assay.

Assay of enzymes: Exo-pectinase: Supernatants from the incubated shake culture flasks at intervals of 8th and 12th days were used as enzyme source. Activity was assayed by DNS method (Miller 1959). The Exo-pectinase activity was determined using 1% pectin as substrate. Reaction mixture containing equal amounts of 1% pectin (1.0 mL) prepared in citrate buffer (0.05 M; pH 5) and partially purified enzyme (1.0 mL).The mixture was incubated at 50°C in water bath for 30 min .The reaction was terminated by addition of 3ml of 3,5-dinitrosalicilic acid DNS reagent and the contents were boiled for 15 minutes. After cooling the color developed was read at 540nm. The amount of reducing sugar released was quantified using galactouronic acid as standard. Standard galacturonic was prepared by taking 100mg galacturonic acid in 100ml standard flask and made up the volume to 100ml. A standard curve of D-Galactouronic (1mg/mL) was prepared under identical conditions to determine the reducing sugars formed. The enzymatic activity was expressed as Unit per ml (U/ml), which is defined as the amount of enzyme that liberates 1µmole of reducing sugar per mL per minute under assay conditions.

Assay of exo-polygalacturonase (Exo-PGase): Supernatants from the incubated shake culture flasks at intervals of 8th and 12th days were used as enzyme source of exo-polygalacturonases, activity was assayed by quantifying reducing sugars using DNS (3,5-dinitrosalicylic acid) method (Miller,1959). The exo-PGase activity was determined using 1% polygalacturonic acid (PGA) as substrate, prepared in sodium acetate buffer (0.1M; pH 4.5). The reaction mixture (2mL) containing equal amounts of enzyme (1.0 mL) and substrate (1.0 mL) and incubating at 50° C for 30 min in a water bath. The reaction was stopped by addition of 3ml of 3,5-dinitrosalicilic acid DNS reagent and the contents were boiled for 15 minutes. The color developed was read at 540nm. A standard curve of D-Galactouronic acid (1mg/mL) was developed under identical conditions to determine the reducing sugars formed. The enzymatic activity of filtrate was expressed as Unit per ml (U/ml), which is defined as the amount of enzyme, which liberates 1µmole of galacturonic acid (reducing sugar) per mL per minute under assay conditions.

Pectin methyl esterase activity (PME): Pectin methyl esterase activity was estimated by the method suggested by Kertesz (1955). Pectin esterase activity was measured by increase in free carboxyl group by titrating against NaOH in the presence of a pH indicator like phenolphthalein. For assaying pectinesterase activity 20ml of 1% pectin was dissolved in 0.15M NaCl (pH-7.0) and 4ml of enzyme extract was taken in a beaker and incubated for 1 hour. After incubation, the solution was titrated against 0.02N NaOH to reach pH 7.0 using phenolphthalein as indicator (colour change from colourless to pink) and the heat killed enzyme extract was used as control.

$$\text{Pectin esterase activity} = Vs - V\,b\,(\text{Normality of NaOH} \times 100/Vt$$

Where, Vs-volume of NaOH used to titer sample (ml), V b-volume of NaOH used to titer blank (ml), V-volume of incubation mixture (ml), t-reaction time (min). Pectin esterase activity was expressed as milli equivalents of NaOH consumed min-1 ml-1 of enzyme extract under the assay conditions

Assay of Endo Poly galacturonase (Endo-PG): Wood's viscometric method (1955) was followed to estimate the endo-PG. Polygalacturonic acid (0.5%) was prepared by dissolving 0.5g of polygalacturonic acid in 100 ml citrate (buffer (pH 5.5). The reaction mixture for the estimation of endo-PG was with polygalacturonic acid (0.5%) substrate, citrate buffer (pH 5.5) and enzyme source in 4:1:2 ratios. The reaction mixture consisting of 12ml of substrate, 4ml of enzyme and 1ml of citrate buffer. The loss of viscosity was measured for every 10 minutes over a period of 30 minutes. The reaction mixture with heat killed (inactivated) enzyme and distilled water served as control. The percentage loss of viscosity was calculated by using the formula.

$$V = \frac{t_i - t_a}{t_1 - t_0} \times 100$$

Where,
V = percentage of loss of viscosity ti = flow time of reaction mixture + inactive enzyme.
t_a = flow time of reaction mixture + active enzyme
t_0 = flow time of distilled water+ active enzyme at "O" time.

The Relative Enzyme Activity (REA) of endo-PG was calculated by dividing 1000 with time required for 50% loss of viscosity (t50) and expressed the enzyme activity in relative viscometric units (RVU).

REA = 1000 / tv50
Where tv50 = time required in minutes taken for 50% loss of initial viscosity

Endo-pectin lyase (Endo-PL): Endo-pectin lyase activity was assayed viscometrically as suggested by Wood (1955). 1% pectin was used as substrate in this assay. 4ml of culture supernatant and 0.8 ml of tris HCl buffer pH (8.0) were added to 12ml of pectin solution. Viscosity of supernatant was determined by using Ostwald viscometer. Initial reading time was noted and incubated for 30 minutes and take all the final reading

time was recorded.The loss of viscosity was measured for every 10 minutes over a period of 30 minutes. The reaction mixture with heat killed (inactivated) enzyme and distilled water served as control. Enzyme activity measured in RVU units (relative viscometric units).

Statistical analysis: The enzyme activities are presented as Mean± SE of all values. Results obtained in this study were subjected to analysis of variance using one way ANOVA and difference between means were separated by Duncan Multiple Range Test using SPSS software 17.0 version.

The effect of temperature on enzyme activity: Production media were prepared in, 50ml/100ml Erlenmeyer flasks, and inoculated by fungal inoculums. Effect of different temperatures on pectinase production was observed by incubating the active culture broth at various temperatures 25ºC, 30ºC, 35ºC, 40ºC and 45ºC for 16 days. The cell suspension was collected on 8th and 12th day and the pectinase production was estimated with the procedure mentioned earlier.

The effect of pH on enzyme activity: Production media were prepared in 50ml/100ml Erlenmeyer flasks. Effect of various pH levels on pectinase production was observed by incubating the culture broth at pH levels ranging from 2.0 to 8.0. The different pH levels were adjusted using 2N NaOH (sodium hydroxide) to the 50 ml production medium taken in 250ml conical flask, inoculated with active fungal culture and incubated at 35oC for 16 days. The pectinase production was estimated on 8th and 12th day using the procedure mentioned earlier. The results are presented in tables and figures.

RESULTS AND DISCUSSION

Effect of incubation temperature on exo-pectinase production: Temperature is directly related to the metabolic activities of the microorganism and it affects the proper growth and product formation by the organism (Lonsane et al. 1985). Enzyme organism has its own optimal temperature at which it grows its maximum and produces the desired products maximally. Hence maintenance of optimal temperature is a must. Many research workers have reported at different temperature for maximum pectinase production in SmF studies, suggesting that the optimal temperature for pectinase production depends on the strain variation of the microorganisms. Temperature of medium have very good influence on enzyme production. To assess the effect of temperature on pectinase production, isolates were grown in production medium at their optimized pH and at different temperatures from 20˚C to 45˚C for pectinase production under submerged fermentation in an incubator shaker with agitation of 140 rpm for 12 days.

Table 1. Effect of temperature on exo-pectinase production by *A. niger* and *A. flavus* on 12th day of incubation.

Temperature	*A. niger*	*A. flavus*
25˚C	0.360^{b}±0.005	0.356^{c}±0.003
30˚C	0.516^{a}±0.008	0.423^{b}±0.003
35˚C	0.110^{d}±0.005	0.460^{a}±0.005
40˚C	0.130^{c}±0.005	0.320^{d}±0.010
45˚C	0.071^{e}±0.004	0.300^{e}±0.000
Control	0.130^{c}±0.005	0.143^{f}±0.003

Values are significant at P< 0.005

Table 2. Effect of temperature on exo-PG production by *A. niger* and *A. flavus* on 12th day of incubation.

Temperature	*A. niger*	*A. flavus*
25˚C	0.580^{b}±0.005	0.123^{c}±0.003
30˚C	0.880^{a}±0.005	0.550^{b}±0.005
35˚C	0.170^{c}±0.005	0.786^{a}±0.003
40˚C	0.090^{d}±0.005	0.106=±0.006
45˚C	0.070^{e}±0.005	0.060^{e}±0.000
Control	0.070^{e}±0.005	0.110^{cd}±0.005

Values are significant at P< 0.005

Temperature play a great role in the enzyme production as it is especially significant in microorganism's growth regulation and physiological activity and microbial product formation and it varies from each microorganism. Optimal temperature is defined as that temperature which results in maximum velocity of the enzymatic reaction above which, the rate of reaction decreases due to thermal inactivation. The very slight changes in the growth temperature, may affect pectinase production (Sandri et al. 2011). Temperature is directly related to the metabolic activities of the microorganism and it affects the growth and product formation of the organism (Sandhya and Kurup 2013).

From the results presented in Table-1 it is evident that *A. niger* showed optimum exo-pectinase activity at 30oC (0.516 U/ml) while the least activity of enzyme at 45oC (0.071U/ml). Exo-pectinase produced by *A. flavus* was optimum at 35ºC (0.460 U/ml). *A. flavus* produced more exo-pectinase at all temperatures under investigation except at 45oC. Similar results were observed in case of *A. niger* which showed best performance at a temperature of 45°C (Bhardwaj and Garg 2014). Temperature of 30°C was observed to yield optimum pectinase production in case of *Bacillus sphaericus* (MTCC 7542), *Aspergillus niger, S. cerevisiae, Aspergillus foetidus* and *Kluyveromyces wickerhamii* (Moyo et al. 2003; Kumar et al. 2012 and Ahmed et al. 2015). Very few species of fungi have the ability to grow vigorously at temperatures between 45°C and 55°C. A temperature of 30°C was reported to be the optimum growth temperature which was similar to the findings of Mathew et al. (2008); Gupta and Kalpana (2011).

Effect of incubation temperature on exo-PG production: Activity of enzymes extracted from the isolates was determined to check out the optimum range of temperature for exo-polygalacturonase enzyme (Table 2). Among all temperatures *A. niger* showed optimum exo-PG activity at 30˚C with 0.880 U/ml and *A. flavus* showed optimum exo-PG activity at 35˚C with 0.786U/ml respectively. When temperature is altered below or above the optimum, the activity decreased. The maximum production of exo-PG enzyme was obtained at 30°C by the *A. niger*. Further increase in the temperature resulted in the decrease of pectinolytic activity by both the organisms under investigation. Ahmed and Sohail (2020) studied on the activity of pectinase by *G. candidum* AA15 revealed that the enzyme shows its maximum activity at 35°C and any further increase in temperature reduces the activity drastically

Table 3. Effect of temperature on Endo-PG production by *A. niger* and *A. flavus* on 8th day of incubation.

Temperature	*A. niger*	*A. flavus*
25˚C	$40.0^{b}\pm0.577$	$11.53^{d}\pm0.290$
30˚C	$43.56^{a}\pm0.881$	$27.40^{b}\pm0.305$
35˚C	$37.26^{c}\pm0.881$	$38.66^{a}\pm0.333$
40˚C	$37.26^{c}\pm0.881$	$26.66^{b}\pm0.333$
45˚C	$23.33^{d}\pm0.666$	$12.70^{c}\pm0.351$
Control	$23.33^{d}\pm0.666$	$11.53^{d}\pm0.290$

Values are significant at P< 0.005

Table 4. Effect of temperature on endo-pectin lyase production by *A. niger and A. flavus* on 8th day of incubation.

Temperature	*A. niger*	*A. flavus*
25˚C	$58.06^{b}\pm0.635$	$45.16^{c}\pm0.166$
30˚C	$91.32^{a}\pm0.695$	$47.66^{b}\pm0.333$
35˚C	$48.0^{c}\pm0.000$	$58.73^{a}\pm0.371$
40˚C	$37.0^{d}\pm0.577$	$42.83^{d}\pm0.440$
45˚C	$37.0^{d}\pm0.577$	$41.80^{e}\pm0.200$
Control	$37.0^{d}\pm0.577$	$13.03^{f}\pm0.333$

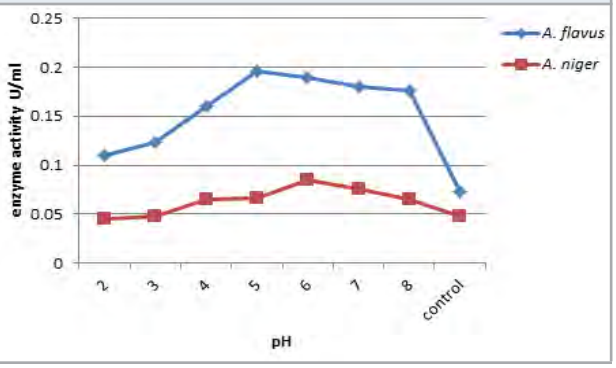
Values are significant at P< 0.005

Table 5. Effect of temperature on PME production by *A. niger* and *A. flavus* on 8th day of incubation.

Temperature	*A. niger*	*A. flavus*
25˚C	$0.031^{b}\pm0.001$	$0.020^{cd}\pm0.000$
30˚C	$0.043^{a}\pm0.001$	$0.031^{ab}\pm0.001$
35˚C	0.028 ± 0.001	$0.035^{a}\pm0.002$
40˚C	$0.022^{c}\pm0.002$	$0.025^{bc}\pm0.002$
45˚C	$0.020^{c}\pm0.000$	$0.025^{bc}\pm0.002$
Control	$0.020^{c}\pm0.000$	$0.020^{cd}\pm0.000$

Values are significant at P< 0.005

Figure 1: Effect of pH on exo-pectinase production by *A. flavus* and *A. niger* on 12th day of incubation.

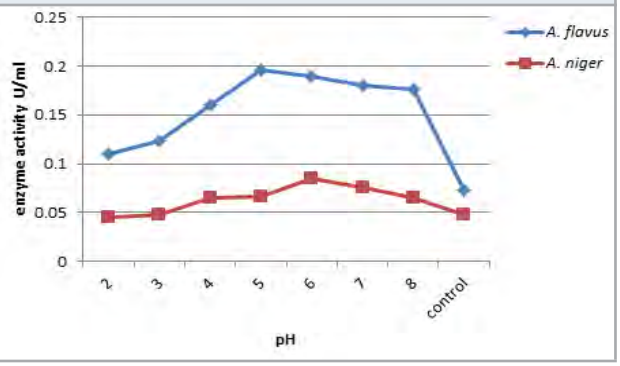


Effect of incubation temperature on endo-PG production: was studied in both the organisms in the range of 25˚C to 45˚C. In both organisms, optimum production was recorded at 30˚C and 35˚C. *A. niger* produced 43.56RVU at 30˚C whereas *A. flavus* produce 38.66RVU at 35˚C (Table 3). Production was started at 25˚C recorded optimum at 35˚C and gradually decreased with subsequent incubation temperature. Maximum enzyme activity at optimum temperature may be due to the faster metabolic activity and increase in protein content and extracellular enzyme production in culture supernatant. Ketipally et al. (2019) reported that at various temperatures between 25°C and 45°C, 35°C was the most suitable temperature for the growth and production of polygalacturonase activity and 30°C was the favourable temperature for the growth and production of pectinase by *A. nomius* MR 103. At very low temperatures, membranes solidify and high temperatures damage microorganisms by denaturing enzymes, transport carriers and other proteins thus lowering enzyme activity.

Effect of temperature on endo-pectin lyase production: Incubation temperature has been found to be a significant controlling factor for enzyme production. Table 4, revealed that the temperature has great influence for the production pectinase. *A. niger* produced maximum endo-pectin lyase activity at 30oC 91.32RVU whereas *A. flavus* produced enzyme at 35˚C (58.73RVU) and lower activity 13.03RVU was showed at control by *A. flavus*. Kamalambigeswari et al. (2018) found the influence of temperature at 30°C, the enzyme activity at high titer (209.04). Celestina et al. (2006) reported the effect of temperature on PG production by Monascus sp. N8 and *Aspergillus sp*. N12 and the optimum temperature were found to be 45°C. Bailey and Pessa (1990) studied lower temperature slows down the hydrolysis of pectin. From above findings it is evident that lower and higher temperature conditions inhibit growth of the microorganisms

Effect of temperature on PME production: Incubation temperature is the most important physical factor which affects enzyme production dramatically and their stability. Maximum PME activity was found at 30°C (0.043 meq. of NaOH consumed/min/ ml) by *A. niger* followed by *A. flavus* produced PME at 35oC (0.035 meq. of NaoH consumed/min/ml). Least enzyme production was recorded at 45oC (0.020 meq. of NaOH consumed/ min/ml) by *A. niger* (Table 5). Gummadi et al. (2007) reported that an optimum temperature of 30°C for pectin lyase production with A. niger NCIM548. Our strain of *A. niger* is a mesophilic fungus and therefore it can grow in the range of temperature between 25°C to 40°C. There was no detectable growth of the microorganism above 45°C. Optimum temperature for PG of *A. fumigatus* by Phutela et al. (2005) was reported to be 50°C. Thakur et al. (2010) reported the same for Mucor circinelloides and the optimum temperature was found to be 42°C. Variations of these results are also studied by Patil and Chaudhari (2012), where the optimum temperature for PG production was found to be 35°C.

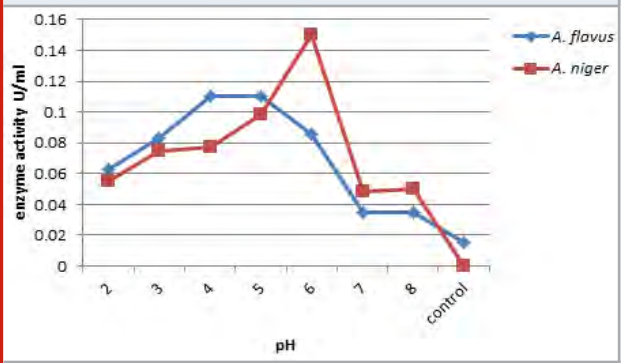
Figure 2: Effect of pH on Exo-PG production by *A.flavus* and *A. niger* on 12th day of incubation.



Effect of pH on exo-pectinase production: Some of the various parameters, the foremost one studied were the pH. Every organism has its own pH. Furthermore to this the production of enzymes also depends on the pH of the medium. Hence pH is a critical factor in the production of microbial enzymes. In the present study hydroxide salts and hydrochloric acids were used for control of pH in the fermentation medium for the production of polygalacturonase. pH of medium have great influence on enzyme production to assess the effect of pH on pectinase production medium was adjusted at 2.0 to 8.0 and incubated at an incubator shaker under submerged fermentation. After incubation fermented medium was centrifuged and supernatant was analyzed for pectinase production.

Exo-pectinase production was tested when the organism was cultured at different pH ranging from 2.0 - 8.0. As shown in fig. 1, Production began at pH 2.0, increased gradually and showed optimum at pH 5.0 and pH 6.0 and decreased subsequently. The optimum pectinase production by *A. flavus* was observed at pH 5 (0.196U/ml) whereas *A. niger* produced exo-pectinase at pH 6.0 (0.085U/ml). This can be an indication that the isolate requires acidic conditions for its optimum enzyme production. *A. flavus* was a good producer in exo-pectinase comparatively than *A. niger*. El Garhy, and Azzaz et al. (2020) reported that the pectinase production by Aspergillus terreus grown on different pH of beet pulp powder medium (BPPM) showed its highest values at pH 4.0. Medium initial pH has great effect on the microbial growth, cell osmotic pressure, nutrient uptake and enzymes production and secretion and concluded that optimum pectinase activity has been given from different fungus within the acidic pH range (Kholif et al. 2018).

Figure 3: Effect of pH on Endo-PG production by *A. flavus* and *A. niger* on 8th day of incubation



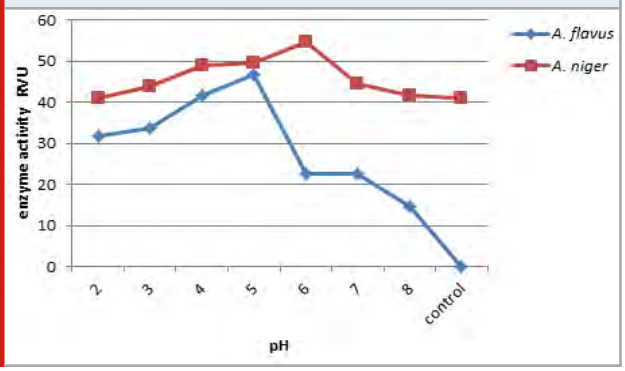
Effect of pH on exo-PG production: The exo-polygalacturonase production was optimized by supplementation using different pH range medium from 2.0 to 8.0 (Fig. 2). In submerged fermentation, maximum pectinase production by *A. niger* was noticed at pH 6.0 (0.150U/ml) followed by *A. flavus* at pH 5.0 (0.110U/ml) on 12th day of incubation respectively. Least enzyme production was recorded at pH 8.0 (0.035U/ml) and control (0.015U/ml) by *A. flavus*. Ahmed et al. (2020) found that the pectinase activity was drastically decreased when enzymatic reaction was carried out at a pH higher than 5 and towards alkaline side, although, a slight increment in the activity was observed at pH 7.5. The present result is similar with the results of (Panda et al. 2012) who observed high pectinolytic activity (0.195U/ml) by *Aspergillus flavus* at pH 6.0. The pectinase activity of 23.9EU/ml was exhibited by FW5 isolate (Mehta et al. 2013) and by *Penicillium chrysogenum* (Laha et al. 2014) at a pH of 6.0, which is similar to the present work

Effect of pH on endo-PG production: Endo-pectinase activity of *A. niger* and *A. flavus* was checked at different pH (2.0 – 8.0). Maximum activity of enzyme produced by *A. niger* was observed at pH 6.0 (58.03 RVU) followed by *A. flavus* at pH 5.0 (58.10 RVU) and minimum endo-pectinase production at control (12.20RVU). Thus pH 5.0 and pH 6.0 are considered as optimum pH for enzyme production (Fig. 3). Kamalambigeswari et al. (2018) reported the effect of pH on the production of pectinase. The optimum pH revealed that the enzyme was highly active at pH 5.5. The reduction of pectinase enzyme activity after increasing medium pH level is may be due to occurring partial or irreversible denaturation in the enzyme protein (Khattab et al., 2019)

Effect of pH on endo-pectin lyase production: Fig. 4, shows that initial pH of 6.0 yielded maximum endo-pectin lyase activity of about (54.86RVU) by *A. niger* whereas *A. flavus* produced at pH 5.0 (46.66RVU) enzyme activity. Minimum enzyme activity produced at pH 8 by *A. flavus* (14.66RVU). Furthermore, optimal pH is important for microbial growth and their metabolic activities. Since the metabolic activities of the microorganisms are quite sensitive to changes in pH, pectinase production by *A.flavus* was affected by varying pH values of the medium. Ketipally and Ram (2018) found that the optimum pH was studied by using varied pH conditions in different flasks ranging from pH 4-9 for the production of pectinase by *Aspergillus oryzae* RR103. The enzyme activity of *Aspergillus oryzae* RR103 begins at pH 4.0 and slightly increased at pH 5.0, the maximum enzyme production 2.071U/ml at pH 6.0 finally stable at pH 6.5. In *Thermoascus aurantiacus* maximum activity was reported at pH 5.0 by Martin et. al (2004). (Muthuprakash and Abraham 2011) found pH 5 was the optimal culture condition for enzyme production by fungal strains including *Aspergillus sp.* under solid state fermentation.

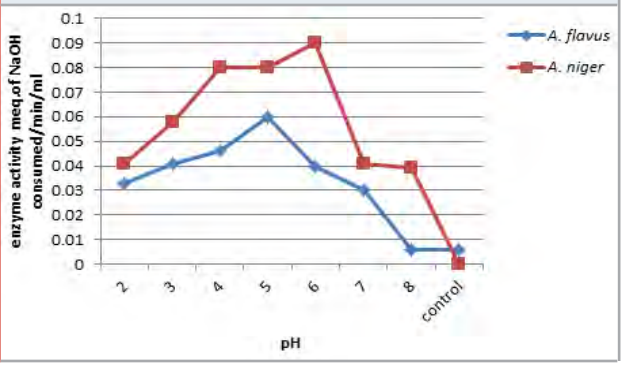
Effect of pH on PME production: Data from the Fig. 5, reveals that pH of 6.0 yielded maximum PME activity of about (0.090 Meq. of NaOH consumed/min/ml) by *A. niger* followed by *A. flavus* at pH 5.0 (0.060 Meq. of NaOH consumed/min/ml) and least enzyme production at pH 8.0 (0.006 Meq. of NaOH consumed/min/ml) by *A. flavus*. *A. niger* could fail to produce PME in control. The enzyme activity decreased with increase in pH of the substrate. El Enshasy et al. (2018) reported that the pH in the uncontrolled cultivation dropped from 5.5 to about 3.6 after 18h, and remained more or less constant until the end of the cultivation. In Mucor circinelloides ITCC 6025 maximum pectinases activity was observed at pH 4.0 (Thakur et al. 2010). Our findings are in similar with the works of Ellaiah et. al. (2002) who found pH 5.0 was the optimal culture condition for enzyme production by fungal strains including *Aspergillus sp.*

Figure 4: Effect of pH on endo-pectin lyase production by *A.flavus* and *A. niger* on 8th day of incubation.



The maximum enzyme activity was observed with an initial pH of 6.5. pH range of 5.5-6.5 has been reported for maximum polygalacturonase production from *A. niger* (Acuna Arguelles et al. 1995), pH 3.5-5.5 for polygalacturonase production by Mucor and A. flavus and pH 4.5-6.0 for polygalacturonase production by *A. awamori* (Abbasi and Mortazavipur et. al. 2011). Kunte and Shastri (1980) reported similar results where maximum PG activity was observed at pH 4.4 and 8.6 for *Alternaria alternata*. It may be due to the presence of two isoenzymes of PG. Variations of these results were studied in *Penicillium frequentans* (Said et al. 1991) where maximum pectinase production was at initial pH 2.5 and *A. niger* CH-Y-1043. Aguilar et al. (1991) showed maximum production at pH 2.5.

Figure 5: Effect of pH on PME production by *A.flavus* and *A. niger* on 8th day of incubation



CONCLUSION

In conclusion, it can be concluded that *A. niger* isolate is a good source of pectinase which is active at pH 6.0 and at temperature 30˚C whereas *A. flavus* showed maximum enzyme production at pH 5.0 and at temperature 35˚C. As there is need of bulk production of enzymes at a cost effective rate and in order to meet this goal, such strategies should be explored by which cost-efficient and ecofriendly method for bulk production can be achieved. In this study, a very assiduous and all-embracing optimization steps are carried out. The production of pectinase was enhanced good fold in submerged fermentation. The potential of agricultural wastes for the production of pectinase using submerged fermentation is highlighted in this study for the highest productivity of pectinase from *A. niger* and *A. flavus* on submerged fermentations. This result conveys the much economized production of pectinase. Attempts should also be made to adapt the enzyme to conditions which make it more useful in terms of commercial applicability in industries such as juice industry, paper, textiles and tea. This study discovers one of economic ways for pectinase production by *A. niger* and *A. flavus* under the optimum fermentation conditions using wheat bran as cheap substrate. The newly produced pectinase enzyme may give feed factories highly effective product with low cost. Application of the newly produced pectinase enzyme in enrichment of the feeding value of diary animal's diet can be useful for animal's breeders who suffering high prices of traditional feed stuff.

ACKNOWLEDGEMENTS

The authors would like to thank the Head, Department of Microbiology, Kakatiya University, Warangal for facilities and encouragement.

REFERENCES

Abbasi, H., Mortazavipour, S. R., & Setudeh, M. (2011). Polygalacturonase (PG) production by fungal strains using agro-industrial bioproduct in solid state fermentation. Chemical Engineering Research Bulletin, 15(1), 1-5.

Abd El Tawab, A.M., Murad, H.A., Khattab, M.S.A., & Azzaz. H.H. (2019). Optimizing production of tannase and in vitro evaluation on ruminal fermentation, degradability and gas production. Int. J. Dairy Sci., 14: 53-60.

Acuña-Argüelles, M. E., Gutierrez-Rojas, M., Viniegra-González, G., & Favela-Torres, E. (1995). Production and properties of three pectinolytic activities produced by *Aspergillus niger* in submerged and solid-state fermentation. Applied microbiology and biotechnology, 43(5), 808-814.

Aguilar, G., Trejo, B. A., García, J. M., & Huitrón, C. (1991). Influence of pH on endo-and exo-pectinase production by *Aspergillus sp*. CH-Y-1043. Canadian journal of microbiology, 37(12), 912-917.

Ahmed, A., & Sohail, M. (2020). Characterization of pectinase from *Geotrichum candidum* AA15 and its potential application in orange juice clarification. Journal of King Saud University-Science, 32(1), 955-961.

Ahmed, I., Zia, M.A., Hussain, M.A., Akram, Z., Naveed, M.T. & Nowrouzi, A. (2015). Bioprocessing of citrus waste peel for induced pectinase production by *Aspergillus niger*; its purification and characterization. J. Radiat. Res. Appl. Sci. 9, 1-7.

Bailey, M. J., & Pessa, E. (1990). Strain and process for production of polygalacturonase. Enzyme and microbial technology, 12(4), 266-271.

Bhardwaj, V. & Garg, N. (2014). Production, Purification of Pectinase from Bacillus sp. MBRL576 Isolate and its Application in Extraction of Juice. Int. J. of Sci. Res. 3(6), 648-652.

Celestino, S. M. C., De Freitas, S. M., Medrano, F. J., De Sousa, M. V., & Ferreira Filho, E. X. (2006). Purification and characterization of a novel pectinase from *Acrophialophora nainiana* with emphasis on its physicochemical properties. Journal of biotechnology, 123(1), 33-42.

Chadha, R., Kumbhar, B.K. & Sarkar, B.C. (2003). Enzymatic hydrolysis of carrot for increased juice recovery. J. Food Sci. Technol. 40, 35-39.

Dominguez, H., Nunez, M.J. & Lema, J.M. (1994). Enzymatic pretreatment to enhance oil extraction from fruits and oil seeds: A review. Food Chem. 49, 271-286.

El Enshasy, H. A., Elsayed, E. A., Suhaimi, N., Malek, R. A., & Esawy, M. (2018). Bioprocess optimization for pectinase production using *Aspergillus niger* in a submerged cultivation system. BMC biotechnology, 18(1), 71.

El Garhy, G. M., Azzaz, H. H., Abd El Mola, A. M., & Mousa, G. A. (2020). Fungal Pectinase Production Optimization and its Application in Buffaloe's Diets Degradation.

Ellaiah P, Prabhakar T, Ramakrishna B, Taleb A.T., & Adinarayana K (2002). Strain improvement of Aspergillus niger for the production of lipase. Indian J. Microbiol. 42: 151-153.

Gummadi, S. N., Kumar, S., & Aneesh, C. N. A. (2007). Effect of salts on growth and pectinase production by halotolerant yeast, *Debaryomyces nepalensis* NCYC 3413. Current microbiology, 54(6), 472-476.

Gupta, R., & Kalpana. (2011). Optimization of production and reaction conditions of polygalacturonase from Byssochlamys fulva. Acta microbiologica et immunologica Hungarica, 58(4), 339-349.

Jayani, R.S., Saxena, S., & Gupta, R. (2005). Microbial pectinolytic enzymes: A review. Process Biochem. 40 (9), 2931-2944.

Kamalambigeswari, R., Yadav, S. A., Sivaswamy, N., & Ushani, U. (2018). Isolation, identification, screening and optimisation of pectinase producing soil fungi (*Aspergillus niger*). International Journal of Research in Pharmaceutical Sciences, 9(3).

Kaur, G., Kumar, S., & Satyanarayana, T. (2004). Production, characterization and application of a thermostable polygalacturonase of a thermophilic mould *Sporotrichum thermophile* Apinis. Bioresource Technology, 94(3), 239-243.

Kertesz , (1955). Z.I; In methods in enzymology/K. Colowick, Academic Press, New York, Vol, I,p.159.

Ketipally, R., & Ram, M. R. (2018). Optimization of pectinase production by *Aspergillus oryzae* RR 103. Current agriculture research journal, 6(1), 37-44.

Ketipally, R., Kumar, G. K., & Ram, M. R. (2019). Polygalacturonase production by *Aspergillus nomius* MR103 in solid state fermentation using Agro-industrial wastes. Journal of Applied and Natural Science, 11(2), 305-310.

Khattab, M.S.A., Azzaz, H.H., Abd El Tawab, A. M., & Murad, H.A., (2019). Production optimization of fungal cellulase and its impact on ruminal degradability and fermentation of diet. Int. J. Dairy Sci., 14: 61-68

Kholif, A.E., Kassab, A.Y., Azzaz, H.H., Matloup,

O.H., Hamdon, H.A., Olafadehan, O.A., & Morsy, T.A. (2018). Essential oils blend with a newly developed enzyme cocktail works synergistically to enhance feed utilization and milk production of Farafra ewes in the subtropics. Small Rumin. Res., 161: 43-50.

Kumar, Y.S., Vijayakumar, P., & Reddy, O.V.S. (2012). Pectinase production from mango peel using *Aspergillus foetidus* and its application in processing of mangojuice. Food Biotechnol. 26, 107-123.

Kunte, S., & Shastri, N. V. (1980). Studies on extracellular production of pectolytic enzymes by a strain of *Alternaria alternata*. Indian Journal of Microbiology, 20(3), 211-215.

Laha, S., D. Sarkar & Chaki, S. (2014). Optimization of production and molecular characterization of pectinase enzyme produced from *Penicillium chrysogenum*. Scholar Academic Journal of Biosciences, 2(5): 326-335.

Lonsane, B. K., Ghildyal, N. P., Budiatman, S., & Ramakrishna, S. V. (1985). Engineering aspects of solid state fermentation. Enzyme and Microbial Technology, 7(6), 258-265.

Martin, N., Souza, S. R. D., Silva, R. D., & Gomes, E. (2004). Pectinase production by fungal strains in solid-state fermentation using agro-industrial bioproduct. Brazilian Archives of Biology and Technology, 47(5), 813-819.

Mathew, A., Eldo, A. N., & Molly, A. G. (2008). Optimization of culture conditions for the production of thermostable polygalacturonase by *Penicillium* SPC-F 20. Journal of industrial microbiology & biotechnology, 35(9), 1001-1005.

Mehta, S. A., Mitali, R., Nilofer, S., & Nimisha, P. (2013). Optimization of physiological parameters for pectinase production from soil isolates and its applications in fruit juice clarification. Journal of Environmental Research and Development, 7(4A), 1539-1546.

Miller, G.L. (1959). International Journal of Science and Nature. Use of di-nitrosalicylic acid reagent for determination of reducing sugars. Anal. Chem. 31, 426-428.

Moyo, S., Gashe, B.A., Collison, E.K. & Mpuchane, S. (2003). Optimising growth conditions for the pectinolytic activity of *Kluyveromyces wickerhamii* by using response surface methodology. Int. J. Food Microbiol. 85, 87-100.

Muthuprakash, K.M.S. & Abraham, J. (2011). A comparative analysis of protease producing microbes isolated from tannery effluent. 2(1), 110-113.

Panda, S. S., Sahoo, K., Das, R., & Dhal, N. K. (2012). Pectinolytic and cellulolytic activity of soil fungal isolates from Similipal bioreserve forest. World Environment, 2(2), 1-3.

Patil, V., & Chaudari, R. Y. (2012). Spectrophotometric method for estimation of cefpodoxime proxetil and ofloxacin in tablet dosage form by simultaneous equation method. Int J Pharm Life Sci, 3, 1982-1984.

Pedrolli, D.B., Monteiro, A.C., Gomes, E. & Carmona, E.C. (2009). Pectin and pectinases: production, characterization and industrial application of microbial pectinolytic enzymes, The Open Biotechnology Journal. 3, 9-18.

Phutela, U., Dhuna, V., Sandhu, S., & Chadha, B. S. (2005). *Pectinase* and polygalacturonase production by a thermophilic *Aspergillus fumigatus* isolated from decomposting orange peels. Brazilian Journal of Microbiology, 36(1), 63-69.

Said, S., Fonseca, M. J. V., & Siessere, V. (1991). Pectinase production by *Penicillium frequentans*. World Journal of Microbiology and Biotechnology, 7(6), 607-608.

Sandhya, R., & Kurup, G. (2013). Screening and isolation of pectinase from fruit and vegetable wastes and the use of orange waste as a substrate for pectinase production. Int. Res. J. Biol. Sci, 2(9), 34-39.

Sandri, I.G., Fontana, R.C., Barfknecht D.M. & Silveira, M.M. da. (2011). Clarification of fruit juices by fungal pectinases. LWT-Food Science and Technology. 44(10), 2217-2222.

Thakur, A., Pahwa, R., Singh, S., & Gupta, R. (2010). Production, purification, and characterization of polygalacturonase from *Mucor circinelloides* ITCC 6025. Enzyme Research, 2010.

Thite, V. S., Nerurkar, A. S., & Baxi, N. N. (2020). Optimization of concurrent production of xylanolytic and pectinolytic enzymes by *Bacillus safensis* M35 and Bacillus altitudinis J208 using agro-industrial biomass through Response Surface Methodology. Scientific Reports, 10(1), 1-12.

vikari, L. M., Tenkanen, A. & Suuranakki (2001). Biotechnology. In Biotechnology in the pulp and paper industry, Eds., Rehm H-J and G. Reed.VCH-Wiley, pp: 523-546.

Voragen, F.H. & Schols Visser, R. (2004). Advances in pectin and pectinase research. Kluwer Academic Publishers, p: 497.

Willats, W.G.T., Mc Cartney, L., Mackie & Knox W. J. P. (2001). Pectin: cell biology for functional analysis. Plant Mol. Biol. 47, 9-27.

Wood, R.K.S. (1955). Pectic enzymes secreted bypathogens and their role in plant infection. In Mechanism of microbial pathogenicity/J.W. Howle and A.J.O. Hea, University Press, Cambridge. p263-293.

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