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Editors Communique

Stay Protected, Stay Safe in the Cradle of Nature

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

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

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

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

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

Amicably yours

Sharique A. Ali, PhD
Editor-in-Chief
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Biomedical Communication

Corona Virus Disease 2019: Origin, Transmission, Diagnosis, Vaccine and Treatment: A Review Article

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ABSTRACT

Corona virus is considered as the major pathogen that primarily threats the human respiratory system. Corona virus has been known to cause a systemic infection, which means affecting the entire body in its specific host. Moreover, natural recombination makes some of them capable to adapt greatly and jump the species barrier, causing pandemics or epidemics. Previous corona virus outbreaks that have been characterized as pathogens, caused a serious problem to public health including the Middle East respiratory syndrome (MERS)-CoV, and the severe acute respiratory syndrome (SARS)-CoV. In December 2019, the first case was reported. The emergence of Novel Corona virus named as Severe Acute Respiratory Syndrome Corona virus 2 (SARS-CoV-2), which is considered the causative agent of Corona Virus Disease 2019 (COVID-19). The genome of SARS-CoV-2 is 29.9 kb. In SARS-CoV-2 the conventional methods were used to detect any viral infectious which mainly depends on the computed tomography, serology and molecular tests. The commonly molecular technique used to detect the presence of SARS-CoV-2 is reverse-transcription polymerase chain reaction (RT-PCR). However, there are several drugs available that have high antiviral activity against viruses especial SARS-CoV-2 as camostat mesylate and umifenovir. The mainly route of transmission is person-to-person contact with either symptomatic or asymptomatic patients. ORF8 is an accessory protein that considers one of the more rapidly evolving proteins in beta corona virus. There are many different functions of SARS-CoV-2 ORF8.

KEY WORDS: CORONA VIRUS, COVID-19, ORF8, RT-PCR, SARS-COV-2, WUHAN CITY.

INTRODUCTION

Corona virus is considered as the major pathogen that primarily threats the human respiratory system and has been known to cause a systemic infection that means affecting the entire body in its specific host (Su et al., 2016; Rothan & Byrareddy, 2020). Moreover, natural recombination makes some of them capable to adapt greatly and jump the species barrier, causing pandemics or epidemics (Bchetnia et al., 2020). This infection may lead to serious symptoms and mortality (Bchetnia et al., 2020). Previous corona virus outbreaks have been characterized as pathogens that caused a serious problem to public health including the Middle East respiratory syndrome (MERS)-CoV and the severe acute respiratory syndrome (SARS)-CoV (Rothan & Byrareddy, 2020). There are other four families of Corona virus known to cause mild respiratory infection in human to include: 229E, OC43, NL63 and HKU1 (Xia et al., 2016). However, those viruses have an envelope characterized by spikes on their surface under the electron microscope like a crown. The presence of these projections gives it the name of Corona virus. These viruses are considered as positive-sense RNA viruses ranging from 60 nm to 140 nm in diameter (Chan-Yeung and Xu, 2003, Walls et al., 2020).

Here we present the appearance of corona virus over time (https://www.who.int). First of all, the emergence of the severe respiratory syndrome was in 2002 in the Guangdong province of southern China, then prevailed to the five continents (https://www.who.int). The new Corona virus origin in bats and then spread to human by the intermediate host of raccoon dogs and palm civet cat (Chan-Yeung & Xu, 2003; Kan et al., 2005; Wang et al., 2006). In 2003, the virus was identified and given the name Severe Acute Respiratory Syndrome Corona virus (SARS-CoV) (https://www.who.int.; Memish et al., 2013). However, SARS-CoV affected 8422 people, and the majority of cases were in Hong Kong and China (Memish et al., 2013). The mortality rate of SARS-Cov reached up to 11% (https://www.who.int.) (Memish et al., 2013). In 2012, the novel Corona viruses of bat origin, emerged in the Kingdom of Saudi Arabia

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(Memish et al., 2020) and then spread to 27 countries (Walls et al., 2020).

It was identified as the Middle East respiratory syndrome corona virus (MERS-CoV), affected 2494 individuals with 858 deaths (the mortality rate of 34 %) (Walls et al., 2020). The inter mediator host of MERS-CoV was found to be the dromedary camels (Memish et al., 2013; Raj et al., 2014; Walls et al., 2020). In late 2019, a group of patients visited a hospital in China, they were suffering from pneumonia of an unknown etiology (Bogoch et al., 2020; Wang et al., 2020; Yang et al., 2020). Epidemiologically these patients associated with wet animals and seafood wholesale market whereas many wildlife species are being sold such as frogs, bats, birds, rabbits and snakes in the Wuhan City of Hubei Province of China (Bogoch et al., 2020; Wang et al., 2020; Yang et al., 2020). However, the potential outbreak of corona virus was early predicted by given the estimate of a reproduction number for the Novel Corona virus which was considered significantly larger than 1 (range from 2.24 to 3.58) (Bogoch et al., 2020; Wang et al., 2020; Yang et al., 2020).

In December 2019, the first case was reported (Yang et al., 2020). Then, from the 18th to the 29thof December 2019, five patients were suffering from an acute respiratory syndrome and one among these patients died (Yang et al., 2020). On January 2ed, 2020, 41 patients have been laboratory-confirmed COVID-19 infection, less than half of these patients were suffering from chronic disease as, cardiovascular disease, diabetes and hypertension (Yang et al., 2020). COVID-19 is rapidly prevalence from the Wuhan City of China to the whole world (Wang et al., 2020) and is capable to infect children with minor effect (Chawla et al., 2020).

The novel Corona virus named as Severe Acute Respiratory Syndrome Corona virus 2 (SARS-CoV-2), which is considered the causative agent of COVID-19 (Al-Tawfiq & Memish, 2020). SARS-CoV2 had generated a new century of pandemic in late 2019 in Wuhan City of Hubei Province, China (Al-Tawfiq & Memish, 2020). On 22 March, 292.142 confirmed cases among them 12,748 death reported by the World Health Organization (Al-Tawfiq & Memish, 2020). Although China was the epicenter of COVID-19, the number of new cases seemed to rapidly decline (Al-Tawfiq & Memish, 2020). Subsequently, the new epicenter was the Eastern Mediterranean region (EMR) and Europe (Al-Tawfiq & Memish, 2020). As in mid-March of 2020, the total number of cases reached 18,060 with 1010 death and 19 out of 22 countries affected reported by the WHO EMR, the majority of cases was in the Islamic Republic of Iran (Al-Tawfiq & Memish, 2020).

On the 12th of July 2020, the number of cases reached 12,698,995 with 564,924 death (2.3 %)(Li & Ren 2020). Until now, SARS-CoV-2 affected 214 countries and territories and the most affected countries were Europe and America with 4,051,387 and 7,748,030 respectively (Li et al., 2020). As on January 30, 2020, SARS-CoV-2 has reported epidemics as a public health emergency of international concern by the World Health Organization (Li et al., 2020). However, on March 11, 2020, WHO declared that SARS-CoV-2 changed from epidemic to pandemic (Li et al., 2020). It was found that SARS-CoV, MERS-CoV and SARS-CoV-2 originated from the bat (Zhou et al., 2020). In SARS-CoV-2 suggest the potential intermediate hosts are snakes and pangolins but this requires more confirmation (Lam et al., 2020; Wan et al., 2020).

The key success of viruses is their evolution (Sanjuán and Domingo-Calap, 2016). Moreover, the type of nucleic acid is one of the main factors that affect the mutation type and subsequently the evolution (Sanjuán & Domingo-Calap, 2016). In addition, RNA viruses and single strain-viruses mutate rapidly than DNA viruses and double-strain viruses (Sanjuán & Domingo-Calap, 2016). These mutations have an impact on the pandemic particularly if they increase the severity of illness as in the severe acute respiratory syndrome corona virus (SARS-CoV-2) (Sanjuán & Domingo-Calap, 2016). The Discovery of novel variant is through sequencing (Leung et al., 2021). The Covid-19 genomics UK consortium performs sequencing for more than 200,000 viruses until date (Tang et al., 2020). January 18th 2021, which revealed the identification of highly infectious variants B1.1.7 and found another similar variant in South African B1.351 (Tang et al., 2020).

Now, the new variant in the UK spread to Australia and Europe (Elfiky & Ibrahim). However , the new variant in the UK reveals nine different mutations for the spike protein (D1118H, D614G, 69–70, S982A, 145, T716I, N501Y, P681H and A570D) (Elfiky & Ibrahim). N501Y mutation present in both variants of SARS-CoV-2 (Elfiky & Ibrahim). N501Y mutation is located in the receptor-binding domain (RBD) of the spikes which is known to interact with the receptor of host cell ACE2 (Elfiky & Ibrahim). The ACE2 is responsible for the recognition and entry into the host cell (Elfiky & Ibrahim). The genome of SARS-CoV-2 is 29.9 kb (Wu et al., 2020). It has 14 open reading frame that encodes 27 proteins (Malik et al., 2020; Wu et al., 2020).

In 5'-terminal region of the genome, 15 non-structural proteins essential for viral multiplication encoded by ORF1 and ORF2 (Malik et al., 2020; Wu et al., 2020). Meanwhile, the 3-terminal region of the genome encodes functional structural proteins as, an envelope protein (E), spike (S), membrane protein (M), nucleocapsid (N) and 8 accessory proteins (Malik et al., 2020; Wu et al., 2020). The computational genomic analysis and phylogenetic revealed that to invade the host's cell, SARS-CoV-2 differs from MERS-CoV which utilizes (DPP4) and shares with SARS-CoV the same human cell receptor (ACE2) (Wan et al., 2020). However, ACE2 is an ectoenzyme receptor attached to the plasma membrane of the host cell, present in many tissues including the kidney, lower respiratory tract, gastrointestinal tract and heart (Imai et al., 2010). The structure pattern analysis suggests SARS-CoV-2 binds with ACE2 by greater affinity about more than 10 folds than SARS-CoV, thus provides more than the threshold desired for viral infection (Wrapp et al., 2020).

The main antigen presented on the envelope of the virus is a spike protein which consists of ~ 150 kDa (Letko et

al., 2020).Consequently, spikes form a transmembrane homotrimer prominent from the surface of the virus to bind to the host's receptor (ACE2) (Qinfen et al., 2004; Weiss & Navas-Martin, 2005). Spike protein consists of two main functional subunits, the first one called subunit S1, plays a role in binding to the host's cell receptor ACE2, and the second is called subunit S2,it is important for viral fusion process to the host-cell surface (Qinfen et al., 2004; Weiss & Navas-Martin, 2005). Since, SARS-CoV-2 are enveloped viruses , the virus is able to enter the host cell by endocytosis (Qinfen et al., 2004; Weiss & Navas-Martin, 2005, Letko et al., 2020).

Phylogenetic analysis revealed that SARS-CoV, SARS-like corona virus and SARS-CoV-2 which was isolated from bats belong to another clade that differs from MERS-CoV (Lu et al., 2020; Zhou et al., 2020). The whole-genome identify between SARS-CoV-2 and SARS-corona virus in bat (SARSr-CoV-RaTG13) is 96% and between SARS-CoV and SARS-CoV-2 is 79.5% (Lu et al., 2020; Zhou et al., 2020). SARS-CoV-2 is distinct from SARS-CoV and MERS-CoV in the rapid spread and that it is more contagious, now SARS-CoV-2 affecting around 214 countries(https://www.who.int.).

Clinical features: The symptoms associated with Covid-19 in the earliest 41 patients varied from major to atypical symptoms (Huang et al., 2020). The major initial symptoms include fever, cough, malaise, in 98%, 76%, and 44% of the 41 patients, respectively while the less common symptoms included headache and diarrhea in 8%, 3% of the patients (Huang et al., 2020). As for a typical symptoms, and according to the epidemiological data from the National Health Commission of China (NHCC), 42 out of 1099 patients have experienced diarrhea and diarrhea may associated with longer duration of more than 10 days (Eastin & Eastin, 2020). Different phases of COVID-19 epidemic have affected an overall clinical features that associated with SARS-CoV-2 (Guan et al., 2020a; Wang et al., 2020). The majority during the first and second phases were old males who had an exposure to the seafood market with a mortality rate ranges from 4.3-15%, which is higher by 1.36% than late phases (Guan et al., 2020a; Wang et al., 2020).

The higher mortality rate in first and second phases is either because of medical conditions and chronic disease, such as diabetes and high blood pressure (Cheng et al., 2020; Guan et al., 2020a). Or because of the high pathogenicity of the virus during its earliest phases (Cheng et al., 2020; Guan et al., 2020b). However, asymptomatic infections were also reported earlier in almost 900 cases (Novel, 2020; Wei et al., 2020).

Routes of transmission: While the main route of SARS-CoV and MERS-CoV transmission is via nosocomial transmission (Elfiky, 2020). The mainly route of transmission is person-to-person contacting with either symptomatic or asymptomatic patients (Elfiky, 2020). It is been estimated that over 31% of the patients have travelled to Wuhan, China and 72.3% have contact with them (Elfiky, 2020). SARS-CoV-2 has been approved to spread mainly through droplets of respiratory system and person-to-person close contact (Qu et al, 2020). Yet, there are growing assumptions that the virus can be spread also through aerosols (Van Doremalen et al., 2020). Furthermore, virus in aerosols perhaps remain infectious on different surfaces for days while in aerosols for minutes or hours (Van Doremalen et al., 2020).

Diagnosis and molecular techniques: Different biomarkers related to specific microorganism known to cause disease, these can be used in the diagnosis of the disease as in the COVID-19 (Taleghani & Taghipour, 2020). However, the biomarker can often be the genetic material of microorganisms, which leads to the development of several molecular tests (Taleghani & Taghipour, 2020). The molecular assay first required to collect the samples from any infected region then extract the genetic material to detect the target gene (Taleghani & Taghipour, 2020). Moreover, there is another biomarker that can be used for diagnosis purpose which is a molecule involved in the body immune response against the antigen (Taleghani & Taghipour, 2020). This biomarker include the immunoglobulin present in the blood that fights against the antigen which in turn leads to the development of serology techniques that detect this interaction (Taleghani & Taghipour, 2020).

Another diagnosis method as well, is to take look at an infected organ which its functions influenced by specific microorganisms then detect the difference between the concentrations of biomarkers (Taleghani & Taghipour, 2020). The abnormalities in the inflammatory markers, chest, kidney markers, liver functions as cystatin C and creatinine are used in the diagnosis of COVID-19 cases (Taleghani & Taghipour, 2020).

Molecular tests (nucleic acid amplification): The commonly molecular technique used to detect the presence of SARS-CoV-2 is reverse-transcription polymerase chain reaction (RT-PCR) (Kelsoe et al., 2012). According to WHO and the Food and Drug Administration (FDA), it's considered as a routine diagnosis to confirm infected cases with SARS-CoV-2 (Kelsoe et al., 2012). Mainly, it's a biochemical reaction (Kelsoe et al., 2012), it is known in the polymerase chain reaction (PCR) technique Deoxyribonucleic acid (DNA) is used as the first template ,while in the case of (RT-PCR) Ribonucleic acid (RNA) is used (Kelsoe et al., 2012). The process of reverse transcription depends on the enzyme reverse transcriptase, which uses a single stranded-RNA to produce single stranded-DNA then this single strand converts to double-stranded DNA before its used in the PCR reaction as a template (Carter & Shieh, 2015; Yan et al., 2020). Even though efforts made to raise the number of conducted PCR test per day (Xue & Jin, 2020). But, there are some limitations consider as an obstacle to this technique among this non-availability of kits and PCR reagents, false-negative detected in a patient has SARS-CoV-2 and it takes a relatively longer time (Xue & Jin, 2020).

The specimen from the lower respiratory tract (broncho alveolar lavage fluid) was used to diagnose cases in the early stage of the outbreak (Huang et al., 2020). The process of sample collection is painful to the patient and requires skilled operator and suction device (Yang et al., 2020). Broncho alveolar lavage fluid is not considered as feasible

for routine diagnosis and SARS-CoV-2 monitoring (Yang et al., 2020). For the time being, the alternative sample collection includes sputum, oropharyngeal swabs and nasopharyngeal swabs which are more safe to patients, simple and rapid (Yang et al., 2020).

A recent study conducted in 2020 has revealed the differences between samples, sputum swap reflected a high-level of positive rate with various degrees of illness severity then nasopharyngeal swabs followed by oropharyngeal swab display low-level of the positive result (Guan et al., 2020b; Huang et al., 2020). In some severe cases, viral RNA is not detectable in the upper respiratory tract sample. As current studies displayed only a small cluster of COVID-19 patients (28%-33.7%) produced sputum (Guan et al., 2020b; Huang et al., 2020). So, the nasopharyngeal swabs are one of the most applicable samples and used worldwide in the COVID-19 diagnosis (Guan et al., 2020b; Huang et al., 2020).

RT-PCR: RT-PCR is a reverse transcription process in which viral RNA converts into complementary DNA (cDNA) then a designed fluorophore-quencher probe and primers are added to amplify the gene of interest –cDNA and detect the existence of SARS-CoV-2(Freeman et al.. 1999; Kojima et al., 2002). To begin with, is RNA extraction from the specimen from the lower or upper respiratory tract (CDC). Moreover, it is recommended to collect specimens from the upper respiratory tract such as, nasal aspirates, oropharyngeal swabs, nasopharyngeal swabs and nasopharyngeal washes (CDC). Meanwhile, specimen collected from the lower respiratory from patients suffering from cough such as , tracheal aspirates, sputum and broncoal veolar lavage (BAL)(CDC). Subsequently, extracted RNA is then added to a mixture consist of probes, primers, precursors, buffers and enzyme, reverse transcriptase, forward and reverse primers, nuclease-free water, nucleotides and a fluorophore-quencher probe (CDC). rRT-PCR cycling conditions are determined by U.S CDC, but two remaining major variables are probe and primer design and choosing target sequence for amplification (Taleghani & Taghipour, 2020).

Particularly, SARS-CoV-2 genome has three main conserved regions: N gene, E gene and RdRP gene which locates in the open reading frame ORF1ab (Wood et al., 2019). In the meantime, most SARS-CoV-2 detection diagnostic kits rely on targeting E gene and RdRP (Wood et al., 2019). These genes have fewer detection limits and high-level of sensitivity in comparison with N gene (Wood et al., 2019). However, any negative result could be an indication to a low viral load and not to virus absence and it may be associated with sampling errors (Wood et al., 2019). The following is a summary of available commercial RT-PCR kits in the market of pharmaceutical companies. On March 13, 2020 , Viractor Erofins released their kit under the name SARS-CoV-2 rRT-PCR test (Eurofins). This test have used upper respiratory samples including: nasopharyngeal swabs, nasal wash, nasal swab, oropharyngeal swabs and nasopharyngeal wash ,also the lower respiratory sample was used as BAL swab (Eurofins).

Later on April 24 , 2020 , BGI Genomics Co. Ltd. (Shenzhen, China) launched their Real- Time Fluorescent RT-PCR detection Kit (BGI, 2020). This kit contains automated specimen preparation system and RNA kit for extraction in addition to the polymerase chain reaction (PCR) which gives the results of 192 samples within 4 hours (BGI, 2020). Both types of lower and upper respiratory specimens can be applied to automated specimen preparation system as BAL fluid, nasopharyngeal swabs, nasal aspirates, oropharyngeal swabs and nasal washes (BGI, 2020). Regarding SARS-CoV-2 kit cross-reactivity challenges was investigated for 50 pathogens without cross-reactivity (BGI, 2020). The fully automated fast test developed by bosch to detect the presence of SARS-CoV-2 with an accuracy of about 95% according to the quality standards of WHO (Global, 2020). This test depends on the micro-array and multiple PCR for SARS-CoV-2 detection (Global, 2020).

However, this device is made up of two main parts: Vivalytic analyser and cartridge including basic reagents (Global, 2020). It's considered the first completely automated test for COVID-19 diagnosis which detects and record the result in less than two hours and a half electronically (Global, 2020). This device can test nine pathogens as influenza B and A simultaneously (Global, 2020). On 21 March 2020, one of the most rapid molecular test has emerged for SARS-CoV-2 , which was developed by Cepheid (Capheid, 2020). In this kit, manual specimen preparation will take less than one minute and the results could be released in about 30 minutes (Taleghani & Taghipour, 2020). Although, previous developed PCR test shared the same step of RNA extraction that is essential prior PCR cycles, but they have few differences in between (Taleghani & Taghipour, 2020). Covid-19 serological test: Serology tests are based on processing blood sample which used to identify infection and distinguish between a recent or previous infection based on immune responses (Bastos et al., 2020). Different serological tests for COVID-19 detection will be discussed below.

The rapid diagnostic test (RDT): It is a rapid and simple test based on the lateral flow immunoassay (LFIA) technology, and it is used in many countries, including the U.S., China, and Singapore (Bastos et al., 2020). The RDT test works by detecting the antigens and antibodies in blood sample (Bastos et al., 2020). In antigen detection, the RDT test directly detects the virus's presence, which indicates that the virus is replicating (there is an active infection) (Espejo et al., 2020). Furthermore, in antibody detection, RDT test detects the immunoglobulin A, immunoglobulin M, and immunoglobulin G (Espejo et al., 2020). The test detects the immune system's response to the virus in the form of antibodies produced in the course of active infection (Espejo et al., 2020). Moreover, the test detects the antibodies that persist following the previous existence of the virus (Ghaffari et al., 2020). The sample that was taken for the antigen test includes oropharyngeal, nasal, or nasopharyngeal swab (Ghaffari et al., 2020).

The antibody test sample is venous blood or finger stick blood (Ghaffari et al., 2020). The antigen and antibody RDT tests may be a priority for different purposes (Ghaffari et al.,

2020). The RDT test results can either be a true positive, a true negative, a false positive, or a false negative (Espejo et al., 2020).

Enzyme-linked immunosorbent assay (ELISA): ELISA test is a lab-based quantitative or qualitative test that uses a patient's serum, plasma, or blood to test for COVID-19 (Alharbi et al., 2020). ELISA test is depending on a plate coated with the viral protein of interest (Alharbi et al., 2020). An example of such a protein, could be the viral spike protein (Alharbi et al., 2020). Later ,The protein is then incubated along with the patient's sample. If the patient's sample contains antibody to the viral protein, the two parts will bind together, forming a complex(Antigen-Antibody complex) (Alharbi et al., 2020). Following the complex formation, detection can be accomplished (Alharbi et al., 2020). The detection can take place by using another wash step of antibodies that a fluorescent or color-based readout (Alharbi et al., 2020).

In COVID-19's context, the test detects for certain antibodies (IgM and IgG) in the patients serum (Alharbi et al., 2020). The test is slower than the RDT test, it requires two to three hours compared to the RDT test, which takes between 15 to 35 minutes (Alharbi et al., 2020). The test is applicable to determine the absence or the presence (quantitative) of the antibodies formed against the virus (Sakamoto et al., 2018). Though the test is applicable in shading light into the presence of the virus and antibodies produced by the immune system against the virus, the test cannot determine whether the antibodies can inhibit the viral replication (Sakamoto et al., 2018). Moreover, the test allows for simultaneous analysis without the pre-treatment of samples (Sakamoto et al., 2018). Furthermore, also the challenge of antibody instability and the high possibility of a false negative, or positive reads (Sakamoto et al., 2018, Alharbi et al., 2020).

Antivirals drugs for SARS-CoV-2 infection:

Fusion inhibitors: Enveloped viruses penetrate the host cell by fusion (Gasmi et al., 2020; Kumar et al., 2020; Matsuyama et al., 2020; Zhang & Liu, 2020). Which could be inhibited by fusion inhibitors which consist of antivirals (Gasmi et al., 2020; Kumar et al., 2020; Matsuyama et al., 2020; Zhang & Liu, 2020). However, there are several drugs available with high antiviral activity against viruses especially SARS-CoV-2 as camostat mesylate and umifenovir (Gasmi et al., 2020; Kumar et al., 2020; Matsuyama et al., 2020; Zhang & Liu, 2020).

Baricitinib: Basically,SARS-CoV-2 like other viruses by the receptor-mediated endocytosis enters inside the host cell (Lu et al., 2020). Moreover, the AP2-associated protein kinase 1[AAK1] used to regulated the endocytosis process (Lu et al., 2020). The process of viral assembly and viral entry can be blocked through the disruption of AAK1 (Lu et al., 2020). Janus kinase inhibitors (JAK) as in baricitinib have a high potential activity to bind and then disrupt AAK1 (Richardson et al., 2020). Subsequently, the baricitinib could be used to disrupt both inflammatory-mediated immune response associated with SARS-CoV-2 and viral entry (Richardson et al., 2020). Therapeutic utilization of baricitinib is related to the occurrence of viral reactivation, neutropenia and lymphocytopenia (Praveen et al., 2020). However, baricitinib may lead to increase the occurrence of co-infection since the patients infected with SARS-CoV-2 suffer from a reduction in the count of lymphocyte (Praveen et al., 2020).

Umifenovir: It also known as arbidol (Kadam & Wilson, 2017). In the fusion mechanisms of influenza viruses, the hemagglutinin envelope glycoproteins can be targeted by the uses of Umifenovir which is considered a nucleoside antivirals (Kadam & Wilson, 2017). A recent study conducted in china demonstrate that Patients treated with umifenovir monotherapy reveal negative viral transformation and SARS-CoV-2 was not detected within 14 days (Zhu et al., 2020).

Camostat mesylate: Also, the fusion step can be inhibited by a serine protease inhibitor as in the Camostat mesylate (Uno, 2020). However, SARS-CoV-2 can enter the host cell through the use of either TMPRSS2 receptors or/and ACE-2 receptor but camostat mesylate inhibits TMPRSS2 receptors (Matsuyama et al., 2020; Uno, 2020). The expression of SARS-CoV-2 spike protein (S) is down regulated which prevent the surface fusion and then block the viral entry into the host cell (Gasmi et al., 2020; Matsuyama et al., 2020). The previous study suggests the entry of SARS-CoV into the human bronchial epithelial cell can be prevented by using camostat mesylate (Kawase et al., 2012). Moreover, an In vitro study found that cysteine protease inhibitor as E-64d and camostat mesylate can efficiently inhibit the binding between SARS-CoV-2 and TMPRSS2 (Wang et al., 2020). Now, in Germany (Bose & Basu, 2020) and Denmark (Sharma et al., 2021) ongoing study taking place to evaluate the effectiveness of combination medication of camostat mesylate and hydroxychloroquine vis-a-vis only single hydroxychloroquine through the clinical trials (Sharma et al., 2021).

Protease inhibitors: Accordingly , COVID-19 protease inhibitors can be used as, atazanavir, lopinavir and darunavirfor treatment (Ferreira et al., 2020; Kadam & Wilson, 2017). Through the use of Computer-aided drug design techniques, a number of drugs such as elbasvir, eravacycline, carfilzomib, lopinavir and carfilzomib are able to disrupt the main viral protease in SARS-CoV-2 (Wang, 2020).

Lopinavir: Lopinavir have showed a possibility to inhibit SARS-CoV-2 at half-maximal effective concentration which is the drug concentration titre that triggers a response midway between the maximum and the baseline after a specified exposure period of 26.36 μM (Choy et al., 2020). The use of lopinavir to treat COVID-19 patients in China leads to an increase in the count of eosinophil (Liu et al., 2020). A recent study found that the combination between ritonavir and lopinavir acts as inhibitors to the main viral protease of SARS-CoV-2 (Liu & Wang, 2020). Moreover, in the clinical trial and in vitro showed that in a combination mixture of lopinavir-ritonavir which is known as Kaletra®, a high antiviral activity against SARS-CoV (Chu et al., 2004). In some countries lopinavir-ritonavir

combination was used as an emergency therapy to treat COVID-19 patients (Cao et al., 2020; Lim et al., 2020). According to WHO options clinical trials for "solidarity" COVID-19, can use the combination of Lopinavir-ritonavir and interferon (INF)-β (Yavuz & Ünal, 2020). Ritonavir-lopinavir combination have resulted in low viral load and improved the symptoms of patients (Zhu et al., 2020).The lung damage could be inhibited by the use of a combination of umifenovir and ritonavir-lopinavir (Deng et al., 2020). A recent study showed the use of lopinavir-ritonavir leads to a better clinical outcomes (Guan et al., 2020b).

Darunavir: Additionally, Darunavir was used as an anti-HIV drug to treat HIV infection but it was recommended to be used for use in Italy to treat COVID-19 infection (Nicastri et al., 2020). In vitro studies showed that the combination use of darunavir with cytochrome P-450 inhibitors as cobicistat or ritonavir, could inhibit SARS-CoV-2 replication activity (Harrison, 2020). Clinical trials are ongoing to assess the efficacy of the combination of darunavir with hydroxychloroquine and other antiviral drugs to treat COVID-19 infection (Kongsaengdao & Sawanpanyalert, 2020). In addition, clinical trials are underway to evaluate the combination of darunavir along with cobicistat (Sarkar et al., 2020). PREZCOBIX® is used as a treatment for COVID-19 which is a fixed dose of the combination of cobicistat and darunavir (Sargent et al., 2021).

Reverse transcription inhibitors: It is another strategy to control SARS-CoV-2 infection by targeting the step of the reverse transcription, through RdRp blocking prevent viral replication (Frediansyah et al., 2020). There are number of inhibitors like nucleoside reverse transcriptase translocation inhibitors (NRTTIs), nucleoside reverse-transcriptase inhibitors (NRTIs), non-nucleoside reverse-transcriptase inhibitors (NNRTIs) and nucleotide reverse transcriptase inhibitors (NtRTIs) (Frediansyah et al., 2020).

Remdesivir: Remdesivir is a nucleotide analogue that has a broad spectrum of antiviral drug properties against most of the single stranded RNA (ssRNA) viruses such as corona viruses (involved both SARS-CoV-2 and MERS-CoV) , Hendra virus , Ebola virus, Nipah virus, Marburg virus , Lassa fever virus, respiratory syncytial virus and Junin virus (Al-Tawfiq, Al-Homoud, & Memish, 2020; Ko et al., 2020). It is designated as GS-5734 , when entering into the host cell the GS-5734 metabolized to GS-441524 which is able to decrease RNA replication of MERS-CoV, SARS-CoV, endemic and zoonotic human delta corona virus at an in vitro conditions (Gordon et al., 2020). Remdesivir is a nucleotide reverse-transcriptase inhibitors (NtRTIs) (Tikkinen ett al., 2020). It is considered an inhibitor for RNA-dependent RNA polymerase (RdRp) (Al-Tawfiq et al., 2020). It acts on changing of viral the exonuclease function that leads to the disruption of proofreading, reducing viral RNA replication and production decline (Al-Tawfiq et al., 2020). It is recommended to treat severe cases of COVID-19 because it can prevent viral replication (Harrison, 2020).

Ribavirin: It is a guanine derivative analogue and has an antiviral activity against hepatitis C virus (HCV) (Graci & Cameron, 2006). In Vitro study revealed that it has antiviral effect in SARS-CoV-2 infection It functions by disrupting the polymerase activity, prevents the step of RNA capping which is important to RNA stabilization then the viral replication will be obstructed (Graci & Cameron, 2006). Moreover, ribavirin disrupts the function of the inosine monophosphate dehydrogenase enzyme, which results in blocking guanosine production then enhances the degradation of viral RNA (Graci & Cameron, 2006). Ribavirin is recommended in combination with either lopinavir-ritonavir or IFN alpha (Drożdżal et al., 2020). In addition, it can be used either by oral or intravenous route (Elfiky, 2020).

Current status and technology used in the development of SARS-Cov2 vaccine worldwide: Corona viruses are single stranded enveloped RNA virus, it consist of few proteins which have a significant role in the virus structure (S protein, E protein, M protein , and N protein) most of vaccine manufacturers target the S protein as a vaccine antigen rather than using an inactivated vaccine (van Doremalen, Lambe, et al., 2020). There are about 48 candidate vaccines in clinical evolution are either in Phase 1, 2, or 3 and about 164 candidate vaccines in preclinical evaluation (Van Doremalen et al., 2020). A number of them have reached advanced stages of development and showed encouraging results (Van Doremalen, Lambe, et al., 2020). In this review, few vaccines that have reached the latest stage of development will be discussed.

ChAdOx1 nCoV-19 Vaccine: It is known as oxford vaccine referring to chAdOx1 nCoV-19 vaccine, additionally, known as AZD1222 referring to the co-development of the vaccine by university of oxford, VACCITECH, and AstraZeneca (Van Doremalen et al., 2020). It is a promising vaccine for SARS-CoV2 from anon-replicating viral vector category. It is a chimpanzee adenovirus vector vaccine; using adenovirus as a gene delivering system by inserting spike protein gene to E1 locus of ChAdOx1 then using human embryonic kidney 293 cell line to increase the number of the viral particle which later was purified to be ready as a vaccine later purified it to be ready vaccine. It is considered a suitable vaccine for covid-19 based on the strength of immune response that it can elicit from one dose and it cannot replicate (Van Doremalen et al., 2020). Therefore, it would not cause an infection which ensures the safety of the vaccine for in elderly and children (Van Doremalen et al., 2020).

It was first tested on mice and Rhesus macaques and showed adequate immunogenicity. Phase I /II clinical trial was randomized controlled, tested on 5 different locations in the UK on 1090 healthy volunteers aged 18-55 y either received ChAdOx1 nCoV-19 (n=543) or MenACWY meningococcal conjugate vaccine (n=534) to compare, both vaccines are single dose delivered intramuscularly. The vaccine showed an increase in antibody titer however, the booster dose had a much efficient response. It reflected enough safety and immunogenicity in this trial (van Doremalen, Lambe, et al., 2020). Phase III was multicenter study in different countries with 30000 participants to ensure the safety and immunogenicity (Van Doremalen et al., 2020).

On November 18, 2020, the preliminary results of phase III were published, and they found that the efficacy of preventing infection reaches an average of 70%, but in the same study, two regimens of vaccination were given (Folegatti et al., 2020). A prime dose followed by a boost dose after a month has an efficacy of 62% (Folegatti et al., 2020).The other regimen includes a half dose followed by a full dose after a month generated an efficacy of about 90% (Ramasamy et al., 2020).

Moderna Vaccine mRNA-1273: Mainly, It is a mRNA-based vaccine (Corbett et al., 2020) .Whereases mRNA is capsulated by lipid nanoparticle (LNP) that codes for S protein to elect immune response in the human body (Corbett et al., 2020). This vaccine is co-developed by Moderna, NIAID, Lonza, Catalent Inc and BIOQUAL (Corbett et al., 2020). The preclinical phase which was applied on rhesus monkey had induced a high level of antibody, and helper T-cells type 1,besides that it showed that there was not viral replicating(Corbett et al., 2020). These results lead to phase I clinical trial, it was accomplished in the USA on 155 healthy participants males and non-pregnant females aged 18-99years (Corbett et al., 2020). The vaccine was sufficient in inducing antibody response (Jackson et al., 2020). Phase II was done on 600 participants aged 18 and above with two doses either 50 μg or 100 μg and it showed a favorable response with higher doses (Jackson et al., 2020). Phase III is currently ongoing with 30000 participants (Jackson et al., 2020). The company announced on 16 November 2020 that the vaccine has 94.5% efficacy in preventing covid-19 based on 95 participants' results (investors.modernatx.com, 2020). On December 18, 2020, the FDA authorized the emergency use of the Moderna COVID-19 Vaccine (Jackson et al., 2020).

Sinovac Biotech Vaccine: This vaccine was developed by Sinovac Biotech in China (Gao et al., 2020). It is a potential vaccine that belongs to inactivated viral vaccines category, by using inactivated SARS-CoV-19, it was isolated from different patient then one strain was chosen CN2 vaccine development (Gao et al., 2020). In the preclinical phase, the vaccine was used in Rhesus Macque to evaluate immunogenicity and protective effect and it showed partial or complete protection (Gao et al., 2020). Phase I/II was generated done on human subjects aged 18-59 years , that resulted by using two doses of 6 μg/0.5 mL or 3 μg/0.5 mL of the vaccine ,which produced specific neutralizing antibodies (Bangash et al., 2020).

Phase III clinical trial introduces in different countries (Bangash et al., 2020). Until now it is in use in china only (Bangash et al., 2020). Many other vaccines have reached an advanced stages of development mainly, located in the USA, China, Russia, Japan, and Europe (Zhou et al., 2020). Cansino biologics is developing a non-replicating viral vector vaccine using adenovirus type-5 (Ad5)-vector (Zhou et al., 2020). The results of Phase I/II showed safety of vaccine and its ability to elicit an immune response after one immunization dose (Zhou et al., 2020). The vaccine is now in Phase III trials in many countries, Saudi Arabia is one of these countries with at least 5000 volunteers (McMurry et al., 2021). BioNTech and Pfizer announced that their mRNA vaccine has an efficacy over 94% and it was approved by the FDA for Emergency Use Authorization (EUA) on December 11, 2020 (McMurry et al., 2021).

The Role of ORF8: ORF8 is an accessory protein that is considered as one of the most rapidly evolving proteins in beta corona virus (Ceraolo & Giorgi, 2020; Cui et al., 2019; De-Sousa et al., 2020; Laha et al., 2020; Lau et al., 2015; Lu et al., 2020; Mohammad et al., 2020). However, the replicating of SARS-CoV-2 and SARS-CoV does not require the expression of ORF8 (Muth et al., 2018). Through the early transmission of SARS-CoV-2 from person to person, 29 nucleotides have been deleted [Δ29], the milder disease is correlated with the splitting of ORF8 into ORF8b and ORF8a (Muth et al., 2018). The decreased occurrence of hypoxia and milder illness in SARS-CoV-2 was associated with the deletion of 382 nucleotides [Δ382] (Gong et al., 2020; Su et al., 2020; Young et al., 2020).

SARS-CoV-2 ORF8 is a protein which consists of 121 amino acids, containing an N-terminal signal sequence ,followed by a predicted Ig-like fold (Tan et al., 2020). The endoplasmic reticulum (ER) can be imported via a signal sequence from the ORF8 protein of both SARS-CoV-2 and SARS-CoV (Gordon et al., 2020). SARS-CoV-2 ORF8 can interact with various proteins of the host inside the ER lumen that involves the interaction with numerous factors including the degradation associated with ER (Gordon et al., 2020). ORF8 supposed to be secreted rather than stored in ER, in SARS-CoV-2 infections the ORF8 antibodies are of the major markers (Hachim et al., 2020). There are different functions about SARS-CoV-2 ORF8. ORF8 over expression in the cells can lead to disrupting the IFN-I signaling (Li et al., 2020). SARS-CoV-2 ORF8 can down regulate MHC class I (MHC-I) inside the cells (Zhang & Holmes, 2020; Zhang et al., 2020).

CONCLUSION

The recent novel coronavirus-19 SARS-CoV-2 spread throughout China and has become a serious public health issue globally. This outbreak poses a heavy burden on both the economic and health status of human beings. However, bats are considered as the key reservoir. Until now, there are no promising prevention strategies or anti-viral drugs have been developed against SARS-CoV-2. However, researchers are working on developing drugs to treat COVID-19 patients. Furthermore, there are many companies working on developing the SARS-CoV-2 vaccine. But it also needs rapid animal-based and human trials because this vaccine needs 3 to 10 months for commercialization.

Case Report (Human Studies) Ethical Clearance Statement: The Current Case Report/ Studies were Conducted as Per the Guidelines of SCARE.

Riaz A Agha et al., (2020). The SCARE 2020 Guideline: Updating Consensus Surgical Case Report (SCARE) Guidelines. doi: 10.1016/j.ijsu.2020.10.034. Epub 2020 Nov 9.

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Dental Communication

An Overview of Applications of Digital Orthodontic Scanners: Unleashing A New Era

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ABSTRACT

Digital intraoral scanners (IOS) have a variety of applications in the orthodontic clinic. Without recommending a particular scanner. In the next decades, this technology will become ordinary in orthodontic practice. Digital dentistry is the future since it provides efficiency and effectiveness for both the clinician and the patient. A number of different intraoral scanners are available on the market. The clinician should be aware of the available device features, maintenance, prices, and advantages. Digital scanning of the dental arch is expected to become routine in the dental clinic, and more progress in the IOS is expected in coming years. The aim of this article is to provide an overview of currently available IOSs for orthodontic use, their advantages and disadvantages over conventional procedures, features of available scanners, and clinical software.

KEY WORDS: INTRAORAL SCANNERS, ORTHODONTICS, DIGITAL IMAGING, DIGITAL MODELS.

INTRODUCTION

Digital orthodontics is in a new era that is progressing rapidly. The availability of this technology has simplified orthodontic practice by reducing inconsistencies when fabricating dental casts, reducing their storage, and reducing the inconvenience of using impressions. Digital intraoral scanners (IOS) have a variety of applications in the orthodontic clinic (Kravitz et al., 2014). The introduction of 3D scanning in dentistry began with the use of computer-aided design and computer-aided manufacturing (CAD/CAM) by Dr. Francois Duret in 1973 (Duret, 1973; Logozzo et al., 2008). Afterwards, Sirona Dental Systems manufactured a chairside scanning device (CEREC) utilizing CAD/CAM technology (Brandestini and Moermann, 1989; Mormann, 2006). Although CEREC had its own limitations and seemed imperfect at that time, it was without competitors until the Cadent iTero digital impression system was launched in 2006, introducing full-arch intraoral scanning (Kravitz et al., 2014; Hwang et al., 2020).

In 2011, Align Technology purchased Cadent, allowing clinicians to submit 3D scans instead of physical impressions for Invisalign fabrication. Since then, dental companies around the world are focused on producing superior IOS. More than 14 scanners were demonstrated at the 2017 International Dental Show in Cologne (Hwang et al., 2020). Digital scanning of the dental arch is expected to become routine in the dental clinic, and more progress in the IOS is expected in coming years. The aim of this article is to provide an overview of currently available IOSs for orthodontic use, their advantages and disadvantages over conventional procedures, features of available scanners, and clinical software.

Advantages and applications of 3D scanners in orthodontics: Impression-taking might be an unpleasant experience for patients, which might cause them discomfort and anxiety, especially with a sensitive gag reflex (Kravitz et al., 2014). Alginate and Polyvinyl siloxane (PVS) impressions have been associated with many drawbacks, such as voids, bubbles and improper tray size, temperature sensitivity, limited working time, poor pouring, and improper trimming, as well as model breakages. Studies have shown that full-arch digital scans are as accurate as conventional impressions (Ender and Mehl, 2011).

In orthodontics, the use of digital scanning has reduced the chair time, expedited submission of the records to the labs, expanded accessibility from different locations, reduced

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storage problems, eliminated model breakages, improved accuracy of the appliances, enhanced the workflow, and facilitated the fabrication of customized appliances, aligners, and retainers (Kravitz et al., 2014; Martin et al., 2015; De Luca et al., 2015; Hwang et al., 2020).

For patients, it has resulted in a better demonstration of their case and the expected results, while reducing chair time, anxiety, and discomfort (Kravitz et al., 2014). Depending on the device, software, and the clinician, IOS can be used for a variety of applications in orthodontics. Usage includes treatment planning (Rheude et al., 2005), indirect bonding (Wiechmann et al., 2003), customized lingual brackets (Wiechmann et al., 2003), clear aligner fabrication (Hilliard, 2006), orthognathic surgery simulation (Gateno et al., 2007; Cousley and Turner, 2014), as well as scoring surgical outcomes in cleft lip and palate patients (Asquith and McIntyre, 2012). The accuracy of IOS has been investigated in several studies.

Although the early version of IOS was not adequate for workflow (Ender and Mehl, 2013), scanning time and accuracy were improved by improving optical technology (Grunheidet al., 2014). Patzel et al. (2014a; 2014b; 2014c) did several studies to verify the accuracies of three to four types of IOS. They found that results in accuracy were comparable and that digital workflow was more time efficient. Other investigators compared the digital with conventional methods, and they found that, even with some local deviation in complete-arch test results, higher precision (range 42.9–82.8 µm; average 50 µm) was achieved with digital scanners than with conventional alginate impressions (162.2 µm) (Mehl et al., 2009; Ender and Mehl, 2013; Ender and Mehl, 2015; Ender et al., 2016 Hwang et al., 2020).

The technical methods of scanners: Scanning performance depends on the technology used in their imaging processors. There are different types of available technologies, but the most common image technologies follow are as:

Confocal laser scanning: The three-dimensional structure is produced by repossessing two-dimensional images at different confocal planes. A filter with a tiny pinhole produces the laser. Only the area within the conofocal plane is captured. This imaging technology is used by TRIOS and iTero (van der Meer et al., 2012; Wong et al., 2017).

Triangulation technique: The Pythagorean theorem is used in this technique to calculate distance and angulation. It is composed of three points: laser emitter, object surface, and sensor. This technique is used by the CEREC system. To produce more details, this technique requires using a radiopaque powder that contains titanium oxide (e.g., Optispray by CEREC) (Logozzo et al., 2008).

3D in-motion video recording: This technology uses high-definition video with three small cameras (trinocular imaging) to capture three precise images of the tooth. A unique characteristic of this technique is the high data redundancy, in addition to high accuracy. This method requires a thin layer of powder to serve as a connector for location references. This technique is used by 3M's Lava C.O.S. and True Definition (Syrek et al., 2010; Kravitz et al., 2014).

Most popular IOS products: Below, I will discuss different types of commercially available scanners, their clinical performance, and their popularity.

iTero scanner by Align Technology: In 2007, Cadent LTD introduced the iTero digital scanner. Later, in 2013, Align Technology restructured and introduced its iTero scanner to the market. The technical method used in this scanner is confocal imaging technology with a red-light laser beam (Babayoff and Glaser-Inbari, 2004). Since the iTero scanner was associated with Invisalign, it became more popular, especially among Invisalign practitioners. The advantage of the iTero scanner is that it is a powder-free scanner, so it provides operators with a real simulation and progress of Invisalign patients. The scan tip can directly contact the tooth surface. The iTero software uses an open-source standard triangulation language (STL), which makes it compatible with different systems, i.e., Invisalign, Incognito, Sure Smile, etc. Although the scanner wand is considered bulky compared with other types of scanners, the manufacturer argues that this was done to take a wider view, which in turn will obtain a shorter scanning time and high accuracy. The scan wand has a reflective mirror design, which, according to the company, will make it easier to scan the most distal tooth. Further, to prevent infection, the wand has a disposable sleeve (figure 1).

Figure 1: iTero Scanner

True definition scanner by 3M ESPE: In 2008, 3M produced Lava C.O.S. using an active wavefront sampling technology. This classic scanner provided good performance at that time. Later, the company improved its specifications and produced the True Definition scanner. In 2016, it launched the Mobile True Definition, which has the same software/hardware as the original. The advantages of this scanner are that it provides a high accuracy and the tip is the size of the hand piece. This type of scanner requires a

thin coat of powder for high accuracy. The scanner doesn't offer real-time full-color scans. The software is an open STL format. The data files are compatible with Incognito, Invisalign customized lingual brackets, Sure Smile, and Clear Correct. Also, the software is designed to work with UnitekTM Treatment Management Portal Digital Model software for treatment planning, such as Bolton and space analysis (figure 2).

CEREC Omnicam by Sirona Dentsply: The CEREC system gained its popularity by manufacturing CAD/CAM. It has a strong name in restorative dentistry. Its scanning technology is the triangulation imaging method. The scanner provides video streaming instead of static images, and powdering is not required in the scanning procedure. In orthodontics, the system has been advanced by the possibility of producing a full-arch scan (figure 3).

Figure 2: True definition Scanner

TRIOS 3/TRIOS 3 wireless by 3Shape: In 2010, 3Shape launched the first edition of TRIOS 3. In the beginning, there were a lot of complaints that there was fogging in the wand, but later, the company solved this problem. The system has the advantage that the operator can perform the scanning as soon as it is turned on, since the new scanner tip is preheated. Moreover, the scanner wand is downsized, and the wand sleeve can be sterilized by autoclave. The scanning technology is the Ultrafast Optical Sectioning, which is based on the conofocal laser principal. The wireless version of this scanner was launched in 2017, offering the operator flexibility and freedom in scanning. The system also has an advanced treatment simulator and a built-in recording feature of dynamic occlusion (figure 4).

Figure 3: CEREC Scanner

Figure 4: TRIOS 3 Scanner

Which one to use?: Suggestion of a specific scanner is not easy, and that is not an aim of this article. A comparison of different features of the popular scanners will be discussed below. One of the most important features in any scanner is the accuracy of the scanning (Goracci et al., 2016). Two factors affect scanner accuracy: the trueness and precision. Trueness means how truly the scanner can replicate the real dimensions, while precision means how reproducible the scanner is (Huang et al., 2020). Though many previous studies suggested that iTero and True Definition have higher accuracy than TRIOS and Omnicam (Ryakhovskiy and Kostyukova, 2016; Amin et al., 2017; Renne et al., 2017), accuracy comparison between iTero and True Definition was not studied. Scanning accuracy is affected by the material being scanned (Nedelcu and Persson, 2014). Powdering improves the scan accuracy. *In vitro* study revealed that lingual brackets are less accurate than buccal ones, though TRIOS and iTero produced accurate scans under these circumstances (Park et al., 2016; Marghalani et al. 2018; Nedelcu et al., 2018).

Scanning time is also important. Kim et al. (2016) found that iTero may have a longer scanning time than the traditional impression method, but as the clinician's experience increases, the time decreases. Moreover, they reported that TRIOS takes a shorter time than the traditional impression and that it is suitable for clinicians with less experience with scanners (Kim et al., 2016). Finally, the machine prices: the IOS prices range from $36,000 (USD) to $90,000 (USD). The price depends on the company, the machine, the software, and annual fees. Investing in IOS is an important step that needs critical thinking. It's a good idea to ask a colleague who has had experience with scanners before buying. Also, ask about the company you are buying from—its reputation, as well as the after-sales service and maintenance.

CONCLUSION

Digital dentistry is the future since it provides efficiency and effectiveness for both the clinician and the patient. A number of different intraoral scanners are available on the market. The clinician should be aware of the available device features, maintenance, prices, and advantages.

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Nutritional Communication

Material and Techniques for Microencapsulation of Probiotics: Literature Based Review

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ABSTRACT

Probiotics are live microorganisms that are introduced to induce positive health benefits in the host. Different species relevant to various genus are used in food in order to enhance the health benefits of the food product. But, the most widely used probiotics are related to lactobacillus and Bifidobacteria genus. The health benefits attributed to the consumption of probiotics include immune system modulation, reduction of symptoms related to irritable bowel syndrome (IBS), diarrhea treatment, reduction of lactose intolerance, serum cholesterol reduction, anti-inflammatory properties, prevention of cancer and mutagenesis, and production of bacteriocins which make environment unsuitable for pathogenic microorganisms specially by lowering the pH. The claimed health benefits are related to the species and even strain of probiotics and achieved when the microorganisms are higher than the minimum satisfactory level. Moreover, the viability of probiotics is of vital importance from the time of production to the time of reaching to the target organ. In order to enhance the survival of probiotics, several techniques have been used among which the results of microencapsulation are outstanding. Microencapsulation is the process of physical protection of probiotics form harsh environmental and hostile conditions. The process is carried out by using different materials like alginate, chitosan, starch and others through different methods such as extrusion, emulsion, spray drying and freeze drying. Alginate in combination with chitosan coating is widely used through extrusion and emulsion techniques. But, in the terms of industrial use the spray drying method is outstanding. In this review, the efforts have been made to gather more relevant information and undertook studies on microencapsulation of probiotics.

KEY WORDS: MATERIALS, MICROENCAPSULATION, PROBIOTICS, REASONS, TECHNIQUES.

INTRODUCTION

Probiotics are beneficial microorganisms and have been extensively used for their beneficial health effects. The term probiotic has been derived from a Greek word which means for life or is a combination of Latin (pro=in favor of) and Greek (bios=life). So far, many definitions have been postulated for the term probiotics, but a more comprehensive and thorough definition has been given by Hill et al. (2014). They define the probiotics as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host". In order to get maximum health benefits from the consumption of probiotics, it is recommended to get various species of probiotics at time of consumption. The most commonly used probiotics are from the genus of lactobacillus and Bifidobacteria. In order to get the claimed health benefits from probiotics, the number of probiotics should be higher than the range of minimum satisfactory level. This level is reported in the range of 10^6-10^7 CFU/mL (Lee and Salminen 2009; Mortazavian et al. 2012; Arihara 2014; Sarao and Arora 2017; Kumari et al. 2020).

The claimed health benefits associated with probiotic consumption are elevation of immune system, improvement in colonic health, cancer prevention, reduction in serum cholesterol level and others (Kumari et al. 2020). Moreover, improvement in sensitivity with foods, neurological activities, diabetes mellitus, *H. pylori* infection, and prevention and treatment of oral infection are also reported to be associated with probiotic consumption (Shafi et al. 2014; Roobab et al. 2020; Chugh et al. 2020). The claimed health benefits for the consumption of probiotics are obtained when the live cells of probiotics reach to the target part of the body. Thus, the survival of probiotics is of importance during storage,

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manufacturing process of food and transit through the stomach and small intestine. Various techniques have been developed for the enhancement of probiotics' survival in food and subsequent process, of which microencapsulation of probiotics are outstanding (Figueroa-Gonz´ et al. 2011; Terpou et al. 2019; Han et al. 2021).

Microencapsulation is a recent method of physical protection of probiotics. The particle size of 0.2-5000µm is considered as microcapsule (Maleki et al. 2015). Alginate, k-Carrageenan, Gellan gum and xanthan gum, Chitosan, Starch, Gelatin, Cellulose acetate phthalate, and Milk proteins are used mainly through different chemical, physical, and physiochemical methods for the microencapsulation of probiotics (Burgain et al. 2011; Hamyouni et al. 2012; Cota and Stanila, 2013; Iravani et al. 2015; Peanparkdee et al. 2016; Yao et al. 2020). Extrusion, emulsion, spray drying, and freeze-drying are broadly used techniques (Burgain et al. 2011; Solanki et al. 2013; Rathore et al. 2013; Serna-Cock and Vallejo-Castillo, 2013; Martín et al. 2015; Pupa et al. 2021).

2. Survival of Probiotics: In order to obtain the maximum health benefits from probiotic products it is necessary that the product should have the minimum certain number of viable cells of probiotics. This number of probiotics is called the therapeutic dose of probiotics (Pupa et al. 2021). Despite of no world-wide anonymous consensus on the minimum viable probiotic cells per gram or milliliter of probiotic product, generally, the concentrations of 10^6 and 10^7-10^8 cfu mL^{-1}(cfu g^{-1}), respectively, have been accepted as the minimum satisfactory levels (Mortazavian et al. 2012; Marinova et al. 2019). It has also been stated that probiotic products should be consumed regularly with an approximate amount of 100 gr /day in order to deliver about 10^9 viable cells into the intestine (Terpou et al. 2019; Pupa et al. 2021). Probiotics' viability, which is crucial for reaching and colonizing the human large intestine, determines their quality in probiotic food products. Probiotics must be viable during three important stages: (1) storage; (2) the functional food's manufacturing process; and (3) stomach and small intestine transit. Thus, probiotic viability is a critical problem from both an economic and technological standpoint. A study found that freezing a probiotic at at−40^{O}C reduced its vitality from 1.8×10^{15} to 1.6×10^{10} CFU mL−1, while freeze-drying followed by storage at 4^{O}C reduced its viability from 8.9×10^{14} to 2.4×10^{9} CFU mL^{-1} (Figueroa-Gonz´ et al. 2011; Pupa et al. 2021).

Many factors have been reported that affect the survival of probiotic bacteria in food throughout the three important stages indicated above. Food parameters (pH, titratable acidity, molecular oxygen, water activity, presence of salts, sugar, and chemical compounds such as hydrogen peroxide, bacteriocins, artificial flavoring, and coloring agents); microbiological parameters (heat treatment, incubation temperature, product cooling rate, packing materials and storage procedures, and manufacturing scale); processing parameters (strain of probiotic, rate and proportion of inoculation)are among the factors important factors (Tripathi and Giri 2014; Terpou et al. 2019). On the other hand, food matrix, very low pH in the stomach, bile salts and gastro-enzymes in the small intestine, Lysozyme in saliva, and colonic conditions (competition with other bacteria including pathogens) are the key factors that determine probiotic survival in the GIT (Mortazavian et al. 2012; Stasiak-Różańska et al. 2021).

Microencapsulation of Probiotics: Encapsulation of probiotics is one of the most effective methods for increasing the viability of probiotics (Dong et al. 2013; Yao et al. 2020). "Microencapsulation is a process by which live cells are packaged within a shell material, which confer them protection by preventing their direct exposure to unfavorable environment, but permits diffusion of nutrients in and out of the matrix, thereby supporting the viability of the cells" (Vivek 2013). Microcapsules are particles with a diameter of 0.2 to 5000 micrometers, while macrocapsules are larger than 5000 micrometers and nanocapsules are smaller than 0.2 micrometers. The procedure is known as coating when the core material is quite large. The enclosed particle is ideally spherical; nevertheless, the structure of the core material influences this (Maleki et al. 2015). The core material is the entrapped components inside the microcapsule, while polymers are referred to as wall materials, shells, coatings, carriers, or encapsulants (Peanparkdee et al. 2016; Pech-Canul et al. 2020).

The reservoir type and the matrix type are two separate types of encapsulations. the reservoir type has a shell around the core material, and therefore it is also known as a capsule. In the later type, the active agent is spread over the carrier material and can also be present on the surface. A third type of capsule is created by combining these two types in which the active substance is retrieved by a coating. Encapsulated probiotics have been employed in a variety of probiotic products so far. Microencapsulated probiotics are most commonly found in dairy products (49%), followed by fruit and vegetable-based goods (28%), meat-based products (13%), and bakery items (11%) (Burgain et al. 2011; De Prisco and Mauriello 2016; Stasiak-Różańska et al. 2021).

Reasons for Microencapsulation: Microencapsulation is primarily used to protect encapsulated materials from extreme environmental conditions so that they can safely reach the point of ingestion and eventually pass-through GIT. The following are some of the most important reasons for encapsulation:

- It enhances probiotic viability by allowing them to pass through the GIT's acidic-enzymatic-bile conditions
- Production of high-viability bacterial starter cultures
- Increase the viability of probiotic microorganisms by protecting them from harsh environmental conditions
- Application in Fermenter: increases microorganisms' endurance to severe the conditions
- Production of food products with a high probiotic viability till its consumption
- Probiotic immobilization
- Fixation and improvement in the sensory properties of probiotic products
- Superior active agent handling
- Improvement in the stability of final product and

throughout processing
- Improved safety (e.g., reduced flammability of volatiles like aroma, no concentrated volatile oil handling)
- Controlled release (Vivek 2013; Razavi et al. 2021)

Materials Used for Microencapsulation of Probiotics: Depending on the substance to be coated and the characteristics needed in the final microcapsules, coating materials, which are essentially film-forming materials, can be chosen from a wide range of natural or synthetic polymers. The coating material's composition is the most important factor in determining the microcapsule's functional characteristics and how it may be utilized to increase the performance of a certain component. The following properties should be present in an ideal coating material (Poshadri and Kuna 2010; Razavi et al. 2021):

1. Good rheological characteristics at high concentrations and simple encapsulation workability
2. The capacity to disperse or emulsify the active ingredient while also stabilizing the resulting emulsion.
3. Non-reactivity with the encapsulated materials throughout processing and long-term storage.
4. The capacity to seal and hold the active ingredients within its structure throughout processing and storage.
5. Under drying or other desolventization conditions, the capacity to entirely release the solvent or other ingredients used during the encapsulation process.
6. The capacity to protect the active substance from adverse environmental conditions (e.g., oxygen, heat, light, humidity).
7. Solvents' solubility that are acceptable in the food industry (e.g., water, ethanol).
8. Chemical nonreactivity with the active core materials.
9. Inexpensive, food-grade status.

A single coating material cannot fulfill all of the aforementioned requirements. In reality, either a mixture of coating materials is used, or modifiers such oxygen scavengers, antioxidants, chelating agents, and surfactants are added (Poshadri and Kuna 2010; Razavi et al. 2021). The following are some examples of encapsulating materials:

Alginate: Alginate is a naturally produced polysaccharide that is made up of two monosaccharide units: α-L-guluronic acid (G) and β-D-mannuronic acid (M), which are linked together by a β (1–4) glycosidic bond. The technological functionality of alginate is determined by M/G ratios. On the other hand, the gel's strength is determined by the large amount of block G (Solanki et al. 2013). Alginate, particularly calcium alginate for its non-toxicity, cheapness, simplicity, and biocompatibility is used extensively in the encapsulation of probiotics (Sarao and Arora 2017). Calcium alginate is used as encapsulation material in different concentrations mainly in the range of 0.5–5% (Martín et al. 2015; Liu et al. 2020).

The use of alginate as the encapsulating material has certain disadvantages as well. The primary drawbacks associated with alginate are: the sensitivity to the acidic environment, problems in the scaling up process, quite porousness of their microcapsules. These pitfalls can be solved by combining alginate with another polymer component or altering the alginate's structural properties. These methods can be blending starch with alginate, mixing alginate with other polymers such as corn starch, resistant starch, mixing of alginate with cryoprotectants as like glycerol in order to improve viability of at -20 °C frozen storage, or to form a semipermeable layer of chitosan around the alginate capsules (Martín et al. 2015; Sarao and Arora 2017). Ji et al. (2019) reported that alginate microcapsule coated with chitosan protected *Bifidobacterium longum* from GIT fluid and elevated temperature conditions (Ji et al. 2019: Liu et al. 2020).

Chitosan: Chitosan is a linear polysaccharide that is randomly made up of -(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine. Chitosan is made commercially by deacetylating chitin, a structural component of crustaceans' exoskeletons (such as crabs and shrimp) and fungi's cell walls. On average, the molecular weight of commercially manufactured chitosan ranges from 3800 to 20,000 Da. It is soluble at pH less than 6 and like alginates forms gel structures by ionotropic gelation. In the presence of anions and polyanions, chitosan can crosslink even more (Călinoiu et al. 2019).

It is most commonly employed as a coat over the produced capsule because to its failure to increase probiotic cell viability. Low-concentration chitosan solutions are usually applied to capsules. Chitosan and hexamethylene diisocyanate, as well as chitosan and glutaraldehyde, have been shown to produce stronger coatings than chitosan alone. Coating of alginate capsules with chitosan is achieved by allowing alginate microcapsules to drip into a chitosan–calcium chloride solution Calcium ions must be present for appropriate coating to occur (Vandamme et al. 2016; Călinoiu et al. 2019).

Gellan Gum and Xanthan Gum: Gellan gum is a microbial polysaccharide, which is produced from *Pseudomonas elodea*. It made up of a repeating unit of four monomers namely glucose, glucuronic acid, glucose, and rhamnose. The use of a mixture of xanthan–gelan gum for encapsulation of probiotic cells is more resistant to acidic environments than the use of alginate for encapsulation (Sarao and Arora 2017). Hoh et al. (2021) documented that the xanthan gum coating improved the survivability of *Lactobacillus rhamnosus* GG in simulated gastric juice and simulated intestinal juice (Hoh et al. 2021).

κ -Carrageenan: κ-Carrageenan is a natural occurring polysaccharide derived from marine macro algae. κ-Carrageenan is made up of repeating D-galactose-4-sulphate units and 3,6-anhydro-D-galactose linked by alternating α1→3 and β1→4 glycosidic linkages (Iravani et al. 2015). The use of κ-Carrageenan necessitates a temperature range of 40 to 50^{O}C. At this temperature, the cells are introduced to the polymer solution. When the mixture is brought to room temperature, it begins to gel formation. The addition of potassium ions stabilizes the microparticles that have formed. When probiotic bacterial

cells are encased in κ-carrageenan, they remain alive (Sarao and Arora 2017; Hoh et al. 2021).

Starch: Maize, potato, barley, oats, and other starchy foods are the most common sources of starch. This polymer is made up of amylose and amylopectin that are linked by a α -1-6 glycosidic bond. Heating is the primary cause of starch gelation. In order to protect bacterial cells and allow optimum diffusion of micronutrients and metabolites, starch is generally combined with alginate microspheres. However, under acidic circumstances and in the presence of pancreatic enzymes in the GIT, it can be destroyed. As a result, resistant starch could be utilized as an encapsulating polymer for probiotics that can be fermented in the colon and are not digested by pancreatic enzymes (amylases) in the small intestine, ensuring the transport of viable and metabolically active probiotics to the colon (Rathore et al. 2013; Hoh et al. 2021).

Gelatin: Gelatin is a kind of gum that may be used to produce a thermo-reversible gel. This has been used to encapsulate probiotic cells, either alone or in combination with other substances. Due to its amphoteric nature, it can create an ideal combination with anionic polysaccharides, such as gellan gum. At a pH greater than 6, the hydrocolloids resist each other due to the fact that they both have a net negative charge. When the pH is reduced below the isoelectric point, gelatin takes on a net positive charge, resulting in a strong interaction with the negatively charged gellan gum (Sarao and Arora 2017).

Cellulose Acetate Phthalate (CAP): CAP is a cellulose polymer in which 50% of the hydroxyl groups are esterified with acetyls and 25% are esterified with one or two phthalic acid carboxyls. At pH 6 or above, CAPs are soluble, while at pH 5 or lower, they are insoluble (Vandamme et al. 2016). When probiotic bacteria are encapsulated in cellulose acetate phthalate, they are well protected in a simulated GI state. Because of its harmless nature, cellulose acetate phthalate is used for controlling drug release in the gut (Sarao and Arora 2017).

Milk Protein: Casein and whey proteins, which are found in milk, are widely regarded as suitable materials for encapsulating probiotic microorganisms. Because of their biocompatibility, whey proteins and their gel matrices are crucial. Because of their structural and physicochemical characteristics, they can be used as a delivery system (Vandamme et al. 2016; Sarao and Arora 2017; Liu et al. 2020).

Microencapsulation Techniques: Several techniques can be used in order to encapsulate food components in coating materials. The contributing factors in the process of technique selection mainly depend on required particle average size, the physical and chemical characteristics of the carrier material, the uses of the encapsulated substance, the required release mechanism, and costs. These factors must be investigated for each probiotic and technique. There are three main stages of encapsulation of probiotics namely: incorporation of the bioactive component in a matrix, microcapsule production, and microcapsule stabilization by a chemical, physicochemical, or physical procedure (Burgain et al. 2011; Serna-Cock and Vallejo-Castillo 2013; Liu et al. 2020). The followings are some microencapsulation techniques:

Extrusion: Extrusion is the oldest and most widely used method for microencapsulating probiotics because of its ease of use, low cost, and mild conditions that enable high entrapment of the microencapsulated probiotics. Extrusion has been effectively used to encapsulate probiotic bacteria by using biopolymers such as alginates and carrageenan in the presence or absence of minerals (calcium, potassium, etc.). In the case of alginate capsules, the extrusion process entails the following steps: production of a cell suspension from probiotic cells and a hydrocolloid solution, extrusion of the produced cell suspension into a hardening solution containing divalent cations such as calcium, and cross-linking of alginate polymers and calcium ion to form a three-dimensional lattice structure (Solanki et al. 2013; Vandamme et al. 2016; Liu et al. 2020).

Prilling is a method that involves the production of droplets in a controlled manner (as opposed to spray-drying). The pulsation or oscillation of the jet nozzle is a good way to achieve this. Another popular method for forming droplets is to employ coaxial flow or an electrostatic field (Burgain et al. 2011). Many parameters impact the size and shape of the beads in this process, including the viscosity of the alginate solution, the distance between the needle and the hardening solution, the diameter of the needle orifice, and the hardening solution's surface tension. This technique produces microcapsules with 2- to 5-mm dimensions, which are bigger than those produced by the emulsion method and, as a result, could affect the sensory properties of the product (Solanki et al. 2013; Vandamme et al. 2016).

Emulsion: Because of vegetable oil is required for emulsion formation, the emulsion process is more costly than extrusion (Iravani et al. 2015). The cell polymer suspension is mixed with a considerable amount of oil in this method. After that, the mixture is homogenized to create a water-in-oil emulsion. The water-soluble polymer is insolubilized (crosslinked) to produce the particles within the oil phase after the water-in-oil emulsion is generated. Finally, filtering is used to extract the beads. The size of the beads is determined by the agitation speed, which can range from 25μm to 2mm. In this technique, vegetable oils are used in food applications. White light paraffin oil and mineral oil have been used in certain experiments. Emulsifiers are also used to produce a better emulsion since these chemicals reduce the surface tension, resulting in smaller particles. In emulsion technique of microencapsulation, carrageenan and its mixes, sodium carboxymethyl cellulose, cellulose acetate phtalate (CAP), alginate and its mixtures, chitosan, gelatin, and chickpea protein can be used (Martín et al. 2015; Razavi et al. 2021).

Spray Drying: Probiotic suspension and dissolved polymer are combined together in the spray drying method. Gum arabic and starches are commonly used as polymer matrices because they tend to produce spherical microparticles during the drying process. The prepared mixture is compressed

and then atomized to produce a ‘‘mist" into the drying chamber in this process. In the drying chamber, heated gas (air or nitrogen) is also blown. This heated gas causes the solvent to evaporate. The capsules are then transferred to a cyclone separator where they will be recovered. The benefits of spray drying include the procedure's speed and cost-effectiveness. The process is extremely repeatable, and, more importantly, it is appropriate for industrial use. One drawback of spray drying is that it has a limited range of applications. However, the main issue is that it uses high temperatures, which are incompatible with the survival of probiotics. Protectants can be applied to the medium before drying to increase probiotic survival. For instance, granular starch improves the viability of culture throughout drying and storage, soluble fiber boosts probiotic viability during storage, and trehalose protects against heat. Furthermore, spray-dried capsules can be coated with an extra layer to provide protection against acidic stomach conditions or to decrease the adverse effects of bile salts (Burgain et al. 2011; Razavi et al. 2021).

Freeze Drying: Freeze drying has been used to make probiotic powders for decades but it has been recently becoming apparent to combine freeze drying and encapsulation. The method is based on sublimation, which occurs in three stages: freezing, first, and then drying. Cells are usually frozen first, then dried by sublimation in a high vacuum. As the processing requirements of freeze drying are milder than those of spray drying, greater probiotic survival rates are attained in this technique.

The solvent is frozen and removed by sublimation in this process. Freezing, on the other hand, damages the cell membrane due to crystal formation and also causes stress due to excessive osmolarity. Various types of protectants, such as skim milk powder, whey protein glucose, maltodextrine, trehalose, and others, have been added to the drying media before freeze drying to protect the viability of probiotics during dehydration (Razavi et al. 2021). Cryoprotectants can also be added to media before fermentation to help probiotics adapt to their surroundings. Cryoprotectants work by accumulating within cells and decreasing the osmotic difference between the internal and exterior environments (Martn et al. 2015).

CONCLUSION

The findings of the present study suggests that probiotics are considered as live beneficial microorganisms that induce positive health benefits in the host. These attributed health benefits are achieved when viable cells of probiotics reach to the target organ. Microencapsulation is one of the promising means for the improvement of probiotics survival in both food matrices and transit throughout GIT. It ensures the delivery of viable probiotics to the intestine. Alginate with chitosan coating is commonly used materials for the microencapsulation of probiotics through extrusion and emulsion. But, for industrial purposes, spray drying especially freeze drying is the promising technique.

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Biomedical Communication

Oral Rehabilitation in An Eight year Child with Rieger's syndrome: A 3-year follow-up Case Report

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ABSTRACT

Axenfeld Rieger's syndrome (ARS) is a rare disease causing genome abnormalities, impeding the development of the alveolar process due to disproportionate jaw growth. This case report presents the interdisciplinary management of an 8-year-old child with ARS exhibiting caries, malformed teeth (conical/microdont teeth), infraoccluded primary molars and hypodontia. After liaising with his paediatrician, treatment was carried out under local anaesthesia which included fissure sealants, fillings, upper overdenture and lower resin-bonded bridge. For aesthetics, an upper overdenture was made to correct occlusion and upper lip support, and also fixed resin-bonded bridge on the lower anterior segment. Early diagnosis and an interdisciplinary approach are necessary to provide the best short- and long-term treatment plans, as well as treatment and follow-up for individuals with the syndrome.

KEY WORDS: AXENFELD RIEGER'S SYNDROME, CARIES, HYPODONTIA, ORAL REHABILITATION.

INTRODUCTION

Axenfeld Rieger's syndrome (ARS) is a rare genetic disorder characterized by malformations of the anterior chamber of the eye (gonio dysgenesis) it may be accompanied by craniofacial, a spectrum of dental and somatic anomalies. Its frequency in the general population has been estimated to be 1 per 200,000. When only the eyes are affected, the condition is termed the Rieger anomaly. At present, two different genes encoding transcription factors (PITX2 and FOXC1) are known to cause the alterations observed in the ARS. In addition, at least two genetic loci involve genes that have not yet been characterized (13q14 and 16q24). Furthermore, two other putative genes have been implicated (PAX6 and MAF), (Alward, 2000, Khatri et al., 2019, Gołaszewska et al., 2021, Siddiqui et al., 2021). This case report aimed to assess the rehabilitation of function and aesthetics, cosmetically improve the appearance of anterior teeth, management of caries primary teeth, monitoring of developing dentition and oral health maintenance and preventive programme.

Case Report: 8- years-old referred by his General Dental Practitioner (GDP) to our hospital regarding missing permanent teeth. T.D had been diagnosed with ARS syndrome, with ventricular septal defect (VSD) which was surgically corrected in his early years. Examination, radiographs and history were carried out. He was diagnosed with caries, malformed teeth (conical / microdont teeth), infraoccluded primary molars and hypodontia. Due to the complexity of his condition a multidisciplinary hypodontia clinic visit was needed for planning because T.D was being bullied because of the appearance of his teeth. After liaising with his paediatrician, treatment was carried out under LA which included fissure sealants, fillings, upper overdenture and lower resin-bonded bridge. T.D and his twin brother live with a foster family, and at each visit, his social worker was updated with the progress of his treatment.

Patient details: A male patient was involved in this study who has followed up for 30 months. The treatment was started when the patient age was above eight years and followed up till at the age of eleven years. The assessment of pre-treatment is as follows: No history of dental pain or abscesses. However, T.D. was concerned about his appearance and his foster mother mentioned he was being bullied at school due to his missing teeth. The patient's relevant medical history was ARS: vision and hearing impairment and ventricular septal defect which was corrected in his early years, Medication: multivitamins, T.D wears hearing aids. The social history was T.D and his twin brother who has the same condition lived with a foster

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family for the last 4 years and recently they were adopted by the same family and they became the legal guardians. The dental history was a regular attendee at general dental practitioner (GDP), No history of operative treatment and tooth-brushing twice daily with children toothpaste (unsupervised). The extra-oral clinical examination was defined as Hard tissue: prominent supraorbital ridges, broad nasal bridge and prominent forehead, Maxillary hypoplasia/prognathic profile (Figure 1), thick glasses and hearing aids, soft tissue, TMJ and lymph nodes: nothing abnormal detected and behaviour: positive toward dental treatment (Frankl Scale:3). The intra-oral examination was as follows:

- Teeth presented intra-orally:
- URD, URC, UR1, UL1, URC, URD
- LR6, LRE, LRD, LRC, LLC, LLD, LLE and LL6
- Good oral hygiene, Caries on LLD and LRD, Conical UR1, UL1
- Infraoccluded primary molars (LRE, LRD, LLD, LLE), Hypomineralised permanent molars

Figure 1: Extra-oral and intra-oral photographs of a patient before the treatment

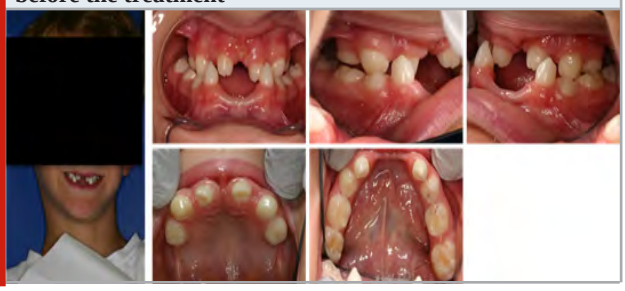
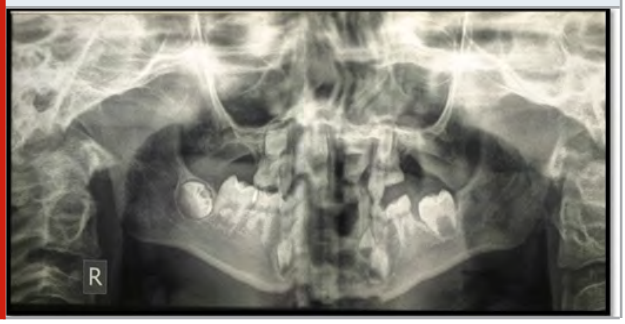


Figure 2: Panoramic radiograph of a patient before treatment



Good oral hygiene, no visible plaque deposits on teeth (Simplified plaque index score). Orthodontic Assessment was confirmed as Mixed dentition stage, Midline diastema (6mm space between upper central incisors), molar relationship: right and left crossbite, upper and lower Arch: anterior segment spacing and skeletal: Class III base (Figure 2). DPT was diagnostically acceptable in the right and left side but not in the middle section presumably because of patient movement. However, caries in primary teeth (LLD, LLE), Infraoccluded (LLD, LLE, LRD and LRE) were found. Also, severe hypodontia (Oligodontia) with upper anterior spacing and skeletal Class III base.

RESULTS AND DISCUSSION

Key stages in treatment progress: T.D.'s physicians were contacted regarding his condition and there were no concerns regarding dental treatment under local anaesthesia (LA). The social worker was contacted and updated regularly with T.D progress. Plaque score 10% and subsequently 0%. The patient was advised to brush twice daily with fluoridated toothpaste that contains at least 1,350 ppm fluoride, and brush at night before sleeping and in the morning. The patient was issued a diet sheet: sweets should be consumed at mealtimes as a dessert rather than between meals. Sugars should not be consumed more than four times per day. Fluoride varnish application on teeth was also advised (2.26% F).

Non-pharmacological behaviour management techniques including tell-show-do and positive reinforcement were used during the provision of preventive care. Local anaesthetic (a pharmacological behaviour management technique) was utilised to provide the planned comprehensive dental treatment (Infiltration of 2% lignocaine with 1:80,000 epinephrine).

Treatment performed under LA and rubber dam: Occlusal composite restoration for LLD and LLE
And resin fissure sealant of LRD, LRE, LR6, LLE, LL6, UR1 and UL1. For aesthetics, an upper overdenture was made to correct occlusion and upper lip support and also fixed resin-bonded bridge on the lower anterior segment. Periodic reviews every 6 months were done over 3 years, they involved monitoring of restorations, enhance oral hygiene, eruption pattern of permanent dentition, monitoring infraoccluded primary molars, preventive and dietary advice, and fluoride varnish application with 2.26% (Figure 3).

Congenital heart defects are common in this syndrome and are often the clinical manifestations leading to diagnosis. Many different defects can occur but the most frequently reported cardiac malformations include ventricular septal defect(Alward, 2000). Dental problems are hypoplasia or hypomineralisation of enamel, this common defect can increase the risk for dental caries (as is the case with T.D.). Other dental problems including delayed tooth eruption and aberrant tooth shape, hence close monitoring of the development of the occlusion and radiographic examination should be undertaken at an appropriate age to exclude hypodontia(Brooks et al., 1989, Fitch and Kaback, 1978, Gołaszewska et al 2021).

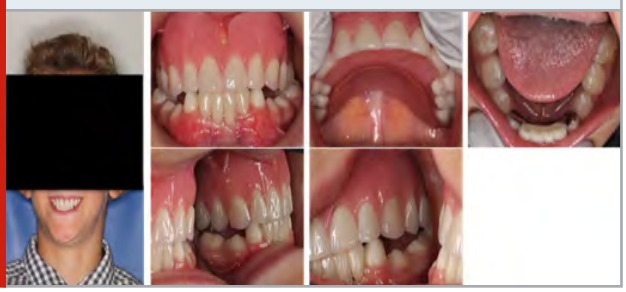
Several non-pharmacological behaviour management techniques like tell-show-do and positive reinforcement were used with the patient during the preventive care visits aiming to create a positive dental attitude and long-term interest in prevention and maintaining oral health (Silva et al., 2021). T.D's hearing and vision impairment made communication challenging; however, improvement in his general behaviour toward dentistry was noticed over subsequent visits, his rating on Frankl scale improved positively. However, T.D. was shy and withdrawn during

the first few visits, he refused photographs to be taken but subsequently agreed.

Management of hypodontia requires multi-disciplinary care which involves the close working relationship of a committed team contributing their expertise to achieve an optimum outcome for the patient and family. Early orthodontic and restorative interception is highly recommended for the best long-term treatment planning. After referring T.D. to the hypodontia clinic, construction of maxillary overdenture was recommended. This was to improve aesthetics and provide better lip support than ordinary removable partial denture (RPD), (Jepson et al., 2003 Silva et al 2021). In addition, a resin-bonded bridge was constructed for the lower anterior segment instead of RPD as children often cope better with fixed appliances rather than removable especially in the lower arch.

Retention of primary molar teeth where the permanent successor is absent is important in cases of hypodontia since they retain bone in an area that may be a site for future transplantation or implant therapy. The prevalence of infraocclusion is 1-9%. Altered pulp pathology determines the most appropriate management but may involve pulp therapy and restoration with pre-formed metal crowns. Where restoration for caries is not necessary, an infraoccluded tooth that is static may have onlays placed in order to facilitate cleaning as well as preventing food packing and its squeal. If seen early enough it may be prudent, in conjunction with orthodontists, to remove the primary molar tooth where there is no successor to achieve an optimum outcome. The longevity of the primary dentition, where there are no permanent successors, is uncertain, (Nunn et al., 2003, Silva et al 2021).

Figure 3: Extra-oral and intra-oral photographs of a patient after the treatment



In this case, over the review period, the infraoccluded teeth were at the same level without any signs of ankyloses. Therefore, the suggested treatment was to monitor.T.D had multiple carious lesions with hypomineralisation enamel defects. Therefore, it was important to establish a preventive regimen aiming to prevent the progress of the disease and reduce the risk of the development of further caries. An evidence-based preventive approach was utilised for T.D. He was placed on 6 months' recall visits, and with the implementation of behaviour management techniques, he received diet advice, oral hygiene instructions, and fluoride therapy, (Gregory, 2014, Gołaszewska et al 2021).

CONCLUSION

Careful planning of treatment while liaising with other colleagues is essential for the successful management of children with Axenfeld Rieger's syndrome. This requires clear pre-treatment and early liaising with orthodontics and prosthodontics in the hypodontia clinic.

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Microbiological Communication

Insights into Biochemical Degradation of the Organophosphate Pesticide Diazinon by Soil Microorganisms and their Mechanisms of Actions

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ABSTRACT

The unregulated, discriminate and increasing applications of pesticides to enhance plant production and reduce vector-borne diseases caused environmental pollution and human health problems. Problems of contamination by pesticide must be alleviated by developing physical, chemical and/or biological methods to detoxify these compounds. Biodegradation is a promising and economic method to remove these compounds by breakdown to small inert end products. Different bacterial genera were active under their favorable environmental conditions due to production of some degradative enzymes. In biological processes, biodegradation of the different pesticides by naturally occurring microorganisms enhance soil fertility and quality, improve human health and preserve life on our earth. Using bacteria for biodegradation is one of the most environmentally important processes because it is cost effective and quick processes which remove pesticide contamination from different environments effectively, easily and quickly. Genetically modified bacteria, immobilization of hydrolytic enzymes and application of the best conditions increased the degradation process. The use of the bacterial cells or their active enzymes with high capacity for pesticide degradation effectively hydrolyzes the toxic materials into less toxic and simple compounds. Diazinon from organophosphate pesticide was used mainly for control of red palm weevil. This review is an interesting attempt to approach bioremediation strategies of Organophosphate pesticide specially Diazinon in order to prevent the increasing earth pollution and contamination by dangerous compounds.

KEY WORDS: BIODEGRADATION, DIAZINON, ENZYME ORGANOPHOSPHATE, PESTICIDE, RED PALM WEEVIL.

INTRODUCTION

The removal of wide range of pesticide groups from the environment requires knowledge of its concentration. In the environment, they can be degraded mainly by the action of indigenous bacteria or fungi through process called biodegradation which required further exploration in relation to total microbial population and their biochemical activities, environmental limiting factors, physiology and genetics of the degrading organisms (Singh and Walker, 2006, Bhattet al., 2019, Kaushal et al., 2021). Bioremediation technology was used to remove toxic materials from the environment. Studying physiologic, biochemical and genetic characters of bacteria may improve pesticide degradation process. Genes encoding for the active enzyme were identified for several pesticides that provide better understanding and inputs to develop a super strain to achieve the desired effect of bioremediation. Organophosphate pesticides constitute a group of widely used, very heterogeneous compounds that share a phosphoric acid derivative chemical structure. The wide use of organophosphate pesticide has created numerous problems, including the pollution of the environment (Singh, 2008, Aly et al., 2017a, b, Sarkar et al., 2021).

The red palm weevil biocontrol: The red palm weevil is a member of Coleoptera which were belonging to family Curculionidae. The adult weevil is reddish brown beetle, capable of undertaking long flights due to strong wings. It was 3 cm long and had characteristic long curved rostrum. It is a serious pest of coconuts, originating in southern Asia and since the mid-1980s, it had advancing westwards very rapidly. It had reached the eastern region of the Kingdom of Saudi Arabia in 1985 and afterwards reached many other areas in the Kingdom (Abozuhairahet al., 1996). It was first

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recorded and spread in the United Arab Emirates since 1985 (El-Ezaby, 1998). The high rate of spread of this pest is by transporting infested young or adult date palm trees and offshoots from contaminated to uninfected areas where this insect infects mainly *Phoenix* dactylifera (Barranco et al., 2000). Mature females put more than 200 eggs at the base of young date leaves.

After infection, palm damage is produced mainly by the larvae which are visible as the first symptoms of the attack result in the death of the tree. No safe techniques for early detection of the pest have been reported. Intensive chemical treatments have been applied to protect or cure the *Phoenix* trees. Despite the difficulty in operating in the public garden's environment, foliage spraying has been conducted with various insecticides. Organophosphate pesticides, especially Diazinon were used mainly to control palm tree pests. Diazinon and preventive treatment of all the palms, even healthy ones, has been repeated once a month outside the tourist season (Gomez and Ferry, 1998). Kaushal et al. (2021) reported that biodegradation of Diazinon by bacterial cells is very important for faster clean of soil and water environments. Now due to harmful effects, many agents recommitted the no use of Diazinon to control palm bests and applied biological biocontrol methods.

Organophosphate pesticides: Due to rapidly growing population, it is necessary to increase food production which leads to increase use of chemical pesticides or xenobiotics to control pests (Ding et al., 2014, Pang et al., 2020). About 50% of annual food production was lost due to the pest attack (Odukkathil and Vasudevan 2013). The term pesticides include algaecides, antimicrobial agents, pheromones, avicides, biopesticides defoliants, desiccants, fumigants, fungicides, herbicides, insect growth regulators, insecticides, mitticide/acaricides, molluscicides, nematicide, ovicides, pesticides, predacides, repellents and rodenticides (Gavrilescu, 2005). More than two million tons/year of pesticides were used. The highest pesticide-consuming countries were Italy, Turkey, Colombia, India and Japan (Verma et al., 2014) whiles USA exports each year big quantities of insecticides to the developing countries (WHO, 2017). According to Garrigouet al. (2019), about 90% of the used fungicides, 60% of herbicides and 30% of insecticides are reported as potent toxics and carcinogens materials (Table 1). Moreover, they decrease soil fertility and useful flora, increase soil acidity, nitrate leaching, floral and faunal resistance and cause groundwater pollution (Kumar et al, 2018, Sarkar et al., 2021).

The most used insecticides were organochlorides, organophosphates, carbamates, and pyrethroids (Aktar et al., 2009). It is clear that to decrease pollution, a single compound with numerous properties may be used like hexachlorophene, methiocarb, coumaphos and Diazinon. Farmers usually used pesticides without looking at their harmful worldwide effects and environmental problems. Now commercially, the degradable organophosphate pesticides are the most used group all over the world and have many worldwide applications (Kaushal et al., 2021). Organophosphate insecticides are like chlorpyrifos, malathion, acephate, phosmet, dicrotophos and Diazinon. They are esters, amides, or thiols forms from derivatives of phosphoric, phosphonic, phosphinic or thiophosphoric acids which were coupled with two organic groups and a side chain consisting of cyanide, thiocyanate, or phenoxy groups (Balali-Mood and Abdollahi, 2014). They are generally regarded as safe for use on crops and animals due to their relatively fast degradation rates. They are soluble in water with low persistence on foliage and are susceptible to degradation in the environment (Dhas and Srivastava, 2010, Ding and Tian, 2014). Due to the increased demand and consumption of pesticides, it is necessary to protect soil, water and air. In this sense, biological or chemical degradation processes have been intensively studied (Pang et al., 2020).

The insecticide Diazinon from the Organophosphate group: The insecticide Diazinon (Figure 1), O, O-diethyl O- [6-methyl-2-(1- methylethyl-4-pyrimidinyl)] ester is highly soluble in water (60 mg/l) and its half-life in soil was about 40 days while it requires 138 days for complete hydrolysis (Di-Bartolomeis et al., 2019). It is generally to control pests and reduce their harmful effects in agriculture sectors. Diazinon was used for inhibition of a wide variety of insects like cockroaches, ants, and fleas in homes, big gardens or farms (Grube et al., 2011). It was found in many formulas as dusts, granules, seed dressings, powders or emulsion. WHO (2009) classified it as hazardous material which showed a moderate toxicity (class II) with IC50 ranged from 26 to300 mg/kg for oral administration and 379 mg/kg for dermal toxicity.

Organophosphat pesticides-Degrading Microorganisms: To mitigate the problem of contamination by pesticide, treatments have been developed to detoxify and/or degrade these pesticides through physical, chemical and/or biological processes (Bhatt et al., 2020a,b,c). In biological processes, biological systems (whole cells or isolated enzymes) are used to catalyze chemical reactions that transform the pesticide into simpler and less toxic compounds or mineralize them into molecules. The search for microorganisms with high capacity for pesticide degradation is a very interesting attempt to approach bioremediation strategies and prevent contamination. To evaluate the potential of agricultural soils and solid organic waste cultures, important strategies for microbial isolation and screening of the potential insecticide degradation organisms must be applied (Singh, 2006, Mesnage and Séralini, 2018; Sarkar et al., 2021).

There is a significant role of metabolic activity of bacteria (including rhizosphere bacteria), fungi, actinomycetes and plants in Organophosphate pesticide degradation process. Bacteria are the potential degraders of complex molecules and most used pesticides for their own metabolism and growth (Hussain et al., 2009 and Sinha et al., 2009). Therefore, under laboratory conditions, the ability of microbes to decrease contamination has been studied and biodegradation techniques were faster than chemical or physical methods (Abraham et al., 2013).

Under environmental limiting nutrient conditions of carbon and/or phosphorus, most microorganisms degrade organophosphates compounds through oxidation, hydrolysis,

and alkylation or dealkylation (Singh and Walker, 2006). Hydrolysis using enzymes like microbial hydrolase, phosphotriesterase, phosphatase and Carboxylesterases of P–O alkyl and aryl bonds were reported for organophosphate compounds biodegradation (Gao et al., 2014; Zuo et al., 2015). Phosphotriesterase degrades the triester bond of organophosphorus compounds and phosphodiesterase and monoesterase are essential to make the phosphorus atom available for uptake as a source of inorganic phosphorus (Cui, 2001, Lu et al., 2013, Sarkar et al., 2021).

Table 1. Types of pesticides and their bad effects on human health (Mesnage and Séralini, 2018)

Pesticide	Class	Health effect
Insecticides	Organophosphates	Neuropathy, myopathy, tremors, irritability, convulsions, inhibiting the enzyme acetylcholine esterase, paralysis
	Carbamates	Inhibition of acetylcholine esterase enzyme, paralysis
	Organochlorines (dichlorodiphenyl methane and cyclodienes)	Stimulation of the nervous system by disrupting the sodium/potassium balance of the nerve fiber, tremors, irritability, convulsions, hyperexcitable state of the brain, cardiac arrhythmias and reproductive problems
Herbicides	Phenoxy, benzoic acids, riazines, ureas and chloroacetanilides	Dermal toxicity, carcinogenic effect, damage to liver, thyroid, nervous system, bones, kidneys, blood and immune system
Fungicides	Substituted benzenes, thiocarbamates, thiophthalimides, organomercury compounds etc.	Damage to liver, thyroid, nervous system, bones, kidneys, blood and immune system, carcinogenic property also

Figure 1: The molecular structure of the organophosphate pesticide Diazinon

Subsequently, many bacteria, fungi, algae, and cyanobacteria are active in biodegradation process of different pesticides, insecticides and herbicides. *Flavobacterium* sp. was isolated and was highly degraders of organophosphate compounds (Singh and Walker, 2006). Fungi such as *Aspergillus niger, A. sydowii, A. fumigatus, Cladosporium cladosporioides* and *Penicillium raistrickii* have been isolated from various contaminated sites and confirmed to be capable of degrading different organophosphate pesticides (Liu et al., 2001, Gao et al., 2014; Pandey et al., 2014, Alvarenga et al., 2014). Also, the algal genera *Scenedesmus, Stichococcus* and Chlorella (Megharaj et al., 1987; Caceres et al., 2009) and, the cyanobacterial genera, Nostoc, Anabaena and Oscillatoria have been established as organophosphates biodegraders (Ibrahim et al., 2014, Salman and Abdul-Adel, 2015, Sarkar et al., 2021).

Degradation of Diazinon by Bacteria: Diazinon is one of the most commonly and widely used commercial insecticides. It is a type of worldwide used organophosphate pesticide. The reported half-life of Diazinon in soil varies from 10-40 days (Aly et al., 2017a,b). Extensive use of Diazinon contaminates air, ground water, rivers and lakes. If the pesticide is not degraded or detoxified rapidly enough, the risk of its offsite migration may pose a health risk to humans. Catabolism and detoxification metabolism occurred when a soil microorganism uses the pesticide as a carbon and energy source. The biodegradation of Diazinon by soil bacteria has been reported by many workers where they use Diazinon as carbon and energy sources. Different bacterial species apparently showed different sensitivity to pesticides and can degrade pesticides with different ability (Hussain et al., 2009, Sinha et al., 2009, Feng et al., 2020b).

In some cases, bio- stimulation is required for in situ remediation. In spite of limitations, naturally occurring or native bacteria can be used for bioremediation process in a certain area while the use of genetically modified bacteria for bioremediation has high impact and long-term effects on the degradation processes. The rhizosphere of date palm trees contained many bacterial isolates that degradation Diazinon to CO2 and clean the environment and soil (Al-aidaroos, 2017). She added that mixed cultures could be relatively more effective in bioremediation of Diazinon from contaminated soil and water compared to bacterial mono-cultures. Proper optimization of treatment like duration and culture volume of bacteria mono- and mixed-cultures, prior to bioremediation process could yield better results. The results of biodegradation by both Pseudomonas

and Bacillus suggested that no toxic intermediates during the degradation of Diazinon was detected and thus could be effectively utilized for the bioremediation process in contaminated soil and water (Kumar, 2016, Feng et al., 2020b).

Arthrobacter sp. was recorded as diazinon degrader by Ohshiro et al. (1996) and it can also use other organophosphate compounds like ethoprophos, fenitrothion, chlorpyrifos, isofenphos and parathion. From petroleum contaminated field, two species of genera *Arthrobacter* and *Mycobacterium* were isolated by Seo et al. (2007) and can efficiently degrade Diazinon. Biodegradation of Diazinon was also studied by Cycon et al. (2009) using *Serratia* liquefaciens, *S. marcescens*, and *Pseudomonas* sp. isolated from contaminated agricultural soil of Poland. Similarly, Cho et al. (2009) and Zhang et al. (2014) reported that *Lactobacillus brevis, L. plantarum*, and *L. sakei* can efficiently use the pesticide Diazinon as carbon and energy sources. From agricultural soil of Saudi Arabia and using enrichment technique, rod-shaped, Gram-negative *Serratia marcescens* was obtained on minimal salt medium. It can completely degrade Diazinon (50 mg/ l) in 11 days and it can use successfully other organophosphate compounds like as chlorpyrifos, coumaphos, parathion and isazofos (Abo-Amer, 2011). Previous results (Table 2) have been reported that several bacterial species such as Pseudomonas sp. (Ramanathan, 1999), *Agrobacterium* sp., *Flavobacterium* sp. and *Serratia marcescenscan* utilize Diazinon as a source of carbon (Ohshiro, 1996, Ghassempour, 2002, Yasouri, 2006, Feng et al., 2020b, Kaushal et al., 2021).

Table 2. Degradation of Diazinon by different microorganisms a and their sources of isolation

Organo-phosphate pesticides	Microorganisms used for Degradation	Source of isolation (country)	Reference
Diazinon	*Arthrobacter* sp. *Mycobacterium* sp.	Petroleum-contaminated soil (Hilo, Hawaii, USA)	Seo et al., 2007
	Trichoderma atroviride	Not mentioned	Tang et al., 2009
	Leuconostoc mesenteroides *L. brevis* *L. plantarum* *L. sakei*	Kimchi during fermentation (Korea)	Cho et al., 2009
	Serratia liquefaciens *S. marcescens* *Pseudomonas* sp.	Agricultural soil (Poland)	Cycon et al., 2009
	Serratia marcescens *Lactobacillus brevis*	Agricultural soil (Saudi Arabia)	Abo-Amer, 2011 Zhang et al., 2014
	Stenotrophomonas sp.	Industrial sludge (China)	Deng et al., 2015
	Bacillus sefensis 7	Rhizosphere of Date palm tree	Aly et al., 2017b

It is well known that Pseudomonas species, known as a very metabolically bacterial species may contribute in biotransformation of other organophosphorus insecticides (Cycon, 2009, Ortiz-Hernandez, 2010). *Serratia* sp. seems to be an active bacterium that may contribute in complete degradation of Diazinon but there is little information concerning the pathways of utilization. Nevertheless, some studies reported the ability of *Serratia* to complete the degradation of other organ phosphorus insecticides, chlorpyrifos, fenitrothion, and parathion at 50 mg/l in 14 days in three different soils (Cycon et al., 2009, 2013). Unfortunately, *S. marcescens* is a facultative anaerobe bacterium and is an opportunistic pathogen. Moreover, from sludge chlorpyrifos manufacturing plant in China, Stenotrophomonas sp. was isolated and showed excellent capacity in Diazinon and other organophosphate pesticides removal (Deng et al., 2015). Moreover, endophytic bacteria may hypothetically helpful for biocontrol pesticides in contaminated areas (Barman et al., 2014, Kaushal et al., 2021).

Diazinon degrading bacteria from date palm soils: The isolation of native bacterial species associated with date palm root soil was performed using different nonspecific media, in order to select a wide range of bacterial genera but presence of the synthetic pestside Diazinon in soil prevents the proliferation of bacteria. To determine the active bacteria, able to use Diazinon, bacterial isolation from different Diazinon contaminated soils must be carried out (Yasouri, 2006, Cycon 2009, Ortiz-Hernandez, 2010). Many attempts to isolate Diazinon degrading bacteria from the rhizosphere of date palm plant have been carried out (Aly et al., 2017a,b). A wide diversity was detected into date palm rhizosphere bacterial community and total of 440 isolates were retrieved from seven analyzed stations of Date palm. To manage such a large set of isolates, 16S rRNA gene were amplified and used for molecular identification which represents a useful molecular tool for the discrimination of bacterial isolates up to the subspecies level (Ferjani et al., 2015, Sarkar et al., 2021).

Significant differences were observed in the degradation ability of the bacterial communities in the rhizosphere of the analyzed area. Presence of bacterial cell obtained from date palm soil enhanced the degradation of Diazinon (60 mg /l) compared to control where it takes 40 days for complete degradation but it was found that it takes 11 days using the isolate BMRF3, 9 days using isolate BMNF7 and 17 days using isolate BMTF8 (Alidros, 2017). Improvement of culture conditions like addition of glucose and yeast extracts, pH, temperature, incubation period and inoculums size increased degradation process. Furthermore, four isolates able to use Diazinon as of carbon and energy sources were obtained from soil samples collected from different sites by using an enrichment culture technique. All four isolates were Gram-negative, rod shaped, oxidase negative bacteria (Alipour et al., 2017, Sarkar et al., 2021).

Factors affecting biodegradation process: It is very difficult to precisely determine the part of particular microorganisms in pesticide destruction, since there are many factors that have an effect on this process. Moreover, microorganism activity may be specific to chemical structure, chemical binding, or a group of selected substances. In soil combination of chemical and biological process was noticed during decomposition synthesized pesticide. Biological degradation is faster and eco-friendly method of converting pesticides by microbial enzymes into simple non-toxic. The success and the failure of biological treatment depends on factors that affect the degradation such as the competitive ability of the suitable microorganisms, moisture level, pH, temperature, salinity, nutrient and water contents, light intensity and oxygen concentration in addition to pesticide structure, molecular weight, functional groups, concentration and solubility in water (Goldstein et al., 1985, Geer and Shelton, 1992, Alexander, 2000, Aly et al., 2017a, Matsuda et al., 2020).

Organic matter content: The organic matter content of the soils had no apparent effect on fenamiphos or chlorpyrifos degradation while a previous report indicated that high organic matter reduced degradation (Karpouzas and Walker, 2000b). High organic matter leads to reduced bioavailability of substrate to the degrading microorganisms. However, many degrading microorganisms produce surfactants or other emulsifiers that desorb chemical compounds from soil and make them bioavailable (Weber and Huang, 1996, Aly et al., 2017b). Presence of glucose enhanced organophosphate pesticide degradation rates. The degradation rate of Diazinon in medium supplemented with succinate or glucose by strain DI101 was increased to 15 days with degradation rates of 29.3/day compared to 11 days and degradation rates of 40.7/ day for control.

The degradation pattern of the strain was greatly affected by the presence of other carbon sources. There was almost no degradation of Diazinon through the first 6 days in the presence of succinate or glucose. However, after 7 days, Diazinon was degraded rapidly in these two modified media. The relative degradation rates of Diazinon in medium without addition of carbon source were significantly different as compared with medium supplemented with carbon sources. Maximum organophosphate compounds hydrolysis of about 84.5% was observed by bacterial isolate in presence of glucose as compared to 73.3% in absence of glucose while fungal isolate had 76% hydrolysis in presence of glucose and only 58% in absence of glucose. The two isolates were resistant to chlorpyrifos at 10 ppm therefore; these isolated could be potential candidates for microbe mediated bioremediation of chlorpyrifos contaminated soils (Hindumathy, 2013, Aly et al., 2017b).

On contrast, the addition of glucose to the soil samples significantly reduced the initial degradation rate but this lag phase was followed by a log phase. This result contrasts with previous findings of Karpouzas and Walker (2000a, b), where addition of glucose stimulated the degradation rate of ethoprophos. However, initial inhibition of pesticide degradation in the presence of glucose can be attributed to the environmental adaptation of bacterial isolates where easily available and rich carbon sources are preferentially utilized. Once the readily available carbon source is depleted, the bacteria begin to utilize the pesticides. This approach gives the bacterial isolates a competitive advantage since they are able to utilize both readily available and less available carbon sources. However, the addition of other carbon sources such succinate or glucose stopped the degradation of Diazinon. When these carbon sources were exhausted, it then degraded Diazinon as a source of carbon. Environmental adaptation of bacteria in natural environment for the competition for carbon sources is massive and the utilization of Diazinon as an energy source by bacteria increased with a significant competitive.

Phosphorus concentration: Degradation rates of Diazinon in MSM supplemented with phosphorus and MSM without phosphorus source were not significant ($p<0.005$). Diazinon in each medium was completely degraded by the strain DI101 on day 11, with degradation rates of 0.241/day for MSM with phosphorus and 0.221 /day for MSM without phosphorus. Therefore, the degradation of Diazinon was quite similar in both media and was not affected by the absence or presence of phosphorus source. Moreover, enzymatic assays of the strain DI101 gave positive results for both phosphodiesterase and alkaline phosphatase activities. The utilization of organophosphorus insecticides as a source of phosphorus by DI101 is a significant observation.

There are few reports in which an organophosphorus compound was used as a source of carbon and phosphorus by a single species such as *Enterobacter* strain B-14, which could use organophosphorus chlorpyrifos as a source of carbon and phosphorus (Eissaet al., 2014). However, *Flavobacterium* species could use parathion as a source of phosphorus but not carbon and diazinon as a carbon source. Nevertheless, a *Flavobacterium* strain was not able to utilize organic phosphorus as a source of phosphorus (Sethunathan, 1973). Moreover, a variety of bacteria that could utilize phosphorothionate or phosphorodithionate compounds as a source of phosphorus were unable to use these compounds as a carbon source (Rosenberg, 1979 Matsuda et al., 2020).

Concentration of pesticide and inoculum density: Concentration of pesticide had no apparent effect on the degradation rate except the longer initial lag phase which may be due to the need for greater numbers of bacteria to initiate rapid degradation of the pesticides. Also, Time is required for propagation of a small population of pesticide-degrading microorganisms to reach the essential level for efficient degradation of pesticide (Karpouzas and Walker, 2000b). Inoculum density had a noticeable effect on degradation of fenamiphos and chlorpyrifos. No degradation of fenamiphos was observed in soils inoculated with o104cfu/g. Similarly, *Enterobacter* sp. was not capable to degrade chlorpyrifos under an inoculum density of 103 cells/g.

Similar results were cited by Ramadan et al., (1990) who found that a *Pseudomonas* sp. did not use p-nitrophenol at a density less than 104 cells/ml. Lower inoculum densities mean the small number of bacteria which was not able to survive the initial competition and population decline. Similar results were obtained by Comeau et al. (1993) who reported degradation by Pseudomonas cepacia. The bioavailability of the pollutant is one of the most important factors. There was no effect of ageing for 60 days on subsequent fenamiphos degradation. In contrast, chlorpyrifos was degraded rapidly in all inoculated soil samples, at the end of the 10 days incubation. Similar results were obtained by Cullington and Walker (1999) and Karpouzas and Walker (2000b). Previous studies with a range of pollutants have demonstrated that increased soil residence time of the compound leads to a reduction in bioavailability (Blair et al., 1990; Chung and Alexander, 1998; Alexander, 2000, Matsuda et al., 2020).

The above findings may explain why bioremediation by microorganisms often does not result in total elimination of target contaminants. The effect of the inoculum density on the degradation of Diazinon is illustrated by many authors (Abo-Amer, 2011, Aly et al., 2017b). The data indicated that there were significant differences in the relative degradation rates of Diazinon between inoculum densities. At the high inoculum density (106 CFU/ml), Diazinon was degraded completely on day 11, with a relative degradation rate of 36.37/day. At moderate inoculum densities of 10^4 and 102cfu/ml, degradation of Diazinon was complete on days 13 and 16, respectively, having relative degradation rates of 30.10and 29.15 mg/day, respectively. However, the relative degradation rate (4.364 /day) was slower at lower density (50 cfu/ml); that is only 37% of Diazinon degradation on day 16 was detected. No apparent Diazinon removal was detected in the control cultures. Abo-Amer (2011) noticed that Diazinon was hydrolyzed in liquid medium within 11 days by phosphotriesterase of cells inoculated at inoculum density of 10^6/ ml.

They added that 10^6 CFU/g of was used to inoculate soils, and this inoculum density appeared to be able to degrade Diazinon completely (As revealed in other reports, inoculum density is a significant factor determining the effective biodegradation of applied pesticides. In other study, lower inoculation densities of strain Sphingomonas chlorophenolica RA^2 (10^4- 10^6 cfu/g soil) resulted in strongly decrease mineralization rate of pentachlorophenol and 10^4 cfu/g soil was not significantly differing from the control (Miethling, 1996). The introduction of 10^4 cfu/g soil of Chelatobacter heintzii resulted in a 3-fold increase of atrazine mineralization capacity (Rousseaux, 2003).

The addition of *Enterobacter* asburiae B-14 (106 cfu/ g) to soil with a low indigenous population of chlorpyrifos-degrading bacteria supplemented with 35 mg/ kg of chlorpyrifos resulted in a higher degradation rate than that was observed in non-inoculated soils (Singh et al., 2003, 2004). In addition, Ramadan et al. (1990) reported that when lower inoculum densities (less than 104 cfu/g soil) were added only a small numbered of bacteria can survive due to competition with other bacteria, thus low numbers contribute in pesticide degradation. At low inoculum density of 103cfu/g soil of *Enterobacter* sp., no degradation of chlorpyrifos was noticed in soil (Singh et al., 2004). A higher initial inoculum density can compensate for the initial population decline, and the survivors can multiply and degrade pollutants. Moreover, these results supported the view that particular species of soil microorganisms fluctuate in their general activity responding to degradation of pesticides.

Some physical factors: The effect of temperature on Diazinon degradation by strain DI101 was significant and the most rapid degradation was recorded at 20˚C, 25˚C, and 30˚C, with relative degradation rates of 28.08, 32.99, and 36.57/day, respectively. On the other hand, the slowest degradation was determined at the two extreme temperatures (10 and 40˚C) with relative degradation rates of 0.724 and 1.624/day, respectively. Complete Diazinon removal was observed on day 11 when incubated at 25˚C and 30˚C, but on day 13 at 20˚C. These results were expected, since most members of the family *Enterobacteriaceae* grow well at 37˚C (Abo-Amer, 2011). Similar results reported that *Serratia marcescens* growth and hexachlorobutadiene degradation were at 25 to 30˚C (Li et al., 2008) while they were 20-40^{o}C and pH 7.5 for Burkholderia sp. FDS-1 (Hong et al., 2007, Matsuda et al., 2020).

Moisture content is important for availability of chemical materials and for movement and proliferation of microorganisms. Degradation was the slowest at low moisture contents (Singh et al., 2004). Also, soil pH had a marked influence on pesticide degradation by the bacterial isolates used for inoculation. Degradation rate for ethorprophos by *Pseudomonas putida* was similar at neutral and slightly alkaline pH (Karpouzas and Walker, 2000b) while Vidali (2001) reported that pH 5.5-8.8 is required for most soil bacteria with optimum pH between 6.5 and 8. The changes in relative degradation rates of bacteria in the presence of different pHs were significant. Diazinon was completely degraded by strain DI101 with relative degradation rates of 32.6, 31.8 and 30.8/day when pH values were 7, 7.5, and 8, respectively. However, the relative degradation rates of Diazinon were low at pH 6 and pH 9 (Abo-Amer, 2011). They added that degradation of Diazinon was negligible at pH 5 and pH 10 as well as

in the control. Diazinon biodegradation was approximately inhibited in relatively acidic (pH 5) and alkaline conditions (Aly et al., 2017b, Matsuda et al., 2020).

Immobilization methods used for biodegradation process: Immobilization of bacteria on different supports increased degradation. Entrapment of enzymes and/or cells in alginate is one of the simplest methods of immobilization. Alginates are available commercially as water-soluble sodium alginates, and have been used for more than 65 years in the food and pharmaceutical industries as thickening, emulsifying, film forming, and gelling agents. Entrapment in insoluble calcium alginate gel is recognized as a rapid, nontoxic, inexpensive, and versatile method for immobilization of enzymes and cells (Datta et al., 2013). The procedure of immobilization in alginate beads is not only inexpensive but also easy to carry out and provides extremely mild conditions, so there is a higher potential for industrial application (Rios et al., 2019, Feng et al., 2020b).

The immobilization strategies were used for some enzymes to facilitate the degradation of organophosphate pesticides. *Enterobacter* aerogenes contained golycero-phosphodiesterase enzyme which was immobilized on a nano-materials and results revealed that the immobilized system worked actively for numerous cycles, thus it may be used commercially for purification of water contaminated with organophosphate pesticides (Daumann et al., 2014). The covalently immobilized enzyme A (Opd A) on porous and nonwoven polyester fabric showed high ability for degradation of organophosphate pesticides (Zhang and Qiao, 2002, Gaoa et al., 2014, Feng et al., 2020b) and immobilization process increased the Km, stability and pH range of the enzyme for methyl parathion. The immobilized enzymes removed 50 µM/cycle and we active for more than two months.

Molecular Biology of Pesticides Degradation: To protect crops from harmful insects and to increase their productivity and yields, pesticides are frequently used and due to the excessive use of pesticides the activity of some microbial enzymes in soil which are indicators of soil health are of great significance. In soil, microbial enzymes had significant roles in degradation of natural and synthetic organic compounds. Enzyme activity is closely correlated to microbial activity. To evaluate the effects of pesticides, molecular techniques were used to detect the microbial community changes in structures and functions (Parween et al., 2016). For large-scale and effective bioremediation, molecular tools help to identify related genes of certain enzymes ineffective bacteria or fungi. In pesticide contamination sites, biodegradation of pesticides by some degrading genes of soil microorganisms were studied using recombinant DNA technology (Parween et al., 2016). The microorganisms degrading ability of toxic pollutants from the environment depend on their genetic content of the cells (Matsuda et al., 2020, Feng et al., 2020b). Deoxyribose nucleic acid coding genes of chromosome or plasmid were activated to form of mRNA which was translated to specific protein act as degradative and detoxifying enzymes (Guo et al., 2020).

Many enzymes in recombinant bacteria were promising for detoxification and biodegradation of organophosphorus pesticides. Methyl parathion hydrolysis occurred 25-fold faster than in wild type *Escherichia coli* while 100% paraoxon and 80% of Malathion were degraded after 45 days by *Escherichia coli, Pseudomonas putida* or *Moraxella* sp. Ice nucleation protein (Hussain et al. (2009). Bacteria may have many copy numbers of degradative enzymes like as esterase, monooxygenases, glutathione S-transferase, and P450s which removed insecticides and toxins by increasing mRNA levels of the important enzymes (Li et al., 2007). Cytochrome P450 is well known in oxidation, hydroxylation or degradation of many industrial toxins (Scott et al., 2008). The gene (ophB) encoding a protein from *Pseudomonas* sp. BF1–3, specific for the organophosphate, Chlorpyrifos degradation was cloned in *E. coli* which degrade 95% of the organophosphate pesticide in 9 days. The previous enzyme showed excellent activity at 35◦C at pH of 8 (Barman et al., 2014, Matsuda et al., 2020).

CONCLUSION

With the knowledge that microbes can degrade xenobiotics such as pesticides, researchers are now focusing on microbial diversity, particularly at contaminated sites. Among all the microbes, bacterial degradation has been extensively studied worldwide. This review summarizes the modes and pathways for microbial degradation of organophosphate pesticides and bacteria degradation of the most commonly used organophosphate pesticides. The conditions under which the bacteria are isolated are crucial not only with the desired degradative enzyme systems, but also with specific regulation mechanisms for the degradation pathways. However, the utilization of Diazinon as a source of phosphorus attributed to the presence of phosphodiesterase and phosphomonoesterase activities in the bacterial strains.

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Ecological Communication

Justification of Choosing and Making Managerial Decisions Concerning Land Reclamation Measures: A Probabilistic Model

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ABSTRACT

In the last decade, the Russian economy has shown a steady trend towards increasing the profitability of agricultural production. The production of agricultural products almost completely ensures the food security of Russia. One of the main agricultural crops is rice. Effective management of the rice irrigation system is impossible without the involvement of a mathematical simulation of the functioning of the water resource management system. Management tasks are multifactorial and multicriterial in nature with probabilistic source information. The peculiarity of evaluating the effectiveness of environmental projects requires taking into account the probabilistic nature of the occurring processes. The authors developed a mathematical (probabilistic) model for the functioning of the irrigation system with a stepwise change in the price of planned activities. The model was tested during 2016-2019 in the Chernoerkovskoe JSC of the Slavyansky district of the Krasnodar Territory (Russia). The use of the developed probabilistic model to manage scheduled activities will allow employees of the agribusiness industry to reduce the risks of uncertainties when making managerial decisions, and take into account the stochastic nature of the impact of natural and climatic factors. To form the options of agricultural crops growing technologies, reclamation measures were considered as the Poisson flow of certain intensity. The article determined the average time necessary to reach the satisfactory state of the system, as well as solved the optimization problem of determining the price change pattern of the satisfactory state of the system that would provide maximum profit taking into account losses caused by the unfavorable state. The mathematical model described in the article can be considered as a reasonable choice for improving the efficiency of land reclamation management.

KEY WORDS: LAND RECLAMATION MEASURES, MANAGERIAL DECISIONS, PROBABILISTIC APPROACH.

INTRODUCTION

Human activity is a powerful factor affecting the ecological state of agricultural landscapes (Safronova and Sokolova, 2017; Degtyareva, et al., 2017). Any agricultural activity consists in obtaining guaranteed high crop yields (Degtyareva, et al., 2009; Kuznetsov, et al., 2017). To obtain such results, it is necessary to use all available material and technical, energy, labor, soil, and natural resources with the greatest efficiency (Safronova and Prikhodko, 2019a). The present study aims at developing a model for managing land reclamation measures that prevent the extension of soil degradation. The implementation of high-performance management information technologies is one of the main areas in the development of high-tech industries (Efrosinin et al., 2018). The key indicators of hydrogeological land reclamation include the depth of groundwater and its mineralization (Kuznetsov et al, 2005). Groundwater is a sensitive indicator of all anthropogenic impacts carried out in reclaimed territories. The accumulation of salts in the aeration zone can be regulated by the amount of water supply and the intensity of drainage runoff (Chebotarev and Prikhodko, 2013; Safronova and Prikhodko, 2019a; Safronova et al., 2020a; ; Prikhodko et al., 2021b).

Therefore, the groundwater level can be considered as a function of these two factors, which, in turn, are determined by the cost of construction and operation of irrigation and drainage networks, as well as the availability of water resources. The reason for the decline in soil fertility and manifestations of degradation phenomena is explained by the overload of drainage and escape network, the deterioration of the oxidizing regime of the soil, leaching of humus and nutrients from the root zone of the soil, as well as by flooding of the surrounding space occupied by both rice and upland rice-based cropping rotations (Dyachenko and Prikhodko,

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2007). Nature management at any stage of its development implies various forms of impact on natural systems and their transformation (Safronova et al., 2020b).

Management can be "hard", grossly violating natural processes, and "soft", using natural mechanisms of self-regulation of the natural environment. Hard management gives a high and quick effect, providing an increase in the volume of production or a decrease in the costs of the activities used. However, over time, environmental and economic losses occur (Degtyareva et al., 2019; Safronova et al., 2020c).

It is necessary to combine both forms of management to ensure environmental protection activities within the framework of the permissible impact on the environment, which is experiencing degradation and can restore its qualities. In the Krasnodar Territory, land resources are subject to the impact of various sources of pollution. Therefore, in the state programs for the development of the region, special attention is paid to measures to restore soil fertility. The more difficult, more expensive the planned event, the less permissible "volitional" decisions in it, and the more important are the methods that allow assessing the consequences of each decision in advance, excluding unacceptable options, and recommending more suitable situations and measures (Prikhodko et al., 2021a).

For example, the task of optimizing meliorative activities with uncertain parameters will increase the efficiency of water, light, air, heat, and food regimes control. In the work, the estimated parameters are considered as random variables (Vladimirov et al., 2019a). This allows the uncertainty associated with the parameter estimation to be estimated in terms of probability distributions. The requirements – to get the maximum effect quickly – contradict each other. Therefore, the researcher first focuses on high efficiency and then gradually reduces it until an acceptable efficiency is achieved. With this formulation of the problem, the rate of reduction in the cost of activities is important (Vladimirov et al., 2019c; Safronova et al., 2020b; Prikhodko et al., 2021b).

MATERIAL AND METHODS

Eight indicators (criteria) of soil reclamation status were selected in the present research:

Groundwater level and salinity;The pH of the soil;The content of humus;The provision with hydrolyzable nitrogen;The provision with mobile phosphorus;The provision with mobile potassium;The content of aggregates:from 0.25 to 10 mm; less than 0.25 mm;Degree and type of soil salinity.

Technological maps for the implementation of an ecological-adaptive complex of technological agro techniques have been developed for each reclamation state of soils. Carrying out an eco-adaptive complex of technological operations during the nonvegetative period on five experimental fields of Chernoerkovskoye JSC in the Slavyansky District of the Krasnodar Territory over four years of research has shown its effectiveness, which was expressed in reducing the rate of leaching of the arable horizon; lowering the groundwater level by 0.4 m; reducing the mineralization by 0.39 g/l; and increasing the content of humus up to 4.5%, mobile potassium – by 8%; mobile phosphorus – by 11 %, soil alkali-hydrolyzable nitrogen – by 7 %, while the increase in crop yield amounted to 10 %.

Assessment of the optimal level of groundwater and its management within each system on reclaimed lands is one of the main tasks of hydrogeological and reclamation service and operation, whose solution requires comprehensive information about the state of the object (Safronova et al., 2019b). The main management principles in land reclamation are based on a systematic approach; consideration of the uncertainty of many influencing factors; basin-based approach and reliance on serious scientific research and reliable data; and analysis of contemporary water resource systems (Safronova et al., 2020c). Another important prerequisite for applying the system-based approach and mathematical simulation is a sharp increase in the number of environmental and economic parameters that must be taken into account when analyzing and making managerial decisions (Vladimirov and Prikhodko, 2019b).

RESULTS AND DISCUSSION

Let us consider one of the most common criteria at the regional level which is the minimum of total reduced costs for agricultural production.

$$\text{Find min } [CQQ + CxX + CyY], \quad (1)$$

where X is the area of cultivated crops (ha), Y is the reconstruction area (ha), Q is the volume of water resources used, thous. m3, CQ is the specific reduced costs for water supply, and flow control, rub/m3, Cx is the agricultural costs, rub/ha, Cy is the specific reduced reconstruction costs, rub/ha.The minimum total losses are sought under the conditions:

$$A_1^1 X + A_1^2 Y \geq B_1 \quad (2)$$

$$A_2^1 X + A_2^2 Y \leq B_2, \quad (3)$$

$$X, Y, Q \geq 0. \quad (4)$$

In inequalities (2) and (3), the A_1^1, A_1^2, A_2^1, A_2^2 matrices include technical and economic standards, namely, agrotechnological coefficients, irrigation standards, and labor costs.

The list of state parameters and factors that determine the difference in the manifestation of ecological functions of soils should be improved, while environmental regulation should provide not only control of the state but also warnings about the onset of the very first signs of significant changes in the soil, leading to its degradation (Vladimirov,

2015; Safronova et al., 2019b; Safronova et al., 2020b; Vladimirov and Alexandrov 2021).

In the article (Safronova and Prikhodko, 2007), the same authors describe a model of continuous changes in the event price. This article considers the case of a stepwise price change. Let Si is the costs associated with the regulation or complete elimination of negative consequences of land reclamation measures. Let us assume that at the start of work, reclamation measures are scheduled for the duration of operation T1, and the price of these measures is S1. If during time T1 the negative consequences of reclamation measures are not eliminated, new reclamation measures are scheduled, whose price is S2, and the duration of operation is T2. If the negative consequences are not eliminated during the time T2 then, new reclamation measures are scheduled with the duration time T3 whose price is S3, etc. Let's call each time period a phase. The duration of the phase depends on the number of scheduled activities.

Let at the initial time moment the price is . Reclamation measures are being put into operation and the number of possible measures is . If the satisfactory result is reached, the process will be finished. If the selected events do not lead to a satisfactory condition, then the following events are scheduled, whose price is S2. If the scheduled activities bring the system to an acceptable state, the process is finished, if not, then other possible activities are selected, whose price is S3, etc. To develop various technology options for growing agricultural crops, the authors consider reclamation measures as a Poisson flow of certain intensity (Kitaeva and Stepanova, 2013; Safronova et al., 2020; Vladimirov and Alexandrov 2021).

Let us determine the average time needed by the system to reach a satisfactory state.
The probability density of time intervals τ has the following form:

$$p(\tau) = \lambda R e^{-\lambda R} \tag{5}$$

If the satisfactory state has been reached within the time interval of length T, then the conditional probability density of the time interval from the beginning of the phase to this time point will be equal to:

$$p(\tau \mid \tau \le T) = \frac{p(\tau)}{P(\tau \le T)}, \quad 0 \le \tau \le T.$$

Presenting in more details, one gets:

$$p(\tau \mid \tau \le T) = \frac{R\lambda e^{-\lambda R\tau}}{\int_0^T R\lambda e^{-\lambda R\tau} d\tau} = \frac{R\lambda e^{-\lambda R\tau}}{1-P}$$

Next, let us write down an expression for the mathematical expectation:

$$M\{\tau \mid \tau \le T\} = \frac{\int_0^T R\lambda \tau e^{-\lambda R\tau} d\tau}{1-P} \tag{7}$$

After calculating the integral in the numerator, one gets:

$$M\{\tau \mid \tau \le T\} = \frac{T}{1-P} \cdot \varphi_1(\lambda RT) \tag{8}$$

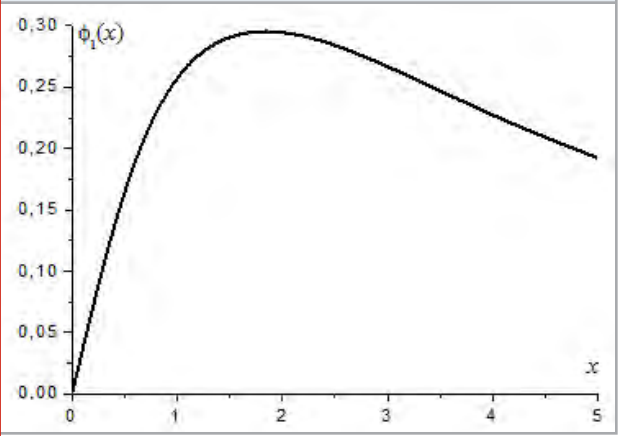
In (8), the following function is used:

$$\varphi_1(x) = \frac{1-(1+x)e^{-x}}{x} \tag{9}$$

The curve corresponding to the function (9) is shown in Fig. 1. Note that

$$M\{\tau \mid \tau \le T\}(1-P) = T \cdot \varphi_1(\lambda RT) \tag{10}$$

Figure 1: Graphical representation of the φ_1 (x) function



Now let us compose an expression for the mathematical expectation of the time needed for the system to reach the satisfactory state. Since the satisfactory state is reached during the period T_n with the probability $Q_n = \prod_{i=1}^{n-1} P_i \cdot (1-P_n)$

$$M\{\tau\} = \bar{\tau} = \sum_{n=1}^{\infty}\left[(T_1 + T_2 + \ldots + T_{n-1})\prod_{i=1}^{n-1} P_i(1-P_n) + T_n\varphi_1(\lambda R_n T_n)\prod_{i=1}^{n-1} P_i\right] \tag{11}$$

which after simplification can be written as:

$$\bar{\tau} = \sum_{n=1}^{\infty}(1-P_n)\prod_{i=1}^{n-1} P_i\left(\sum_{k=1}^{n-1} T_k\right) + \sum_{n=1}^{\infty} T_n\varphi_1(\lambda R_n T_n)\prod_{i=1}^{n-1} P_i \tag{12}$$

By rearranging the sum in the first term, one gets:

$$\sum_{n=1}^{\infty}(1-P_n)\prod_{i=1}^{n-1} P_i\left(\sum_{k=1}^{n-1} T_k\right) = \sum_{k=1}^{\infty} T_k \sum_{n=k+1}^{\infty}(1-P_n)\prod_{i=1}^{n-1} P_i \tag{13}$$

But

$$\sum_{n=k+1}^{\infty}(1-P_n)\prod_{i=1}^{n-1} P_i = \left(\prod_{i=1}^{k} P_i - \prod_{i=1}^{k+1} P_i\right) + \left(\prod_{i=1}^{k+1} P_i - \prod_{i=1}^{k+2} P_i\right) + \ldots = \prod_{i=1}^{k} P_i,$$

since $\prod_{i=1}^{\infty} P_i = 0$. And now one can write:

$$M\{\tau\} = \bar{\tau} = \sum_{n=1}^{\infty} T_n P_n \prod_{i=1}^{n-1} P_i + \sum_{n=1}^{\infty} T_n\varphi_1(\lambda R_n T_n)\prod_{i=1}^{n-1} P_i = \sum_{n=1}^{\infty} T_n\psi_1(\lambda R_n T_n)\prod_{i=1}^{n-1} P_i \tag{14}$$

Where

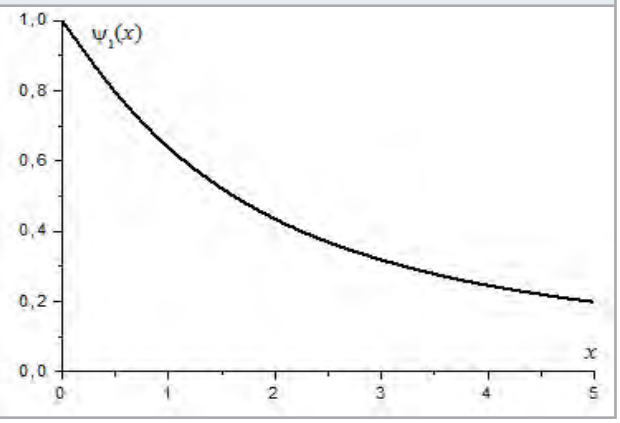
$$\psi_1(\lambda R_n T_n) = P_n + \varphi_1(\lambda R_n T_n) = e^{-\lambda R_n T_n} + \varphi_1(\lambda R_n T_n) \tag{15}$$

From the latter one gets the following expression for the function:

$$\psi_1(x) = e^{-x} + \varphi_1(x) = \frac{1-e^{-x}}{x} \tag{16}$$

which can be used to calculate the average time needed by the system to reach the satisfactory state. A curve representing the average time needed to reach the satisfactory state is shown in Figure 2.

Figure 2: Plotted function for finding the average time needed to reach the satisfactory state by the irrigation system



Thus, ultimately, the following expression is obtained:

$$\bar{\tau} = \sum_{n=1}^{\infty} T_n \psi_1(\lambda R_n T_n) \cdot \exp\left(-\sum_{k=1}^{n-1} \lambda R_k T_k\right),$$

where it is believed that

$$\sum_{k=1}^{0} \lambda R_k T_k = 0 \qquad (17)$$

Let us consider the optimization problem of reclamation activities at each phase. Let us compose an expression of the mathematical expectation of total income from the use of the agricultural landscape:

$$\Phi = \sum_{n=1}^{\infty} (S_n - K_n)(1 - P_n) \prod_{i=1}^{n-1} P_i \qquad (18)$$

Here, K_n is the loss, if the satisfactory state is reached at the n-th phase (Kitaeva and Stepanova, 2013). The problem of optimizing ϕ based on $\{S_n\}$ is reduced to solving the following system of equations:

$$\frac{\partial \Phi}{\partial S_m} = 0, \quad m = \overline{1, \infty}.$$

Consider the explicit form of these equations. Initially, the value occurs in the summand equal to:

$$(S_m - K_m)\left(1 - e^{-\lambda R(S_m)T_m}\right) \prod_{i=1}^{m-1} P_i$$

Since $\prod_{i=1}^{m-1} P_i$ does not include S_m, the derivative of this term is equal to:

$$\left[1 - e^{-\lambda R(S_m)T_m} + (S_m - K_m)\lambda R'(S_m)T_m e^{-\lambda R(S_m)T_m}\right] \prod_{i=1}^{m-1} P_i \qquad (19)$$

When $n > m$, the value S_m in the summands is present only in the cofactor $P_m = \exp(-\lambda R(S_m)T_m)$, and the corresponding derivative is equal to:

$$P'_m = -\lambda R'(S_m)T_m \cdot P_m \qquad (20)$$

Writing down $\frac{\partial \Phi}{\partial S_m}$, one gets:

$$\left[1 - e^{-\lambda R(S_m)T_m} + (S_m - K_m)\lambda R'(S_m)T_m e^{-\lambda R(S_m)T_m}\right] \prod_{i=1}^{m-1} P_i - \lambda R'(S_m)T_m \cdot \sum_{n=m+1}^{\infty} (S_n - K_n) \prod_{i=1}^{n-1} P_i (1 - P_n) \qquad (21)$$

After equating this expression to zero, dividing by $\prod_{i=1}^{m-1} P_i$, the following expression is obtained:

$$\left[1 - e^{-\lambda R(S_m)T_m} + (S_m - K_m)\lambda R'(S_m)T_m e^{-\lambda R(S_m)T_m}\right] - \lambda R'(S_m)T_m \cdot F_m = 0, \qquad (22)$$

where

$$F_m = \sum_{n=m+1}^{\infty} (S_n - K_n) \prod_{i=m}^{n-1} P_i (1 - P_n) \qquad (23)$$

To find a recurrent relation for F_m, it is necessary to extract the first term:

$$F_m = (S_{m+1} - K_{m+1})P_m(1 - P_{m+1}) + P_m \sum_{n=m+2}^{\infty} (S_n - K_n)(1 - P_n) \prod_{i=m+1}^{n-1} P_i.$$

The comparison allows recording the recurrent ratio for F_m:

$$F_m = (S_{m+1} - K_{m+1})P_m(1 - P_{m+1}) + P_m F_{m+1} \qquad (24)$$

Now, a recurrent relation for S_m can be derived. Let us write the expression (23) as follows:

$$\frac{1 - e^{-\lambda R(S_m)T_m}}{\lambda R'(S_m)T_m} + (S_m - K_m)\lambda R'(S_m)T_m e^{-\lambda R(S_m)T_m} = F_m \qquad (25)$$

By analogy:

$$\frac{1 - e^{-\lambda R(S_{m+1})T_{m+1}}}{\lambda R'(S_{m+1})T_{m+1}} + (S_{m+1} - K_{m+1})\lambda R'(S_{m+1})T_{m+1} e^{-\lambda R(S_{m+1})T_{m+1}} = F_{m+1} \qquad (26)$$

Write (26), given (24), and after multiplying by $e^{\lambda R(S_m)T_m}$ one gets:

$$S_m - K_m + \frac{e^{\lambda R(S_m)T_m} - 1}{\lambda R'(S_m)T_m} = S_{m+1} - K_{m+1} + \frac{1 - e^{-\lambda R(S_{m+1})T_{m+1}}}{\lambda R'(S_{m+1})T_{m+1}} \qquad (27)$$

The expression (27) connects S_m and S_{m+1}. This recurrent relation is used as follows. Having been given, S_o it is possible to find numerically S_1, then, knowing S_1, one can find S_2, etc. As a result, the value of ϕ, which depends only on S_0, is obtained. Further, examining the function for the extreme point, the value is determined numerically (Φ – total income from the use of the agricultural landscape).

The iterative planning process proposed in the work can be the basis of a program that corresponds to the optimal production activity, provided that the state of the external environment is maintained at a given level while choosing the optimal combination of various environmental protection measures that meet the requirements of environmental balance. When choosing a control action, the question often arises of replacing some control factors with others that have a similar effect on the transfer of the control object to a given state. The outlined approach allows solving this problem and finding the optimal ratio of measures to prevent environmental damage. The

considered concept of the price of sustainability of the agricultural landscape allows one to objectively choose the optimal solution for the use of resources. During the development of the project, several of its variants are compared. Different versions of the project may differ not only in technical parameters but also in the organizational and economic mechanism of implementation. Therefore, if we consider design as a process of developing the best version of the project, then it should also provide for the development of various options for the organizational and economic mechanism, the most rational of which will be accepted for implementation.

CONCLUSION

The authors have developed a mathematical (probabilistic) model of the irrigation system operation at a stepwise change in the scheduled event price. The model makes it possible to study the features of the system's operation in any real situation, predict the system's behavior when environmental conditions change, as well as reduce the risks of uncertainty when making managerial decisions. The optimization problem of finding the price change pattern of the satisfactory state of the system that would provide maximum profit taking into account losses from the unfavorable state, is considered. A recurrent ratio for total revenue is obtained, linking the scheduled activities at the m-th and (m+1)-th phases. This recurrent relation is used as follows. Having been given S_o, it is possible to find numerically S_1, then, knowing S_1, one can find S_2, etc. As a result, a total revenue function can be obtained that depends only on S_o. Next, examining the function concerning the extreme point, maximum profit is calculated numerically.

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Agricultural Communication

Development of Small forms of Farming in the Agricultural Sector: Trends and Outlook

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ABSTRACT

The purpose of the article is to analyze trends and prospects for the development of small forms of management in agriculture of the Russian Federation. Statistical and economic, monographic, computational, and constructive research methods were used in the course of the study, as well as an information base including data from the Federal State Statistics Service of the Russian Federation, the Ministry of Agriculture of the Russian Federation, articles in peer-reviewed Russian and foreign periodicals. The paper analyses the current state and developmental trends of small forms of farming in the agricultural economy. There is a tendency in the Russian Federation to reduce their number, except for individual entrepreneurs, with an increase in the area of agricultural land in them. The size of agricultural production in small forms of farming varies sharply. Small agricultural enterprises are the largest, and households are the smallest. The production of labor-intensive products is mainly concentrated in small forms of management. They produce 83.4% of potatoes, 78.8% of vegetables, 81.8% of fruits and berries, 84.8% of wool, and almost 99% of honey. The study identified the aspects, forms, and scope of government support of small forms of farming and determined its role in the development of agriculture. A typical description of small forms of farming in agriculture is small commodity production, moderate level of mechanization and high labor intensity, high quality (organic) production with minor use of mineral fertilizers and chemical pesticides, challenges in marketing the product, and low levels of government support. Priorities were substantiated in the development of small businesses, peasant (private) farms, and individual entrepreneurs.

KEY WORDS: AGRICULTURE, COOPERATION, GOVERNMENT SUPPORT PRODUCTION SIZE.

INTRODUCTION

One of the priorities of agricultural policies in the Russian Federation today is the development of small forms of farming, which make the basis of sustainable rural development (Ushachev et al., 2021). However, the core of agricultural production is made up of major agricultural organizations developing as agroindustrial structures involved in the production and deep processing of agricultural commodities and marketing finished products (Egorov et al. 2020). Meanwhile, the role of small forms of farming is big in specific product types. Moreover, they help to address employment problems in rural regions, relieving tensions in the labor market. They also show improved adaptivity to changes in the external environment compared to bigger businesses (Sosenkov 2019). 18.5 thousand small agricultural businesses are operating in the Russian Federation, representing 67.3% of the total number. Of those, 12.1 thousand, or 44.0% of the total, are micro-businesses. They consolidate 43.5 million ha of agricultural land, or 48.2% of the area operated by agricultural organizations, and employ 378.8 thousand people, which equals 27.3% of the workforce. Small businesses accommodate 2,923 thousand heads of cattle, or 34.0% of the total livestock of agricultural organizations, including 1,332 heads of cows (39.5%). Between 2006 and 2016 (National agricultural census years), the number of small businesses declined by 1.9 thousand, or 9.3%, meanwhile, their respective area of agricultural land in operation grew by 19.8 million ha, or 83.5% (Laktionova & Samsonenko, 2019; Mandrova et al., 2020; Grishina et al., 2021).

There are 18.8 million private subsistence farms and other individual operations supported by individuals engaged

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in agriculture operating on 12.2 million ha of agricultural land and counting 8,177 thousand heads of cattle, including 4,044 heads of cows. Over the analyzed period, the number of such operations declined by 1.4 thousand, or 6.9%, while the respective agricultural land in use rose by 3.4 million ha, or 38.6%. Accordingly, small forms of farming include farms engaging in business operations (small agricultural businesses in various legal forms, peasant (private) farms and individual entrepreneurs) and non-business-related farms (private subsistence farms and other individual operations, non-profit gardening (horticulture) communities). A distinction should be drawn between the notions of small forms of farming and small agribusiness, where the latter refers to businesses run for a profit. The small business segment in agriculture comprises small businesses, peasant (private) farms, and individual entrepreneurs.

The problem of the development of small forms of farming in the agriculture of the Russian Federation is devoted to numerous studies concerning various aspects of this problem: state support of agricultural small business (Dzhadan & Nevdakh, 2019; Mandrova, 2019; Kotranova & Dolgusheva 2019; Akhmetzhanova, 2019), various forms of financial support (Belousova, Dorogoichenko, Goncharov, 2019; Alentieva, 2020), regional problems and prospects for the development of small business in the agrarian sector of the Russian economy (Bessarabova, 2020; Chistyakova & Shmidt, 2021), the development of small forms of management in agriculture in Russia in general (Laktionova & Samsonenko, 2019; Mandrova et al., 2020; Grishina et al., 2021), as well as problems and restrictions associated with this (Yakimenko, 2019; Fazliev, 2019; Mandrova et al., 2020; Grishina et al., 2021).

MATERIAL AND METHODS

Information and evidence for this paper were based on data from the Federal State Statistics Service and the Ministry of Agriculture of the Russian Federation and articles in Russian and foreign periodicals. Research methods included statistical and economic, monographic, calculation and design methods, etc. The statistical and economic method served to provide a detailed description of the analyzed phenomenon based on mass digital data; therefore, it was used to analyze the state and developmental trends of small forms of farming (Grishina et al., 2021). The monographic method was used to review the operation of peasant (private) farms with outstanding economic performance (Bessarabova, 2020; Mandrova et al., 2020). The calculation and design method were used to substantiate the priorities in advancing small forms of farming for the future (Yakimenko, 2019).

Table 1. Sizes of small forms of farming in the Russian Federation*

	Small agricultural businesses	Peasant (private) farms	Individual entrepreneurs	Private household farms
Number of farms, thousand	18.5	136.7	25.4	23,497
Area of agricultural land, thousand ha	43,486	35,047	4,531	12,898
Employees, thousand of people	378.8	301.2	76.2	40,723
Average per farm:				
Agricultural land, ha	1,791	256	119	0.5
Employees	21	3	3	2
Cattle, head	416	66	43	4
including cows	185	34	21	2
Pigs	2,363	62	61	4
Poultry	39,575	952	1,075	26

*According to the National agricultural census of 2016.

RESULTS AND DISCUSSION

A typical description of small forms of farming in agriculture is small commodity production, moderate level of mechanization and high labor intensity, high quality (organic) production with minor use of mineral fertilizers and chemical pesticides, challenges in marketing the produce, low levels of government support and social orientation in countering rural unemployment by engaging local residents in productive processes (Guliaeva & Volobueva 2014; Oganian 2015; Ushachev 2011). Small forms of farming largely consolidate the production of labor-intensive products. They produce 83.4% of potatoes, 78.8% of vegetables, 81.8% of fruit and berries, 84.8% of wool, and almost 99% of honey (Mandrova et al., 2020). The scale of agricultural production varies significantly among small forms of farming (Table 1). The biggest in scope are small agricultural businesses. On average per farm, the parameters are as follows: 1,791 ha of agricultural land, 416 heads of cattle, including 185 heads of cows, 2,363 heads of pigs, 39,575 heads of poultry, and 21 employees.

Peasant (private) farms and individual entrepreneurs operate on a somewhat smaller scale. Specifically, the respective parameters on average per farm are 256 and 119 ha of agricultural land, 66 and 43 heads of cattle, including 34

and 21 heads of cows, 62 and 61 pigs, 952 and 1,075 heads of poultry, and three employees in each case. The smallest are private household farms: the area of agricultural land is 0.5 ha, livestock equals four heads of cattle and pigs, 26 heads of poultry. The size of farms depends on production objectives. The objective of small agricultural businesses, peasant (private) farms and individual entrepreneurs is to make a profit. Private household farms are meant to supply the household's food requirements from local onsite production with only excessive supplies sold in the market. Accordingly, the rate of commercial agricultural output is high (75-98%) for the former, but very low for the latter (15-30%) (Minakov & Nikitin 2019; Kulikov & Minakov 2020).

The State Programme of Agricultural Development and Regulation of Agricultural Products, Commodities, and Food Markets has contributed to the development of agriculture, although not all categories of farms have benefitted from the effects. Successful development in the sector is observed among big agricultural organizations, though production declines are registered in certain types of products among small businesses (Table 2). Over 2016-2019, production declined by 16.7% in sugar beet, 11.7% in livestock and poultry for slaughter (live weight), and 3.1% in grain. Meanwhile, the levels rose by 65.7% in eggs, 40.8% in oil crops, and 6.5% in milk.

Table 2. Development of agriculture in small businesses in the Russian Federation

	2016	2017	2018	2019
Cropped area, million ha	24.8	25.6	24.2	23.5
Livestock, thousand head:				
Cattle	2,846	2,806	2,574	2,544
including cows	1,222	1,187	1,093	1,072
Pigs	1,386	1,312	1,295	1,196
Sheep and goats	3,198	2,884	1,777	1,691
Gross production, million tons				
Grain	32.7	38.3	30.1	31.7
Oil crop produce	4.9	5.3	6.0	6.9
Sugar beet	6.8	7.1	4.9	5.7
Potatoes	2.2	2.4	2.2	2.2
Vegetables	1.2	1.2	1.3	1.3
Production, thousand tons				
Livestock and poultry production for slaughter (live weight)	754	915	775	666
Milk	4,254	4,407	4,363	4,529
Eggs, millions	1,995	2,843	3,101	3,307

Source: calculated according to data from Rosstat

Over the analyzed period, small businesses saw a decline in their resource potential equivalent to 5.2% in terms of cropped areas, 10.6% in livestock of cattle, 12.3% in livestock of cows, 13.7% in pigs, and 47.1% in sheep and goats. The declines in cropped areas, livestock, and production volumes of certain types of commodities are due to the low levels of government support of small businesses. Many would withdraw from the production of low-margin or loss-making animal farming products and switch to more profitable crop farming operations. Growing output in certain types of products is largely due to growing yields of crops and productivity of livestock and poultry (Kulikov & Minakov 2018; Solopon & Minakov 2018; Mandrova et al., 2020; Grishina et al., 2021).

The relative share of small businesses in the total output of many types of products declined as a result of slower production growth compared to that of bigger operations. The share of small businesses declined to 37.3% from 37.9% in the production of grain, to 11.8% from 15.1% in sugar beet, to 47.8% from 51.3% in potatoes, to 32.5% from 39.8% in vegetables, and to 26.7% from 27.9% in milk. Government support measures have contributed to the development of peasant (private) farms and individual entrepreneurs in the Russian Federation (Table 3).

Over 2016-2019, this segment saw an increase of production of oil crop produce by 55.6%, potatoes by 11.5%, vegetables by 16.7%, grain by 5.7%, livestock and poultry for slaughter (live weight) by 14.4%, and milk by 23.0%. Growing agricultural output in the segment of peasant (private) farms and individual entrepreneurs is due to growing cropped areas, livestock, yields of crops, and animal productivity. Over the discussed period, the cropped area grew by 2.3 million ha, or 10.5%, livestock of cattle – by 300 thousand heads, or 12.5%, including cows – by 200 thousand heads, or 16.7%. As a result of production growth in the segment of peasant (private) farms and individual entrepreneurs, there was an increase in their relative share in the agricultural production structure. The production share of such farms rose to 29.2% from 27.7% in grain, to 30.8% from 27.4% in oil crop produce, to 13.1% from 11.8% in potatoes, to 19.9%

from 18.1% in vegetables, to 3.4% from 3.0% in cattle and poultry for slaughter (carcass weight), to 8.5% from 7.3% in milk and to 38.2% from 36.2% in wool (physical weight) (Laktionova & Samsonenko, 2019; Grishina et al., 2021).

Table 3. Development of peasant (private) farms and individual entrepreneurs in the Russian Federation

	2016	2017	2018	2019
Cropped area, million ha	22.0	23.1	23.6	24.3
Livestock, million head:				
Cattle	2.4	2.5	2.6	2.7
including cows	1.2	1.2	1.3	1.4
Pigs	0.5	0.4	0.4	0.4
Sheep and goats	9.1	9.1	8.7	8.7
Gross production, million tons				
Grain	33.5	39.5	32.8	35.4
Oil crop produce	4.5	4.7	5.7	7.0
Sugar beet	6.0	6.0	4.5	5.9
Potatoes	2.7	2.5	2.8	2.9
Vegetables	2.4	2.6	2.6	2.8
Production, thousand tons				
Livestock and poultry production for slaughter (live weight)	487	513	542	557
Milk	2,174	2,375	2,511	2,675
Wool (physical weight)	20.3	20.9	19.7	19.2

Source: calculated according to data from Rosstat

Over the past years, production growth achieved by peasant (private) farms has outpaced that of agricultural organizations. E. g., the 2019 agricultural production index for peasant (private) farms equaled 106.6% vs. 105.8% for agricultural organizations. Further agricultural production growth through the expansion of agricultural areas is almost impossible for peasant (private) farms, as land is scarce and fixed (Dubovitskii & Klimentova 2019). Therefore, production growth can be only achieved through a transition to an innovation-driven method of agricultural development. Private household farms have observed a decline in agricultural production (Table 4). Over 2016-2019, production declined by 7.1% in potatoes, 5.2% in vegetables, 8.2% in livestock and poultry for slaughter (live weight), 7.0% in milk, 3.5% in eggs, 11.7% in wool and 7.8% in honey. Production decline in this category was due to the decrease of cropped areas and livestock. The decrease in production narrowed the share of private household farms in the agricultural production structure. Despite this decline in the production share, they remain major suppliers in many product categories. In 2019, private household farms produced 65.9% of potatoes, 51.8% of vegetables, 65.7% of fruit and berries, 91.5% of honey, 46.7% of wool, and 37.4% of milk in the Russian Federation.

The development of agriculture in private household farms is dragged back by the lack of real government support, challenges in marketing the produce, inadequate agricultural consumer cooperation, low supply levels of animal feeds, spread of contagious animal diseases (such as African swine fever), and age profile of rural populations (Kulikov & Minakov 2019b). Further development of agriculture in private household farms would be driven by the establishment of marketing and procurement, processing, and other consumer cooperatives handling procurement, processing, and marketing, which would help to considerably reduce losses and increase commercial output levels (Kulikov & Minakov 2019a). An important requirement for the development of small forms of farming is the refinement and increase of government support. A predominant part of investment under the program (more than 90%) is assigned to big businesses (Palatkin & Afanaseva 2014; Bessarabova, 2020; Chistyakova & Shmidt, 2021).

The State Programme for Development of Agriculture and Regulation of Agricultural Commodity, Materials and Food Markets sets forth government support measures aimed at small forms of farming, such as Agrostartup grants for peasant (private) farms, support for startup farmers, for the development of a family farm and agricultural consumer cooperatives. Under the federal project of the System of Farming Support and Rural Cooperation Development as part of the State Programme, funding was assigned in 2019 in the form of Agrostartup grants to support farmers, and subsidies were provided for advancing agricultural consumer cooperation. Spending on the project from the federal budget stood at 5.4 billion roubles. The average grant size equaled 2.42 million roubles, the average subsidy per cooperative was 2.4 million roubles (Kotranova & Dolgusheva, 2019; Alenteva, 2020).

In 2019, support of small forms of farming was also provided under the departmental project for the development of agricultural industries enabling accelerated import substitution of certain types of agricultural products,

commodities, and food within the State Programme for Agricultural Development. Specifically, two types of grants were used to support startup farmers and animal farming and grant support of consumer cooperatives for building up resource and equipment capabilities. Grants to support startup farmers are provided to co-fund the costs of setting up and running a peasant (private) farm and creating new permanent jobs in rural areas based on the calculations of 2 million roubles for two or more new permanent jobs or less than 2 million roubles for one permanent job. In 2019, the actual size of funding to support startup farmers from the budgets of the federal subjects of the Russian Federation stood at 659.2 million roubles. The average grant per farm of a startup farmer equaled 2.14 million roubles, which is 3.4% more than in 2018 (Dzhadan & Nevdakh, 2019; Alentieva, 2020).

Table 4. Development of agriculture in private household farms of the Russian Federation

	2016	2017	2018	2019
Cropped area, million ha	2.6	2.5	2.4	2.3
Livestock, million head:				
Cattle	7.6	7.5	7.4	7.3
including cows	3.4	3.4	3.4	3.3
Pigs	3.1	2.8	2.5	2.4
Sheep and goats	11.4	11.3	10.7	10.4
Gross production, million tons				
Potatoes	15.6	15.0	15.2	14.5
Vegetables	7.7	7.5	7.5	7.3
Fruit and berries	2.2	1.8	2.1	2.3
Production, thousand tons				
Livestock and poultry production for slaughter (live weight)	3,246	3,135	3,050	2,981
Milk	12,600	12,100	11,900	11,722
Eggs, billion	8.5	8.4	8.3	8.2
Wool (physical weight)	26.5	26.8	25.8	23.4
Honey	65.1	61.2	61.1	60.0

Source: calculated according to data from Rosstat

Grants for the development of a family farm are provided for the development and creation of new permanent jobs in rural areas based on the calculation of at least three new permanent jobs per grant. With that, the projected breeding stock should not exceed 300 heads of cattle and 500 heads equivalent of sheep (goats). In 2019, the average size of grants provided to family farms in animal breeding equaled 8.33 billion roubles, which is 7.9% more than in 2018. Spending on support for peasant (private) farms from the federal budget of the Russian Federation in 2019 equaled 7,959 million roubles, including 3,299 million roubles for startup farmers and 4,660 million roubles for the development of family farms in animal breeding. Within a year, grant support helped peasant (private) farms to create 5,826 new permanent jobs, while agricultural production growth reached 35.8% (The Order of the Government of the Russian Federation 2020; Mandrova, 2019; Alentieva, 2020).

Their development is contingent on the measures and size of support provided to peasant (private) farms under the State Programme for Development of Agriculture and Regulation of Agricultural Commodity, Materials, and Food Markets. E. g., the respective gross output, and new permanent job creation levels were the highest for the years with higher levels of government support. That is why the refinement of government support of peasant (private) farms constitutes a principal reserve for consolidating agricultural output levels. Support is also provided to consumer cooperatives in the form of grants for building up resource and equipment capabilities and permanent job creation in rural areas based on the calculation of at least one permanent new job for each 3 million roubles of the grant amount, but not less than one job per grant (Mandrova, 2019; Mandrova et al., 2020).

In 2019, grant support of agricultural consumer cooperatives for building up resource and equipment capabilities from the federal budget equaled 2,341 million roubles. The average size of grants per cooperative equaled 16.08 million roubles. 1,138 new jobs were created; the increase of agricultural production volume marketed by cooperatives receiving grant support reached 26.3% (The order of the Government of the Russian Federation 2020). Besides, the State Programme of Agricultural Development and Regulation of Agricultural Products, Commodities and Food Markets provides for financial support of small forms of farming arranged as subsidies from the federal budget to partially compensate for interest costs on loans attracted:

- by individuals engaging in private subsistence farming under loan agreements entered into on or before December 31, 2016, for a term within five years, – for purchasing agricultural animals, animal farming equipment, and agricultural processing equipment

or for repairs, reconstruction, and construction of premises for animal farming, acquiring gas equipment and connections given that the principal amount drawn for the year should not exceed 700 thousand roubles per farm (Dzhadan & Nevdakh, 2019);

- by peasant (private) farms under loan agreements entered into a) on or before December 31, 2012, for a term within 8 years, – for purchasing agricultural machinery or equipment for animal or poultry farming, feed production, machines, units, and devices of sprinkling, irrigation and pumping stations; b) on or before December 31, 2016, for a term within 8 years, – for storage and processing of agricultural products, purchasing of breeding materials, construction, reconstruction and modernization of storages for potatoes, vegetables, fruit, for greenhouse or animal farming complexes, animal farming facilities, feed production, and flax processing, construction, and reconstruction of bud complexes for perennial plantings, for planting perennial crops and vinery, given that the principal amount drawn for the year should not exceed 10 million roubles per farm (Dzhadan & Nevdakh, 2019).
- Agricultural consumer cooperatives under loan agreements entered into:
- on or before December 31, 2012, for a term within 8 years, – for purchasing machinery and equipment manufactured by Russian and foreign producers;
- on or before December 31, 2016, for a term within 8 years, – for purchasing special technology equipment, refrigeration equipment, agricultural animals, breeding materials, for construction, reconstruction, and modernization of storage and production facilities, storages for potatoes, vegetables, fruit, for greenhouse or animal farming complexes, feed production, and flax processing facilities, for construction and reconstruction of agricultural markets, marketplaces, points of the transaction, initial processing and storage of milk, meat, fruit and vegetable, and other agricultural products, for planting perennial crops and vinery given that the principal amount drawn for the year should not exceed 40 million roubles per cooperative (Kotranova & Dolgusheva, 2019).

Subsidies from the federal budget to partially compensate for interest costs are provided based on two-thirds of the key rate (official discount rate) of the Central Bank of the Russian Federation over the whole term of such loan agreements. In 2019, the size of such subsidies equaled 7.6 billion roubles, which is 2.6% less than in 2018 (Chernykh & Goncharenko 2020; 21). Of all small forms of farming, peasant (private) farms, individual entrepreneurs, and small businesses are expected to show the biggest advance. The best conditions are created for these forms of farming. Efficient operation of small forms of farming would require wider adoption of innovation and the most advanced agricultural production technology, development of agricultural cooperation (across marketing, servicing, procurement, processing, and other types), and integration with major agricultural organizations developing as agroindustrial structures to cut losses and preserve the quality of products in storage and processing.

Small business is a traditional and integral part of the agricultural economy. However, demonstrating a high sensitivity to the state of the business environment, dependence on the availability of credit resources, instability to long-term crisis phenomena, it needs systemic government support (Kotranova & Dolgusheva, 2019). In this regard, the prospects for its development are associated with the support of sectoral priorities and an increase in the efficiency of state regulation mechanisms in two key areas: firstly, the solution of general sectoral problems (implementation of technical and technological modernization, the creation of modern social and industrial infrastructure, formation and improvement of staffing in the industry, stimulation of seed and pedigree breeding, which are basic for effective agriculture) (Akhmetzhanova, 2019); secondly, the creation of conditions for increasing the efficiency and sustainable development of small businesses (mainly through the development of cooperation, which will contribute to the technical re-equipment of small businesses and provide reliable access to markets for agricultural products, and increasing the availability of short and long-term credit resources to fill the deficit of working capital and the possibility of capital investments) (Dzhadan & Nevdakh, 2019). Therewith, the development of small businesses in the agricultural sector is possible only if a set of measures is developed in each region and municipal formation aimed at creating a favorable investment climate for them that stimulates competition (Mandrova, 2019).

CONCLUSION

Small forms of farming constitute a major sector of the agricultural economy largely defining the state of national food security. Advancing small forms of farming contributes to the development of a multi-structural agricultural economy and revival of abandoned rural settlements, as well as provides the environment for competition between agricultural producers in the agrifood market and conditions for socio-economic progress in rural areas.

Ethical Issue: Authors are aware of and comply with, best practices in publication ethics specifically concerning authorship (avoidance of guest authorship), dual submission, manipulation of figures, competing interests, and compliance with policies on research ethics. Authors adhere to publication requirements that submitted work is original and has not been published elsewhere in any language.

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Dental Communication

Dental Management in a Child with Ectodermal Dysplasia: A Case Report Along with Review

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ABSTRACT

Ectodermal dysplasia (ED) is a rare hereditary syndrome that is categorized by the nonstandard development of certain tissues and structures of ectodermal origin. This disorder is important to dentists because it affects teeth and results in hypodontia or anodontia. Young children who are affected by ED and anodontia face functional and aesthetic difficulties due to the deficiency of teeth. Restoring lost vertical dimension and teeth with a removable prosthesis improves speech, aesthetics, and appearance and thus boosts the patient's self-confidence. We report a case of hypohidrotic ED with an oligodontia (absence of all primary teeth except upper second primary molars) in a 5-year-old female patient who was successfully rehabilitated with conventional removable dentures in the maxillary and mandibular arches using multidisplinary approach. The goal of the treatments was to improve psychological development in addition to promoting aesthetics, speech, and eating effectiveness, as well as the overall improvement of the stomatognathic system. Previouslay, most of the ED cases were treated with removable prosthesis, either in form of partial pr complete removable prosthesis in both maxilaary as well as mandibular arches. These types of prosthesis were allowed for relaining and rebasing as most of the ED patients were receiving treatments during young ages. These days, with the improvements of dental materials and equipments, advance treatments with over denture implants have been introduced for replacement of missing teeth which are caused by ED The present case report along with an updated review discusses the successful dental management of the rare disorder.

KEY WORDS: ANODONTIA, ECTODERMAL DYSPLASIA, MOUTH REHABILITATION, PROSTHODONTIC TREATMENT, REMOVABLE PROSTHESIS.

INTRODUCTION

Ectodermal dysplasia (ED) syndrome is a rare heterogeneous group of inherited disorders that share main defects in the development of two or more tissues derived from the ectoderm (Ul Bari and Ber Rahman 2007). Clinically, ED can be broadly classified into hypohidrotic (X-linked recessive) and hidrotic (autosomal inherited) types. Anhidrotic (hypohidrotic) ED, also known as Christ–Siemens–Touraine syndrome, is the utmost common ED (80%). It affects males and is inherited from female carriers. It is a triad of sparse hair (atrichosis or hypotrichosis), abnormal or missing teeth (anodontia or hypodontia), and inability to sweat due to the lack of sweat glands (anhidrosis or hypohidrosis) (Naveen et al. 2012; Ladda et al. 2013 Mascolo et al. 2018). Defects in materials originating from other embryologic layers are not unusual. These disorders are congenital, diffuse, and nonprogressive (Ul Bari and Ber Rahman 2007). Patients with ED have a normal life expectancy and intelligence (Al-Araimi et al. 2019 Wimalarathna et al. 2020).

However, if ED goes unrecognized in early infancy, hyperthermia due to the lack of sweat glands may lead to brain damage or death. Anodontia, which represents the

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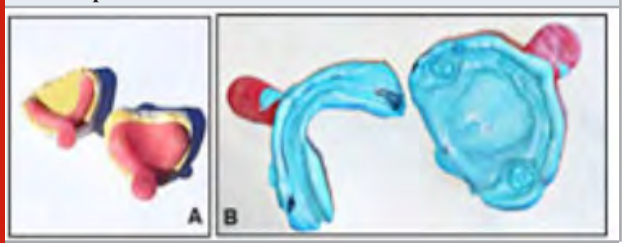
congenital absence of all teeth in the primary dentition and/or the permanent dentition, is a rare dental manifestation that associates with ED (Chugh et al. 2016; Al-Araimi et al. 2019). Vaidya et al. (2013), observed EDs in 11 patients over two generations in one family. Severe teeth abnormalities; mild fingernail deviations; and toenail abnormalities were seen in all the patients and were considered as abnormalities that affected individuals in a one family. Many case reports have been published worldwide. Table 1 provides a summary of the studies, including the following information: author(s) names and year of publication, country and family history, gender, age, arches involved, teeth present (primary or permeant), dental abnormality associated (if any), treatment received by the patient, and other associated clinical manifestations (Al-Araimi et al. 2019).

The lack of teeth and special appearance are reported as the major concerns of patients with ED. The sequence and type of treatments differ from patient to patient. However, it is important ti address address the complaints of most patients with ED which include restoring function, speech, facial appearance, and tooth aesthetics by normalizing the vertical dimension and supporting the facial soft tissues. This case report describes a multidisciplinary approach for dental managnent of a child with ED which involved pediatric dentistry and prosthodontic specialties.

Figure 1: Extra-oral lateral views (A-B), Skin of the hand and nail fingers (C), Pre-operative intra-oral view (D).

Figure 2: Panoramic View of the patient (A), an alginate impression for maxillary arch (B).
Figure 3: Primary cast with special tries (A), Max and mand final impression.



METHODOLOGY

A 5-year-old female patient reported to the Department of Pedodontics and Preventive Dentistry, College of Dentistry, Sanaa University, Yemen. Her chief complaints were the absence of teeth in the oral cavity, the dryness of the mouth, and difficulty during eating. On general and extraoral examinations, the patient presented the classical triad of hypohidrosis, hypotrichosis, and hypodontia. Apart from these manifestations, the patient presented dry skin, very thin eyebrows, thin brittle nails, saddle nose, thick everted depressed lips, nasal bridge, and sunken cheeks with prominent supraorbital ridges. The lower third of the face was shorter than the middle and upper thirds (Fig I A–C). Intraoral examination revealed a large tongue and complete anodontia in both arches (except for the two primary second molars in the maxillary arch) (Fig 1D). Radiographic examination revealed the presence of thin and resorbed bone in both arches and two maxillary primary second molars (Fig 2A).

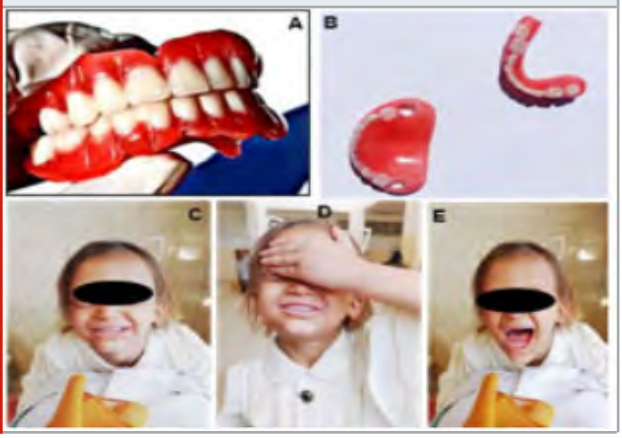
Treatment options were discussed with prosthodontic colleage and the parents.The preferred treatment option was a removable prosthesis (partial removable denture for the maxillary arch and removable complete denture for the mandibular arch). At this appointment, oral prophylaxis was performed for the two existing maxillary teeth, then maxillary and mandibular alginate primary impressions were taken (Fig 2B). From these impressions, a study cast (Fig 3A) was poured, and then special maxillary and mandibular custom tries were prepared for the final impression (Fig 3B).

Figure 3; Max and Mand master caste (A), Occlusal rimes on the casts (B), right, left, and frontal views after arrangement of teeth and before to teeth try-in (C-E).

The final impressions of the maxillary and mandibular arches were recorded accurately after border molding by using medium-viscosity elastomeric impression materials. After the beading and boxing of the final impressions, the casts were poured with an improved die stone for increased strength and abrasion resistance (Fig 4A). Then, the maxillary and mandibular occlusal rimes were prepared (Fig 4B). Subsequently, jaw relation was done in the conventional manner, and tooth arrangement was done with acrylic resin teeth and was followed by the

final try-in of the waxed dentures (Fig 4C–E). Finally, the wax dentures were processed with a heat-polymerized denture base resin (Fig 5A-B). The complete dentures were delivered, and the patient was instructed on the maintenance of oral hygiene and dentures. Extraoral photographs after the insertion of the dentures were taken (Fig 5 C–E). Recall appointments were done after 1 week and 3 and 6 months for adjustments if needed.

Figure 4: Processed denture is ready for insertion (A-B), extra-oral photographs after insertion of the dentures (C-E)



RESULTS AND DISCUSSION

Our patient is a 5-year-old female Yemeni with oligodontia caused by ED. She was managed by the fabrication of a maxillary Removable partial denture (RPD) and mandibular Complete Denture (CD). With this prosthesis, occlusion, masticatory function, aesthetics, speech, and overall quality of life were restored. The early replacement of missing teeth results in a positive effect on growth and aids in restoring masticatory function and aesthetics; maintains healthy supporting tissues and speech; and boosts the patient's self-esteem, thus improving the patient's overall quality of life (Murthy and Vaze 2010; Al Nuaimi and Mansoor 2019). The use of RPD will allow relining in the future given that the growth of the patient is continuous. It accommodates jaw growth in both arches, reduces the cost and frequency of remaking the prosthesis, and maintains typical oral occupations, thus resulting in an overall improvement in aesthetic outcome (Vieira et al. 2007; Murthy and Vaze 2010; Al Nuaimi and Mansoor 2019).

The clinical expression of ED depends on the structures and organs that are affected during embryonic development. In the present case, a female with ED exhibited several abnormalities that included the skin, scalp, body hair, gland, finger- and toenail, craniofacial, oral, and tooth abnormalities. The facial characteristics of young patients disturb their emotional condition and social life. Therefore, the management of orofacial and teeth abnormalities has a positive effect not only on masticatory and phonetic functions but also greatly influences the aesthetics and self-confidence of the patient and considerably increases their social confidence, self-esteem, and quality of life (Aragon 2020; Wimalarathna et al. 2020).

A pedodontist, maxillofacial surgeon, orthodontist, prosthodontist, and speech therapist should be involved in the treatment of such patients. Malformed, decayed, or stained teeth should be managed with composite materials (Patel et al. 2010; Prithviraj et al. 2014; Mittal et al. 2015). In patients with ED, establishing the correct maxillo–mandibular relations and the normal function of the dento–facial system (chewing, swallowing, and speaking) is important. Prosthodontic treatment has a major effect on aesthetics and functions, facilitates psychological development, and improves the emotional condition and social life of the patient (Mittal et al. 2015; Mascolo et al. 2018). Alginate hydrocolloid (irreversible hydrocolloid) impression material is used for primary impression and sometimes for final impressions with special trays because it is considered as clean, biocompatible, and pleasant for the patient. In addition, this material has a short setting time and thus causes limited discomfort to the patient and can be easily removed from the undercut area presented by malformed teeth (Wimalarathna et al. 2020).

Although no absolute time for the start of treatment exists, Pigno et al. (1994), stated that an initial prosthesis should be provided before the patient reaches school age. Kupietzky and Houpt (1995), reported that a Removable Denture RD can be fabricated for a patient as young as 3 years old. As the child grows, the RD has to be modified and substituted because longitudinal studies on anodontia have indicated that the growth of both jaws is independent of the presence of teeth. At least three replacements are needed between the period of early and late mixed and permanent dentition. When the patient is in the last period, the RD may be replaced with a fixed-type restoration by using osseointegrated implants. Guckes et al. (1991), recommended that this approach should be postponed till the age of 13 because of possible implant movement caused by jaw growth (Guckes et al. 1991; Pigno et al. 1994; Kupietzky and Houpt 1995; Wimalarathna et al. 2020).

This clinical report describes the types, characteristic features, and treatment options for a young female patient with ED. With proper multidisplinary treatment, the patient can enjoy a relatively normal life. The options for a definitive treatment plan may include implant-supported fixed or removable prosthesis singly or in combination (Murthy and Vaze, 2010). New alternatives, such as the use of implants, for the rehabilitation for children with hypohidrotic ED must be carefully considered given the presence of underdeveloped, thin alveolar bones, and age (Vieira et al. 2007; Mascolo et al. 2018; Al-Araimi et al. 2019).

CONCLUSION

The findings of this study suggests that a multidisplinary management of ED should be considered as this multisystemic disorder affects numerous parts of the

body. Early analysis is vital for taking essential preventive actions and enhancing the patient's quality of life. This review and case report contributed to the information on ED and to the education of undergraduate students and general dentists. The treatment was supported in accordance with the patient's requirements and support (symptomatic management depending on the affected ectodermal structures). Dental professionals play a vital role in contributing to the overall appearance and wellbeing of the affected patients.

Table 1: Summary of worldwide published case reports arranged alphabetically

Author(s)/ Year	Country, Family History	Gender /Age/ Arches	Teeth Present	Treatment Received	Dental Up-normality Associated
Current case	Yemen No	Female/5-Years/ Max & Mand	2 Max primary 2nd molars	Maxillary RPD & Mandibular CD	Abscess of most primary teeth and all permeant teeth
DI LANARO et al., 2017	Brazil No	Male/ 6-Years/ Max & Mand	Most of teeth were present	Reshaping of Max anteriores with composite & Mand RPD	Absence of permanent teeth Peg shape of all anteriores
de QUEIROZ et al. 2017	Brazil. Yes	Female/10-year-oldl 0000000000000 female patient,	13, 15, 17, 18, 23, 25, 27, 28, 31, 35, 37, 38, 41, 45, 47, 48.	Deciduous teeth 55, 65 and 75	Maxillary and mandibular canines, maxillary premolars Symmetry of present teeth
Velazque and da Silva, 2015	Brazil NO	Male/ 11-Years/ Max & Mand	All Maxillary and Mandibular anteriors	No treatment	Impacted Max & Mand canines
Vieira et al., 2007	Brazil No	Male /5-Years/	No teeth present	Maxillary CD & Mandibular CD	Maxillary and Mandibular with supposed 2nd primary molar
Nakayama et al., 2015	Japan Yes	Male/14-Years/ 5 Female; age range, 12.7–27.2 years)	Most of teeth present	Referred to ortho, and FPD, Implant treatment	Most effected anterior teeth Present of most permanent teeth Premolar most absent teeth
		4 Female/18-27-Yeras	5-14 teeth present	Referred to ortho, and FPD, Implant treatment	----------
Chugh et al., 2016	India	Male/ 5.6-Years/ Max & Mand	No teeth present	Maxillary & Mandibular CD	----------
Mittal et al., 2015	India Yes	Male/5-years/ Max & Mand	Maxillary 3 teeth	Maxillary RPD Mandibular CD	Conical shape of maxillary canine Absence of permeant teeth
	India Yes	Male /9-Years/ Max & Mand	No teeth	Maxillary RPD and Mandibular Implant Over Denture	----------
Srivastava VK, 2011	India	Male/11-Years/ Max & Mand	Maxillary 1 tooth conical canine	Maxillary RPD & Mandibular CD	----------
Moothedath, et al., 2018	India	Male/20-Years/ Max & Mand	Maxillary canine & Posterior primary teeth	Maxillary RPD & Mandibular CD	Conical shape of Maxillary canines
Sood & Mishra, 2020	India	Male/ 11-Years/ Max & Mand	Maxillary bilateral canines and 1st Molars	Maxillary & Mandibular RPD	Maxillary peg shape and bilateral primary molars
Kumar et al., 2012	India	Male/ 14-Years/ Max & Mand	Maxillary bilateral canines	No treatment	Absence of all permanent teeth
Hekmatfar et al. 2012	Iran No	Male/3-Years/ Max & Mand	4-peg-shaped anterior in Max Complete edentulism in Mand	Maxillary & Mandibular RPD	2-unerupted molar in Max 2-unerupted incisors in Mand Presence of permanent 1st molar
	Iran No	Female/10-Years/ Max & Mand	teeth maxillary anterior 4 primary molars, 5 permanent molars, 3 incisors,	Maxillary & Mandibular RPD	Absentees of 3rd molar teeth ↓ vertical height of facial lower 1/3 & prominent chin lips Unilateral Max contraction Semi-erupted molar, 3 Impacted premolars
Emilija et al., 2015	Macedonia	Male/6.5-Years/ Max & Mand	Maxillary and Mandibular Anterior teeth	Maxillary & Mandibular RPD	Sever bone loss of both arches Absent of all permanent teeth
Emilija et al., 2015	Macedonia	Male/6.5-Years/ Max & Mand	Maxillary and Mandibular Anterior teeth	Maxillary & Mandibular RPD	Sever bone loss of both arches Absent of all permanent teeth
Mascolo et al., 2018	Malta	Female /20 years/ Max & Mand	Most of teeth are present	Maxillary FPD, Madibular CD	Absent of all permanent teeth
AlNuaimi & Mansoor, 2019	Middle Eastern	Male/ 5-Years/ Max & Mand	All Maxillary and Mandibular anterior with left unilateral molar	Maxillary/ Functional space maintainer Mandibular Fixed PD	Absent of all permanent teeth
Al-Araimi et al., 2020	Oman Yes	Male/ 5-Years/ Max & Mand	--------	No Treatments	Mandibular peg shape canine
Ul Bari and Ber Rahman, 2007	Pakistan No	Male/ 9-Years/ Max & Mand	Centerals and canines are malformed	Refer to Orthodontic	----------
Wimalarathna et al. 2020	Sri Lanka No	Male/11-years/ Max & Mand	----------	Orthodontic Direct and Indirect restorative methods Max and Mand RPD	Absentees of Max, third molar maxillary and mandibular canines Crowded Mand teeth, Peg shaped Mand laterals
Koyuncuoglu et al., 2014	Turkey	Male/12-Years/Max & Mand	2 Max anteriores & 1 Mand	Maxillary FPD & Casted RPD, Mandibular Implant Over Denture	Presented 3 teeth were peg shape Abscess of most primary teeth and all permeant teeth
Yildirim et al., 2012	Turkey	11 Males/5-40 Years/Max & Mand 12 Females	Carious teeth in Maxilla	Depend upon the case	Early diagnosis is vital, patient's quality of life can be improved.
Aragon C, 2020	Turkey	Male/18-Years/Max & Mand	Maxillary 4 anterior & 2 Molar teeth	Maxillary FPD and Casted RPD, Mandibular Implant Over Denture	Semi erupted Maxillary canines No mandibular teeth

Abbreviations: Max-Maxillary; Mand- Mandibular; RPD-Removable partial denture; CD-Complete denture; FPD-Fixed partial denture; 1st – First; 2nd – Second; 3rd - Third

From the physical, emotional, social life, aesthetic, speech, and psychosocial standpoint, early prosthodontic treatment is of great importance to patients with the systemic condition of missing teeth. The psychological environments of the parents and the patient were highly improved particularly with simple and conservative prosthodontic therapy being the rational, reasonable, acceptable, and cost-effective option. Maxillofacial surgery, implant placement, and fixed prosthesis, which will provide results with increased comfort and aesthetics, could be considered later on when the patient have completed skeletal growth.

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

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Biomedical Communication

Vitamin D Supplementation Can Prevent and Treat Multiple Types of Respiratory Illness – An Updated Review

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ABSTRACT

Vitamin D is synthesized by human skin cells exposed to sunlight and also obtained from nutritional sources. It has dual nature and serves as a vitamin and an immunomodulatory hormone. Metabolically, vitamin D is responsible for calcium and phosphate homeostasis, bone resorption, and maintenance of a healthy and mineralized skeleton. As a hormone, its activated form (1,25-dihydroxyvitamin D), binds with the vitamin D receptor (VDR), triggers the regulation of more than 100 genes, many of those associated with the immune system, Hence, it plays a critical role in the regulation of the key components of both, the innate and adaptive immune systems. Deficiency of 25-hydroxyvitamin D has been linked with an increased risk of autoimmune and respiratory diseases such as rheumatoid arthritis, type 1 diabetes, multiple sclerosis, tuberculosis, and influenza. Recent pharmacogenomic studies have shown that variation in vitamin D receptor gene expression alters the response of different individuals to treatment with vitamin D. Introduction of vitamin D promotes the synthesis of antimicrobial and antiviral proteins in the cell and improves the cellular levels of calcium and phosphorus, eventually promotes autophagy to remove viruses and bacteria from the cells. This review specifically aims at establishing a concrete relationship between vitamin D deficiency and increased susceptibility to various respiratory diseases. We also aimed to explore the possibility of using vitamin D supplementation programs to improve immunoprotection in individuals prone to respiratory illnesses.

KEY WORDS: VITAMIN D, RESPIRATORY ILLNESS, IMMUNOPROTECTION, PHARMACOGENOMICS, SUPPLEMENTATION.

INTRODUCTION

Vitamin D is one of the four fat-soluble vitamins which are required for the general physiology of human body. Vitamin D is found naturally in some food materials, added as nutritional supplements with calcium and phosphorous (Lamberg-Allardt, 2006; Bruins and Létinois, 2021). However, the major contribution of vitamin D is by photosynthesis in the human skin exposed to ultraviolet B radiations (UVB) (Oonincx et al., 2018). Physiologically, vitamin D, functions as a vitamin and a hormone, it is responsible for the homeostasis of calcium and phosphorous in the human body, bone formation, health and functions (Ono-Ohmachi et al., 2021).

Vitamin D is obtained or synthesized in 2 natural forms including vitamin D2 and vitamin D3, both types are inactive. After synthesis in the skin from dietary cholesterol in the skin cells exposed to UVB, the cholecalciferol enters blood stream, it is subsequently activated in the liver and kidneys to active form of vitamin D known as calcitriol or 1,25-(OH) 2 D (Panfili et al., 2021). Calcium and phosphorous homeostasis is considered as one of the main functions of vitamin. It promotes the bone resorption by increasing the calcium absorption resulting the better bone structure and physiology (Hanel and Carlberg, 2020). Supplementary intake of vitamin D has been reported to decrease the chances of many diseases including hypertension, osteoporosis, cancer, and many autoimmune diseases. Vitamin D serves to strengthen the innate and adoptive immune systems by increasing the number and activity of cells and proteins associated with the immune response (Gilani et al., 2021).

The vitamin D receptor (VDR) that interacts with vitamin D is present in most of the body cells and tissues. The interaction of vitamin D with its corresponding receptor as a hormone triggers the regulation of 100s of genes.

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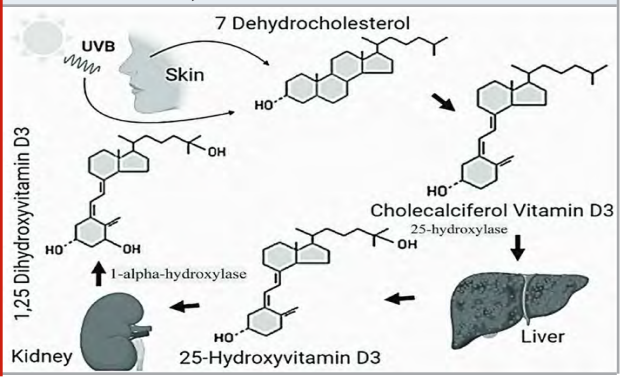
Most of the regulated genes are involved in the promotion of innate and adoptive immune systems. These findings suggest the hypothesis that vitamin D can play a crucial role in strengthen the immune response against bacterial and viral infections such as influenza, pneumonia and respiratory diseases. Vitamin D supplements are especially recommended to boost the components of immune system against such disease. It also leads to the hypothesis about the vital role of vitamin D against the severity and mortality of diseases (Giménez et al., 2021).

Looking at the important role of vitamin D as a protective agent and in the treatment of many virus and bacteria, based respiratory infections; we have aimed to conduct a review article representing the association of vitamin D deficiency with the onset and mortality rates of a few respiratory diseases including influenza, COVID-19, tuberculosis and pneumonia.

Synthesis and metabolism of vitamin D: In the human body, diet and photosynthesis in the skin provide all the vitamin D required for the better physiology. The pigmented substance melanin absorbs UVB from sunlight that interacts with 7-dihydrocholesterol, and synthesize vitamin D. The vitamin D3 produced by photosynthesis is an inactive compound that needs hydroxylations in the liver and kidneys to produce an active form of vitamin known as 1,25 dihydroxyvitamin D or calcidiol. The reaction to activate vitamin D is calalyzed by the enzyme 1-α-hydroxylase (CYP27B1). The active form of hormone also interacts with the interstinal cells to stimulate calcium reabsorption, to promote osteoblast differentiation and matrix calcification. After its utilization the 1,25-OH-D form is metabolized to the 1,24,25-OH vitamin D by 24-hydroxylase (CYP24) (Khairy et al., 2021). The binding interaction of active vitamin D with its relevant receptor (VDR) regulates the genes associated with the vitamin D-VDR combination (Marozik et al., 2021). The synthesis and activation of vitamin D has been described in the Figure 1.

Figure 1: A schematic representation of synthesis and activation of vitamin D in the human body (adopted from Gilani et al., 2021)



Vitamin D and immunity: The immune system provides a defense against the invading pathogens, microbes, viruses and unwanted hazardous bodies or substances. It helps in the maintenance of healthy status of body by protecting against diseases. Recently, the vitamin D has been well implicated with the human immune system. Vitamin D serves as the promoter of immune system and its deficiency leads to an increased susceptibility, increased severity and mortality by many infectious diseases (Carpagnano et al., 2021). The immune system consists of a set of cells, with receptors and soluble proteins. All of these components of immune system are affected, either individually or in the form of a signaling cascade by the deficiency of vitamin D. As for example, vitamin D inhibits the proliferation of beta cells, blocks B cell immunoglobulin secretion by these cells (Bui et al., 2021).

Vitamin D promotes the shift of Th1 to Th2 cells as a suppressor of T cell proliferation (Laird et al., 2020). It also helps in the maturation of T cells by twisting them away from Th17 phenotype that has inflammatory effects, and triggers the formation of T regulatory cells (Fakhoury et al., 2020). All these activities result in the reduced production of IL-17 and IL-21 cytokines that have inflammatory impact, and enhance the production of IL-10 cytokines that have been associated with anti-inflammatory response of cells (Leal et al., 2020). The physiology of dendritic cells (DCs) and monocytes is also regulated by vitamin D by an inhibition in the production of inflammatory cytokines by monocyte such as TNFα, IL-1, IL-6, IL-8, and IL-12 (Nastri et al., 2020). Differentiation, maturation, and preservation of cellular phenotypes are inhibited by a reducing the expression of co-stimulatory molecules, MHC class II molecules, and IL12 (Aygun et al., 2020). The deficiency of vitamin D has also been associated with the onset of autoimmune diseases. In such diseases, vitamin D has found to have an ameliorative effect indicating the beneficial role of vitamin D supplements.

Vitamin D and respiratory diseases: In context with the available information about the role of vitamin D on the calcium homeostasis, bone formation, bone health and strengthening of immune system, the present review article aims at highlighting the possible correlation between vitamin D levels and susceptibility to respiratory disease.

Vitamin D and tuberculosis: Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (MTB) is an infectious disease that spreads by aerosols. The disease generally affects respiratory system, especially the lungs, it can also damage other parts of body. The disease is associated with poor living conditions, and prevalent worldwide, causing more than 2 million deaths every year. Deficiency of vitamin D has been linked with the spread of infection in the human body, its severity and mortality. Level of vitamin D has also been associated with the successful treatment and time of recovery. The genetic variability can also affect the level of susceptibility for infection among the world populations. may influence host susceptibility to developing active tuberculosis and treatment response (Ganmaa et al., 2020).

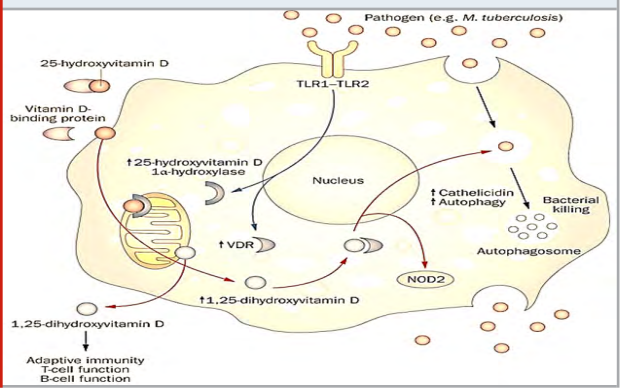
A study from Pakistani populations has shown that the low levels of vitamin D has been found to increase the risk of tuberculosis infections by 5-times than those having normal levels of plasma vitamin D levels (Jaimni et al., 2021). Vitamin D deficient populations have risk of rapid spread

of disease among healthy individuals. One of the similar studies has shown that the African populations migrated to Australia with low levels of vitamin D had high probability of tuberculosis infections than those with normal vitamin D levels (Acen et al., 2021). The findings from other studies have shown a rapid spread and progression of infection among the people with low levels of vitamin D (Faniyi et al., 2021).

The mechanism adopted by vitamin D to prevent the onset of infection or to inhibit the rapid progression of disease has been clearly described. According to the findings, vitamin D limits the *Mycobacterium tuberculosis* infection by binding to the VDR receptor, later is a polymorphic nuclear receptor responsible to manage the regulation of many genes for their expression. Most of these genes are involved in the strengthening of human immune system by regulation of the production of cytokines (Bishop et al., 2021).

VDR is found in almost all types of cells and it is up regulated by specific toll–like receptors. The receptor (VDR) is essential component of immune cells, and cells of pulmonary epithelial walls. The vitamin D based mechanism is recognized for the production of many antimicrobial proteins including cathelicidin LL-37 and β defensin (Acen et al., 2021). The mechanism is also responsible to suppress the activity of metalloproteinase enzymes to prevent the degradation of extracellular matrix of pulmonary tissues (Sutaria et al., 2014; Meca et al., 2021).

Figure 2: The TLR receptors interact with pathogen (*M. tuberculosis*) and results in the induction of transcrption of genes coding for vitamin D receptor (VDR), the enzyme responsible to activate vitamon D named 25-hydroxyvitamin D-1α-hydroxylase, upregulating the expression of these genes. Once the vitamin D enters a monocyte with the help of vitamin D binding protein (DBP), the vitamin gets activated, by mitochondrial 25-H-D-1α-hydroxylase and subsequently binds with the VDR. The vitamin-VDR binding triggers the upregulation of many proteins including cathelicidin, intracellular pathogen recognition receptor NOD2 and defensin beta 2 protein. These proteins and enzymes promote autophagy and clearance of bactria from the cells (adopted from Liu et al., 2006).



Vitamin D status has been found to be affected by many variants of VDR in the cells (Le Pavec et al., 2008). The difference of vitamin D levels in the populations from different geographic areas and with different ethnicity have also found to affect the susceptibility to disease and resistance of bacteria to the drugs (Griffin et al., 2021). Many studies from Ethiopia, Tanzania, Uganda and other countries have positively correlated the vitamin D deficiency with the onset, spread and mortality rate due to tuberculosis (Jovanovich et al., 2014). These investigations have also found that the low BMI has also been associated with the deficiency of vitamin D and both have been associated with the onset of TB (Rhodes et al., 2021; Lungu et al., 2021). Trials based on vitamin D supplementation have also shown an improvement of human immunity against tuberculosis (Xiong et al., 2021). A vitamin D based mechanism involved in the in the autophagy and removal of M. tuberculosis has been described (Figure 2).

Vitamin D and pneumonia: Pneumonia is the infection of alveolisacs of respiratory tract. The main signs and symptoms include dry, productive cough or combination of both, difficulty in breathing, chest pain and fever. The headache and body ache are also observed as common symptoms, but these are common with many other conditions. The intensity and severity of symptoms variable depending upon the human immune response and degree of infection (Jovanovich et al., 2014). The causative agents of disease can be usually a virus, bacteria or other microorganisms.

Pneumonia can be hospital-acquired, community-associated, and healthcare-associated depending upon the site of infection. Smoking history, sickle cell disease, chronic obstructive pulmonary disease (COPD), asthma, cystic fibrosis,heart failure, diabetes, weak immune system, and poor ability to cough are common risk factors of pneumonia (Zhou et al., 2019). Recently, there has been sufficient evidence indicating the association of vitamin D deficiency with the community-acquired pneumonia (CAP) (Bergman et al., 2013), it resulted in the extensive research on the applications of vitamin D on the human defense mechanisms and immune responses (Camargo et al., 2011). It has been reported by many studies that the blood levels of vitamin D < 37nmol/L has been associated to an increase in the susceptibility and severity of disease (Hashemian and Heidarzadeh, 2017; Oktaria et al., 2021).

The studies including 5660 individuals of all age limits, investigated in 11 randomized placebo-controlled trial have shown a significant decrease in the risk of respiratory diseases especially pneumonia in case of vitamin D supplementation (Oktaria et al., 2021a; Labib et al., 2021). The populations with low vitamin D levels have significantly higher (2.5 times) risk of contracting pneumonia as compared to those with normal levels of vitamin. Low levels of vitamin D in the umbilical cord has been linked with the high risk of respiratory infections in the new borns in the early 3 years of their life childhood (Oktaria et al., 2021 b). A direct relationship has been reported between the vitamin D (Leow et al., 2011). The normal levels of vitamin D has shown a preventive role against all kinds of pneumonia among the age groups of 24 to 60 months (Chowdhury et

al., 2021), similar findings have been reported by a study in Bangladeshi children of 1 to 18 months age group.

According to many studies, the infection leads to the synthesis of antimicrobial proteins and peptides in the mucosal and epithelial surface cells in multicellular organisms. These molecules or peptides serve as the first line of defense against viral or bacterial infections. Some of these peptides have a role in the modulation of immune responses. Defensins and cathelicidins are the most widely studied proteins that are responsible to fight against pneumonia infections (Aygun et al., 2020). The mechanism followed by the defense system is similar to that described in the figure 2. In short, the TLRs activate the defense cells, subsequent upregulation of vitamin D receptor and CYP27B1 enzyme. The enzyme is also associated with the conversion of 25-hydroxyvitamin D to 1,25- dihydroxyvitamin D, the latter being an active form of vitamin D (Parsanathan and Jain, 2019; Campolina-Silva et al., 2021).

Vitamin D and influenza: Influenza, is a respiratory infectious disease also known as flu or common cold. The causative agent of is a virus known as influenza virus. The complicated forms of influenza are known as viral pneumonia, sinus infections, if the infection is caused by bacteria, it is also named as secondary bacterial pneumonia. The disease is associated with worsening of existing respiratory problems including heart diseases and asthma. With more than 290,000 infections and about 650,000 annual deaths, the disease is found worldwide, especially common during winters (Goncalves-Mendes et al., 2019). Vaccine is available against influenza, but the whole world population is not vaccinated yet. According to the estimates, about 10% unvaccinated adults and 20% unvaccinated children are infected every year (Martineau et al., 2017; Pham et al., 2021). In the northern and opposite hemispheres, the infection occurs mostly in winters. However, in the rest of the world and near to the equator, the outbreaks can be observed at any time of the year (Zhou et al/. 2018; Goncalves-Mendes et al., 2019).

Very young, old populations and those with existing respiratory and heart diseases are populations at high death risk by influenza. As the antiviral medicines and vaccines are limited in availability and efficacy against influenza, non-pharmaceutical interventions including herbal medications and supplements of immune boosting vitamins and minerals are essential to control the spread and severity of infection. By stimulating the naturally produced antimicrobial proteins and peptides, vitamin D serves as an important promoter of innate and adoptive immune systems that lead to the destruction of invading pathogens (Chung et al., 2020; Bleakley et al., 2021).

Particularly, in the cell lining of upper and lower respiratory tract, these peptides and proteins are able to fight the viruses and bacteria directly. Vitamin D is responsible to shift the Th1 (T helper 1) to Th2-mediated cells in their response, and reduce the inflammatory responses (Briceno Noriega and Savelkoul, 2021). This results in the suppression of major symptoms of common cold or flu. Vaccination is often recommended to fight against flu infections. However, the vaccines have very low (17–53%) efficacy in old people and 70–90% among the young adults. Vitamin D has been also reported to increase the levels of TGFβ in response to influenza vaccination and promote the defense mechanisms (Singh et al., 2020). Also, the populations subjected to vitamin D supplementation along with vaccination have shown high levels of protection against flu as compared to those with vaccination and no vitamin D supplements (Jolliffe et al., 2021).

Studies on infants have shown that the high dose with up to 1200IU per day of vitamin D has proven significantly useful in the prevention against seasonal influenza, rapid decrease in viral load, decreased intensity of symptoms and early recovery. The high dose was found safe for children (Abioye et al., 2021). Less or unavailability of sun light has been linked to the deficiency of vitamin D and consequently the susceptibility to influenza. Vitamin D supplementation is therefore, recommended in that particular season or areas (Urashima et al., 2010; Ma et al., 2021). Studies involving 11,000 participants of all age groups (0 and 95 years), has shown that the daily dose of vitamin D reduced the frequency of flu infections and supported the general health of respiratory tract. As described in the previous two sections, vitamin D activates the synthesis of antimicrobial peptides, strengthens the immune system and improves the immune responses by significantly lowering the inflammatory events of infection process.

Vitamin D and COVID 19: The world population is threatened by coronavirus disease 19 (COVID-19) that is a respiratory infection with high rate of transmission and severe health consequences. The disease is caused by a zoonotically transmitted virus known as SARS-CoV-2. The disease started in December 2019 from Chinese city Wuhan (WHO, 2020) and spread all over the globe very rapidly. SARS-CoV-2 has more than 80% genome similarity with the causative agent of recent coronavirus disease in the last decade (2003-2004) (Rajapakse and Dixit, 2021).

There is no specific cure for the COVID-19 and only protective and supportive managements are being conducted. Most of the deaths caused by COVID-19 are due to acute respiratory distress syndrome (ARDS) and respiratory failure and multiorgan failure. Vaccination has been initiated in many countries in the recent times. However, the efficacy of these vaccines is still under question. Moreover, the demand for the vaccines is very high, especially in third world countries which neither have the technical know how to produce them nor the economic means to procure them. In the wake of this situation, it would be interesting to probe the association between vitamin D deficiency and susceptibility to COVID-19 as well as recovery rate.

In COVID-19 patients the presence of pneumonia/acute respiratory distress syndrome (ARDS), microvascular thrombosis and/or cytokine storm, myocarditis, all of which involve underlying inflammation are main indicators of disease severity. While the COVID-19-specific CD8 T cells and the specific antibodies produced by B cells are critical for eliminating the virus, uncontrolled non-specific

inflammation and cytokine release can cause catastrophic injury to the lungs and other vital organs. Consequently, decreasing this early non-specific inflammation during COVID-19 may provide time for the development of specific acquired immunity against COVID-19 (Nadeem et al., 2020).

T regulatory lymphocytes (Tregs), are responsible to provide a principal defense line against high intensity inflammatory response in vital infections. The levels of Tregs have been reported to be low in one group of COVID-19 patients, and 'markedly lower in severe cases (Leila et al., 2020; Weir et al., 2020). In a study of older nursing home patients, high Treg blood levels were found to be associated with a reduced level of respiratory viral disease. These observations suggest that if Treg levels can be increased, this might be of benefit in diminishing the severity of viral disease and perhaps of COVID-19. Treg levels can be increased by vitamin D supplementation (Ali, 2020; Gilani et al., 2021).

One of the major devastating effects on immune system produced by covid 19 is the cytokine storm that leads to rapid deterioration of lung cells. It is a well-known fact that vitamin D is capable of reducing the inflammatory cytokine production. A study of healthy women in the USA found a significant inverse relationship between the serum levels of 25(OH)D and TNF-alpha (Khemka et al., 2020). Thrombotic complications are common in COVID-19 patients. Of those with severe disease, over half have been found to have elevated D-dimer levels. Interestingly, vitamin D is also involved in the regulation of thrombotic pathways, and vitamin D deficiency is associated with an increase in thrombotic episodes (Mohan et al., 2020; Gilani et al., 2021). An increased risk of death with COVID-19 is also observed in black, Asian and minority ethnic (BAME) groups. As melanin reduces the production of vitamin D asociated with exposure to the ultraviolet radiation in sunlight, this may help to explain the observed frequent occurrence of vitamin D deficiency in BAME groups.

CONCLUSION

Vitamin D, one of the fat-soluble vitamins has been reviewed for its association with the onset and severity of respiratory diseases. Vitamin D serves as a double-edged sword, one one hand it operates as an immunomodulator and on the other side it promotes autophagy to remove the pathogenic viruses and bacteria from the cells. It influences both the innate and adaptive immune systems, and reduces inflammatory cytokines. The molecular pathways of these immunomodulatory effects have been well established, thereby making Vitamin D and important candidate for development of therapeutic as well as prophylactic applications against various infectious diseases.

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Dental Communication

The Radix Entomolaris: Management of a Failed Root Treatment : A Case Report

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ABSTRACT

Among all the causes of root canal failure, incapability to identify and negotiate additional canals is one which is of prime value, from treatment point of view. Mandibular molars display anatomical variations in the form of an additional root located lingually (radix entomolaris) or buccally (radix paramolaris). It is critical to understand the root morphology and canal patterns of teeth indicated for canal treatments for successful treatment outcomes. This case report presents a re-root treatment of a failed root canal treatment of a first molar tooth with Radix Entomolaris. Correct interpretation of angled radiographs and careful examination of the access cavity chamber floor and use of recent concepts in access cavity preparation along with the sound knowledge of the variable anatomy helps clinician to locate and treat the root canals in case of Radix Entomolaris. The findings of the present case report suggests that correct interpretation of angled radiographs and careful examination of the access cavity chamber floor is pivotal in managing Radix Entomolaris. The use of recent concepts in access cavity preparation along with the accurate understanding of variable anatomy helps clinician to locate and treat the root canals in case of Radix Entomolaris

KEY WORDS: FAILURE, MOLAR TEETH, RADIX ENTOMOLARIS, ROOT CANAL TREATMENT, TREATMENT SUCCESS.

INTRODUCTION

The healing of periapical pathology is influenced by comprehensive cleaning via chemo-mechanical means and shaping of the infected radicular canals (Parashar et al. 2015). Among all the causes of root canal failure, incapability to identify and negotiate additional canals is one of them. Thus, the understanding of the dentist towards the unusual root canal anatomy is an important aspect during an endodontic treatment. The mandibular first molar tooth because of its age, position and pattern of eruption is the most commonly involved tooth with endodontic requirement (Campos 1989; Calberson et al. 2007). It displays the most significant morphological variation related to number of canals and the roots among the entire dentition (Sarangi and Uppin 2014; Lima et al. 2020).

Carabelli in (1844), accidently found mandibular molar a supernumerary third root which is found to be located on disto-lingually side of the tooth and named it as "radix entomolaris" (RE) (Pai et al. 2014). RE can be found in any of the mandibular molars with least occurring in second molars (Sinha et al. 2016). Literature revealed that RE most commonly displayed vertucci type 1 canal configuration (Meidyawati et al. 2016). It occurs most frequently in Mongoloid race with a frequency of 5-30%. Whereas, in white Caucasian, African, Eurasian and Indian populations frequency was less than 5% (Kuzekanani et al. 2017; Duman et al. 2020). Radiographic analysis is a prerequisite to identify and instrument the extra canals, which if overlooked will later be responsible for future failure of root canal treatment (RCT). Previous studies rely on an additional angulated periapical radiograph by tube shifting mesially and distally at 20–30 degrees angle (Parallax technique). This technique helps to identify extra root and canal manifestation and its position using Buccal Object Rule. Nowadays, cone beam volumetric tomography (CBVT) has gained more importance due to its highly informative nature when compared to tube shifted periapical radiographs.

Case Report Presentation: A 26-year-old female patient visited the dental OPD with a complain of spontaneous pain

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on biting in lower right area from 1 month. Medical history revealed that patient is not allergic to any drug. BP and pulse was in the normal range i.e. 124/85, 70 respectively. On the basis of medical history patient was classified as ASA I which corresponds to normal healthy patient. Patient also revealed that she had undergone RCT, 5 years ago from a general dental practitioner. An extra-oral examination showed a symmetric face with no head and neck lymph node involvement. TMJ examination revealed no clicking sound with limited mouth opening. Upon intraoral clinical examination it was found that that tooth was previously restored with PFM crown and it is sensitive to percussion and palpation. Gingiva around the tooth appeared infected and swollen.

Figure 1: Pre-operative radiographs of the tooth

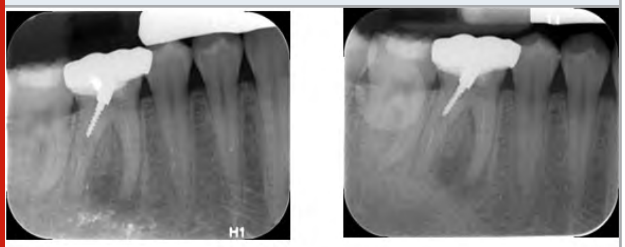
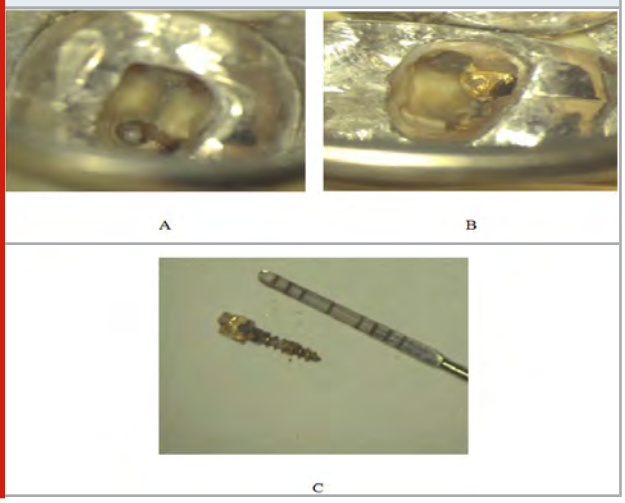


Figure 2: Clinical Removal of post form the tooth prior to root canal treatment.



In order to get a clearer picture a periapical radiograph was performed. From the routine radiographic examination it was found the roots were poorly obturated with metal post on the distal canal and displaying PDL widening (Fig 1 A). On detailed examination of the periapical radiograph it was found that there was a third root present between the mesial and distal roots, which was confirmed by additional, angulated periapical radiograph (Fig 1B). A periapical radiolucency was observed at the disto-lingual canal and mesial canal of the tooth (Fig.1A). Re-endodontic treatment was planned and patient was informed regarding the treatment for informed consent. Before starting the dental treatment, local anesthetic was injected and access cavity was prepared (Fig 2A). Removal of the metal screw post was achieved using ultrasonic tip (Fig 2B & C).

All the canal orifices were negotiated with the help of DG-16 explorer. Four canals were found under operating microscope magnification (M320 dental microscope, Leica Microsystems, Germany). The apex locator (Root ZX II, J Morita, Germany) was used to determine the working length. After the conventional hand filing, universal protaper was used to prepare all the four canals. The canals were constantly irrigated using 6% sodium hypochlorite (NaOCL) After instrumentation was completed, calcium hydroxide was placed for 7 days and patient was recalled. On the patient visit after 7 days intra canal medicament was removed by irrigating the canal with NaOCL and hand instrument followed by final rinse with 17% Ethylenediaminetetraacetic acid (EDTA). The canals were dried using paper points and obturated using warm vertical compaction with Gutta-percha and AH Plus sealer (Maillefer Dentsply, Ballaigues, Switzerland) (Fig 3 & 4). The patient was reviewed regularly at 12 months interval and a radiograph was taken after two-year follow up (Fig 5).

Figure 3: Working Radiograph taken for assessing the working length for root filling

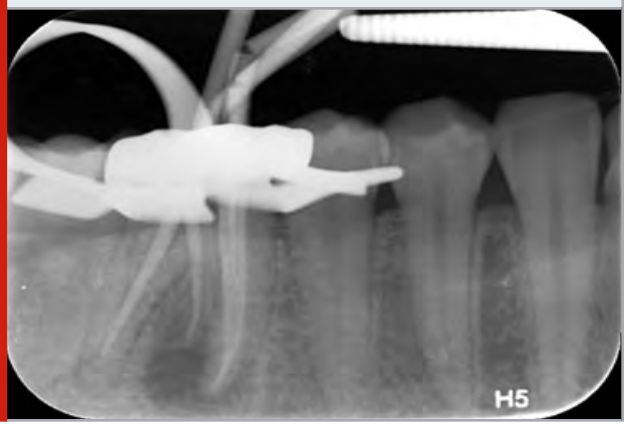
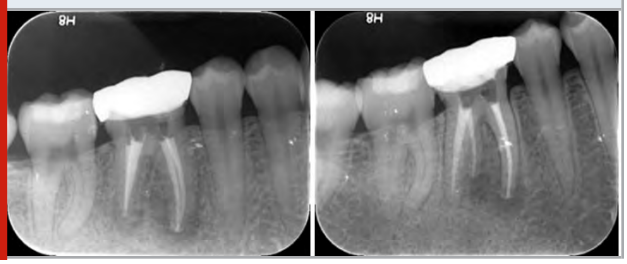


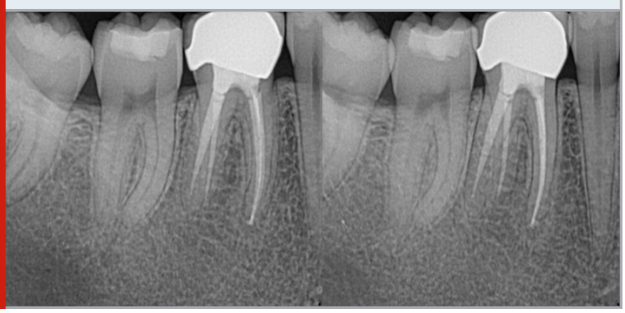
Figure 4: Final Radiographs of the root treated tooth after completion of re-root treatment



Comparative Analysis: A thorough and deep understanding of canal morphology is an important aspect which determines the clinical success of the RCT. With the introduction of latest techniques and expertise related to access cavity preparation, location of canals in atypical chamber morphology becomes less challenging (Tu et al. 2019). Missed canals due to inadequate knowledge of anatomy usually leads to root canal failure. Available

literature revealed that approximately 97.8% of lower first molars exhibit two roots (Vertucci 1984; Christie and Thompson 1994; Pattanshetti et al. 2008). Out of which, 64.4% displayed three canals with two within the mesial root and one in the distal root. However, 28% of cases showed two canals in the distal root (Iwanaga et al. 2020; Alkahtany et al. 2021). RE which is comparatively a rare finding during root dental procedure. The actual causes of its occurrence are still not discovered. However, some researchers assumed that this may be due to some disruption of pathway during odontogenesis or it may be due to genetic predisposition (Carlsen and Alexandersen 1990; Iwanaga et al. 2021).

Figure 5: Radiogrpahs of the re root treated tooth at 2 years follow up with no radiological signs of pathology.



Accessory roots and canals can be identified using conventional periapical radiographs at different angulations (Kang et al. 2014). There are multiple ways including law of symmetry, dentinal map visualization, bleeding point from the orifice, ultrasonic tips, 1% methylene blue dye for chamber staining, champagne bubble test, and CBCT to identify the accessory or missed canals (Rouas et al. 2007; Oliveira et al. 2018; Lima et al. 2020). If RE is identified before initiation of endodontic procedure it is preferred to prepare a modified trapezoidal access cavity for canal location (López-Rosales et al. 2015). De Moor et al. in their study revealed that in most of the RE cases the canals were curved (De moor et al. 2004).

Therefore, exploration of canals with K patency file (size 10 or less) followed by working length and curvature determination using periapical radiograph to avoid procedural accident is preferred (Chaganti, 2017; Duman et al. 2020). Most of the time it was observed that, radix ento-molaris canal orifice are covered and occluded with secondary dentinal tissue. The preparation of access cavity in such cases may result in perforation (Attam et al. 2012; Różyło et al. 2014). Hence, it should be kept in mind that secondary dentin is mostly white and opaque in color whereas pulp chamber is comparatively darker and gray in appearance (Gupta et al. 2013). The visual access and greater control of instruments during access preparation will help to prevent any mishap (Mahendar et al. 2013; Duman et al. 2020).

CONCLUSION

The findings of the present case report suggests that correct interpretation of angled radiographs and careful examination of the access cavity chamber floor is pivotal in managing Radix Entomolaris. The use of recent concepts in access cavity preparation along with the accurate understanding of variable anatomy helps clinician to locate and treat the root canals in case of Radix Entomolaris

Conflict of Interests: Author declares no conflict of interest to disclose.

Case Report (Human Studies) Ethical Clearance Statement: The Current Case Report/ Studies were Conducted as Per the Guidelines of SCARE.

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Biomedical Communication

Parental Awareness of Bruxism in Saudi Children: A Public Health Concern

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ABSTRACT

Bruxism is defined as a non-functional rhythmic and/or spasmodic gnashing, grinding, and clenching of the teeth. Parents are often unaware of bruxism in children. The aim of this study is to assess the awareness of bruxism among parents of children in Saudi Arabia. Across-sectional study approved by the (IRB) was conducted using a questionnaire, which was developed to assess the awareness of bruxism among parents based on the American Association of Sleep Medicine criteria. The questionnaire included questions distributed among the following elements: a) demographic data, b) prevalence of self-reported bruxism by parents, c) parental history of bruxism, d) child's sleep habits, e) the seeking of professional medical help, and f) parents' knowledge regarding bruxism. Data were analyzed using SPSS 24.0 version statistical software. Fifty-five of the children were male (n = 824) and 45% were female (n = 675). The children's ages ranged from 6-10 years. The parent-reported prevalence of bruxism among their children was 45.7%. About 38% of parents were aware of what bruxism is, 34.7% were doubtful, and 27.8% were not aware of it, which is statistically significant ($p<0.0001$). Almost 52% of parents expressed a positive response to the question of whether or not "bruxism could endanger their child's health". Fifty-two percent of participants selected psychological causes as a trigger for bruxism. In Conclusion the majority of parents lack awareness of bruxism. A multidisciplinary approach including dentistry should be considered to increase parental awareness about bruxism in children.

KEY WORDS: AWARENESS, BRUXISM, CHILDREN, PUBLIC HEALTH.

INTRODUCTION

Bruxism is defined by rhythmic and/or spasmodic gnashing, grinding, and clenching of teeth involuntarily and in a non-functional manner. These mandibular movements can occur both nocturnally and diurnally and may occasionally cause occlusal trauma (Shetty et al., 2010). Temporomandibular joint (TMJ) disorders often arise from bruxism; they are accompanied by a number of signs and symptoms, including tension headaches, wear on teeth, a clicking jaw, facial muscle fatigue, and difficulty chewing and yawning. Furthermore, reduction in sleep quality is also associated with bruxism (Carra et al., 2011). Lavigne et al. (2003) found that complaints about sleep quality decline with age, from 14% in children, 8% in adults, and 3% in patients over 60. Bruxism's prevalence in children and adolescents varies widely among the available studies due to heterogenous data collection methods and targeted groups (Prado et al., 2018).

Despite that, a recent systematic review conducted by Melo et al. (2019) reported that the prevalence of bruxism among the young population (children and adolescents) ranges between 3.5% and 49.6%. Diagnosing bruxism is often challenging for health care providers. The diagnostic markers are based upon the following criteria: a history of clenching teeth at least 3-5 nights a week for a period of six months or more, muscle soreness in masticatory and mandibular regions upon waking up, tension headaches, tooth wear beyond a normal level, masseter muscle hypertrophy, evidence of regular cheek or tongue biting, hypersensitivity in the TMJ area, or unusual sounds/clicking in the jaw. While polysomnography (PSG, or sleep study) serves as a standardized method of diagnosis, it is yet considered as a costly diagnostic procedure (Kato et al., 2001, Alves et al., 2019, Soares et al., 2020).

The etiology of bruxism is multifactorial including the psychosocial (anxiety, stress, personality disorders), the

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pathophysiological (oral health), and the morphological (birth defects) (Lobbezoo et al., 2006). Clenching may be triggered by fear or anxiety, and these psychosocial factors have recently been shown to be particularly impactful in diurnal forms of bruxism (Manfredini, & Lobbezoo, 2009). Oral health, like all forms of health, is an important feature of a parent's or caregiver's responsibility for a child. Unfortunately, knowledge of oral health and oral pathologies is lacking in many such caregivers, limiting their understanding of conditions like bruxism. Dentists, therefore, play an essential role in raising awareness of these conditions and informing patients of their effects and symptoms (Silva et al., 2017).

Furthermore, a small number of studies have been carried out on the nature and level of public knowledge regarding bruxism among parents and caregivers. Serra-Negra et al. (2013) evaluated the parents/guardian knowledge about the bruxism of their children and reported that 95.5% of the responses from 221 participants correctly described bruxism. Moreover, Silva et al. (2017) assessed the knowledge of parents/guardians about nocturnal bruxism in children and adolescents and found that only 38.1% of the respondents among a sample of 134 were able to correctly define the condition.

Recently, the results of the study conducted by Alves et al. (2019) on the knowledge of parents/caregivers about bruxism in children showed that 67% of the caregivers responded in the affirmative when asked whether they knew what bruxism was, but only 52.4% of those could define the habit correctly. In addition, nearly three quarters of the study's respondents could identify the causes of bruxism, while 16.5% attributed the condition to emotional factors. Monitoring and measuring parents' and caregivers' level of knowledge about bruxism is important in assessing the wellbeing of children with the disorder. Not only will such an assessment aid us in understanding the problem, but it will also provide dentists with relevant information and allow them to take informed action in raising awareness among their patients and society. Additionally, it contributes to early diagnostic and preventive measures to reduce the oral consequences of the bruxism, and a deeper clinical exploration of possible associated co-factors. Therefore, the aim of this study is to assess the parental awareness about bruxism in children in Saudi Arabia.

MATERIAL AND METHODS

A cross-sectional study was conducted starting April 2020 and ended in July 2020 to assess parental knowledge regarding bruxism in children (6-10 years old) in Saudi Arabia. This study was reviewed and approved by Institutional Review Board (IRB) (no. E-20-4860) of King Saud University in Riyadh, KSA. One thousand four hundred and ninety-nine (1499) participants agreed to participate in the study. A validated electronic questionnaire which is based on the American Association of Sleep Medicine criteria was used and the parent's agreement to participate at the beginning of the questionnaire considered as a consent for participating in the study. Parents of Saudi, healthy, living in Saudi Arabia and aged (6-10 years old) were included in the study. Parents of non-Saudi, medically compromised/special needs children, not living in Saudi Arabia and younger than 6 years and older than 10 years were excluded from the study.

Acknowledgments & Ethical Approval: This work was part of grant number E-20-4860, supported by King Saud University upon the recommendation of the Research Committee following a review of the Institutional Research Board on the ethical aspects of the proposal. Prior to filling the questionnaires, participants were given the choice of whether to participate, and they were informed that the study results would be used in publications. The questionnaire was developed to assess the parental knowledge and awareness about bruxism in children in Saudi Arabia. It included 22 questions and divided into 6 main sections: a) demographic data, b) prevalence of self-reported bruxism by parents, c) parental history of bruxism, d) child's sleep habits, e) the seeking of professional medical help, and f) parents' knowledge regarding bruxism. A power analysis was done to specify the targeted sample size. The sample size was calculated to be 300 subjects to achieve a significance level at the 95th percentile confidence level and power of 80 percent, with 0.5 estimated effect size.

The electronic survey was distributed using the institution's social media platforms. Additionally, participants were encouraged to share the survey with others. Moreover, prior to the main questionnaire distribution, a pilot study was performed to test the clarity and the understanding of the questionnaire by parents which was assessed by one of the authors (ZH). Additionally, parents with multiple children in the same age range answered the survey based on their eldest child. Data were entered in MS Excel and analyzed using SPSS 24.0 version statistical software (IBM Inc., Chicago USA). Descriptive statistics (frequencies and proportions) were used to describe the categorical variables. Furthermore, Pearson's chi-square test was used to compare the distribution of responses and categorical variables in order to observe the association between the categorical variables. A p-value of ≤ 0.05 was used to report the statistical significance of results.

RESULTS AND DISCUSSION

One thousand five hundred eighty-four parents of children aged between 6-10 years old completed the online questionnaire. Of those, 1,499 study subjects agreed to participate in the study. 55% of the children were male (n = 824) and 45% were female (n = 675). The children's ages ranged from 6-10 years, where 30.4% were six years old. The majority of participants (69.2%) reported that they were located in the central region of Saudi Arabia. The parent-reported prevalence of medical/psychological/emotional conditions in their children was 11.1% (Table 1).

Table 1. Characteristics of study subjects (n=1499)

Characteristics	No.(%)
Gender of the child	
Male	824(55)
Female	675(45)
Age of the child (in years)	
6	455(30.4)
7	214(14.3)
8	234(15.6)
9	225(15.0)
10	371(24.7)
Region of residence	
The middle region	1038(69.2)
The east region	193(12.9)
The west region	149(9.9)
The north region	65(4.3)
The south region	54(3.6)
Does your child have any medical or psychological or emotional condition?	
Yes	167(11.1)
No	1332(88.9)

The parent-reported prevalence of bruxism among their children was 45.7%; additionally, 12.9% reported that more than one child in their household was affected by bruxism. It was of interest to evaluate the parental history of bruxism. 8.5% of fathers and 8.3% of mothers reported having the problem of bruxism. Regarding the need to address their child's bruxism, 57% of parents responded positively toward the idea of seeking help for their bruxer child; 70.6% of them wanted to seek help from a dentist, and 79.5% wanted to know more information regarding bruxism in children (Table 2).

The assessment of knowledge towards bruxism among parents shows that 37.5% of parents were aware of what bruxism is, 34.7% were doubtful, and 27.8% were not aware of it, which is statistically significant ($p<0.0001$). A higher proportion (91.5%) of parents reported the concept of bruxism as "when a person clenches hard on their teeth, making an audible sound which can be heard by others," which is highly statistically significant ($p<0.0001$). About 51.6% of parents expressed a positive response to the question of whether or not "bruxism could endanger their child's health," whereas 37.2% answered "I don't know," which produced a statistically significant ($p<0.0001$) result. The multiple responses towards possible bruxism triggers show a statistically significant difference; 51.9% of participants selected psychological causes as a trigger for bruxism, 29.5% selected "emotional," 28.3% selected unknown causes, 28.2% selected dental problems, and 20.5% selected neurological issues ($p<0.0001$) (Table 3).

The distribution of parents' knowledge in relation to the reported prevalence of child bruxism shows a statistically significant association for the three items (what is the concept of bruxism, bruxism could endanger a child's health, and reasons that trigger bruxism). A higher proportion of parents who reported that their child did not have bruxism mentioned three items as the concept of bruxism ("when a person clenches hard on their teeth, making an audible sound which can be heard by others," "closing the teeth together," and "pressing on the teeth due to a habit like a thumb-sucking or tongue-pressing") when compared with parents who reported that their child did have bruxism ($p=0.005$).

Table 2. Prevalence of self-reported bruxism by parents and response towards the level of help required

Items of prevalence	No. (%)
Does your child have bruxism?	
Yes	685(45.7)
No	814(54.3)
Do you have other children with bruxism?	
Yes	193(12.9)
No	1219(81.3)
I don't know	
Does the father have bruxism?	
Yes	87(5.8)
No	128(8.5)
Maybe	
Does the mother have bruxism?	
Yes	1258(83.9)
No	113(7.5)
Maybe	
Would you seek help for your bruxer child?	
Yes	125(8.3)
No	1253(83.6)
Maybe	121(8.1)
Who will you seek help from?	
Physician	854(57.0)
Dentist	175(11.7)
Maybe	470(31.4)
Who will you seek help from?	
Physician	364(24.3)
Dentist	1059(70.6)
Alternative medicine practitioner	76(5.1)
Do you think you need more information regarding bruxism in children?	
Yes	1191(79.5)
No	88(5.9)
Maybe	220(14.7)

Also, there is a statistically significant association between the responses to the item of knowledge ("Do you think bruxism could endanger child's health?") and prevalence of child bruxism; 52.4% of parents who responded negatively to this question also reported that their child had bruxism, while 57.2% of parents who responded positively to this question reported that their child did not have bruxism, which is statistically significant

(p=0.039). Also, there is a statistically significant association between the responses toward reasons that trigger bruxism and parent-reported prevalence of their child's bruxism (p<0.0001) (Table 4).

Table 3. Distribution and comparison of parent's knowledge regarding bruxism

Items of knowledge	No. (%)	χ^2-value	p-value
Are you aware of what bruxism is?			
Yes	562(37.5)	22.28	<0.0001
No	417(27.8)		
Maybe	520(34.7)		
What is the concept of bruxism?			
When a person clenches hard on his teeth that makes an audible sound, which can be heard by others	1372(91.5)	3548.68	<0.0001
Closing the teeth together	15(1.0)		
Pain in the joints of the jaws	19(1.3)		
Pressing on the teeth by a habit like a thumb sucking and tongue	93(6.2)		
Do you think bruxism could endanger a child's health?			
Yes	773(51.6)	376.48	<0.0001
No	168(11.2)		
I don't know	558(37.2)		
What do you think triggers bruxism? (Multiple responses)			
Emotional	442(29.5)	1058.30	<0.0001
Psychological	778(51.9)		
Physical issues	81(5.4)		
Unknown causes	424(28.3)		
Dental problems	423(28.2)		
Neurological issues Parasites	307(20.5)		
	34(2.3)		

The factors associated with the reported prevalence of bruxism were found to be the following: age of child, region, the child's existing medical/psychological/medical conditions, the way the child sleeps at night, whether the child sleeps alone, and whether either of the parents has bruxism. A higher proportion (55.6%) of the children with bruxism were six years old, 54.4% were located in the western region, 61.1% were reported to have a medical/psychological/emotional condition, 64% were reported to have a restless sleep, 52.7% did not sleep alone, 68% of the fathers had bruxism, and 63.2% of the mothers had bruxism, all of which are statistically significant (Table 5).

The primary outcome of this study was the level of knowledge among parents concerning bruxism. Of those queried in the present study, 47.5% have claimed to know what bruxism is, which is a lower figure than the results found in the study done by (Serra-Negra et al., 2013) who evaluated the family knowledge about bruxism and 95% of the parents reported that they know what bruxism is. (Alves et al., 2019) evaluated the knowledge of the parents/caregivers about bruxism in children attending the pediatric dental clinics and their results showed that 67% of the participants reported knowing bruxism which is almost similar to the results of the present study.

Inspite of that almost 38% of the participants in this study reported knowing what bruxism is, 91.5% of the respondents defined bruxism properly by choosing the correct definition in the questionnaire, which is in contrast with the results of (Silva et al., 2017; Clementino et al., 2017; Alves et al., 2019) who received a lower rate of correct definitions, at 52.4% and 38.1%, and 35.8% respectively. This difference can be explained that most of our participants (69%) were from the central region of Saudi Arabia which is known to be one of the highest educated regions and has a lot of medical and dental educational campaigns and programs at different areas in the region. Among all participants, 253 parents (16.8%) and 685 children (45.7%) reported that they have bruxism. The prevalence of self-reported bruxism in parents and children in the present study is in agreement with the (Serra-Negra et al., 2013) (16.5% of caregivers and 48% of children). However, the prevalence of bruxism in children in the current study is higher than that reported by (Clementino et al., 2017) (32.4%) and (Alves et al., 2019) (25.2%).

The differences in the prevalence of bruxism in children among studies could be attributed to the diversity found in the studied age groups and study designs. The present study did not find a significant association (P=0.501) between gender and bruxism, which is in accordance

with the findings reported by (Bharti et al., 2006; Manfredini et al., 2009; Suguna et al., 2020). However, in the research conducted by (Clementino et al., 2017) the female children were statistically associated with bruxism (64.5%). Whereas (Alves et al., 2019) found that bruxism was more prevalent in male children. These differences can be explained by the low number of participants in both studies and the predominance of one participating gender over the other. Brancher et al. (2020) reported that children who had greater emotional symptoms according to their caregivers' evaluation had a higher prevalence of bruxism.

Table 4. Association between the self-reported prevalence of bruxism and parent's knowledge towards bruxism

Items of knowledge	Child has bruxism		χ2-value	p-value
	Yes	No		
Are you aware of what bruxism is?				
Yes	267(47.5)	295(52.5)	2.61	0.271
No	177(42.4)	240(57.6)		
Maybe	241(46.3)	279(53.7)		
What is the concept of bruxism?				
When a person clenches hard on his teeth				
that makes an audible sound, which can be heard by others	644(46.9)	728(53.1)	12.80	0.005
Closing the teeth together	6(40)	9(60)		
Pain in the joints of the jaws	9(47.4)	10(52.6)		
Pressing on the teeth by a habit like a thumb-sucking or by the tongue	26(28)	67(72)		
Do you think bruxism could endanger a child's health?	331(42.8)	442(57.2)	6.48	0.039
Yes	88(52.4)	80(47.6)		
No	266(47.7)	292(52.3)		
I don't know				
What do you think triggers bruxism? (Multiple responses)	232(52.5)	210(47.5)	105.56	<0.0001
Emotional	272(35)	506(65)		
Psychological	29(35.8)	52(64.2)		
Physical issues	254(59.9)	170(40.1)		
Unknown causes	140(33.1)	283(66.9)		
Dental problems	126(41)	181(59)		
Neurological issues	16(47.1)	18(52.9)		
Parasites				

χ^2-value = Pearson's chi-square

The recent research performed by Soares et al., (2020) indicated that children with bruxism often have other oral habits, such as lip biting, that may result from emotional tension. Moreover, in the present study, it was of interest to assess parents' knowledge about the risk factors of bruxism, including emotional factors and 29.5% of the participants identified emotional factors as the etiology for bruxism. Alves et al. (2019) reported that 16.5% of the participants believes the main cause of bruxism in children is related to emotional factors. On the other hand, the percentage of participants who reported emotional factors to be the main factor causing bruxism was considerably higher 63.8% in the study by (Serra- Negra et al., 2013). The contrasting results indicating that emotional factors are the causal reason for bruxism in children might be due to differences in the educational level of the participants in the studies.

The results of our study showed a statistically significant association with sleep type (restless sleep) and bruxism ($p < 0.001$), which is in agreement with the results reported (Serra-Negra et al., 2013) who found a significant association between childhood bruxism and restless sleep. Additionally, (Clementino et al., 2017) reported a significant relation between agitated sleep and bruxism. In contrast, (Alves et al., 2019) found no such correlation. Our findings are coinciding with the International Classification of Sleep Disorders (ICSD-3), which consider bruxism a sleep-related movement disorder associated with arousals during the night. In close agreement with (Serra-Negra et al., 2013), this study found that 68% of fathers and 63% of mothers with bruxism had children with bruxism. This provides considerable evidence for the heritability of bruxism, or at least heritability in the parafunctional habits which lead to bruxism and in agreement with the literature review done by (Lobbezoo et al., 2014) who found that all the reviewed studies concluded that bruxism appears to be (in part) genetically determined.

The current study had a convenient sample from the Saudi population, which makes generalization of the results difficult to apply on similar populations. Therefore, further studies with representative samples should be conducted as a community-based studies. The assessment of the parents' knowledge will enable the health providers

to formulate policies, to educate and clarify the habit to the parents. Furthermore, parents will be able to identify the presence of bruxism in their children and seek early treatment if necessary, that will lessen the complications that might take place as consequences to this parafunctional habit. The limitations of the present study include a possible reporting bias involved in the data, as the reported emotional conditions were based on the parents' evaluation. Furthermore, each child's sleep quality was subjectively assessed by parents, and was not measured using a numerical scale. Future research including clinical examination for the pediatric dental patients is needed to better understand the associated factors of bruxism and their possible health impact in children.

Table 5. Factors associated with the self-reported prevalence of bruxism

Factors	Child has bruxism		χ^2-value	p-value
	Yes	No		
Gender of the child				
Male	383(46.5)	441(53.5)	0.453	0.501
Female	302(44.7)	373(55.3)		
Age of the child (in years)				
6	253(55.6)	202(44.4)	28.89	<0.0001
7	90(42.1)	124(57.9)		
8	98(41.9)	136(58.1)		
9	82(36.4)	143(63.6)		
10	162(43.7)	209(56.3)		
Region of residence				
The middle region	477(46)	561(54)	19.36	0.001
The east region	93(48.2)	100(51.8)		
The west region	81(54.4)	68(45.6)		
The north region	20(30.8)	45(69.2)		
The south region	14(25.9)	40(74.1)		
Does your child have any medical or psychological or emotional condition?				
Yes	102(61,1)	65(38.9)	17.92	<0.0001
No	583(43.8)	749(56.2)		
How many hours does your child sleep at night?				
Less than 8 hours	109(42.1)	150(57.9)	1.65	0.199
More than 8 hours	576(46.5)	664(53.5)		
How does your child sleep at night?				
Restless (moves a lot, frequently wakes up fearful-crying-worried, sleeping hours are not smooth and continuous)	171(64.0)	96(36.0)	69.51	<0.0001
Normal (continuous hours of calm sleeping)	444(39.5)	680(60.5)		
Other	70(64.8)	38(35.2)		
Does your child sleep alone?	161(34.4)	307(65.6)	39.40	<0.0001
Yes	404(52.7)	362(47.3)		
No	120(45.3)	145(54.7)		
Sometimes	87(68)	41(32)	35.28	<0.0001
Does the father have bruxism?	535(42.5)	723(57.5)		
Yes	63(55.8)	50(44.2)		
No				
Maybe	79(63.2)	46(36.8)	20.96	<0.0001
Does the mother have bruxism?	542(43.2)	711(56.7)		
Yes	64(52.9)	57(47.1)		
No				
Maybe				

χ^2-value = Pearson's chi-square

CONCLUSION

The importance of parents gaining general knowledge of bruxism cannot be overstated. Bruxism is usually first identified in children by their parents. The present study shows that the majority of parents lack awareness of bruxism, its causes, where to seek help for the condition and the possible complications. A multidisciplinary approach with dentistry which has a major role in diagnosing and treating bruxism should be considered to improve educational tools to increase parental awareness about bruxism in children.

Conflict of Interest: The authors declare that they have no conflicts of interest.

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Agricultural Communication

Dynamics of Agrochemical Properties of leached Chernozems in the Republic of Mordovia

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ABSTRACT

There is an acceleration, deceleration, or distortion of elementary soil processes under the influence of anthropogenic impact on the soil cover, which dictates the formulation of a state strategy for the rational use of soil resources. The object of the study was the leached chernozems of the Republic of Mordovia, where reference sites with state status were located on permanent spaces and which reflect the level of anthropogenic loads to the maximum extent. Soil sampling was carried out annually in spring before the start of fieldwork. Samples were taken to the depth of the arable layer. The following investigations were carried out in soil samples: humus by the Tyurin method with a photometric end; pHkcl with the preparation of a salt extract and determination of mobile phosphorus and potassium by the method of CINAO by the method of Kirsanov in the modification of CINAO with a photometric end; potassium by the flame photometric method. The trend of humus change indicates that its content in the plowed layer is decreasing. The greatest decrease was noted at reference plots 5 and 21, where in the 80s and 90s high doses of organic fertilizers were applied. Changes in the reaction of the soil solution for 1994–2012 indicate that this indicator is decreasing in all reference plots. The differences between individual plots are relatively small. The content of absorbed bases in the upper layer was quite high. In the arable layer, except for reference plot 5, an increase in mobile phosphorus was observed. The importance of soil-ecological monitoring is increasing immeasurably, which allows identifying deviations from optimal indicators to see a retrospective of natural complexes.

KEYWORDS: HUMUS, LEACHED CHERNOZEM, PHOSPHORUS, POTASSIUM, REFERENCE PLOTS, THE REACTION OF SOIL SOLUTION.

INTRODUCTION

The importance of soil-ecological monitoring of chernozems increases in conditions of active agro-technological impact, i.e., constant monitoring of changes in soil properties, the concept of which was developed in the second half of the 20th century (Lisetskii, 2009). Scholars carry out monitoring of the soil cover by comparing the studied objects with their analogs, establishing their age or the period during which they were in different conditions (Mukha, 1988; Galeeva, 2012). The possibility of assessing modern trends in the development of soils and geosystems, in general, is widely used in the scientific literature, using detailed studies that were carried out by scholars at different times (in the 19th – 20th centuries) (Khitrov, 2008; Eryashev et al., 2017).

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The anthropogenic influence on the development and fertility of chernozems was studied by various methods, the conclusions of scholars are contradictory. According to some researchers (Ivanov, 2002; Eryashev et al., 2015), the involvement of soil in agricultural use leads to a significant decrease in the amount of humus, especially in the arable horizon. A decrease in the humus content of the soil occurs only in the initial period of its plowing, later the loss of humus is less intensive, and then stabilization of its reserves in the arable layer of the formed agrocenosis occurs. Other researchers (Mukha, 2004; Ivanov et al., 2020) point to the preservation of the nature of humus formation and the quality of humus in arable soils (Ivanov et al., 2020).

Under the influence of anthropogenic impact on the soil cover, an acceleration, deceleration, or distortion of elementary soil processes can happen (Lisetskii, 2009; Kashtanov, 2011). This requires a formulation of a state strategy for the rational use of soil resources (Chekmarev & Lukin, 2013; Sukhanovskii et al., 2013). Under the influence of long-term agricultural use, both improvement

and deterioration of soil fertility can occur (Ivanov et al., 2013; Eryashev et al., 2016; and changes in the conditions for the growth and development of living organisms (Kargin and Zaharkina, 2016 Kargin et al., 2019).

The main factors causing degradation changes are reduced to hydrological, erosional, chemical, radiological, and mechanical factors (Kashtanov, 1974; Zaidelman, 2009). They can lead to desertification, the development of water and wind erosion; hydromorphism, soil compaction with heavy equipment, contamination with heavy metals and radionuclides, accumulation of soluble salts in the soil, increased salinity of surface and ground waters, destruction of organic matter with the removal of fine soil fraction (Shuvaev et al., 2009). Degradation of ecosystems, a significant decrease in the content of humus, total nitrogen, elements of ash nutrition of plants in soils, and deterioration of agrophysical properties occurs under conditions of intensive use of soils in agricultural production. In such conditions, the importance of soil-ecological monitoring increases immeasurably to identify undesirable deviations from optimal indicators and preserve the potential soil fertility.

MATERIAL AND METHODS

The object of the study was leached chernozems of the Republic of Mordovia, where reference plots (RPs) that had a state status and reflected the levels of anthropogenic loads to the maximum extent were located on permanent sites. The observations were carried out in 1994–2013. by the "Mordovskiy" State center of agrochemical service. The results of those observations were used in the preparation of this paper (Reports of the State center of agrochemical service Mordovskiy for 1994–2013, 2014). Cultivated crops and fertilizers applied at RPs are presented in Table 1.

Table 1. Cultivated crops and applied fertilizers on RPs

District and nearby settlement, RP No.	Year of the layout	Sown crops, number of years	Applied fertilizers, kg/ha of active ingredient per year			
			Nitrogen	Phosphorus	Potassium	Total
Oktyabrsky, Nikolaevka village, 5	1994	Grain crops (10 years), corn (4), perennial grasses (5), complete fallow (1 year)	38.7	26.6	27.2	92.5
Oktyabrsky, Saransk, 8	1994	Grain crops (17 years), annual herbs (2), potatoes (1 year)	23.1	23.2	16.4	62.7
Lyambirsky, Lyambir village, 12	1994	Grain crops (15 years), complete fallow (3), annual herbs (2 years)	16.3	11.1	11.1	38.6
Chamzinsky, Sabur-Machkasy village, 21*	1994	Grain crops (13 years), sugar beet (1), corn (2), perennial grasses (2), annual grasses (1), complete fallow (1)	34.1	23.5	24.0	81.6
Romodanovsky, Maloe Chufarovo village, 22	1994	Grain crops (13 years), complete fallow (3), perennial grasses (3), corn (1 year)	23.1	15.8	12.7	51.6

Note: * at RP 21, manure was introduced in 1997 for winter wheat at a dose of 40 t/ha

Soil sampling was carried out annually in spring before the start of fieldwork. Samples were taken to the depth of the arable layer. The following studies were carried out in soil samples: humus content according to State Standard (GOST) 26213–91 by the Tyurin method with a photometric end; pHkcl with preparation of a salt extract and determination by the Central Research Institute of Agrochemical Services for Agriculture (CINAO) method (GOST 26483-85), mobile phosphorus and potassium content by the Kirsanov method in the CINAO modification with a photometric end (GOST 26207-91); potassium content by flame photometric method (GOST 30504–97) (Kargin et al., 2017; Ivanova et al., 2019; Solodovnikov & Levkina, 2020).

RESULTS AND DISCUSSION

1994-1998 to 2009-2012 the humus content decreased from 4.0 to 22.8% to the original content (Table 2). The greatest decrease was noted on leached chernozems of RP 5 and 21, where in the 80s and 90s high doses of organic fertilizers had been applied. The trend of changes in humus in the arable layer indicates that in the arable layer of the studied soils there is a decrease in the content of soil organic matter. Trends in the change in the percentage of humus indicate a decrease in the content of humus in all RPs:

$Y = 9.61–0.1•X$ (RP 5);
$Y = 8.0–0.05•X$ (RP 8);
$Y = 8.69–0.04•X$ (12);
$Y = 12.38–0.22•X$ (RP 21);
$Y = 7.69–0.05•X$ (RP 22);

During the study period, there was slight acidification of the reaction of the soil solution. The negative trend in the change in the reaction of the soil solution remained until the end of the observations. The increase in acidity can largely be associated with infiltration losses of exchange bases and the absence of liming during the years of observation. The differences between individual sites are relatively small between different sites in terms of pHsol.

Table 2. Humus content, the reaction of soil solution, phosphorus, potassium, 1994–2012.

Indicators	Years of observations	RP numbers				
		5	8	12	21	22
Humus content, %	1994–1998	7.9–9.6	7.3–8.2	7.65–9.0	10.4–11.48	6.9–8.4
		9.04±0.30	7.68±0.16	8.45±0.24	11.12±0.2	7.62±0.25
	1999–2003	9.0–9.9	5.8–6.1	8.2–9.3	10.4–12.7	7.2–7.8
		9.34±0.15	5.9±0.05	8.84±0.21	11.36±0.37	7.58±0.12
	2004–2008	7.2–9.2	5.86–6.6	7.3–8.3	6.9–10.2	6.4–6.8
		8.46±0.42	9.04±0.23	7.75±0.24	8.28±0.66	6.62±0.09
	2009–2012	6.1–8.5	6.00–7.1	7.1–8.4	7.1–9.40	5.8–8.0
		7.65±0.53	6.72±0.25	7.87±0.29	8.58±0.51	7.0±0.46
	1994–2012	6.1–9.9	6.0–8.4	7.1–9.3	6.9–12.7	5.8–8.40
		8.67±0.22	7.46±0.14	8.27±0.15	9.9±0.40	7.22±0.15
The reaction of the soil solution pHsol	1994–1998	5.3–6.7	5.8–7.5	5.6–7.2	7.1–8.3	5.4–6.9
		6.1+0.3	6.5+0.4	6.1+0.3	7.6+0.2	6.3+0.3
	1999–2003	5.3–7.2	5.8–6.1	6.7–6.9	7.1–7.4	5.3–5.6
		6.2+0.4	5.9+0.05	6.8+0.04	7.2+0.06	5.4+0.05
	2004–2008	5.1–5.6	5.5–6.6	5.5–5.8	6.8–7.2	5.2–5.7
		5.3+0.1	5.9+0.3	5.6+0.07	7.0+0.08	5.5+0.09
	2009–2012	5.9–6.4	5.5–6.0	5.5–6.0	5.1–6.8	5.5–6.9
		6.1±0.12	5.8±0.12	5.7±0.11	6.3±0.41	5.9±0.34
	1994–2012	5.2–7.2	5.5–7.5	5.5–7.2	5.1–8.3	5.2–6.9
		5.95±0.14	6.1±0.14	6.1±0.14	7.1±0.15	5.8±0.13
P_2O_5, mg/kg of soil	1994–1998	225–349	205–239	110–204	209–260	97–153
		259.2+23.3	227+6.36	153.4+17.7	237.6+8.9	125.2+9.2
	1999–2003	202–595	211–309	162–227	276–391	107–132
		353.2+68.7	254+16.5	197.6+13.0	305.0+22.1	121.4.5
	2004–2008	182–443	225–339	210–260	306–470	113–213
		313.4+46.7	258.8+22.7	232.7+10.5	408.4+35.6	165.4+20.2
	2009–2012	202–364	230–380	233–431	378–507	124–179
		252.0+38	310.0+31	325.5+43.6	435+30	154.8+11.6
	1994–2012	182–595	205–380	110–431	209–507	97–213
		292.9±25.2	262.9±11	221.6±18.4	342.6±22.0	141.1±7.41
K_2O, mg/kg of soil	1994–1998	128–236	212–354	117–164	262–449	163–210
		184.0+18.6	285.4+27.4	117–164	327.2+31.8	188.6+7.9
	1999–2003	126–306	197–286	108–181	324–541	122–204
		211.0+33.3	237+14.9	139.4+12.8	420+49.0	160.6+13.3
	2004–2008	114–174	229–279	142–168	482–558	138–239
		142.8+9.9	243.4+9.3	155.7+6.8	514.2+12.3	191+20.4
	2009–2012	127–190	170–374	158–217	336–985	142–220
		165+13	279+51	178+13.2	657+169	175+17.3
	1994–2012	114–306	170–374	108–217	262–985	122–239
		176.4±12.2	260±13	151.5±6.4	470.3±44.5	178±7.59

Note: the numerator is the oscillation interval, the denominator is the arithmetic mean + average deviation

Cultivated chernozems (RP 8, 12, 21) are characterized by an increased content of phosphorus and potassium. The farms where these soils are located function on intensive farming. Mineral fertilizers are annually applied to the crops growing in these areas, so the fertility of the soil does not decrease. RP No. 21 has had a high content of phosphorus and potassium at approximately the same high level (461 and 336 mg/kg) for a long period of observations, which can be explained by the high content of natural fertility (leached chernozems, with a humus content of 8.7%, the use of fertilizers and protective equipment). On the same benchmark, the pH value of 6.8 practically has not changed from year to year, which can be explained by the fact that the plot is located near a process plant (a cement plant of Mordovcement OJSC) and during the production process of the plant cement dust settles on the soil, neutralizing soil acidity.

The maximum amount of mobile phosphorus was observed on average over the years of the study. This indicates that only in the Oktyabrsky district (RP 5) its content was at the same level. In all other areas, there was an increase in this indicator. It is known that the content of potassium in soils depends primarily on their mineralogical and granulometric composition. The minimum content of potassium was found in the soils of Cheremishevskoye LLC of the Lyambirsky region, and the maximum content was observed at Saburmachkasskoye LLC of the Chamzinsky region.

Summing up, we can conclude that the evolution of the properties of chernozem soils as a result of their long-term agricultural use is determined by the balance of the production and cultural soil-forming process. Therefore, continuous monitoring is an integral part of national control. Its significance increases even more in the conditions of active agro-technological impact. During the 1994-2012 observations, the humus content in the arable layer decreased by 4.0-22.8% to the original content. The greatest decrease was noted on leached chernozems of RP 5 and 21, where in the 80s and 90s high doses of organic fertilizers had been applied. The trend of changes in humus in the arable layer indicates that in the arable layer of the studied soils there is a decrease in the content of soil organic matter. The reaction of the soil solution in 1994–2012 indicates that in all RPs a decrease in this indicator can be noted. The negative trend in the change in the reaction of the soil solution remained until the end of the observations. The increase in acidity can largely be associated with infiltration losses of exchange bases and the absence of liming during the years of observation. The differences between individual plots are relatively small (Solodovnikov & Levkina, 2020).

In the arable layer of RPs, except for RP 5, an increase in mobile phosphorus was observed. The potassium content is lower than the phosphorus content. Its high content against the background of a negative balance is obvious since high doses of phosphorus fertilizers have been used for a long time (70–80s of the last century). This made it possible to form a reserve fund of this element, which still maintains the content of its mobile forms at a very high level. There was a decrease in the level of mobile phosphorus, while its increase in the upper ones in the process of agricultural use in the lower layers of the soil profile. We have found a significant ($r = 0.72$) relationship between the content of mobile phosphorus and the accumulation of humus. In our observations, the content of potassium is lower than that of phosphorus, which is associated with much higher mobility and, accordingly, lower ability of potassium to accumulate in the soil, as well as a large removal of this element with the crop. A significant relationship of this indicator with the applied potash fertilizers and humus content was revealed. The concentration of mobile potassium decreases to a depth of 140 cm under conditions of insufficient application of potassium fertilizers, which is associated with the use of potassium by plants from deep layers (Ivanov et al., 2020).

The study showed that against the background of relatively favorable agrochemical characteristics of the arable layer of leached chernozems, there are negative trends associated with dehumification, deterioration of physicochemical characteristics, and a decrease in the content of available forms of nutrients. The degree of development of unfavorable processes is not critical, which is associated with the high buffering capacity of leached chernozems and their resulting stability.

CONCLUSION

The use of soils in agricultural production in recent years has led to a deterioration in their condition, a significant decrease in the content of humus, phosphorus, and potassium in them, which requires constant monitoring of their condition. The importance of soil ecological monitoring is increasing immeasurably, as it allows identifying deviations from optimal indicators to see a retrospective of natural complexes.

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Environmental Communication

Justification for Effective Water Planning and Management in the North of the Sinai Peninsula, Egypt

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ABSTRACT

Groundwater is virtually the only source of water supply to the north of the Sinai Peninsula. Quaternary aquifers are promising for organising reliable drinking water supply at the peninsula. Propagation conditions for Quaternary aquifers were characterised. Information was updated for all existing hydrogeological wells in the North Sinai area. Well logs for the development of the main Quaternary aquifers were compiled; statistical analysis of permeability parameter values for water-bearing rocks was carried out, based on which the zoning was identified according to the permeability coefficient values. Distribution of aquifers with significant thickness in Quaternary deposits is limited only to the coastal plain along the Mediterranean Sea. Based on testing of 52 wells, all Quaternary deposits are characterised by very high permeability coefficients, which on average are tens and even hundreds of metres per day. Analysis of correlation dependences between the permeability coefficient and the depth of the tested wells showed that there is no unambiguous dependence for all Quaternary deposits or for each of the individual samples. Consequently, ranges of change in the permeability coefficient values for various Quaternary sediments are determined not by the geostatic pressure, but by the packing density of loose rocks and degree of their secondary cementing. Based on the conducted zoning of the area according to the permeability coefficient, the prime areas for underground water production for each aquifer were identified. Performed studies do the groundwork for water that is resource that is more efficient planning and management.

KEY WORDS: GROUNDWATER RESOURCES, HYDROGEOLOGICAL ZONATION, QUATERNARY AQUIFERS, SINAI PENINSULA.

INTRODUCTION

Although surface water is the most accessible type of water resources for industrial and agricultural use, it is the most vulnerable to depletion in quantity and quality. Since the river runoff accounts for 89% of the total annual inflow of inland waters into the World Ocean, the rivers play a crucial role in the global water balance. The data on water resources and their use in selected countries and world regions, which were obtained as a result of long-term studies of the State Hydrological Institute (Saint Petersburg) within the framework of the International Hydrological Program of UNESCO and WMO, have shown that the total inflow of river waters from the continents (excluding Antarctica) to the World Ocean is 39,500 km^3 per year (Lisetskii 2021).

In addition, the World Ocean receives approximately 2,400 km^3 of underground water that are not drained by rivers. In total, the World Ocean receives approximately 44,200 km^3 of continental fresh water per year or 300 mm for the entire land area (Shiklomanov 2008). An important feature of any river basin is its hydrological characteristic, which allows calculating the water balance and simulating the spatial moisture content of the entire surface of the catchments

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based on the received precipitations and their flow rate at the outlet (Ivanov and Lisetskii 1995; Moskalenko et al. 2012; Gusarov 2019; Dragunova et al. 2020; Yermolaev et al. 2021).

The watershed parameters, which are distributed in space and time, are included in the design solutions for the development of the territory, using modern technologies, such as GIS modeling, remote sensing, creation of geoportals (Shtompel et al. 1998; Lisetskii et al. 2014; Yermolaev et al. 2018). A list of top-priority scientific issues in the field of rational use of water resources begins with estimated water stocks on the Earth available to the human race, water consumption rate, and awareness of freshwater shortage as a global problem, it is later transformed into a set of measures needed to address the problems of unsustainable water consumption, water pollution, environmental impacts on other natural environments, and development of design and practical solutions for soil and water protection arrangement of the catchment area (Buryak and Marinina 2020).

In addition, it is nationally important to develop and evaluate procedures for using objective environmental and economic indicators of anthropogenic impacts on water resources, including the ratio of water used to water resources, taking into account the environmental component of runoff; the rate of dilution of wastewater with the river runoff; and indicators of the effectiveness of runoff regulation. While the potential for necessary research, which ultimately aims to overcome the blue water-groundwater and surface water scarcity, is now time to gradually move towards the limits to the green water footprint, the green water flow allocated to human society (Schyns et al. 2019). Today, there is an urgent need to find additional sources to solve the problem of projected water shortages in the Arab Republic of Egypt. Groundwater is one of these sources. Now the country's fresh groundwater resources comprise of less than 20% of the total balance of water resources used (Abdel-Shafy and Kamel 2016; Koronkevich et al. 2020).

To cope with problems involving the population growth and the need for its social support, the Government of Egypt focused on the development of the Sinai Peninsula, which is believed to have a high potential for exploration of mineral resources, tourism, and agriculture. The Sinai Peninsula covers an area of 61,000 km^2; its population is 559,071 people (Abdel-Shafy and Kamel 2016). At the same time, the peninsula, which is located far from the major water source, the river Nile virtually has no other water supply sources, other than underground water, at least during the most part of year, beyond of the high-water period. The area of Sinai is completely represented by desert lands, with the exception of its north-eastern part. In general, the Sinai Peninsula is characterised by arid climate: annual precipitation in various regions is estimated at 40 to 200 mm (the latter is north-east) (Mohamed et al. 2020).

Until now, the development of surface waters in Sinai has been implemented in some areas through construction of dams and organisation of temporary reservoirs, functioning only during flood periods. Numerical modelling is successfully used to assess effectiveness of creating artificial aquifers in body of earth dams. In some areas, such as El-Arish in the north-east and El-Tor in the south-west, groundwater is used for drinking or irrigation, extracted mainly from Quaternary sediments at a depth of 50 to 100 m, or more. At the same time, excessive pumping in recent years has significantly deteriorated their quality due to intrusions of sea salt water (Mohamed et al. 2020). This paper studies the Quaternary aquifers, which are promising for organisation of reliable drinking water supply at the Sinai Peninsula.

MATERIAL AND METHODS

The conditions for propagation of Quaternary aquifers in the north of the Sinai Peninsula are characterised by the totality of the results of all studies conducted earlier in this area, including those conducted in recent years, etc. (Arad and Kafri 1980; Shata 1982; Aggour et al. 2007; Elewa and Qaddah 2011; El Samanoudi et al. 2013; Ghoubachi 2013; Raouf 2014; Arnous 2016; Khaled et al. 2016; Ibrahim et al. 2021). The main evidence bases of studies became information on hydrogeological wells contained in the summary report 'North Sinai Groundwater Resources Study in the Arab Republic of Egypt (JICA, 1992), as well as data provided by the Water Resource Research Institute (WRRI) under the Ministry of Water Resources and Irrigation of the Arab Republic of Egypt (Ibrahim et al. 2021).

As part of this paper, information was updated for all existing hydrogeological wells in the North Sinai area. The result of this systematisation was represented by the corresponding database, which in total includes information on 180 wells, 52 of which open Quaternary aquifers. The authors compiled the well logs for the development of the main Quaternary aquifers and carried out a statistical analysis of permeability coefficient values using the SPSS software, based on which the territorial zoning of North Sinai in terms of the development of Quaternary aquifers in accordance with permeability coefficient values was determined (Ibrahim et al. 2021).

RESULTS AND DISCUSSION

Quaternary formation deposits were widespread in the area under investigation. Nevertheless, the spread of significant aquifers there was limited only by the coastal plain along the Mediterranean Sea. Relatively thick Quaternary deposits stretched along the coastal plain with a line 10 to 15 km wide from the estuarine part of the El-Arish wadi to the Rafah region (Figure 1) and from Bir al-Abd to Rohman, with a line width of about 10 km. Within the indicated line, the overall width of Quaternary deposits varied in a rather wide range, increasing from a few metres at the south boundary of the specified line to 80 to 100 metres in the north (Figure 2). In general, the Quaternary cover thickness to the west of the El-Arish wadi was less than to the east of the same.

The main water-bearing rocks of Quaternary deposits of the considered area are sand, gravel, and kurkar. Kurkar is limestone sand deposits that are almost ubiquitous in the coastal plain. In the coastal zone, the upper layer is generally represented by sand with a thickness of 20 to 40

m. The sand stratum is composed of sand dune deposits and ancient beach sand. Differentiation of these formations in the lithological profile of the wells is rather difficult. At the same time, ancient beach sand, covered by dune sands, is usually considered one of the promising aquifers. Gravel or kurkar deposits underlie the sand stratum. The distribution of sand thickness in the El-Arish wadi valley and in the coastal plain as a whole, from Sheikh Zuweid to Rafah, is shown in the figure (Figure 3a).

Figure 1: Coastal plain map within the Quaternary deposits development

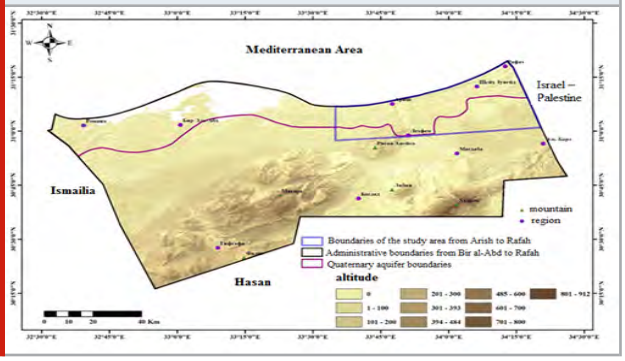
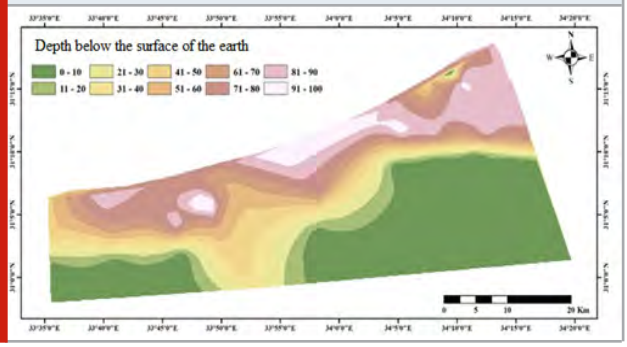


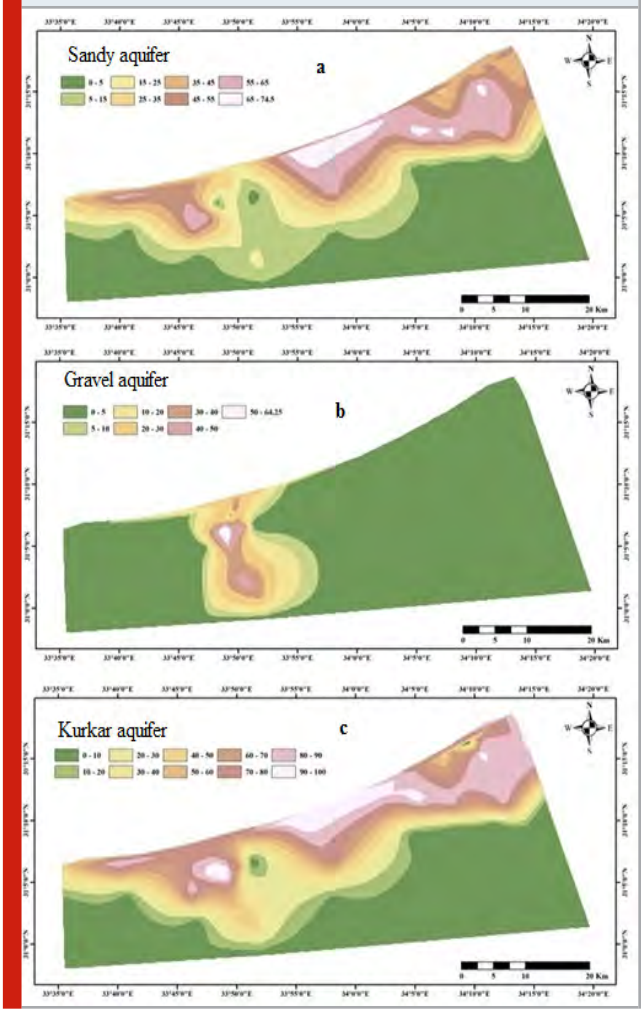
Figure 2: Isopach Map (Total Thickness) of Quaternary Formations



Gravel strata occur in many areas of the considered area, but as an independent aquifer, this stratum is concentrated mainly in the alluvial valley in the El-Arish wadi area: it stretches for about 10 km along the El-Arish wadi bed. The origin of this gravel is debatable. However, it is clear that it extends beyond the current alluvial valley of the El-Arish wadi, at least north of the El-Arish airport. The distribution of the gravel aquifer thickness is shown in the figure (Figure 3b). In the coastal plain as a whole, from Sheikh Zuweid to Rafah, gravel layers are observed only in local areas. Obviously, these local deposits have accumulated in old wadis. In most cases, it is noted here that groundwater levels in the wells are established below of the gravel stratus bottom. For this reason, the water abundance of gravel is considered low here.

Kurkar deposits are widespread within the coastal plain, in the area from Sheikh Zuweid to Rafah, under the deposits of sand dunes. The absolute elevation of the kurkar deposit base ranges from -60 to -20 m. Its thickness varies from 10 to 40 m. In some parts of the coastal zone of sand dunes, kurkar is pinched out. The distribution of these deposits by thickness is shown in the figure (Figure 3c). In wells of the alluvial valley of the El-Arish wadi, kurkar is traced for 10 km along the wadi bed and is overlain by gravel. In this zone, filters of most wells are fitted precisely for the kurkar deposits.

Figure 3: Isopach Map of the Aquifer Represented by: A) Sands; B) Gravel; C) Kurkar

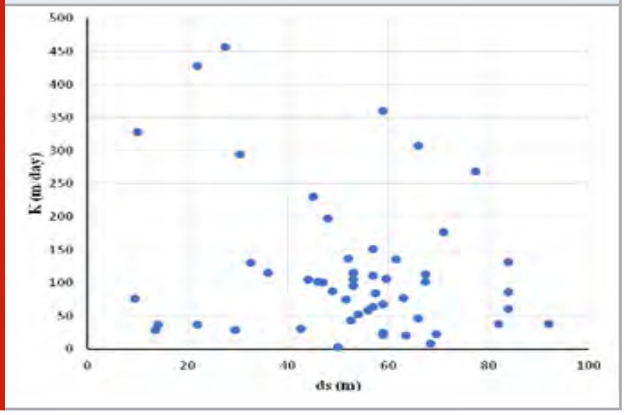


Using the SPSS software, statistical estimates for the distribution of values of the permeability coefficient, K (m/day), obtained because of field-testing of wells, were carried out. For Quaternary deposits, different data samples were considered depending on the sampled aquifers and sampling areas: 1) general population for all Quaternary deposits (Qtotal); 2) joint sampling (single filter) for gravel and kurkar deposits: in the El-Arish wadi valley (kurkar + gravel_A); 3) kurkar deposits on the north-eastern coast of the study area, in the districts of El-Arish, Sheikh Zuweid, and Rafah (kurkar_ASR); 4) sandy deposits on the north-eastern coast of the study area in the same areas of El-Arish, Sheikh Zuweid, and Rafah (sand_ASR); and 5) sandy deposits in the north-western region of Bir al-Abd (sand_BaA). The calculated results for the statistical parameters are shown in Table 1.

Table 1. Statistical parameters of distribution of the permeability coefficient values (K, m/day) in the Quaternary deposit aquifers

Indicator	Qtotal	Kurkar + Gravel_A	Kurkar _ASR	Sand _ASR	Sand _BaA
Number of measurements	52	31	11	6	4
Average	118	120	75	45	327
Median	92	102	77	37	361
Standard deviation	106	91	54	19	149
Minimum	2.7	21	2.7	29	131
Maximum	457	360	177	77	457

Figure 4: Correlation Dependence between the Permeability Coefficient (K, m/day) and the Well Depth (ds, m) for all Wells Fitted for Quaternary Deposits



As can be seen from Table 1, according to the results of testing of 52 wells, all Quaternary deposits are characterised by very high permeability coefficients, on average tens and even hundreds of metres per day, with an absolute single minimum of 2.7 m/day (kurkar). At the same time, kurkar and gravel deposits are characterised by a very large scatter of permeability coefficients: the minimum and maximum values differ by tens (practically up to a hundred) times. Most probably, this is caused by different degrees of cementing of these rocks at different sampling areas.

On the contrary, sandy deposits are simpler in terms of permeability: minimum permeability coefficients differ from maximum ones only by 2.5 to 3.5 times. But at the same time, the average value of the permeability coefficient for the sands to the west of the El-Arish wadi (in the Bir al-Abd region) was on the whole the highest among all the tested land sites of Quaternary deposits (327 m/day), and about 7 times more than in the sands of the eastern area, in the areas of El-Arish, Sheikh Zuweid, and Rafah. Conclusions made in relation to sands should be considered now to be conventional requiring further clarifications due to rather small number of samples. The correlation field and the relationship between the permeability coefficient (K) and the depth of the investigated well (ds) for all wells adapted for Quaternary deposits are shown in the graph in Figure 4.

No unique dependence (trend) for all Quaternary deposits either as a whole or for each individual sampling was found. Thus, it should be assumed that within the development of Quaternary rocks to the established depths not exceeding 100 metres, there was no change in permeability properties of rocks with the depth increase. Therefore, the stated above value ranges for permeability coefficients of different Quaternary deposits were determined not by the rock pressure, but by the packing of loose rocks and by the degree of their secondary cementing, which developed both directly during the deposition and during the following secondary processes, apparently associated with ground water filtration. It is also important to note that, as shown earlier of the spatial and temporal analysis of the unconfined aquifer (Nema et al. 2017), there is a good hydraulic connection of groundwater level with the rainfall. Investigating hydraulic connections in complex hydrological systems shows the key role of geological heterogeneity in water-bearing horizons (Pang et al. 2020; Wang et al. 2020).

It is also important to note that, as shown earlier of the spatial and temporal analysis of the unconfined aquifer, there is a good hydraulic connection of groundwater level with the rainfall (Nema et al. 2017). Based on the results of statistical data processing, areal (in plan) zoning of the development area for Quaternary deposits was conducted according to the values of permeability coefficients of water-bearing rocks, within the coastal plain up to 15 km wide, which is extremely important from the point of view of assessing the prospects for water supply of the area under consideration. It should be noted that aquifers with the participation of kurkar (local term for aeolian carbonate-cemented, quartz sandstone) have significant specificity in terms of permeability compared, for example, with sands (Galili and Zviely 2019; Bao et al. 2021; Tasalloti et al. 2021).

Permeability zoning of the coastal plain was carried out, similar to statistical estimates, separately for the areas of development of various rock types: 1) for combined aquifer represented by kurkar deposits and gravel (single filter) in the El-Arish area and aquifer of kurkar deposits on the north-eastern coast of the study area (in the western part of the coastal plain, these deposits were not sampled) (Figure 5), and 2) for aquifer, composed of sands and developed both on the north-eastern coast of the study area, in the areas

of El-Arish, Rafah, and Sheikh Zuweid, and in the north-west, in the area of Bir al-Abd (Figure 6). It should be noted that aquifers with the participation of kurkar (local term for aeolian carbonate-cemented, quartz sandstone) have significant specificity in terms of permeability compared, for example, with sands (Galili and Zviely 2019; Bao et al. 2021; Tasalloti et al. 2021).

Figure 5: Zoning Map of Development Area for the Aquifer, Represented by Kurkar and Gravel Deposits, according to the Permeability Coefficient, K, m/day

Figure 6: Zoning Map of Development Area for the Aquifer, Represented by Sand Sediments, according to the Permeability Coefficient, K, m/day

As shown in Figure 5, in the most north-eastern part of the area under consideration, near the border of the Republic of Egypt with the Gaza Strip and Israel, the permeability coefficient in the aquifer represented by the kurkar deposits will be less than 100 m/day (K < 100 m/day), and in the rest of the region, in the El-Arish wadi area and in the inner parts of the study area, where gravel aquifer is additionally developed, the permeability coefficients will generally be more than 100 m/day (K > 100 m/day). Consequently, the development of water supply at the expense of horizons, represented by kurkar and gravel deposits, is more promising in the El-Arish wadi area than in the north-eastern regions of North Sinai. As shown in Figure 6, in the aquifer represented by sands, in all north-eastern regions of North Sinai (both El-Arish, Rafah, and Sheikh Zuweid), the permeability coefficients are less than 100 m/day (K < 100 m/day), and in the north-west of North Sinai, in the Bir al-Abd region, they are more than 100 m/day (K > 100 m/day).

Thus, it is preferable to organise water supply from the sand aquifer in the regions of the north-western part of North Sinai in comparison with the north-eastern part. Discussing these results, one cannot fail to mention the interaction of groundwater with the sea areas of the Mediterranean Sea. As shown by previous research (Gad and Khalaf 2015; Elshinnawy and Almaliki 2021), the difference between the maximum and the minimum water table is in the tide range of the eastern region of the Mediterranean Sea, and the groundwater resources, in particular in the North Sinai area, were found to be affected by salt water due to over-pumping phenomenon.

CONCLUSION

The findings of the present study suggests that an important and still underestimated aspect of the problem of water resources management in the catchment area is to study the underground and surface water relationship, which tends to be modified due to geological structure differences for any given territory. This is, in particular, due to the fact that when the quality of river water is reduced according to the drinking water supply standards, there is a transition to the use of underground water as the main source for domestic drinking, industrial, and agricultural water supply. In arid regions, such as the north of the Sinai Peninsula, groundwater is virtually the only water source used by local communities. Obviously, extraction of groundwater from aquifers in areas with high water availability will be easier and cheaper than in other areas, and, therefore, the development of these areas will be more intensive. Therefore, the studies conducted do the groundwork for more efficient water planning and management, since in fact they form the basis for further zoning of the area in terms of groundwater resources.

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Dental Communication

Orthodontic Management of Traumatized Teeth: Saudi Orthodontists' Perspectives

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ABSTRACT

The purpose of the present study is to undertake an up-to-date assessment of the knowledge and skills of Saudi orthodontists, regarding the time frame required before the commencement of orthodontic tooth movement, and their views of best orthodontic management of traumatized teeth. A cross-sectional study was performed by utilizing survey research techniques. The short self-administered questionnaire used here consisted of demographic data, clinician's opinion of the most appropriate time frame to receive orthodontic treatment, and the clinician's approach and management for patients with different types of traumatized teeth cases. This questionnaire was mailed electronically to all Saudi orthodontists. A total of 166 orthodontists participated in the study. A period of three months was recommended by most participants as necessary before moving crown and crown/root-fractured teeth, root canal treated teeth, and teeth with injury to the periodontium. On the other hand, the survey shows that the best time to initiate orthodontic movement for patients with root-fractured teeth was after twelve months. The majority of respondents prefer performing regular radiographic examination, opted for reducing the applied orthodontic forces, and performing regular pulp vitality tests as the best follow-up management of a traumatized teeth with different types of dental trauma. The study reveals the imperativeness of performing an accurate history documentation of any previous dental trauma and to make such an inquiry a major part of every orthodontic diagnosis. Lastly, when dealing with traumatized teeth, it is important to perform a thorough clinical and radiographic evaluation before and during the orthodontic treatment.

KEYWORDS: DENTAL TRAUMA, TRAUMATIZED DENTITION, ORTHODONTIC MANAGEMENT, ORTHODONTIC TOOTH MOVEMENT.

INTRODUCTION

Dental trauma is a common, inevitable and serious oral health problem among both adolescents and children with boys being more affected than girls (Chadwick and Pendry 2004; Al-Malik 2009; Damé-Teixeira et al. 2013; Tewari et al 2020; Abdel Malak et al 2021). Although there are limited reports available on the epidemiology of dental injuries in Saudi Arabia, a review of the literature shows various prevalence rates for traumatic dental injuries in individuals younger than 18; with some arguing for as low a range as 5%, and others as high as 17.5% occurrence (Chadwick and Pendry 2004; Azami-Aghdash et al. 2015; Van Gorp et al. 2020).

Furthermore, dental trauma is a frequent finding in patients with orthodontic treatment needs. It is estimated that over 10% of young patients admitted for orthodontic treatment has a history of dental trauma (Bauss et al. 2004). Since the malocclusion is considered as the third common oral health problem in dental public health priorities worldwide, specific malocclusions, such as occlusal relationship, increased overjet with prominent incisors, insufficient lip closure are predisposing factors that require early orthodontic intervention necessary to reduce the risk of dental trauma (Petti 2015; Alhammadi et al. 2018; Batista et al. 2018; Van Gorp et al. 2020).

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Such kinds of dental trauma, most frequently, involve the upper incisors that usually cause esthetic and phonetic problems, which negatively impacts the quality of life and psychosocial distress of individuals (Kindelan et al. 2008; Fakhruddin et al. 2008; Enabulele et al. 2016; Van Gorp et al. 2020). Nonetheless, orthodontic tooth movement (OTM) has possible undesirable effects such as root resorption. Thus, excessive number of orthodontic forces combined with previous dental trauma may further increase the susceptibility to root resorption (Poi et al. 2007; Kindelan et al. 2008). Dental injuries vary from simple fractures affecting the enamel to a more complex fractures that could complicate and delay orthodontic treatment. Furthermore, since the rate of trauma in patients seeking an orthodontic treatment is increasing, a comprehensive dental history along with radiographic and clinical examinations are essential to a successful orthodontic movement of traumatized teeth before and during the course of any orthodontic treatment. Each case should be evaluated and treated based on several factors including the patient's age, dental and anatomical development status, general health of the patient, previous orthodontic treatment, severity, time, and type of the trauma (Bauss et al. 2004; Poi et al. 2007; Kindelan et al. 2008; Owtad et al. 2015; Joybell et al. 2019).

Although no standard guidelines have been established in regards to the time required before the initiation of OTM and for the appropriate management of traumatized teeth cases, the literature agree that there is a different observation period for each type of trauma, depending on the severity of the injury and the knowledge and skills of the physician (Bauss et al. 2004; Malmgren and Malmgren 2007; Kindelan et al.2008; Owtad et al. 2015). However, there has been limited research conducted to further explore possible adverse effects of traumatized teeth undergoing OTM, thus making definitive conclusions hard to draw. Hence, the aim of this study is to obtain an up-to-date assessment of the knowledge and skills of Saudi orthodontists towards the time required before the initiation of OTM, and the best orthodontic management of traumatized teeth.

MATERIAL AND METHODS

For the purpose of data collection, a survey is designed as a useful method in the present cross-sectional study. The study was approved by the Institutional Review Board (IRB) at the College of Dentistry, King Saud University [E-20-5111]. The short-self-administered questionnaire consisted of 11 questions, including demographic data questions such as age, job title, years of practice, and type of practice, followed by descriptive questions to evaluate the clinician's opinion on the most appropriate time for receiving orthodontic treatment of patients with different types of traumatized tooth cases. Furthermore, additional questions are designed to analyze the clinician's approach and treatment management for different dental trauma cases. This questionnaire was mailed electronically in a Google Forums format, a web-based survey, with a link provided to all of orthodontists and orthodontic resident members of Saudi Commission for Health Specialities and the Saudi Orthodontic Society for a period between July, 2020 to Octoer, 2020.

A Pilot study consisting of 20 participants was conducted to validate the questionnaire and modifications were done accordingly and was reviewed and assessed by an orthodontist. The entire procedure and the aim of the study were thoroughly explained via the email to participants who voluntarily agreed to participate in the study. The study ensured the anonymous identity of the participants and data was kept protected to ensure confidentiality. The responses were assessed to determine the knowledge of the clinicians regarding orthodontic management and movement of traumatized teeth.

For statistical analysis, the collected data were recorded and analyzed by using SPSS version 26.0.0 (IBM Corporation, Armonk, NY, USA). G*Power software analysis was used to calculate the statistical power and estimate the sample size for the studied group. A sample size of 148 out of the total number of Saudi orthodontists was needed to achieve a 95% confidence level (AlBaker et al. 2017). However, to avoid a low response rate which may affect the sample size, a survey of a larger sample was conducted which should be more than that calculated in the assumption. Frequency distributions and descriptive statistics for age, gender, years of practice, type of practice, number of patients and patient age range with dental trauma treated in their practice were calculated.

In addition, a comparison and cross tabulation of participants' job title and inquiry about previous dental trauma during initial orthodontic assessment of patients was conducted. Furthermore, another cross tabulation of participants' job title and their responses regarding the most appropriate time to initiate OTM for patients with different types of dental trauma were investigated. Both investigations were conducted by using Pearson's chi-squared test. In all statistical assessments performed, the level of significance was recognized at 95% level of confidence ($p<0.05$) to indicate the statistical significance between the studied variables.

RESULTS AND DISCUSSION

A total of 166 Orthodontists participated in this study consisting of 91 females and 75 males, with a response rate of 70%. All trainees with less than one year of experience were excluded. Demographic characteristics of the participants were presented in Table 1. More than half of the participants were females (54.8%), and the majority were in the age group of 31-40 years (43.9%), followed by the age group of 41-50 years (22.3%). The university hospital setting was the main practice area of the participants (33.1%). Furthermore, participants were categorized according to their job title and the number of years they have been practicing as shown in Table 1. Thus, those who were consultant orthodontists and have been practicing for 6-10 years were the majority of the participants (48.8%, and 23.5%, respectively). On the other hand, the lowest percentage of participants were among general practitioners (GPs) with interest in orthodontics, and those who have been practicing for 1-2 years (1.8%, and 12.6%, respectively). In addition, most of the participants

reported seeing patients with history of dental trauma in the previous 12 to 18 months (87.3%).

The largest group of participants reported treating a range of 1 to 3 patients with history of dental trauma in their previous 12 to 18 months (59%). The frequency distribution of patients' age, at the start of orthodontic treatment following trauma revealed that the patients from 11 to 15 years old with a history of dental trauma had the highest percentage of orthodontic visitations than any other patients groups, which was 52% (n=79). On the other hand, patients who were more than 31 years of age had the lowest percentage (2%, n=3) (Table 1).

Table 1. Demographic characteristics of the participants

Socio-demographic Participants Characteristics		N	%
Gender	Female	91	54.8%
	Male	75	45.2%
Age Groups	30 and less	28	16.9%
	31-40	73	43.9%
	41-50	37	22.3%
	51-60	23	13.9%
	Above 60	5	3%
Job Title	Consultant orthodontists		
	Specialist orthodontists	81	48.8%
	Orthodontic residents	47	28.3%
	GP with training in	23	13.9%
	orthodontics	12	7.2%
	GP with interest in orthodontics	3	1.8%
Years of Experience	1-2 years	21	12.6%
	3-5 years	38	22.9%
	6-10 years	39	23.5%
	11-20 years	36	21.7%
	More than 20 years	32	19.3%
Working Sector	University clinical setting	55	33.1%
	Hospital setting	52	31.3%
	Private practice	48	29%
	Primary care	7	4.2%
	Other	4	2.4%
Number of patients seen	None	21	12.7%
with history of dental trauma	1-3 patients	98	59%
in previous 12-18 months	4-6 patients	25	15.1%
	7-12 patients	10	6%
	More than 12 patients	12	7.2%
patients' age, at the start of	7-10 years old	26	17%
orthodontic treatment following	11-15 years old	79	52%
trauma	16-20 years old	36	23.7%
	21-30 years old	8	5.3%
	Above 31 years old	3	2%

It is clear from the results that the majority of the participants reported a routine inquiry about any previous dental trauma during their initial orthodontic assessment (65.1%, n=108) as shown in the pie chart (Figure 1). On the other hand, 25.9% of the participants inquire about history of dental trauma only for patients with signs of trauma. Other participants inquired about history of dental trauma only for patients with clear signs of trauma and an increased overjet (6.6%). The minority of the participants were those who do not ask about any previous dental trauma and those who ask only if an increased overjet was clearly present, and both had equal percentages of 1.2% (n=2) (Figure 1).

According to the survey, responses regarding the most appropriate time to initiate OTM for patients with different types of dental trauma were presented in different charts in Figure 2. These responses were as follows: most of the participants prefer to wait for three months before moving crown and crown/ root-fractured teeth (n=83; 50%). Similarly, the majority of the participants (n=101;

60.8%) reported waiting three months before moving traumatized teeth with minor damage to the periodontium such as concussion, and before moving traumatized teeth with moderate to severe injury to the periodontium such as intrusion luxation (n=64; 38.5%). On the other hand, eighty-nine participants believed that the best time to initiate OTM for patients with root-fractured teeth was after twelve months (53.6%). For teeth treated with root canal treatment (RCT) due to trauma, the majority of the participants prefer to wait three months before moving a tooth (n=71; 42.7%), followed by a number of participants who prefer an immediate orthodontic movement of RCT teeth (n=62; 37.3%).

Figure 1: Pie chart showing frequency distribution of participants' enquiry about a history of previous dental trauma during initial orthondoctic assesment of patients

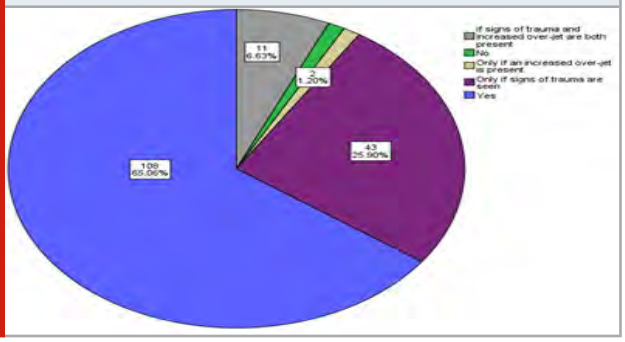


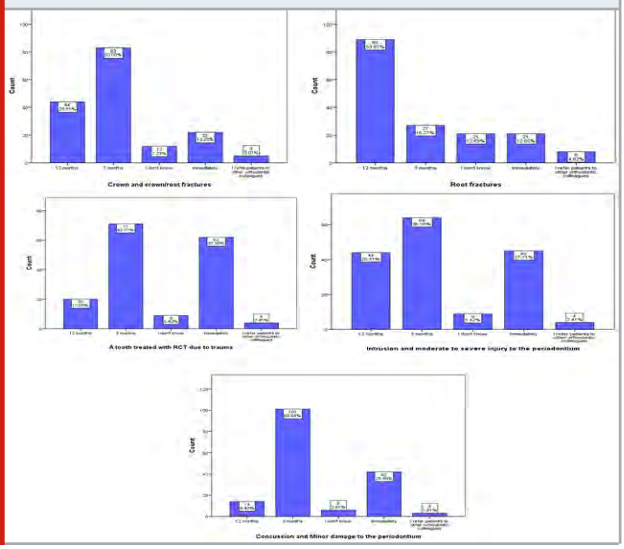
Table 2 shows that there is statistically significant relationship between participants' job title and the predisposition to inquire about dental traumas during initial orthodontic assessment of patients ($\chi 2$ = 34.1, P = 0.005, P <0.05). It revealed that orthodontic residents, specialists, and consultants are more inclined to ask about previous dental trauma. Similarly, the results also showed that there was a statistically significant association between participants' job title and their responses regarding the most appropriate time to initiate OTM for patients with different types of dental trauma. The majority of orthodontic residents, specialists, and consultants were in agreement in most of their responses as shown in Table 3.

Table 2. Cross Tabulation of participants' job tile and inquiry about previous dental trauma during initial orthodontic assessment of patients.

	Inquiry about any previous dental trauma during initial orthodontic assessment of patients.					Chi-Square Tests	
Job Title	No	Only if signs of trauma are seen	Only if an increased over-jet is present	if signs of trauma and increased over-jet are both present	Yes	Value	P.value
GPs interested in Orthodontics	0	1 (33.3%)	2 (66.7%)	0	0	34.1	0.005**
GPs training in orthodontics	2(16.7%)	4 (33.3%)	0	0	6 (50%)		
Consultant Orthodontist	0	22 (27.2%)	0	6 (7.4%)	53 (65.4%)		
Specialist Orthodontist	0	12 (25.5%)	0	5 (10.6%)	30 (63.8%)		
Resident Orthodontist	0	4 (17.4%)	0	0	19 (82.6%)		
Total	2 (1.2%)	43 (25.9%)	2 (1.2%)	11 (6.6%)	108(65.1%)		

(*) significant P ≤ 0.05, (**) highly significant P ≤ 0.01, (***) very highly significant P ≤ 0.001.

Figure 2: Different charts for the participants' responses regarding the most appropriate time to initiate orthodontic tooth movement for patients with different types of dental trauma.



The participants' responses regarding the best management of a traumatized tooth with different types of dental trauma during OTM were presented in Figure 3. The majority of the participants preferred to perform regular radiographic examination, followed by reducing orthodontic forces when treating cases with crown and crown/root fracture (65.7%, and 57.8%, respectively), root fracture (70%, and 42.8%, respectively), concussion (56%, and 54.8%, respectively), and intrusion luxation (69.3%, and 57.2%, respectively). In addition, performing regular pulp vitality test was reported among the participants when managing a traumatized tooth with crown and crown/root fracture (39.2%), root fracture (34.3%), concussion (28.9%), and cases with intrusion luxation (43.4%). For management of traumatized teeth treated with RCT, more than half of the participants preferred to perform regular radiographic examination (53%), while 44% of the participants preferred to treat patients with RCT the same way they treat non-traumatized cases. Other methods of management, with lesser percentages, were reported among participants. Such methods included reducing the orthodontic forces (41%), short recall intervals (19%), regular pulp vitality tests (16.3%), and leave the tooth off the arch wire (8.4%).

For management of ankylosed teeth, more than half of the participants opted for leaving the tooth off the arch wire (n = 94, 56.6%), and performing regular radiographic examination (n= 48, 28.9%).

Table 3. Cross Tabulation of participants' job tile and their responses regarding the most appropriate time to initiate orthodontic tooth movement for patients with different types of dental trauma.

Trauma Type	Job Title	Immediately	3 mon.	12 mon.	I don't know	Refer patients to other orthodontists	Chi-square	Sig.
Crown and crown/root fractures	GP interested in orthodontics	2 (66.7%)	0	0	0	1 (33.3%)	37.889a	0.002
	GPs training in orthodontics	2 (16.7%)	4 (33.3%)	2 (16.7%)	2 (16.7%)	2 (16.7%)		
	Consultant Orthodontist	7 (8.6%)	41 (50.6%)	24 (29.6%)	7 (8.6%)	2 (2.5%)		
	Specialist Orthodontist	7 (14.9%)	27 (57.4%)	13 (27.7%)	0	0		
	Orthodontist Resident	4 (17.4%)	11 (47.8%)	5 (21.7%)	3 (13%)	0		
Root Fractures	GPs interested in orthodontics	2 (66.7%)	0	0	0	1 (33.3%)	29.684a	0.02
	GPs training in orthodontics	1 (8.3%)	1 (8.3%)	5 (41.7%)	3 (25%)	2 (16.7%)		
	Consultant Orthodontist	7 (8.6%)	12 (14.8%)	49 (60.5%)	10 (12.3%)	3 (3.7%)		
	Specialist Orthodontist	6 (12.8%)	8 (17%)	28 (59.6%)	4 (8.5%)	1 (2.1%)		
	Orthodontist Resident	5 (21.7%)	6 (26.1%)	7 (30.4%)	4 (17.4%)	1 (4.3%)		
Minor damage to the periodontium (concussion)	GPs interested in orthodontics	2 (66.7%)	0	0	0	1 (33.3%)	36.859a	0.002
	GPs training in orthodontics	1 (8.3%)	7 (58.3%)	1 (8.3%)	2 (16.7%)	1 (8.3%)		
	Consultant Orthodontist	18 (22.2%)	54 (66.7%)	7 (8.6%)	1 (1.2%)	1 (1.2%)		
	Specialist Orthodontist	14 (29.8%)	28 (59.6%)	4 (8.5%)	1 (2.1%)	0		
	Orthodontist Resident	7 (30.4%)	12 (52.2%)	2 (8.7%)	2 (8.7%)	0		
Moderate to severe injury to the periodontium (Intrusion)	GPs interested in orthodontics	2 (66.7%)	0	0	0	1 (33.3%)	58.243a	0.000
	GPs training in orthodontics	6 (50%)	0	1 (8.3%)	3 (25%)	2 (16.7%)		
	Consultant Orthodontist	19 (23.5%)	37 (45.7%)	21 (25.9%)	3 (3.7%)	1 (1.2%)		
	Specialist Orthodontist	16 (34%)	13 (27.7%)	17 (36.2%)	1 (2.1%)	0		
	Orthodontist Resident	2 (8.7%)	14 (60.9%)	5 (21.7%)	2 (8.7%)	0		
A tooth treated with RCT due to trauma	GPs interested in orthodontics	2 (66.7%)	0	0	0	1 (33.3%)	50.926a	0.000
	GPs training in orthodontics	2 (16.7%)	4 (33.3%)	1 (8.3%)	3 (25%0	2 (16.7%)		
	Consultant Orthodontist	36 (44.4%)	38 (46.9%)	4 (4.9%)	2 (2.5%)	1 (1.2%)		
	Specialist Orthodontist	13 (27.7%)	22 (46.8%)	10 (21.3%)	2 (4.3%)	0		
	Orthodontist Resident	9 (39.1%)	7 (30.4%)	5 (21.7%)	2 (8.7%)	0		

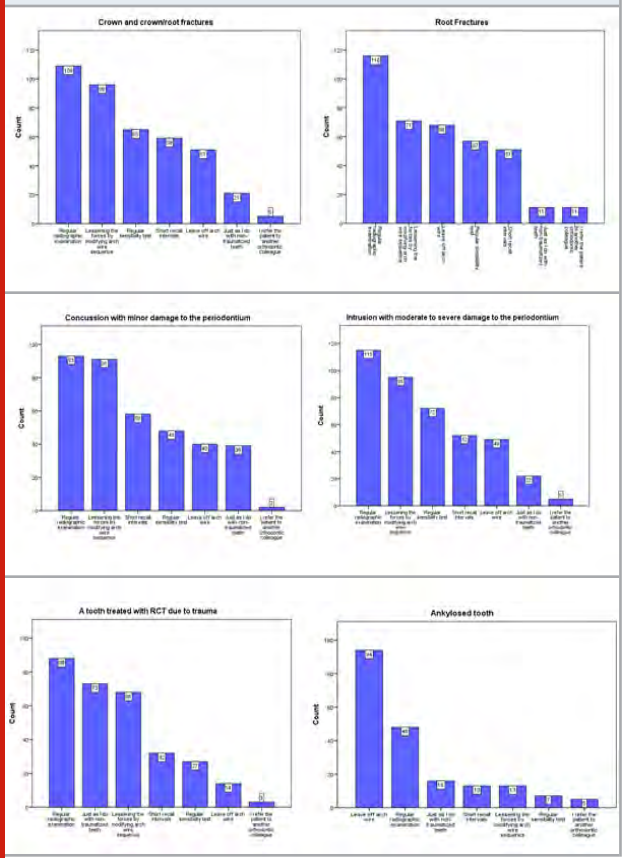
*Sig - approximate significance, where P-value at 0.05 level

Furthermore, the participants who answered the two questions (the most appropriate time to initiate orthodontic movement, and the best orthodontic management of a traumatized tooth with different types of dental trauma) with answers such as "I don't know" or "I refer patients to other orthodontic colleagues", were asked to kindly state their reasons. It was found that 38% of the participants referred patients to their colleagues because they believed that interdisciplinary treatment was needed, 20% of responses were due to lack of knowledge, 16% of responses were due to lack of experience, and the last reason for referral was due to lack of clinical practice which was 12% (Figure 4).

The evaluation and rather careful monitoring of the traumatized tooth before and during OTM are very important in cases in which emergency management is required to enhance the prospects of successful prognosis. There are limited number of researches available on orthodontic management of traumatized teeth in Saudi Arabia. Hence, the purpose of the present study is to obtain current assessment of the knowledge and skills of Saudi orthodontists towards the time required before the initiation of OTM, and the best orthodontic management of traumatized teeth. The evaluation of the traumatized tooth must include: timing of the injury, any previously

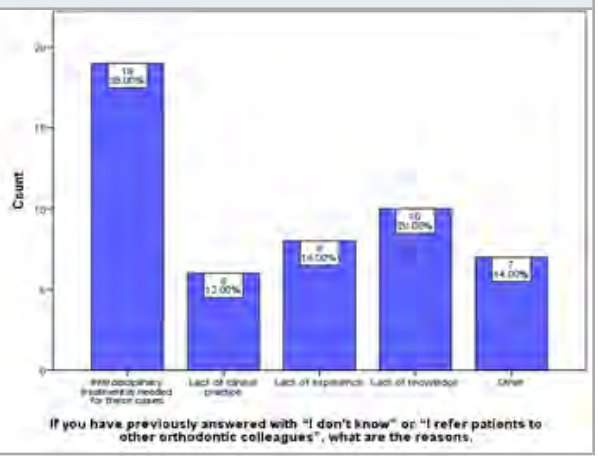
performed treatment, pulp vitality, and pulp sensibility tests (thermal and electric) to determine the state of pulpal health by assessing the condition of the dental pulp nerves and blood flow. In addition, thorough evaluation including transillumination of enamel infraction lines in traumatized teeth, examination for any sinus tracts or swelling, investigation of tooth mobility, in both a horizontal and vertical directions, palpation over the tooth apex for any tenderness, and percussion tests diagnosing ankylosis and root fracture are all needed and imperative for comprehensive diagnosis and treatment plan (Kindelan et al. 2008; Batista et al. 2018).

Figure 3: Different charts for the participants' responses regarding the best management of a traumatized tooth with different types of dental trauma during orthodontic movement.



In the present study, most of the participants reported having treated patients with history of dental trauma in the previous 12-18 months. In the research design, this period was categorized as a relatively long enough to ensure that orthodontists were able to see patients with traumatic cases, as this survey was carried out during the Covid-19 lockdown. Furthermore, it was found that the patients from 11 to 15 years old with a history of dental trauma had the highest percentage of orthodontic visitations than any other patients' groups. This finding is consistent with the conclusion drawn by other researchers who found that the dental trauma is common among children and adolescents seeking orthodontic treatment (Bauss et al. 2004; Kindelan et al. 2008; Damé-Teixeira et al. 2013; Azami-Aghdash et al. 2015).

Figure 4: The participants' responses for the reasons why they refer patients with a history of different types of dental trauma to other orthodontic colleagues.



During the initial orthodontic assessment of patients, more than half of the participants routinely inquire about their patients' dental trauma history, which underscores the importance of appropriate history investigation, and hence enabling orthodontists to provide an adequate management course customized according to each case's need, to better prepare for any possible future complications, and to enhance the prognosis of traumatized teeth during OTM as reported in the literature (Kindelan et al. 2008; Duggal et al. 2015). Moreover, 25.9% of the participants do inquire about their patients' dental trauma history only if signs of trauma are evident and observable. Similar finding was illustrated by Sandler et al. (2019) who concluded a wide variation in the orthodontic management of traumatized teeth among UK-based orthodontists (Sandler et al., 2019). Fewer participants in the study inquire about history of trauma if both signs of trauma and increased overjet are present (6.6%), while others inquire only if there is an increase in overjet (1.2%).

This finding could be attributed to the fact that an increased overjet of more than 3mm is one of the predisposing factors that require early orthodontic intervention in order to reduce the risk of dental trauma (Petti 2015; Alhammadi et al. 2018; Batista et al. 2018). The significant relationship in the present study between participants' job title and their tendency to inquire about previous dental trauma during initial orthodontic assessment of patients indicates that those with more experience are more inclined to ask about previous dental traumas more frequently compared to those with less experience or less training in orthodontics. Some of the consequences of dental trauma investigated in this research included crown fracture, root fracture, RCT of traumatized teeth, ankylosis, concussion, and intrusion luxation. In regards to the time required before the initiation of OTM for traumatized teeth, it has been agreed upon in the literature that there is a different observation period for each type of trauma, depending on the severity of the injury

based on the expert opinion (Malmgren and Malmgren 2007; Kindelan et al. 2008; Duggal et al. 2015; Sandler et al. 2019).

When the participants were asked about the time required before the initiation of OTM in cases of crown, crown\ root fractures, teeth with concussion, and teeth with intrusion luxation, the majority agreed that three months are sufficient observation period prior to the initiation of an active OTM in order to avoid any serious complication such as inflammation and pulpal necrosis. Moreover, most of the participants reported that teeth with root fractures and ankylosed teeth due to severe traumatic injuries should have 12-months observation period prior to the orthodontic treatment. These findings are consistent with the guidelines and recommendations reported by several researchers, except for intrusion luxation, for which the recommended observation period prior to OTM is 6-12 months if no ankylosis can be detected (Zachrisson and Jacobsen 1974) Malmgren and Malmgren 2007) (Kindelan et al. 2008; Hermann et al. 2012) (Duggal et al. 2015) (Sandler et al., 2019).

On the other hand, different responses were reported when participants were asked about the observation period prior to OTM needed for root canal treatment of closed apex teeth due to trauma. Most of the participants (42.7%) believed that a 3-months of observation following a well-cleaned, good quality obturated root filling, and good seal are adequate to commence OTM, while 37.3% of the participants recommended an immediate OTM of root canal treated teeth. These findings are consistent with previous findings in the literatures (Drysdale et al.1996; Sandler et al. 2019). They, however, contradict other studies that concluded a period of one year was recommended prior to commencement of orthodontic treatment and necessary to increase the possibility of complete healing without ankylosis (Duggal et al., 2015) Malmgren and Malmgren 2007). In the present study, statistically significant differences were found among the participants opinions regarding the recommended observation period prior to the initiation of an active OTM for patients with different types of dental trauma. In contrast to GPs interested and trained in orthodontics, the majority of orthodontic residents, specialists, and consultants were in agreement of most of their responses as observed in Table 3. This could be attributed to the lack of knowledge and clinical judgment of the GPs compared to those who have specialized and experienced in the field of orthodontics.

The majority of responses in this study opted toward performing regular radiographic examination, reducing the applied orthodontic forces, and performing regular pulp vitality tests as the best follow-up management of a traumatized tooth with different types of dental trauma, including crown and crown/root fractures, root fracture, minor and severe damage to the periodontium, during OTM. This finding is consistent with the conclusion of several studies and it could be explained and supported by the fact that traumatized teeth or teeth showing signs of pretreatment root resorption undergoing OTM are known to have a low response to vitality testing, high rate of pulpal canal obliteration, and high risk of increased root resorption as a result of orthodontic forces.

Hence, it has been recommended to follow-up with these patients after being treated orthodontically with regular radiographic examination and pulp vitality tests at consecutive intervals (Brin et al. 1991; Malmgren and Malmgren 2007; Kindelan et al. 2008; Duggal et al. 2015; Jaradat and Rahhal 2016; Sandler et al. 2019). Furthermore, most of the participants responded that taking regular radiographic examination is the proper management of traumatized teeth treated with RCT. This finding corresponds with Malmgren et al. (2007), who advocate radiographic monitoring by taking radiographs of root filled teeth before OTM begins, and to be repeated in six months after the start of orthodontic therapy (Malmgren et al. 2007). In contrast, 44% of the participants reported that managing traumatized teeth treated with RCT should be dealt with same way they treat non-traumatized teeth. Similar finding was reported by Sandler et al. (2019). This can be attributed to the conclusion that there is no significant difference in the root resorption in both root canal treated teeth and vital teeth subjected to the same orthodontic forces (Esteves et al. 2007; Sandler et al. 2019).

Nonetheless, when the participants were asked about the management of root fractures and ankylosed teeth due to severe traumatic injuries, the majority of responses were to perform regular radiographic examination for root fractured tooth, and to leave off arch wire for ankylosed tooth (70%, and 56.6%, respectively). These findings are similar to the previously reported studies which concluded that a long-term follow-up of applying light orthodontic forces and radiographic evaluation are recommended for root fractured and ankylosed cases (Kindelan et al. 2008; Bauss et al. 2008; De Souza et al.2015; Sandler et al. 2019). In contrast to the previous groups that answered with specific treatment plans and clear management course of OTM, fifty participants (30%) have answered with "I don't know" or "I refer patients to other orthodontic colleagues". In addition, 38% of the participants preferred to refer such patients to their colleagues due to the fact that interdisciplinary treatment is needed for such cases, while 20% and 16% of participants either had insufficient knowledge or lack of sufficient experience, respectively.

This illustrates the lack of guidelines in the literature regarding orthodontic movement of traumatized teeth, and highlights the necessity for further increasing the orthodontist' awareness regarding the management techniques required for different cases of traumatized teeth. Finally, it is necessary to mention that the present study has some limitations. Most importantly is the small sample size and sample distribution representing the Saudi orthodontists' knowledge and skills towards the time required before the initiation of OTM, and the best orthodontic management of traumatized teeth. Therefore, further studies are required to increase the sample size and improve sample distribution to include other regions of Saudi Arabia and larger number of Saudi orthodontists. In addition, another study is needed to assess how to orthodontically manage different types of traumatized teeth such as alveolar fractures, extrusion

luxation, avulsed teeth, immature traumatized teeth, and auto-transplanted teeth.

CONCLUSION

The findings of the present study suggests that it is imperative for any orthodontist to perform an accurate history investigation about any previous dental trauma, and make such an inquiry a major part of every orthodontic diagnosis. In addition, when dealing with traumatized teeth, it is necessary to perform a thorough clinical and radiographic evaluation before and during the orthodontic treatment. Some of the traumatized teeth require endodontic approaches, while others need splinting or even surgical or orthodontic repositioning such as intruded traumatized teeth before initiating the orthodontic treatment. It is; therefore, essential to evaluate any traumatic teeth for pulp vitality, root resorption, and signs of ankylosis that may complicate the course of orthodontic treatment. In regard to the time required before initiating orthodontic movements of traumatized teeth cases, it has been concluded that there is a different observation period for each type of trauma, depending on the severity of the injury based on expert evaluation.

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Conflict of Interest: None we certify that this work has not been published previously and is not under consideration by another journal.

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Biomedical Communication

The Role of Pro-Inflammatory Cytokines in Sickle Cell Disease Saudi Patients

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ABSTRACT

Sickle cell disease (SCD) is an inherited disorder of hemoglobin structure and synthesis, with chronic hemolysis, repeated infections, and recurring microcirculation occlusions. Cytokines play a role in hemopoiesis control, immune function suppression, and the development of growth deficits. This study aimed to determine and analyze some important pro-inflammatory cytokine (IL-8, IL-18, IL-1β, and TNF-α) levels and compare them in patients with SCD versus healthy males and females of the general Saudi population. This comparison helps to explain the possible role of cytokines in the development of SCD and the effect of hydroxyurea on the cytokines. A cohort study was performed that included 56 patients Sickle cell anemia patients with hydroxyurea (SCAHU) and Sickle cell anemia patients without hydroxyurea (SCA) and 22 controls (healthy group). Plasma cytokine concentration was detected using ELISA and compared in the different groups. The results of this study showed that there were significant differences in the levels of cytokines between the control, SCA, and SCAHU groups. Data showed that plasma cytokine levels were significantly higher in the SCAHU group compared to the control. Additionally, plasma cytokine levels were significantly higher in SCA group compared to the control individuals. However, there was no significant differences between the SCA and SCAHU groups. The current observations suggest that cytokine levels are associated with SCD, and hydroxyurea does not have an effect on controlling the pro-inflammatory cytokine levels in patients. The persistently elevated levels of pro-inflammatory cytokines significantly contribute to the pathogenesis and the severity of pain in Sickle cell anemia.

KEY WORDS: HYDROXYUREA, PRO-INFLAMMATORY CYTOKINES, SICKLE CELL DISEASE.

INTRODUCTION

Chronic hemolysis, repeated infections, and persistent occlusions of the microcirculation are all symptoms of sickle cell disease (SCD), a hereditary disorder of hemoglobin (Hb) structure and synthesis. These complications cause painful crises and eventually result in chronic organ damage, disability, and death (MJ, 2004). Vaso-occlusion is a direct cause of morbidity and mortality in patients with SCD (Keikhaei et al., 2013). SCD is caused by a mutation in the sixth codon of the beta globin gene, which causes the nitrogenous base adenine to be replaced by thymine; thus, the glutamic acid is replaced by valine, resulting in hemoglobin (HgbS) (Connes et al., 2018). This mutation can be homozygous (SS), resulting in sickle cell anemia, a disorder of increased severity and frequency (Macharia et al., 2018), or a mutation in combination with other hemoglobin defects—including Hb C, D, E, and beta-thalassemia (Allali et al. 2020). SCD refers to all symptomatic variants of this gene (Sílvia et al., 2019).

Cytokines have been linked to several possible mechanisms vaso-occlusion in SCD: vascular endothelial activation, red-cell adhesion to vascular endothelium, neutrophil adhesion to endothelium, development of vascular intimal hyperplasia, platelet activation, endothelin-1 synthesis, and endothelial apoptosis dysregulation. Additionally, cytokines are believed to play a role in hemopoiesis control, immune function inhibition, and the production of growing deficits. The study of cytokines in SCD patients will shed light on the pathogenesis and symptoms of the disease, as well as

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aid in determining the nature and prognosis of the disease (Darbari et al., 2020).

The use of Hydroxyurea (HU) to treat people with SCA has been shown to lower white blood cell (WBC) counts and myeloperoxidase function (Gueye-Tall et al., 2020), as well as cell adhesion properties. Despite evidence of the important role of leukocytes in modulating clinical and pathophysiological aspects of SCA, the mechanism by which leukocyte numbers rise in SCA patients remains unknown, though changes in leukocyte-producing cytokines have been suggested to participate in this phenomenon (Allali et al., 2020). The aim of this study was to compare IL-8, IL-18, IL-1β, and TNF-α pro-inflammatory cytokine levels between patients with SCD, SCAHU and a control group of healthy men and women in the general Saudi population. This would help understand the possible role of pro-inflammatory cytokines in disease development and the effect of hydroxyurea on these cytokines.

MATERIAL AND METHODS

The study was performed after obtaining the ethical approval number (243-19) from The Biomedical Research Ethics Committee, Ministry of Higher Education, King Abdulaziz University, Faculty of Medicine (Reference No 243-19). The study included 78 males and females, 22 of whom were in the control group, and 56 of whom were divided into two groups: steady-state patients and SCA patients under hydroxyurea therapy (SCAHU) who were diagnosed and who followed up with the Hematology and Hemotherapy Center of King Abdulaziz University Hospital. Each of them was issued a written and informed consent for their participation in the study.

Blood samples were collected from the patients (5 mL of venous blood) in ethylenediaminetetraacetic acid (EDTA) tubes. The following parameters were measured: IL-8, IL-18, IL-1β, and TNF-α using ELISA Kits in plasma to estimate the concentration of the pro-inflammatory cytokines. The following ELISA kits were used in the study: Human IL-18 (Interleukin 18) ELISA Kit, catalog number: E-EL-H025396T; Human IL-1β (Interleukin 1 Beta), catalog number: E-EL-H014996T; Human TNF-α (Tumor Necrosis Factor Alpha) ELISA Kit,catalog number:E-EL-H010996T; and Human IL-8 (Interleukin 8(ELISA Kit, catalog number: E-EL-H004896T. The data was analyzed using the IBM Statistical Package for Social Science (SPSS), version 22, for Windows. The results were expressed as mean ± SEM. Both the Kruskal-Wallis test and Mann-Whitney U test were used, and a p-value less than 0.05 was considered significant (Domingos et al., 2020).

Table 1. Characteristic and hematological parameters of the participants (The results expressed as mean ± SD). WBC = white blood cells, RBC = red blood cells, NS = not significant at p < 0.05.

Parameters	Control (n=22) A	SCAHU (n=30) B	SCA (n=26) C
Age (years)	28.3 ± 5.2	26 ± 7.9	29 ± 9.2
Male/female	15-Jul	18-Dec	16/10
WBC (10∧3 /µL)	5.4 ± 1.2	11.8 ± 3.4	11.4 ± 4.7
RBC (10∧6 /µL)	4.5 ± 0.6	2.4 ± 0.2	3.2 ± 0.9
Hemoglobin (Hb) [g/dl]	12.8 ± 1.2	7.5 ± 0.7	8.8 ± 1.2
Hematocrit (HCT) [%]	38.2 ± 3.2	21.9 ± 1.8	26.7 ± 4.6
Mean corpuscular hemoglobin (MCH) [pg]	27.9 ± 2.8	30.5 ± 2.1	27.9 ± 4.5
Mean corpuscular volume (MCV) [fL]	83.2 ± 6.8	93.3 ± 9.7	83.3 ± 9.7
Platelets (10∧3/µL)	287.7 ± 45.6	342.6 ± 144.6	515.8 ± 142.5
Neutrophils (10∧3/µL)	2.5 ± 0.7	5.9 ± 2.1	5.53 ± 2.5
Lymphocytes (10∧3/µL)	2.2 ± 0.5	4.01 ± 0.9	4.66 ± 2.6
Monocytes (10∧3/µL)	0.5 ± 0.1	1.1 ± 0.4	0.99 ± 0.46
Eosinophil (10∧3/µL)	0.17 ± 0.12	0.4 ± 0.4	0.38 ± 0.27
Basophil (10∧3/µL)	0.04 ± 0.02	0.1 ± 0.08	0.085 ± 0.04

RESULTS AND DISCUSSION

Table 1 described the participants' hematological characteristics for the control group, the SCA group, and the SCAHU group. Looking into the hematological characteristics of all three groups, a significant statistical difference was reported as follows: there were statistically significant differences between the control group and the SCA group for WBC, RBC, hemoglobin (Hb), hematocrit (HCT), platelets, neutrophils, lymphocytes, monocytes, eosinophils, and basophils, where the p-value was less than 0.05. Additionally, there were significant differences between the control group and the SCAHU group for WBC, RBC, hemoglobin (Hb), hematocrit (HCT), mean corpuscular hemoglobin (MC), mean corpuscular volume (MCV), neutrophils, lymphocytes, monocytes, eosinophils,

and basophils, where the p-value was less than 0.05. Additionally, there were significant differences between the SCA and SCAHU groups for RBC, hemoglobin (Hb), hematocrit (HCT), and platelets, where the p-value was less than 0.05.

The data showed that the plasma IL-8, IL-18, TNF-α, and IL-1β levels were significantly higher in the SCAHU group compared to the control group (p-value = 0.001). Also, IL-8, IL-18, TNF-α, and IL-1β levels were significantly higher in the SCA group compared to the control group (p-value = 0.001), while no significant difference between the SCA and SCAHU groups was detected for all previous cytokines as described in Figures 1-4.

Figure 1: Comparison of IL-8 plasma level in control, SCA steady-state and SCAHU. The data showed that SCAHU has the highest level of IL-8 compared to control and SCA. A significant higher level was recorded in SCAHU and SCA compared to control group (p = 0.001), but no significant difference between the other groups recorded.

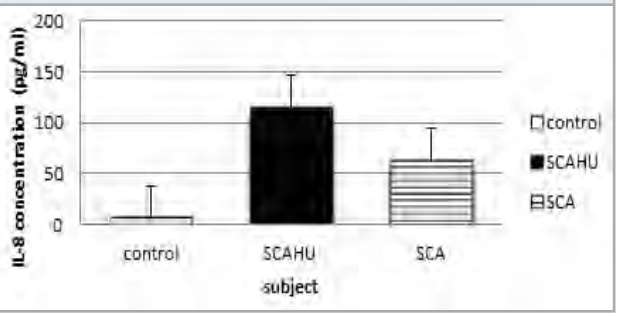
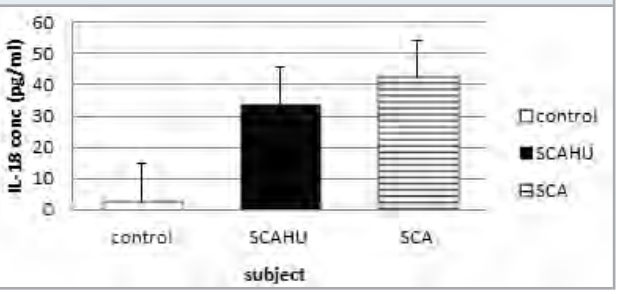


Figure 2: Comparison of IL-18 plasma level in control, SCA steady-state and SCAHU. The data showed that SCA has the highest concentration of IL-18 compared to control and SCAHU. IL-18 level was significantly higher in SCA group than the control group (p = 0.001), and also significantly higher in SCAHU group than the control group (p = 0.001). However, there is no significant differences in the level of IL-18 between SCA and SCAHU.



In the present study, our findings showed that levels of pro-inflammatory cytokines (IL-8, IL-18, IL-1β, and TNF-α) were higher in SCAHU patients compared to healthy men and women in the control group. The elevated levels of cytokines observed may be because of the increased secretion by the leukocytes and platelets that were significantly elevated in the SCAHU patients compared to the control. The data from this study showed a significant difference in the concentration of the studied cytokines IL-8, TNF-α, IL-18, and IL-1β. All of the cytokines were higher in the SCA and SCAHU groups compared to the control group. More specifically, the data from IL-8 and TNF-α suggest a higher concentration in the hydroxyurea-treated group. Significantly, the elevated TNF-α found in the SCA classes matches the results from Lanaro et al. (2009) and Goncalves et al. (2001).

Figure 3: Comparison of TNF-α plasma level in control, SCA steady-state and SCAHU.
The data showed that SCAHU has the highest level of TNF-α compared to control and SCA. Significant higher levels were recorded in SCA group and SCAHU than the control group (p = 0.001). However, there is no significant differences in the level of TNF-α between SCA and SCAHU.

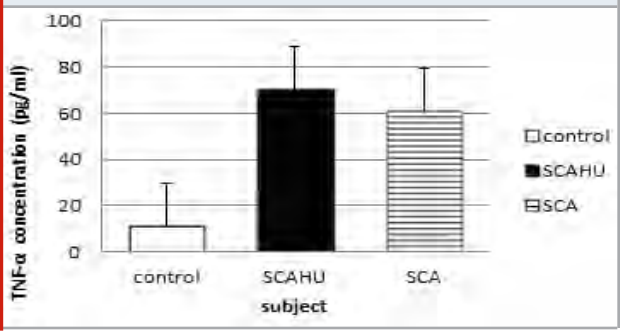
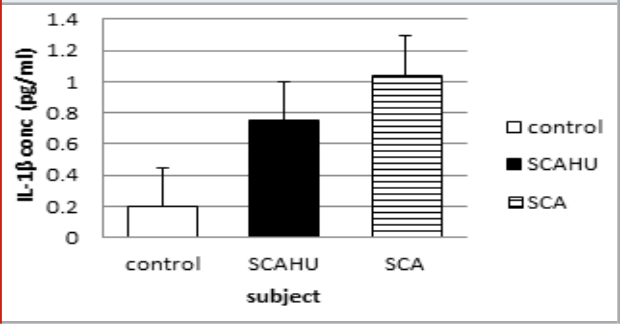


Figure 4: Comparison of IL-1β plasma level in control, SCA steady-state and SCAHU. The data showed that SCA has the highest concentration of IL-1β compared to control and SCAHU. A significant higher level as recorded in SCAHU than the control group (p = 0.001), also a significant higher level in SCA group than the control was recorded (p = 0.001). However, there is no significant differences in the level of IL-1β between SCA and SCAHU.



Both groups of researchers found that SCA patients had higher levels of circulating TNF-α at a steady-state and during the crisis (Gonçalves et al., 2001, Lanaro et al., 2009). However, these studies differed from a study by Laurentino et al. (2014), who found that patients receiving HU treatment had lower TNF-α levels compared to patients who were not receiving HU (Laurentino et al., 2014). TNF-α stimulates leukocytes and the coagulation cascade, resulting in an increase in the plasma levels of acute-phase plasma proteins, including fibrinogen, which aids erythrocyte adhesion to endothelium. In SCA patients, these factors

contributed to the growth of vascular occlusion (Eggestøl et al., 2020).

The neutrophils react to IL-8 as a chemotactic component. IL-8 is thought to be the mediator of the neutrophil activation seen in SCA patients during Vaso-occlusive crises (VOC) and is supplemented by other pro-inflammatory mediators (Liu et al., 2020). The findings of higher plasma IL-8 levels in patients with SCAHU and SCA agree with those of other researchers who discovered higher IL-8 levels in patients with acute chest syndrome and patients with vaso-occlusion (Domingos et al., 2020). IL-8 promotes inflammation through the adherence of sickle red cells to the endothelium through the α4β1 integrin receptors on sickle reticulocytes (Etienne-Julan et al., 2004). The passive mechanical obstruction that leads to vaso-occlusion in SCA is aided by the active mechanism of endothelial adhesion (Grunenwald et al., 2021).

TNF-α levels in the blood were higher in SCD patients in steady-state trials (Tavakkoli et al., 2004) and during crisis events (Pathare et al., 2004). On the other hand, circulating IL-8 was historically only found in SCD patients who were in a state of distress or who presented with an acute syndrome (Abboud et al., 2000). TNF-α, a pro-inflammatory cytokine, has been implicated in a variety of inflammatory disorders, including sepsis, rheumatoid arthritis, and Crohn's disease, where it tends to play a role in upregulating the cytokine cascades that trigger inflammation (Clark, 2007). IL-8 is an important mediator in neutrophil-mediated acute inflammation as well as a central player in a variety of chronic inflammatory disorders and lung pathologies. Pro-inflammatory cytokines—TNF-α and IL-1β, as well as other inflammatory molecules, such as bacterial products—can induce IL-8 production at the level of gene transcription and mRNA stability (Tsai, 2020).

In SCD, IL-1β is thought to play a role in vaso-occlusion through its involvement in inflammation, cellular adhesion, signaling, transmission, and coagulation. In our data, IL-1β levels were high in both the SCA and SCAHU groups. One study observed that cytokine IL-1β was significantly higher in steady-state SCD patients when compared to control group (Pathare et al., 2003). IL-18 is a pleiotropic cytokine that promotes neutrophil accumulation and induces acute inflammation in the innate immune system. These functions may cause the effects seen in the vascular events in SCA, but previous research has not found a function for IL-18 in SCA patients. The IL-18 levels in the blood have been shown to increase during a serious sepsis episode, implying that this cytokine plays a role in both host defense and possible host damage because of tissue lesions. In this study, the plasma IL-18 levels were significantly higher in both the SCAHU and SCA groups versus the control, but there was no significant difference between the SCA and SCAHU groups. According to Cerquera (2011), SCD and vaso-occlusion are linked to IL-18, also typical red blood cell deformation-related disease and endothelium activation are heavily related to the same cytokines.

VOC is a multi-factorial mechanism causing hematological, immunological, and thrombotic abnormalities, according to several studies (Brittain et al., 2008) (Uwaezuoke et al., 2018). The endothelial defects increased sickle red cell adherence to the vascular endothelium, plasma proteins and cytokines, and leukocyte activation, especially during neutrophil activation. All these play important roles in SCD vaso-occlusion and pathophysiology (Canalli et al., 2004). Further activation of the endothelial cells through inflammatory cytokines released from activated endothelial cells (IL-1β, IL-8, and TNF-α), activated platelets (IL-1β and TNF-α), and monocytesmacrophages (IL-1β and TNF-α) improve the interaction between HbSS erythrocytes and the vascular endothelium. As a result, investigating the impact of cytokines on the vascular endothelium and adhesion molecule expression, as well as their possible function in clinical outcomes in SCD patients, is a fascinating and important field of research (Vilas-Boas et al., 2010) (Barbu et al., 2020). In one study, the evaluation of serum cytokines in all SCD patients was compared to normal controls. All inflammatory cytokines, except IL-1β, showed an elevated levels in both steady-state and VOC. All cytokines showed substantial variations between VOC patients and controls, with the exception of IL-1β, which had no significant, detectable levels in the serum of patients in the steady-state condition or normal controls (Silva-Junior et al., 2021).

HU, the first drug with any clinically proven efficacy for SCA, can affect cell adhesion through several possible mechanisms, such as reduced HbSS erythrocyte adhesion, VCAM-1 down-regulation, and endothelin-1 expression, which are likely to contribute to the reduction of VOC episodes (Osunkwo 2020). Lanaro et al. (2009), reported that TNF-α levels in the blood were shown to be slightly lower in patients taking HU. TNF-α and IL-8 gene expression levels were also shown to differ significantly between patients who received and did not receive HU therapy (Lanaro et al., 2009). In line with the afore mentioned study, we observed the possibility for a higher concentration of TNF-α and IL-8 in the HU group, but there was no significant difference in HU-receiving patients compared to those who did not receive the HU treatment. However, the beneficial effects of HU in SCD are regulated in part by elevated Hb F levels, which suppress HbS polymerization in red cells and are linked to a milder type of the disease (Croizat and Nagel, 1999). Our results agree with a study that reported the beneficial effects of HU-induced increases in TNF-α levels in SCD patients (Tavakkoli et al., 2004). While previous findings suggest that HU may play a role in the reduction of vaso-occlusive episodes in SCD patients, further research is required to determine the exact mechanism of action for HU in relation to adhesion molecules and other inflammatory mediators, including cytokines (Osunkwo et al., 2020).

CONCLUSION

In conclusion, the current observations suggest that changes in cytokine levels are associated with SCD. The persistently elevated levels of these pro-inflammatory cytokines have further confirmed that SCA is a chronic inflammatory state and contributes significantly to the pathogenesis and the severity of pain in SCA. Additionally, HU treatment did not show any improvement in the control of the elevated levels of the pro-inflammatory cytokines investigated, but

this needs further testing for confirmation. Therapies that target these inflammatory peptides could help to ameliorate or forestall bone pain crises in SCA. The results of this study suggest several interesting avenues of investigation that could be studied in the future.

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Biomedical Communication

Increase in the use of Electronic Applications – An impact analysis of COVID – 19 Pandemic

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ABSTRACT

This paper endeavors to research the part of COVID – 19 spreads in expanding the utilization of electronic applications. The examination covers the period from June 15, 2020 to July 15, 2020. Very much organized poll used to gather the information from the respondents. Garrett's Ranking Technique and F - test were received to test the exploration questions. The aftereffects of the positioning shows that amusement classifications of electronic applications are most downloaded class among different classes. The F-test examination results demonstrated that the spread of Covid prompted expanding the utilization of electronic applications. In this article, the utilization for the electronic applications for respondents of the financial elements are considered. The statistical analysis is attained on the observed results of the respondents. The statistical analysis is accomplished to observe the variance among the data and it explains the outcome of the respondents.

KEY WORDS: COVID – 19, RANKING, ELECTRONIC APPLICATIONS.

INTRODUCTION

Covid – 19 directly is a usual pandemic. Struck at domestic for the duration of the Covid pandemic the large portions of the matters were closed. Thusly, social orders spending an extra noteworthy measure of their continues with on the web. With incredibly tons all open social affairs dropped, Americans are searching out delight on digital highlights like Netflix and YouTube, and wanting to interface with one another through on line news sources like Facebook (Reyes, 2020). Since we are experiencing our days at home, with PCs close enough, social orders are come into center to recall how lousy it might be to squint at these little telephone screens. With the climb of social isolating, we are looking for out better approaches to deal with interface, typically thru video visit. While developed online media areas have been creating, it has all the earmarks of being that we have to gain some exclusive option from interface via illuminating and text — we have to see one another (Alexopoulos et al., 2020).

This has given a great raise to applications that used to stay at the back of in relative cloudiness, like Google's video travelling application, Duo, and House party, which provides get-togethers of allies to be part of a lone video speak and jumble around together. We have similarly grown in truth more stressed in our passing condition, and how it is moving and responding to the disorder and the confine techniques. This has provoked a re-established energy for Next-door, the online media web page targeted in on companion shut by way of neighborhoods. We have out of the blue gotten reliant on corporations that license us to work and addition from domestic the working environments and schools of wherever on over the world have all moved into our parlours. Nothing is having an extra shrewd have an effect on online development than this change. School undertakings are being given out on Google Classroom. Get-togethers are taking place on Zoom, Google Meet and Microsoft Teams (Kristóf, 2020, Haleem et al., 2020).

The rush to these companies is that as it might, have invited new evaluation on safety practices. The ride for invigorates on the contamination has pushed up readership for neighbourhood and set up papers, with the amount of useless increasing continually -society are show up to require hardly ever any things extra than the today's news on the Covid. Among the excellent beneficiaries are neighbourhood information objections, with big bounces in busy time gridlock as humans endeavor to make sense of how the

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pandemic is affecting the spots where they grew up. Social orders have in like manner been searching for extra settled media brands for records on the average prosperity disaster and its money associated results. The enterprise information website has viewed readership take off. The locations for top most associations have both created site visitors more than 50 percentage for the duration of the today's month, as shown with the aid of Similar Web.

PC games have been getting whilst sports have surpassed up a top-notch probability with all major-affiliation games dropped; there haven't been a variety of games to smash through. At the identical time, a couple of PC game districts have had floods in busy time gridlock, as have objections that let you watch others play. Jolt, the principal website for streaming sport play, has had traffic shoot up 20 percent. Saudi's net use rose by way of 33% on the grounds that the cross-country lockdown used to be set up to check the spread of Covid-19, as shown by correspondence and Information development fee regular step via step use extended 33% higher than the year back. The selection through media associations to handicap outstanding video and improve bandwidth utilization helped networks fulfil the extended need, consisting of that the chairmen also started out using unused phone towers. Saudi's usage fees have considered a predictable augmentation during the modern couple of years.

Speed Test, a website that assessments internet get entry to execution over the globe, in its cutting-edge file on following COVID-19's have an effect on speeds all over which was revived on April 15, proven a 6% decline in fixed line speeds and 18% in compact speeds when diverged from the multi day stretch of March 2 As indicated by the report, Saudi's current broadband speed is a normal of 69.86 mbps and handy down load pace is 71.73 mbps. Saudi's month to month dynamic internet consumer base is evaluated to exhibit up at 30.47 million previous the completion of 2020, in light of the Covid lockdown that has pushed human's interior residences with nearly no else to do. By and by way of evaluated at 29.91 million, the amount of month-to-month dynamic web clients has enrolled a every year development of 24%, indicating an average invasion of 41% in 2019.

About 84% clients get right of entry to the internet for preoccupation purposes. The 12 months 2019 saw a flood in OTT (over-the-top online), both sound and video, pushed through exclusive substance and cricket (both the IPL and the cricket World Cup spouted on OTT stages) other than the supportive openness of substance across devices and in a rush insignificant exertion of web services which finished big improvement in redirection usage consistently. This depended upon to proceed in 2020 also, especially considering the Covid lockdown, the report says. Kantar reviews enhancement of over 60% in step through step Internet customers over the modern one year; and practically 9 out of 10 unique Internet customers had been getting to the Internet for satisfaction and correspondence needs.

At 38%, school-going teenager's area, of 15 years of age or below has tested a promising improvement in Internet usage. Permission to statistics and preparing, on line media, gaming and preoccupation, especially, sports, is driving the gathering. With demonstrating this speculative institution, the examination makes an assignment to find which grouping of digital applications through and giant desired to use and the respondent's utilization level of the digital applications (Amin et al., 2020, Whitelaw et al., 2020). In this article, the utilization for the electronic applications for respondents of the financial elements are considered. The statistical analysis is attained on the observed results of the respondents. The statistical analysis is accomplished to observe the variance among the data and it explains the outcome of the respondents.

Review of Literature: The world health organization has released the 175 situational report and it highlights the COVID-19 reality across the world. The COVID-19 has made complicated situation among the people and also made huge impact on various domains. The context of pandemic has ruined diversified aspects of every individual, which also necessitated numerous contacts less application that is largely incorporated in education, business and in hospitals as telemedicine (Singh, 2020). This context necessitated a detailed review on numerous applications and scenario of COVID-19. Iyengar et al., (2020) examined the COVID 19 and uses of cell telephone innovation in the contemporary pandemic.

They feature in the examination mobile phone improvements evade eye to eye discussions that convey congruity of medical services for the duration of the pandemic, phone cell phone advances supply progression of care by means of staying away from bodily contact and maintaining up social removing. Cell smartphone innovations will count on indispensable characteristic later on for medical offerings conveyance. Haleem et al., (2020) examined " Effects of COVID – 19 Pandemic in day through day life", COVID – 19 has quickly influenced our everyday life, organizations, upset world alternate developments. Distinguishing proof contamination at commencing segment is a fundamental to manipulate the unfold of the contamination due to the fact it rapidly spreads from the individual to individual.

In their investigation the consequences of COVID – 19 in day via day lifestyles are vast and expansive effects into three considerable lessons they are Healthcare, Economic, social. Ayittey at al., (2020) gauge that, without urgent international things to do to abridge the Wuhan 2019-nCoV internal the most short achievable time, China is relied upon to lose up to $62 billion21 in the fundamental quarter of the year, while the world is probable going to lose over $280 billion internal the equivalent period.15 This cease thinks

about near the World Banks evaluation that even an extra fragile influenza pandemic, for example, the2009 H1N1 infections, may want to at current wipe 0.5% off global GDP, which provides up to round $300 billion. Tasnim, et al., (2020) predicted to discover Impact of gossipy titbits or deception on Covid illness (COVID-19) in web-based media. The investigation verified.

The COVID-19 pandemic has no longer simply prompted noteworthy difficulties for well-being framework in all places on over the globe but in addition energized the flood of a variety of bits of gossip, deceptions and falsehood, with recognize to etiology, results, avoidance, and restoration of the malady. This falsehood is concealing solid practices and advancing mistaken practices that growth the unfold of the contamination and eventually carry about bad bodily and psychological wellness effects amongst people. Horde episodes of incidents added about by means of these gossipy tidbits was once accounted for over the world.

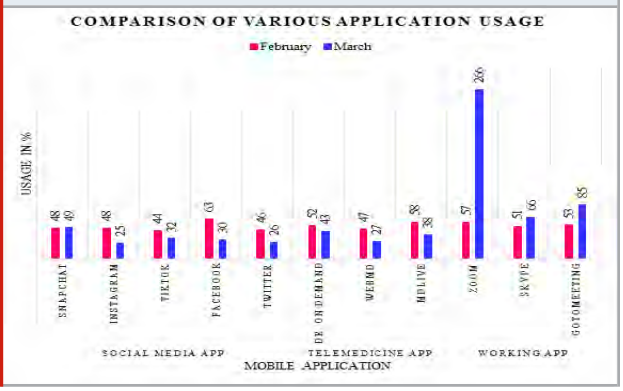
Branscombe, (2020) examined the corporation impact of the Global Covid 0 19 pandemic. In this investigation. The lockdown has come about that a massive element of the folks searching for internet and net based administrations to impart, Interact and proceed with their responsibilities from home. Internet carriers have considered ascends in use from 40% to 100% contrasted with prelock down levels. Video conferencing administrations like zoom have ten times increment in use, (Branscombe, 2020). Elsayed and Elrhim, (2020) to observe the impacts of the unfold of COVID-19 on global on-line business organizations, where the five biggest internet based totally commercial enterprise companies on the planet were picked as some distance as incomes and market worth, and they had been as per the following: American Amazon, Chinese Alibaba, Japanese Rakuten, German Zalando, United realm ASOS.

The investigation results endorse that the level of the effect of Covid spread shifted beginning with one employer then onto the next, contingent upon the state to which it had a place, the place the American company Amazon and the United realm company ASOS had been "the total instances of disorder are the most persuasive and this is regular with that they are the most influenced countries of the Covid at some stage in the time of examination, and the Chinese business enterprise Alibaba and Rakuten organisation Japanese "Covid cases" have been the most effective in their provide value returns, and the German agency Zalando used to be the most compelling variable "total passings".

Mohammad (2016) made an investigation with the desires of to apprehend the elements that have an effect on purchasers for choosing net based shopping; to be aware of the shoppers' inclinations in regards to items/administrations they typically store on the web; to distinguish and seem at the mentality of male and female respondents for loving or loathing the web shopping. The facts have been gathered through a prepared survey from 83 respondents arbitrarily from a range of regions of Chittagong City, Bangladesh from the period of September to November, 2014. The investigation uncovers that web page publicizing (45.78%), T.V. Promotion (20.48%), loved ones (15.66%) are the substantial media through which they got comfy about internet shopping. The accessibility of assortments (31.33%), much less time (30.12%) and low fee (20.48%) are the widespread explanations behind favouring net primarily based shopping. The educational fields, industries, and other business fields have demanded the renovation in the way of individual's work nature and collaboration within the working atmosphere that potentially influence the daily actions Barnes, (2020).

Whilst abundant companies and educational institutions forced to shut down or minimize the performance where the condition is subjective to social distancing Hellewell et al., (2020). Every industry and company across the world adapt the atmosphere of COVID-19 by instigating the altered patterns of work, remote working techniques, and establishment of communication with the assistance of a digital technology Leidner, (2020). During the pandemic several mobile based applications are highly used and widely utilised in the education, telemedicine and in the business environment. The application downloaded and utilised in the month of February and March is given in Figure (1) (Inmobi 2020). Purpose of this study to the primary target of the examination is to investigate the expansion in the use of electronic applications as for financial elements of the respondents.

Figure 1: Usage of Mobile Applications



Research Methodology: The current investigation is an endeavour to look at COVID - 19 in expanding the utilization of Electronic Applications. In this current investigation, to know the most downloaded classification of electronic Applications in this period utilizing Garrett's positioning strategy (Schliesser, 2011). To realize the utilization level and it was contrasted and financial components are having any effect on it utilizing F test. The necessary essential information has been gathered from 175 example respondents due to in sufficiency of information

160 reactions were taken for definite examination with an all-around organized and pre tried survey.

Sample Profile: Increase the utilization of Electronic Applications in this pandemic period segment factors assume a significant job. The segment highlight of this examination is shown in the Table 1.

Table 1. Sample Profile of the Respondents

	Percentage	Categories	Count
Gender	Male	95	59.3%
	Female	65	40.7%
Age	Young	27	16.8%
	Middle aged	87	54.3%
	Old	46	28.75%
Marital Status	Married	128	80%
	Unmarried	32	20%
Education	School	28	17.5%
Qualification	College	62	38.7%
	Professional	70	43.7%
	Student	24	15%
	Business	39	24.4%
	Employee	55	34.4%
	Profession	42	26.2%
Nature of Family	Joint	95	59.4%
	Nuclear	65	40.6%
Size of Family	Small	43	26.8%
	Medium	69	43.1%
	Large	48	30%

Most Downloaded Category Of Applications In This Pandemic By The Respondents: To distinguish most downloaded Application class in this pandemic period, Garrett's Ranking Technique has been utilized. Initially, 20 additional Applications have been remembered for the pilot study. By utilizing thing examination method, that 20 additional applications changed over into 6 significant classes to tending to the right one. At last, the Categories like Entertainment Applications, Educational Applications, Social Media Applications, Gaming Applications, and News Applications and Professional Applications were remembered for the timetable. All the major Apps have gone under this one of the classifications. The respondents were approached to give rank I to the most significant Category, rank II to the second significant class, etc. The positions given by the respondents are changed over into scores. Score esteem has been determined for the rank allotted by the respondents with the assistance of Garrett's positioning method. The subtleties are appeared in Table 2.

From the Table 2 it is surmised that the Entertainment applications with high mean score of 65.21 is 'gave to be the most downloaded application classification that utilized by the greater part of the advanced mobile phone clients in this pandemic period. Online Media Applications classification is positioned as second with the mean score of 63.61, Instructive Applications is positioned as third with the mean score of 62.91, Professional Applications is positioned as fourth with the mean score of 61.01, trailed by Gaming Applications and News Applications. Subsequently, it is reasoned that Entertainment classification applications has been positioned as most downloaded applications classification.

Table 2. Garrett's Ranking

Categories	Rank X	1 81	2 70	3 63	4 57	5 52	6 47	Total Score	Mean Score	Rank
Entertainment Apps	F	49	31	19	22	15	24	160	65.21	I
	Fx	3969	2170	1197	1254	780	1128	10498		
Education Apps	F	27	36	26	28	21	22	160	62.91	III
	Fx	2187	2520	1638	1596	1092	1034	10067		
Social media Apps	F	32	42	20	17	23	26	160	63.61	II
	Fx	2592	2940	1260	969	1196	1222	10179		
Gaming Apps	F	25	17	17	33	20	48	160	59.14	V
	Fx	2025	1190	1071	1881	1040	2256	9463		
News Apps	F	10	15	25	42	41	27	160	57.68	VI
	Fx	810	1050	1575	2394	2132	1269	9230		
Professional Apps	F	17	19	53	18	40	13	160	61.01	IV
	Fx	1377	1330	3339	1026	2080	611	9763		
Sample Size		160	160	160	160	160	160	160		

Quantification Of Data To Measure The Usage Level: A rundown of 20 articulations identifying with expanding the utilization of electronic applications is at first arranged. Based on result of the pilot study and by applying thing investigation strategy, 6 explanations are dropped out lastly 14 proclamations were remembered for the last

examination. Rensis Likert's summated 5-point scaling method is applied to discover the total expanding use level of the respondents. It is normal that financial attributes of the example respondents would impact the expanding the utilization level of respondents towards the electronic applications. To analyze the relationship between the expanding the utilization level and financial attributes, the accompanying invalid theory has been encircled.

Table 3. Age And Increasing Usage: 'F' Test.

Cause of Variation	Sum of Square	Degrees of Autonomy	Mean Square	'F' Value
Between Samples	1197.54	2	598.77	6.65
Within Samples	14140.65	157	90.06	
Total	15338.19	159		

Table 4. Gender And Increasing Usage: 'F' Test

Cause of Variation	Sum of Square	Degrees of Autonomy	Mean Square	'F' Value
Among Samples	118.08	2	59.04	0.61
Within Samples	15220.11	157	96.94	
Total	15338.19	159		

H_0: There is no critical relationship between the individual factors (age, sex, conjugal status, instructive capability, occupation, size of family, and nature of group) of respondents and their expanding utilization level.

Table 3 uncovers that the determined estimation of 'F' is 6.65 which is higher than the Table estimation of 2.99. Along these lines, it tends to be inferred that the relationship between the normal score of various age gathering and utilization level electronic uses of the example respondents is noteworthy. Table 4 uncovers that the determined worth (0.61) of 'F' is not exactly the Table worth (2.99). Accordingly, it tends to be presumed that the relationship between the normal score of Gender and expanding use level of electronic uses of the respondents is immaterial. Table 5 shows that the determined worth (2.52) of 'F' is not exactly the Table worth (2.99). Thusly, it very well may be presumed that the relationship between the normal score of conjugal status and expanding the utilization level of electronic uses of the respondents is discovered to be unimportant.

Table 5. Marital Status And Increasing Usage: 'F' Test

Cause of Variation	Sum of Square	Degrees of Autonomy	Mean Square	'F' Value
Among Samples	475.18	2	238.09	
Within Samples	14862.01	157	94.66	2.52
Total	15338.19	159		

Table 6. Education And Increasing Usage: 'F' Test

Cause of Variation	Sum of Square	Degrees of Autonomy	Mean Square	'F' Value
Among Samples	101.65	2	33.88	
Within Samples	15338.19	157	97.67	0.35
Total	15338.19	159		

Table 6 uncovers that the determined worth (0.35) is not exactly the Table worth. Appropriately, it is surmised that "F" esteem is immaterial. Subsequently, it tends to be inferred that that the relationship between the midpoints score of instruction and expanding utilization level of the example respondents is irrelevant. Table 7 uncovers that the determined worth (1.55) of 'F' is not exactly the Table worth (2.37). Subsequently, it tends to be inferred that the relationship between the normal score of occupation and expanding utilization level of electronic uses of the respondents is unimportant. Table 8 uncovers that the determined worth (4.64) of 'F' is higher than the Table worth (2.60). In this manner, it very well may be presumed that the relationship between the normal score of nature of the family and expanding use level of the electronic utilizations of the respondents is discovered to be critical.

Table 7. Occupation And Increasing Usage: 'F' Test

Cause of Variation	Sum of Square	Degrees of Autonomy	Mean Square	'F' Value
Among Samples	589.03	4	147.26	
Within Samples	14749.16	155	95.16	1.55
Total	10207.95	159		

Table 8. Nature Of The Family And Increasing Usage: 'F' Test

Cause of Variation	Sum of Square	Degrees of Autonomy	Mean Square	'F' Value
Among Samples	1225.53	3	418.51	
Within Samples	14082.66	156	90.27	4.64
Total	10207.95	159		

Table 9. Size Of Family And Increasing Usage: 'F' Test

Cause of Variation	Sum of Square	Degrees of Autonomy	Mean Square	'F' Value
Among Samples	15.13	2	7.565	
Within Samples	15323.06	157	97.60	0.08
Total	15338.19	159		

Table 9 uncovers that the determined worth (0.08) of 'F' is not exactly the Table worth (2.99). It tends to be inferred that the relationship between the normal score of size of family and expanding utilization level of electronic uses of the respondents is immaterial.

CONCLUSION

The current paper attempting to tend to the Increasing utilization of electronic applications in this pandemic period. And furthermore, decides the relationship between the financial qualities and expanding the use level. Expanding the utilization of electronic applications – an effect of COVID – 19 spread was estimated by the all-around organized poll comprises 14 explanations to address the expansion. The consequences of the F examination, the spread doesn't have critical relationship with Age of the respondents, Nature of the family. Interim, Gender, Marital status, educational capability, Occupation of the respondent, size of the family such financial elements are has relationship between the expanding the utilization level of electronic applications due to COVID – 19.

Amusement applications with high mean score of 65.21 is 'gave to be the most downloaded application classification that utilized by the majority of the advanced cell clients in this pandemic period. Online Media Applications class is positioned as second with the mean score of 63.61, Educational Applications is positioned as third with the mean score of 62.91, and Professional Applications is positioned as fourth with the mean score of 61.01, trailed by Gaming Applications and News Applications.

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Biomedical Communication

Hematological Parameters in Mature Age Men Who Have Begun Regular Sports Walking

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ABSTRACT

Rheological features of erythrocytes significantly determine the processes of microcirculation in all vessels of the body. In people of mature age, even without obvious pathology, there is a gradual deterioration in the rheological properties of erythrocytes. In this regard, they need regular exercise to improve the properties of red blood cells important for microcirculation. The work was carried out on 38 men of mature age without obvious pathology and who have not been involved in any kind of sport during their life. During the course of the study, they began to regularly engage in race walking 4 times a week. The control group consisted of 35 men of mature age, who were engaged in race walking for at least 5 years at least 4 times a week. To obtain scientific information, traditional biochemical, hematological and statistical research methods were used. After six months of sports walking in the blood of physically untrained men, the level of arachidonic acid metabolites was optimized, the content of cholesterol and lipid peroxidation products decreased in the composition of erythrocyte membranes with an increase in phospholipids in them. After six months of physical training in previously untrained men, the level of altered types of red blood cells increased. For men of mature age with an initial low muscle activity, who began regular sports walking, gradual optimization of the surface characteristics of erythrocyte membranes is characteristic, which improved the course of their microcirculation processes.

KEY WORDS: MEN, MATURE AGE, RACE WALKING, PHYSICAL ACTIVITY, ERYTHROCYTES, SURFACE PROPERTIES OF THE CELL MEMBRANE.

INTRODUCTION

Poor physical fitness is increasingly common among modern people of mature age (Filippov and Petrov, 2015; Bespalov et al., 2018). This circumstance provides conditions for the development of various pathologies to which a person has a predisposition (Drapkina and Shepel, 2015; Kotova et al., 2017). Such a danger was revealed when examining different categories of the population in many countries of the world (Skoryatina and Zavalishina, 2017; Zavalishina, 2018a). In conditions of low physical activity in humans, the functional reserves of internal organs decrease, metabolism is inhibited and the overall resistance of the body weakens (Zavalishina, 2018b; Zavalishina, 2018c, Checinska-Maciejewska et al., 2019; Karpov et al., 2020).

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Long-term low muscle training provides a gradual aggravation of the course of existing diseases and the development of their dangerous complications (Zavalishina, 2018d; Tkacheva and Zavalishina, 2018a). Very quickly, weak muscle activity impairs the work of the cardiovascular system and the blood system (Carrizzo et al., 2013). The situation developing in the body leads to functionally extremely unfavorable changes (Zavalishina, 2018e; Zavalishina, 2018f). Low physical fitness provides, especially in men in adulthood, a high risk of rheological disorders in their vascular bed. This is largely due to the deterioration of the surface properties of blood cells and especially erythrocytes. This contributes to the formation of hypoxia in all organs (Zavalishina, 2018g). The chronic oxygen deficiency in cells arising under these conditions further weakens anabolic processes and reduces the body's defenses (Zavalishina, 2018h). The resulting conditions lead to the formation of permanent vasospasm, which impairs the function of all cells (Tkacheva and Zavalishina, 2018b, Zavalishina et al., 2021b).

It has been noticed that with poor physical fitness, conditions are quickly formed to increase the risk of atherosclerosis, surges in blood pressure and the appearance of persistent arterial hypertension (Zavalishina, 2018i; Tkacheva and Zavalishina, 2018c, Zavalishina et al., 2021b). Under these conditions, the rheological properties of the main population of blood cells, erythrocytes, are actively violated. In adulthood, this may additionally be facilitated by chronic pathology of varying severity (Zavalishina, 2018j; Vorobyeva et al., 2018). Considering the large negative consequences of the low level of muscle training in relation to the functioning of the whole organism, it is urgent to continue the search for options for eliminating hypodynamia in people of mature age with an improvement in the state of the parameters of their blood and, first of all, erythrocytes. The purpose of the study: to establish changes in the rheological properties of erythrocytes in physically inactive mature men who began regular training in the framework of sports walking.

MATERIAL AND METHODS

The observation group consisted of 38 men of mature age (average age was 47.5±1.1 years). All of them during their life did not experience physical exertion higher than household. All of these men began regular free-pace race walking for an hour a day, 4 times a week. The control group consisted of 35 healthy men of mature age (the average age was 48.2±1.3 years), who regularly engaged in race walking for at least 5 years at least 4 times a week. The duration of one training session was at least 1 hour. The blood levels of thromboxane B2 and 6-keto-prostaglandin F1α were determined in the subjects taken under observation using an enzyme-linked immunosorbent assay using a set of reagents manufactured by Enzo Life Science (USA). After washing and resuspension of erythrocytes in the composition of erythrocyte membranes, the cholesterol content was assessed using an enzymatic colorimetric method using a kit manufactured by Vital Diagnosticum (Russia) and the concentration of total phospholipids by the amount of phosphorus present in erythrocytes (Kolb and Kamyshnikov, 1982).

The intensity of the processes of lipid peroxidation inside erythrocytes was assessed after washing erythrocytes in the course of recording the amount of malondialdehyde and acyl hydroperoxides in them by using traditional research methods (Volchegorskiy et al., 2000). In the blood of the examined, the number of discoid and altered erythrocytes was recorded using light phase-contrast microscopy using a traditional technique. Representatives of the observation group were examined at baseline, after 3 months and 6 months of systematic race walking. The entire control group was examined once. Statistical processing of the results of the observation were carried out by the student's t-test.

RESULTS AND DISCUSSION

Physiologically unfavorable changes in the ratio of the products of arachidonic metabolism were revealed in the surveyed, who at the beginning had poor physical fitness. The level of thromboxane B2 in their plasma was higher than that in the control group by 25.2% ($p < 0.01$), while the concentration of 6-keto-prostaglandin F1a in them was lower than the level in the control group by 14.6% ($p<0.01$) (table). In the examined untrained men in the erythrocyte membranes, the concentration of cholesterol was initially higher than that in the control group by 15.2%, while the number of total phospholipids was initially lower than the control by 17.7% ($p<0.01$). In mature men who did not regularly experience physical exertion, the outcome of the level of acyl hydroperoxides and the amount of malondialdehyde were higher than those in the control group by 35.6% ($p<0.01$) and 34.5% ($p<0.01$), respectively.

When taken under observation in the blood of men with poor physical fitness, the content of normal discoid erythrocytes was lower than in the control by 17.1% ($p<0.01$) (table). The number of erythrocytes in them, which had a reversibly and irreversibly disturbed shape, at the time of taking under observation was higher than in the control group, by 42.2% and 4.1 times, respectively ($p<0.01$). As a result of regular sports walking in the group of men with initial low training, the imbalance of the metabolic products of arachidonic acid decreased. At the end of the study, the amount of thromboxane B2 in the blood of these men decreased by 23.0% ($p<0.05$). This was accompanied by an increase in blood concentration of 6-keto-prostaglandin F1α by 13.6% ($p<0.05$) by the end of the study. In the membrane structures of erythrocytes in initially untrained men under conditions of regular sports walking, the cholesterol level decreased by 12.8% by the end of the entire observation. This was accompanied by an increase in the content of total phospholipids in their erythrocytes by 16.1% ($p<0.05$). By the end of the study, the levels of acyl hydroperoxides decreased by 33.0% ($p<0.01$) and the amount of malonic diadehyde by 34.5% ($p<0.01$) in men who began to experience physical activity in the structures of erythrocytes.

Against the background of systematic physical training in the framework of sports walking, the level of normal erythrocytes-discocytes increased by 16.7% in the blood of mature men compared to the initial level ($p<0.05$) (table). During the observation period, the number of reversibly disturbed erythrocytes and the number of their irreversibly damaged varieties in the blood of those who started regular sports walking decreased by 39.1% ($p<0.01$) and 4.0 times ($p<0.01$), respectively. Long-term maintenance of the normal level of any parameters in a person can be only in conditions of regular dosed physical activity (Zavalishina, 2020a). With prolonged low muscle activity, numerous disorders in the body always develop with the realization of a hereditary predisposition to pathological conditions (Karpov et al., 2020).

The negative influence of low physical activity on the state of blood parameters and, first of all, its rheological properties is very strongly manifested (Sungurova et al., 2018). It has long been established that prolonged low muscle activity contributes to the formation of any violations of the rheological parameters of the blood and, above all, their largest group - erythrocytes. Under these conditions, a strong increase in the level of lipid

peroxidation products occurs, which leads to biologically unfavorable rearrangements in erythrocyte membranes and significantly disrupts their work (Zavalishina, 2018k). This is further aggravated by the appearance in conditions of weak muscle activity by changes in the ratio of lipid fractions in the composition of erythrocyte membranes. The current situation can significantly worsen the parameters of these blood cells (Zavalishina, 2020b).

Disturbances in the level and ratio of phospholipid molecules and cholesterol molecules in their membranes form highly biologically unfavorable changes in erythrocytes (Zavalishina, 2018l). This disrupts the permeability of erythrocyte membranes and impairs the function of their membrane proteins due to the appearance of defects in their secondary and tertiary structure. The emerging situation has an extremely negative effect on all life processes in the membranes of the bulk of erythrocytes in the blood (Karpov et al., 2021).

Table 1. Levels of indicators taken into account in the surveyed

Blood indicators	Started training, n=38, M±m			Long-term practitioners (control), n=35, M±m
	start of observation	3 months of observation	6 months of observation	
Discoid erythrocytes, %	75.2±0.33 P<0.01	82.0±0.29 P<0.05	87.8±0.22 p1<0.05	88.1±0.16
Reversibly altered red blood cells, %	12.8±0.24 p<0.01	10.9±0.14 p<0.01 p1<0.01	9.2±0.10 p1<0.05	9.0±0.14
Irreversibly altered erythrocytes, %	12.0±0.16 p<0.01	7.1±0.11 p<0.01 p1<0.05	3.0±0.05 p1<0.01	2.9±0.23
Thromboxane B_2, pg / ml	197.1±0.45 p<0.01	172.3±0.60 p<0.05	160.2±0.71 p1<0.01	157.4±0.74
6-keto prostaglandin F1a, pg/ml	82.4±0.38 p<0.01	88.0±0.29 p<0.05	93.6±0.22 p1<0.05	94.4±0.38
Erythrocyte cholesterol, µmol / 1012 erythrocytes	1.06±0.010 p<0.01	0.99±0.016 p<0.05	0.94±0.009 p1<0.05	0.92±0.014
Total phospholipids of erythrocytes, µmol / 1012 erythrocytes	0.62±0.014 p<0.01	0.67±0.018 p<0.05	0.72±0.006 p1<0.05	0.73±0.016
Acylhydroperoxide of erythrocytes, D233 /1012 erythrocytes	4.15±0.012 p<0.01	3.47±0.022 p<0.01	3.12±0.018 p1<0.01	3.06±0.017
Malondialdehyde of erythrocytes, nmol /1012 erythrocytes	1.91±0.007 p<0.01	1.64±0.010 p<0.05	1.42±0.016 p1<0.01	1.42±0.019

Note: p is the statistical reliability of the differences between the indicators of the observation group and the indicators of the control group; p1 - statistical reliability of changes in the level of indicators in the observation group during the study.

An increase in the level of erythrocytes with a reversible disruption of the shape, and an increase in the number of erythrocytes that have irreversibly lost their normal shape, lead to an increase in the number of aggregates formed by erythrocytes in the blood of untrained men, which can greatly impair their implementation of microcirculation. With prolonged low physical fitness in the vascular walls of mature men, there is a low activity of formation of biologically active compounds important for hemostasis and blood rheology. Under the conditions that have arisen, the level of proaggregants in the blood of these men significantly increases. There comes a pronounced intensification of thromboxane synthesis and the synthesis of its functional antipode - prostacyclin is suppressed. Due to this, there is a pronounced physiological imbalance of metabolites of arachidonic acid with a predominance of the activity of proaggregants. This situation is characterized by a violation of the microrheological parameters of erythrocytes, and then rheological processes in small vessels.

This has a very negative effect on metabolism in all organs and contributes to the formation of pathology in them. For the general improvement of the whole organism of men of mature age during their life, who avoided regular physical exertion, systematic training in the framework of sports walking was recommended. Against their background, in the erythrocytes of the surveyed men there was a decrease in the level of peroxidation products of lipid molecules. This situation provided the optimization of the structure and function of their cell membranes. It is clear that the

improvement in the parameters of erythrocytes in those who began to train for race walking developed largely due to the positive dynamics of the lipid composition of erythrocyte structures. This created conditions for positive changes in the surface characteristics of the membranes of their erythrocytes (Zavalishina et al., 2021a).

Normalization of the level of phospholipids and cholesterol molecules in erythrocyte membranes is considered functionally very preferable. It promotes the normalization of the level of regulatory substances in the composition of erythrocytes, the degree of permeability and rigidity of their membranes and promotes the stimulation of the function of their membrane proteins acting as receptors (Zavalishina et al., 2021b). With regular sports walking, previously poorly physically trained men demonstrated a decrease in the content of altered types of red blood cells in their blood and an increase in the number of their normal forms. It is clear that a decrease in the content of altered erythrocytes in the blood in mature men leads to a significant weakening in their blood of the process of aggregation between erythrocytes. This circumstance significantly facilitates perfusion in all tissues of their body.

CONCLUSION

Low muscle activity often leads to an increase in the number of malformed red blood cells. This can impair blood circulation, especially in the capillaries, and inhibit metabolism. It was found that in mature men with low training, who began to regularly engage in race walking, the activity of lipid peroxidation processes decreased in erythrocytes. Against the background of regular physical training, the number of damaged red blood cells in their blood decreased, thereby improving microcirculation. Taking into account the optimization of the properties of the erythrocyte membrane in mature men after six months of sport walking, it is legitimate to widely recommend this type of physical activity to mature men to optimize their physical condition.

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

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Genetical Communication

Kmeans-Pillar-Salpepi: Genetic Interactions Detection Through K-Means Clustering with Pillar and Salp Optimization Techniques in Genome-Wide Association Studies

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ABSTRACT

The detection of gene-gene interactions (GGIs) is essential in determining disease susceptibility of complex human diseases. Epistasis (genetic interactions) is defined as interactions between varied Single-Nucleotide Polymorphism (SNPs). Numerous computational strategies for detecting SNP interactions have been suggested. However, currently available methods are overwhelmed by a high degree of computational complexity caused by the explosion of high-order interactions. This research aims to develop a multi-locus epistasis model that accelerates the detection of disease-related SNP-SNP interactions. This paper introduces a two-stage method for epistasis detection based on K-Means clustering and optimization strategies to find genetic interactions of complex human diseases. K-Means clustering algorithm segments the genotype dataset into different clusters during the screening stage. The accuracy and performance of the K-Means clustering algorithm are highly dependent on the selection of the initial centroids. The initial centroids are usually randomly chosen in K-Means algorithm which leads to closest possible local minima, rather than the global optimum. To address the above issue, we introduced a hybridized technique that is built on the K-Means algorithm and the Pillar algorithm to avoid local optima as well as all the SNPs falls into a unique collection of clusters for different runs. Salp optimizations with a single objective function (Salp-SO) and Salp optimization with multiple objective functions (Salp-MO) have been applied to the clusters during the search stage to identify disease-associated SNP combinations. Experimental findings indicated that the KMeans-Pillar-SalpEpi-MO method yielded superior performance than traditional K-Means with the Salp optimization technique. This study is expected to become a suitable milestone for future studies by becoming a credible source of updated information on Kmeans-Pillar-Salpepi.

KEY WORDS: EPISTASIS; GENETIC INTERACTIONS; K-MEANS, PILLAR, SINGLE NUCLEOTIDE POLYMORPHISM.

INTRODUCTION

The principle of epistasis played an influential role in the research field of genomics and genetics for over a century. A genetic interaction is a set of genetic changes that may cause an unexpected loss or benefit of cell viability (Skwark et al. 2017). A single-nucleotide polymorphism (SNP) is a mutation in a single nucleotide present in most human genomes. Variation in SNPs are responsible for identifying diseases susceptibility in human (Leaché and Oaks 2017). A genome-wide association analysis (GWAS) investigates various genetic variants among the whole genome to identify those that have statistically significant connections with a particular disease manifestation (Elliott et al. 2018).

GWAS researchers strive to identify significant genotype variants for various disease categories, such as high blood pressure, arthritis, leukemia, chronic disease, heart disease, obesity, psoriasis, etc. GWAS examined multiple SNPs and phenotypic biomarkers related to human disease cases and controls. Identifying epistasis is a common way to discover the aetiology of complex disorders (Tam et al. 2019). Typically, several possible techniques have been used to discover epistasis: stochastic search, exhaustive search, statistical-based techniques, and optimization-based strategies. Researchers employed both parametric and

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nonparametric statistical approaches in epidemiological research to identify genetic associations. Stochastic search methods use random sampling strategies to identify the combinations of epistasis statistically correlated to disease effects. Stochastic search is subject to randomness, so it takes too little time to complete the search than the exhaustive search (Sun et al. 2019; Priya and Manavalan 2020).

It is possible to discover all disease correlated SNP combinations through exhaustive research, but its computational cost is exorbitant. It analyzes each SNP interaction by estimating interaction's score and select the interactions that correspond with a disease using the user-specified threshold. The epistasis-based algorithms such as MDR, GMDR, BOOST, ES-MDR, PLINK, GMDR-GPU are evaluated based on exhaustive analysis (Priya and Manavalan 2020). An exhaustive and stochastic algorithm demands substantial computational expenses and a proneness only to specific disease models. Recently, evolutionary algorithms for epistasis identification have significantly been concerned with finding low-cost solutions to address computational problems. Evolutionary strategies reduce search time complexity, and scoring functions aid them to detect the best SNP combinations. The epistasis techniques like MACOED, CSE, epiACO, epiBat, and EIMOABC/D can be employed as an optimization strategy (Manavalan and Priya 2021).

We presented a novel epistasis identification strategy using a two-stage approach called hybridization of K-Means clustering with Pillar algorithm and Salp Swarm Algorithm (SSA) for Epistasis detection (KMeans-Pillar-SalpEpi) to focus on candidate SNP combinations. The main issues with currently available epistasis detection algorithms often have higher computational cost, low detection power, and almost all models are only designed for looking at two-locus interactions rather than addressing multiple loci interactions. In contrast to existing methods, the proposed approach aims to identify disease-associated SNPs with high detection power and focus on multi-locus interactions (Manavalan and Priya 2021). This research aims to develop a multi-locus epistasis model that accelerates the detection of disease-related SNP-SNP interactions. The proposed approaches' efficiency is tested over the 2-locus and 3-locus disease models with marginal effects (DMEs) and disease models with no marginal effects (DNMEs).

MATERIAL AND METHODS

The proposed KMeans-Pillar-SalpEpi algorithm was divided into two stages: the screening stage and the search stage. At the screening stage, the SNPs were categorized into three clusters using the Pillar based K-Means clustering technique. K-Means clustering was fused with pillar algorithm to address the issue of random initial centroid, and determine the optimal initial centroid. The outcome of pillar-KMeans was a set of clusters passed as input into the search stage to identify disease-associated SNP combinations. K-Means clustering is a widely used approach for cluster analysis. The main aim of this algorithm was to divide n number of unlabeled observations into k number of clusters. The degree of similarity between two objects was measured using Euclidean distance (Su and Dy 2004). Pillar algorithm was based on the pillars position strategy used in the construction of a stable building. The pillars can support the roof's weight and stabilize the building when placed as far apart from each other as possible in the roof's pressure distribution. As a result, this algorithm chose initial centroids at the furthest distance apart in the given data (Barakbah and Kiyoki 2009).

Two search strategies were followed during the search stage. When the size of the cluster was small (less than ten), an exhaustive search technique found disease-related SNP combinations within the cluster. In contrast, the salp optimization technique found disease-related SNP combinations within the cluster for a large cluster. The Salp Swarm Algorithm (SSA) is a population-based optimization method. SSA mimics the social behavior of salps that are chained together when sailing and foraging the food in the sea. There are two types of agents in SSA; the leader is located at the top of the chain, while the other salps are designated as followers. The leader is responsible for guiding the population's movement direction, while the supporters obey the leader (Mirjalili et al. 2017).

In this research, two variants of the Salp optimization techniques, single-objective (SO) salp optimization and multi-objective (MO) salp optimization, were proposed to identify disease-associated SNP combinations. The G-test served as a fitness function in SalpEpi-SO, whereas K2 score and AIC score act as fitness functions in SalpEpi-MO, and the Pareto optimal front method selected non-dominated SNPs from these two fitness functions. Then, the chosen non-dominant SNPs were evaluated using G-test to identify disease-associated SNP combinations in 2-locus and 3-locus models. Pseudo code for screen stage is presented hereunder.

Step 1: Utilize the Pillar algorithm to initialize cluster centroids; the pillar technique took a simulated dataset as input and generated optimal centroids as outputs.

Step 2: For Each SNP, Euclidean distance was calculated between Si and centroid of cluster Cm (m = 1, 2, 3). Then, SNP (Si), Si (i=1,2, 3, 100) is divided into the mth ($1 \leq m \leq k$) group.

Step 3: Each individual SNP was assigned to one of the k clusters. Then, the centroids of each clusters were updated for each iteration.

Step 4: Steps (2) and (3) were repeated until the centroids of k clusters no longer changed or the maximum number of iterations were reached.

The algorithmic step for Search Stage for SalpEpi-SO is given below.

Step 1: Initialize the salp positions.

Step 2: Assign each salp with a random position based on the SNPs in the clusters

Step 3: While t < max_iter do
For every salp in the solution space, combination of SNPs was chosen. Then, SNP combinations were selected and local solutions were generated based on the G-test statistic. The leader and follower position were updated based on the condition.

End while

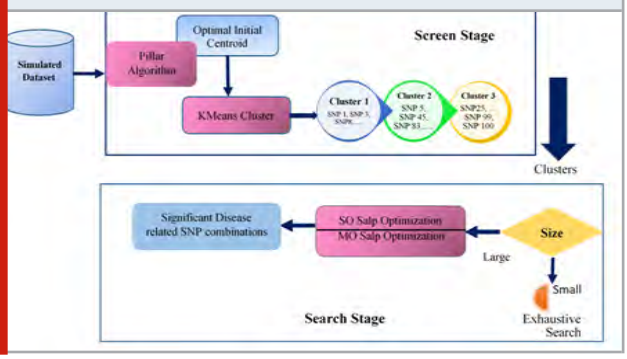
The Search Stage for SalpEpi-MO is given below.
Follow the steps Step 1 to Step 3 in SalpEpi-SO
Step 4: Pareto optimal front return the Non-dominated SNPs
Step 5: For i=1 to size (non-dominated SNPs)
For j =i+1 to size (non-dominated SNPs)
Epistasic_pair = G-test (xi, xj)
End For

Finally, the performance of KMeans-Pillar-SalpEpi-SO and KMeans-Pillar-SalpEpi-MO were tested and compared to SalpEpi-SO, SalpEpi-MO and traditional K-Means with salp techniques like KMeans-SalpEpi-SO and KMeans-SalpEpi-MO. The pseudo-code for screen and search stages of KMeans-Pillar-SalpEpi is presented in Figure 2 and Figure 3, respectively. The pseudo-code for screen and search stages of KMeans-Pillar-SalpEpi is presented in Figure 2 and Figure 3, respectively.

RESULTS AND DISCUSSION

The general architecture of the proposed approach is presented in Figure 1. The proposed epistasis models were implemented using MATLAB R2018(b) software. Section 4.3 exposes the experimental outcome of epistasis disease models. The architecture of the proposed approach is exhibited in simulated datasets.

Figure 1: General Architecture of KMeans-Pillar-SalpEpi



Simulated Datasets: The proposed approach was evaluated over the simulation dataset for genotype created using widely adapted software called GAMETES_2.0 (Urbanowicz et al. 2012). In this research, we generated two-locus and three-locus disease models. Two distinct types of epistatic disease models, such as Disease Loci with Marginal effect (DME) models and Disease Loci without Marginal Effects (DNME) models, were generated for two-locus and multi-locus disease analysis (Tuo et al. 2017).

The description of DME and DNME models chosen for experimental analysis is exposed in Table 1.

Table 1. Simulated Dataset Details

Dataset Name	Disease Model	No. of Models	SNP Details	Description
3-Locus Dataset	DME Models - Additive, Multiplicative, Threshold Models	5 Models	3 Pathogenic SNPs 97 Non-Pathogenic SNPs	No. of Datasets - 100 No. of Samples – 1600 with 800 cases and 800 controls
	DNME Models	10 Models		
2-Locus Dataset	DME Models - Additive Model, Multiplicative, Threshold models	4 Models	2 Pathogenic SNPs 98 Non-Pathogenic SNPs	
	DNME Models	10 Models		

Performance Metrics: The efficacy of the proposed epistasis detection model was evaluated using evaluation metrics power. Power is defined as the statistical measure of detecting true disease loci by rejecting the null hypothesis, and the same is expressed as

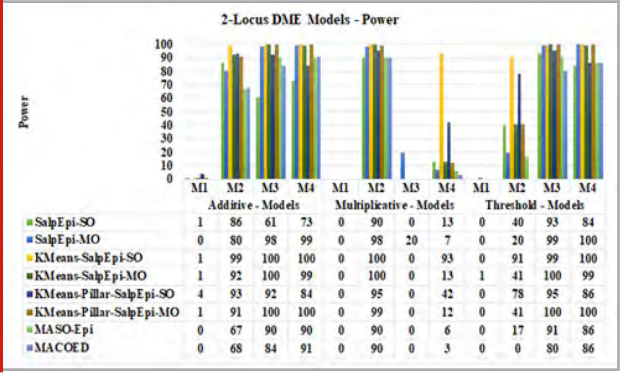
$$\text{Power} = \frac{\#\text{Dcount}}{\text{TDS}}$$

where #Dcount represents the number of datasets containing successful detection of disease-related SNPs among the Total number of Datasets (TDS) (Chen et al. 2019).

Simulation Results and Interpretation: The primary focus of GWAS was to identify associations between SNP and phenotype for the essential of epistasis detection. In this section, the performance of proposed approaches was compared with epistasis detection ability of SalpEpi-SO and SalpEpi-MO with G-test fitness function using DNME and DME models. In addition, the superiority of the proposed methods was compared to the previous research work Multi-Objective Ant Colony Optimization for Epistasis Detection (MACOED) and Multi-Objective Atom Search Optimization for Epistasis Detection (MASO-Epi) (Jing and Shen 2014; Priya and Manavalan 2021).

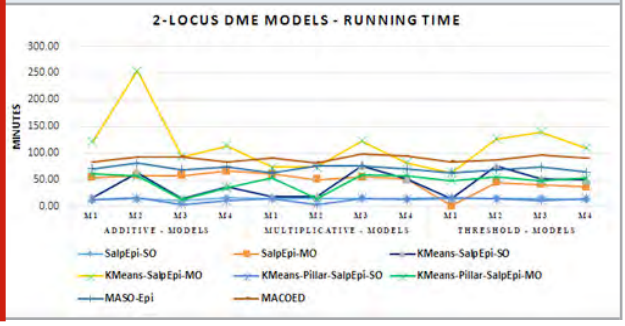
Experimental Results of 2-Locus DME Models: The power of Salp-SO, Salp-MO, KMeans-SalpEpi-SO, KMeans-SalpEpi-MO, KMeans-Pillar-Epi-SO and KMeans-Pillar-Epi-MO for twelve 2-locus DME models is exhibited in Figure 2. KMeans-Pillar-SalpEpi-MO, KMeans-Epi-SO and KMeans-Pillar-Epi-MO achieved 100% power for the additive model 3. KMeans-Pillar-SalpEpi-MO and KMeans-SalpEpi-SO obtained power of 100% for the additive model 4, which were superior to others. In additive model 1, KMeans-Pillar-Epi-SO obtained the power of 4%, whereas the remaining methods found only a single disease causative SNP pairs among 100 datasets. None of the methods found any disease causative SNP pairs for the multiplicative model 1.

Figure 2: Power Analysis of 2-locus DME Models



	M1	M2	M3	M4	M1	M2	M3	M4	M1	M2	M3	M4
SalpEpi-SO	1	86	61	73	0	90	0	13	0	40	93	84
SalpEpi-MO	0	80	98	99	0	98	20	7	0	20	99	100
KMeans-SalpEpi-SO	1	99	100	100	0	100	0	93	0	91	99	100
KMeans-SalpEpi-MO	1	92	100	99	0	100	0	13	1	41	100	99
KMeans-Pillar-SalpEpi-SO	4	93	92	84	0	95	0	42	0	78	95	86
KMeans-Pillar-SalpEpi-MO	1	91	100	100	0	99	0	12	0	41	100	100
MASO-Epi	0	67	90	90	0	90	0	6	0	17	91	86
MACOED	0	68	84	91	0	90	0	3	0	0	80	86

Figure 3: Running Time Comparison of 2-locus DME Models

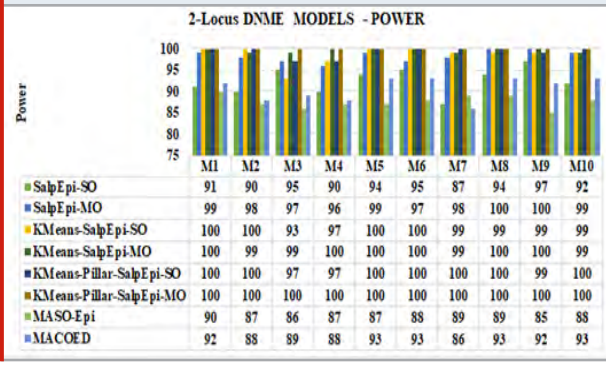


In multiplicative model 2, KMeans-Epi-SO and KMeans-Epi-MO achieved 100% power, whereas KMeans-Pillar-SalpEpi-MO achieved the power of 99%. In threshold model 3, KMeans-SalpEpi-MO and KMeans-Pillar-SalpEpi-MO yielded 100% power. In threshold model 4, KMeans-SalpEpi-SO, Salp-MO and KMeans-Pillar-SalpEpi-MO achieved 100% power. The experimental finding proved that one of the proposed methods, KMeans-Pillar-SalpEpi-MO yielded superior detection power compared to others. Among the 12 DME models MACOED and MASO-Epi didn't yield 100% detection power even for a model, whereas KMeans-Pillar-SalpEpi-MO produced 100% detection power for 4 models. It was clearly observed that KMeans clustering with salp optimizations performs superior to the existing algorithms MACOED and MCASO-Epi for all DME models (Jing and Shen 2014; Priya and Manavalan 2021).

The running time of twelve 2-Locus DME models is exposed in Figure 3. For all the 12 DME models, the approaches KMeans-Pillar-SalpEpi-SO and KMeans-Pillar-SalpEpi-MO take the lowest running time. The highest running time required for 2-locus DME models was KMeans-SalpEpi-MO. It was noticed that our proposed single objective models KMeans-Pillar-SalpEpi-SO, KMeans-SalpEpi-SO took minimum running time compared to others. Further, the time consumption of one of the proposed multi-objective model KMeans-Pillar SalpEpi-MO models was lower than the state-of-art methods MACOED and MASO-Epi (Jing and Shen 2014; Priya and Manavalan 2021).

Experimental Results of 2-Locus DNME Models: Figure 4 exposed the detection power of proposed approaches for all the 2-Locus DNME models. Among the 10 DNME models, KMeans-Pillar-SalpEpi-MO achieved 100% power for all the models, which was superior to others. The KMeans-Pillar-SalpEpi-SO achieved 100% detection power for seven models, such as model 1, model 2, model 5 to model 8, and model 10. KMeans-SalpEpi-MO obtained 100% power for 6 DNME models such as model 1, model 4 – model 6, model 8, and model 9. The SalpEpi-MO yielded 100% power for four models such as model 1, model 2, model 5 and model 8. The SalpEpi-SO achieved the highest detection power of 97% for model 9. MACOED obtained 93% detection power for 4 DNME models, whereas proposed model KMeans-Pillar-SalpEpi-MO gained 100% detection power in all DNME models. The MASO-Epi achieved the minimum detection power of 85% for model 9 and, maximum detection power of 90% in model 1. Hence, the outcome revealed that our proposed models were superior to MACOED and MASO-Epi in all 10 DNME models (Jing and Shen 2014; Priya and Manavalan 2021).

Figure 4: Power Analysis of 2-Locus DNME Models



	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10
SalpEpi-SO	91	90	95	90	94	95	87	94	97	92
SalpEpi-MO	99	98	97	96	99	97	98	100	100	99
KMeans-SalpEpi-SO	100	100	93	97	100	100	99	99	99	99
KMeans-SalpEpi-MO	100	99	99	100	100	100	99	100	100	99
KMeans-Pillar-SalpEpi-SO	100	100	97	97	100	100	100	100	99	100
KMeans-Pillar-SalpEpi-MO	100	100	100	100	100	100	100	100	100	100
MASO-Epi	90	87	86	87	87	88	89	89	85	88
MACOED	92	88	89	88	93	93	86	93	92	93

Figure 5: Running Time of 2-Locus DNME Models

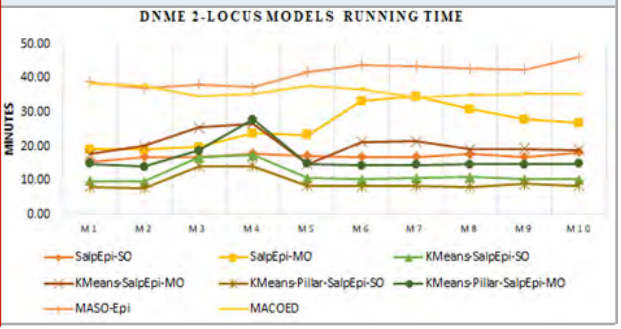
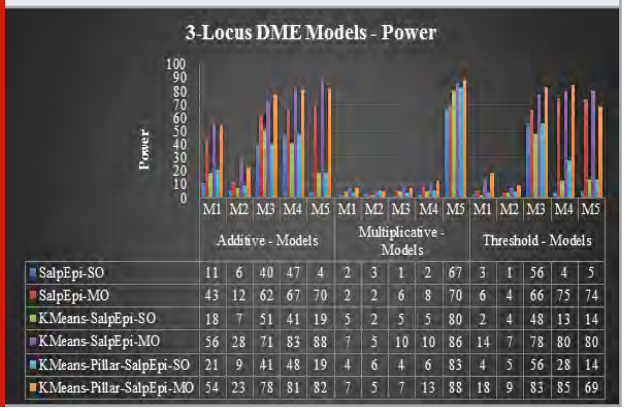


Figure 5 presented the running time of 2-locus DNME models. For all the ten models, the KMeans-Pillar-SalpEpi-SO and KMeans-SalpEpi-SO require minimal running time compared to others. The SalpEpi-MO method spent the highest running time for all ten models. The state-of-art methods such as MACOED and MASO-Epi consumed more running time, whereas the proposed models KMeans-Pillar-SalpEpi-SO and KMeans-Pillar-SalpEpi-MO took less running time for all DNME models. The state-of-art approaches such as MACOED and MASO-Epi was tested

over 2-locus interactions only due to the computational overhead. Hence, these two models were not considered as comparative methods in this research for the analysis of 3-locus disease models (Jing and Shen 2014; Priya and Manavalan 2021).

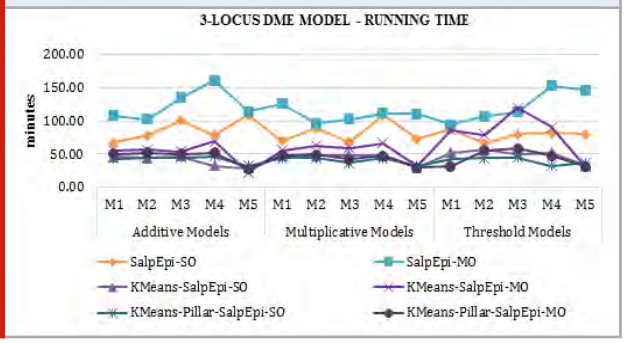
Experimental Results of 3-Locus DME Models: The power of proposed approaches for fifteen 3-Locus DME models was exhibited in Figure 6. For the additive model 1, KMeansEpi-MO and KMeans-Pillar-SalpEpi-MO achieved 56% and 54% of power, respectively, which were superior to other approaches. The KMeans-Pillar-SalpEpi-MO yielded the power of 78% for additive model 3. In additive model 4, KMeans-SalpEpi-MO obtained the highest power of 83%, which was superior to the other five approaches. KMeans-SalpEpi-MO gained the power of 88% for model 5, which was superior to Salp-SO, Salp-MO, KMeans-SalpEpi-SO, KMeans-Pillar-SalpEpi-SO, and KMeans-Pillar-SalpEpi-MO, respectively. KMeans-Pillar-SalpEpi-MO obtained the highest detection power of 88% for multiplicative model 5.

Figure 6: Performance Evaluation of 3-Locus DME Models



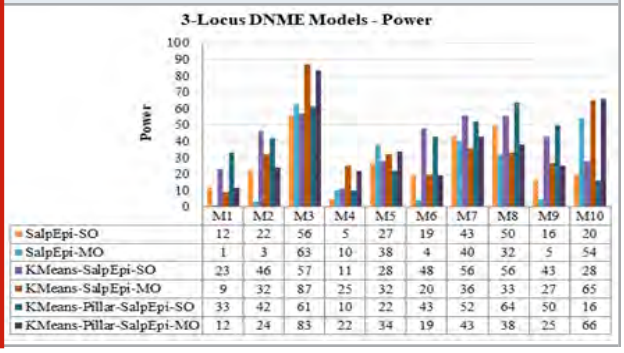
	M1	M2	M3	M4	M5	M1	M2	M3	M4	M5	M1	M2	M3	M4	M5
SalpEpi-SO	11	6	40	47	4	2	3	1	2	67	3	1	56	4	5
SalpEpi-MO	43	12	62	67	70	2	2	6	8	70	6	4	66	75	74
KMeans-SalpEpi-SO	18	7	51	41	19	5	2	5	5	80	2	4	48	13	14
KMeans-SalpEpi-MO	56	28	71	83	88	7	5	10	10	86	14	7	78	80	80
KMeans-Pillar-SalpEpi-SO	21	9	41	48	19	4	6	4	6	83	4	5	56	28	14
KMeans-Pillar-SalpEpi-MO	54	23	78	81	82	7	5	7	13	88	18	9	83	85	69

Figure 7: Running Time of 3-Locus DME Models



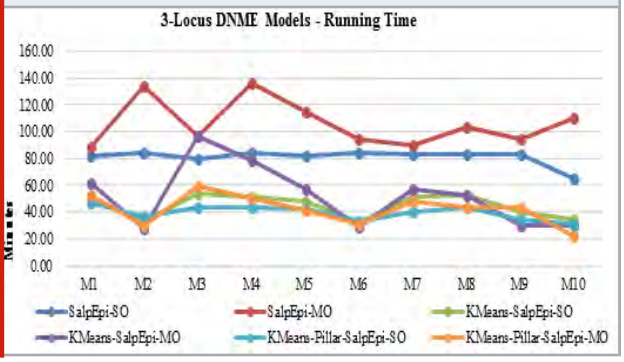
KMeans-SalpEpi-MO obtained the highest detection power of 80% for threshold model 5, whereas SalpEpi-SO gained the lowest detection power of 5%. In threshold model 4, KMeans-Pillar-SalpEpi-MO yielded the power of 85%, which was 5% higher than KMeans-SalpEpi-MO. KMeans-Pillar-SalpEpi-MO obtained the highest detection power of 83%, whereas KMeans-SalpEpi-SO achieved the lowest detection power of 48% for the threshold model 3. The efficacy of the methods highly dependent on the nature of the dataset, and its dimension and the simulated parameter settings. The DualWMDR approach was proposed to find high-order epistasis interactions and tested over two 3-locus disease models with and without marginal effects. The DualWMDR achieved 82% power for a DME model (Cao et al. 2020).

Figure 8: Performance Comparison of 3-Locus DNME Models



	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10
SalpEpi-SO	12	22	56	5	27	19	43	50	16	20
SalpEpi-MO	1	3	63	10	38	4	40	32	5	54
KMeans-SalpEpi-SO	23	46	57	11	28	48	56	56	43	28
KMeans-SalpEpi-MO	9	32	87	25	32	20	36	33	27	65
KMeans-Pillar-SalpEpi-SO	33	42	61	10	22	43	52	64	50	16
KMeans-Pillar-SalpEpi-MO	12	24	83	22	34	19	43	38	25	66

Figure 9: Running Time comparison of 3-Locus DNME Models



The running time of 3-Locus DME models was exposed in Figure 7. KMeans-Pillar-SalpEpi-SO taken the lowest running time compared to Salp-SO and Salp-MO, KMeans-SalpEpi-SO and KMeans-SalpEpi-MO. The SalpEpi-MO approach taken the highest running time for all the 15 DME models.

Experimental Results of 3-Locus DNME Models: The detection power of ten 3-Locus DNME models is presented in Figure 8. The highest accuracy of 87% is obtained for Model 3 by KMeansEpi-MO, whereas KMeans-Pillar-SalpEpi-MO achieved 83% for the same model. The second highest detection power was 66% yielded by KMeans-Pillar-SalpEpi-MO for the model 10. Salp-SO obtained the lowest detection power of 1% for Model 1. The experimental outcome revealed that clustering-based approaches were superior to Salp-MO and Salp-MO for the 3-Locus DNME models. The Running time of ten 3-Locus DNME models is shown in Figure 9. The line chart clearly proved that the KMeans-Pillar-SalpEpi-SO and KMeans-Pillar-SalpEpi-MO were taken the lowest running time for 10 DNME models compared to others.

CONCLUSION

The findings of the present study highlight the discovery of epistatic interactions aids in the detection of complex human diseases in GWAS. In this paper, we proposed a two-stage method called KMeans-Pillar-SalpSO and KMeans-Pillar-SalpMO. The proposed methods were more suitable for finding higher-order SNP interactions during the search stage through exhaustive search or optimization-based search. Exhaustive search was applied to a small clustered dataset, and salp based search was used for a large candidate set. The proposed method had the capability for discovering high-order epistatic interactions with a minimal computational effort. The results from the experiment on simulated datasets showed that KMeans-Pillar-SalpSO and KMeans-Pillar-SalpMO outperform SalpEpi-SO and SalpEpi-MO.

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

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Ecological Communication

Role of Socio-Geographical Parameters in Natural Resource Management of Belgorod Region, Russia

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ABSTRACT

The relevance of the research corresponds to modern trends in the development of science and technology and is determined by social tasks in the meeting human needs in providing a comfortable environment within the common lands of specific localities. Man is a part of nature itself and his whole life is inextricably linked with nature. Man, and his work become an active principle that transforms nature. It is necessary to pay attention to the changes in the landscape, the violation of the integrity of natural complexes, and the environmental pollution. In addition, prevent the deterioration of the environmental situation, predetermined by the intensification of anthropogenic pressure on the environment. The article describes the approaches to the study of patterns of the social-geographical parameters of regional environmental management. The variant of the author's methodology for the study of social-geographical aspects of public nature management and assessment of aesthetic and consumer parameters of the environment is proposed and tested. The case study was conducted in the rural village Streletskoe, Krasnogvardeisky district of Belgorod region. Based on the results of the study, we obtained significant results of social-geographical parameters of regional environmental management. In conclusion the population of the Streletskoye rural settlement is definitely characterized by its own specific socio-geographical parameters of natural resource management. Thus, the results obtained can be used in the territorial scheme of nature protection, in the General plan of settlements, during the environmental assessment, and also in the future can serve as a basis for the implementation of landscape planning. Research materials can be used as local history material.

KEY WORDS: ATTRACTIVENESS, ENVIRONMENT, IMPLEMENTATION, MANAGEMENT, RURAL TERRITORY, SUSTAINABLE DEVELOPMENT.

INTRODUCTION

In recent decades, there has been an increased interest in cultural landscapes, their historical, cultural, and social and geoecological study. Research on landscape aesthetics and natural resource management is becoming increasingly urgent (Borsuk, 2000; Likhacheva and Timofeev, 1978; Nazarov and Postnikov, 2002; Chervan et al., 2016; Carlson, 1994; Trikart, 1979). Any landscape has its own unique appearance, i.e., a scenery that acts as a carrier of information about the culture, traditions and customs of the people living there. In addition, landscapes are of interest both economically and in terms of influence on people, their psychophysiology and health, beautiful sceneries help to create a locality trademark – for tourist business (Nikolaev, 1999; Nikolaev, 2002; Rodoman, 1995; Frolova, 1994; Eringis and Budryunas, 1971; Bunting and Gueelke, 1979; Downs and Meyer, 1978; Haeyrynen, 1996; Penning-Rowsell, 1974; Rushton, 1979; Lopina et al., 2018; Zelenskaya et al., 2019; Ivanov et al., 2020 Yee et al., 2021).

A human is a part of nature itself and his/her whole life is inextricably linked with the natural environment. A human and his/her work become an active initiator, which transforms nature, and therefore it is essential to pay attention to any landscape change, violation of natural complexes integrity and environmental pollution (Chervan et al., 2016; Lopina et al., 2018; Silchenko and Semeniuk, 2019; Yee et al., 2021). Finally, yet importantly, it is important to prevent any degradation of the ecological situation predetermined by intensified anthropogenic pressure on the environment. Thus, there is a long-felt need to improve and enrich the modern rural visual environment and enhance the aesthetic effect. The purpose of this study is to establish consistent

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patterns for socio-geographical parameters of regional natural resource management. The study was conducted in the Streletskoye Rural Settlement of the Krasnogvardeysky Municipal District of the Belgorod Region.

MATERIAL AND METHODS

Taking into account the currently available theoretical and methodological developments by domestic and foreign scientists, the proprietary methodology titled "Study of socio-geographical aspects of public natural resource management and assessment of aesthetic and consumer parameters of the environment" was developed (Kireeva-Genenko and Senkina, 2015; Kireeva-Genenko et al., 2013; Lopina et al., 2015; Lopina et al., 2017; Lopina et al., 2018). The procedural framework of the study was supplemented by traditional geographical research methods: sociological, comparative geographical, cartographic, mathematical and statistical ones.

The Belgorod region is a region of intensive economic development, in which environmental problems have accumulated in the use of land, water, forest, recreational and other resources (Zinchenkou et al., 2013; Lisetskii et al., 2014; Zelenskaya et al., 2019; Ivanov et al., 2020). The findings of the studies conducted by the authors during 2015-2019 (questionnaire results) as well as periodicals, library and archive funds of the Administration of the Streletskoye Rural Settlement in the Krasnogvardeysky District of the Belgorod Region were used as source material.

RESULTS AND DISCUSSION

The proposed version of the methodology to be used to study socio-geographical aspects of natural resource management, consistent patterns of socio-geographical parameters, and estimated aesthetic and consumer parameters of regional natural resource management includes the following stages and their content: 1. Preparatory stage:– Justification of the relevance and objectives of the study.– Justification of evaluation methods.2. Organizational stage: – Development of routes with relevant number of settlements of different types in all districts of the Belgorod Region.– Representative sample calculation for a locality.3. Evaluation stage:– Development of personal questionnaire.– Selection of respondents based on two indicators: by age group and by the length of residence in the study area. – Conducting a socio-geographical survey (questionnaire) in the selected localities. 4. Synthesis stage:– Summarizing the results into a consolidated table in Microsoft Excel format.– Analysis of field research results.5. Design stage:– Scheme development for a visual representation of results.– Design of visual materials – development of maps. 6. Final stage.

The territory of the Krasnogvardeysky District where the Streletskoye Rural Settlement is located is in the southern part of the Central Russian Upland, in the eastern part of the Belgorod Region. The Krasnogvardeyskoye Settlement, which is the centre of the district, is located at 38º24 east longitude and at 50º39' north latitude, 145 km west of Belgorod, and the regional centre. The area of the Krasnogvardeysky District is 1,762.6 km2 (17,6263 ha), the area of Krasnogvardeyskoye district centre is 13.7 km2 (1,371 ha) within the settlement limits. On the territory of the district, there are 86 settlements with an area of 13,481 hectares (7.6% of the district's area); they are under the jurisdiction of 14 rural and one-settlement administrations.

The industrial land in the district occupies a total area of 918 hectares (0.5% of the district's area) and Valuyskaya Maintenance Section No. 11 of the South-Eastern Railway, Department of Public Roads, RAO United Energy System of Russia, the Belgorodenergo Eastern Electric Networks and the Mostransgaz Main Gas Pipeline, represents it. In addition, it includes 12 gas stations and oil depots of Belgorodnefteprodukt JSC. The main direction of the district's economy is agriculture. The agricultural lands occupy 132,759 ha, including arable land – 70.6%, gardens – 1.1%, and hayfields – 4.3% and pastures – 24.0%.

The district's terrain relief is primarily characterized by its location on the south-eastern spurs of the Central Russian Upland. It occupies a watershed plateau between the basins of the Oskol River and the Tikhaya Sosna River, both flowing into the Don. The entire territory is largely dissected by the well-developed gully-ravine system. There are individual elevated or lowered places as clearly contrasted with the even land. The most elevated part is located on the lands belonged to Bolshebykovo and Verkhososna villages, it rises up to 200–220 m above sea level while the lowest part is the floodplain of the Palatovka River. The watershed slopes are subject to erosion processes, they can reach up to 2–4 km in length, and the ravines can sometimes extend up to several tens of kilometres. In the district, there are quite a lot of gullies, i.e., deep depressions with steep exposed slopes and a narrow bottom. The glaciation of the Russian Plain, which started in the Quaternary period, affected the Don River valley with its eastern wing. The deposits made on the territory of the district during this period are represented by significant reserves of various clays and sand (Lopina et al., 2015; Zelenskaya et al., 2019)

There are pottery clays near the villages of Sadki and Nikolskoye, and approximately Palatovo, Livenka and Filkino, there are reserves of refractory and high-melting clays. Local clays are used to make bricks. In the quarries located approximately the villages of Gorovoye, Chermenevka and Malobykovo, near the Biryuch railway station, and in some other places sand mining is underway for the needs of the district. The climate of the region is specifically characterized by the influence from the Asian continent during spring and summer periods, from there some continental hot air masses, i.e., dry winds, tend to come, thus causing atmospheric and often soil drought. Drought events occur every 3-4 years. In the district, the average monthly temperature varies from +20oC to +22oC in summer (July) and between 8 and 9 oC in winter (January). The duration of the warm period is 230–240 days, and that of the cold period is 120–130 days. The amount of precipitation is insignificant – 450 to 470 mm per year. This is slightly less than the average number for the region. The river network of the district is sparse. All rivers run into the basin of the Sea of Azov (Lopina et al., 2017)

The district is latitudinal dissected by the largest river, i.e., the Tikhaya Sosna. The total length of this river in the district is 43.5 km. The following left-bank tributaries of the Tikhaya Sosna flow in the meridian direction: the Userdets (26 km) and the Sukhaya Sosna as well as the Valuy (28 km within the district) with tributaries Palatovka (19 km) and Sennaya. All of them are low water. There are nine other small rivers and seventeen streams, sometimes unnamed, on the district's territory. Most of them dry up in summer time. The different-depth underground waters are not yet fully understood. Swamps occupy 380 hectares. The water bodies of the district cover only 1,222 ha or (0.6%) of the total area. The Tikhaya Sosna and the Userd are the major rivers. There are ponds on rivers Renevod, Palatovod, Valuy, Sennaya and Userd. The ponds are mainly used for commercial fishing and irrigation (Lopina et al., 2018; Ivanov et al., 2020; Butkaliuk et al., 2021).

The main pollution sources for the district's rivers are as follows:

- The Tikhaya Sosna (it is polluted by rain-storm runoff from the settlement, enterprises and organizations as well as by poorly treated wastewater from the settlement's sewage facilities).
- The Userd (it is considerably subject to contaminations from the Krasnogvardeysky Fish Farm and it is polluted by manure-containing storm drains from the livestock farms).
- The Valuy (it is polluted by rain-storm runoff from Mashinostroitel OJSC and the Nikitovsky Fish Farm).

The ecological condition of surface waters in the water protection zones of the Tikhaya Sosna, Userd and Palatovka rivers is affected by violations of their use conditions by Pokrovka, Zasosenskaya, Streletskaya and Krasnogvardeyskaya settlement administrations. These are territories where there are cases of land plot ploughing in the water protection zones of the above-mentioned rivers. The Krasnogvardeysky District is rich in springs. There are more than 120 springs there, 55 of them are provided with proper infrastructure. It is planned to develop the springs located on the bank of the River Sosna in the village of Veseloye near the Belgorod – Rossoshch highway. The well-developed springs are all operational and they are maintained in a good sanitary condition. The coverage of centralized water supply in the district is 58%. There are 61 wells to supply water to the population of the district. The total length of water supply networks is more than 200 km. There are operational treatment facilities for household and industrial effluents located in the settlement of Krasnogvardeyskoye and the village of Livenka respectively, the treated wastewater is discharged into the Tikhaya Sosna River and the Valuy River (Lopina et al., 2018; Ivanov et al., 2020; Yee et al., 2021).

In the settlement of Krasnogvardeyskoye and the village of Livenka the treatment facilities have been long used without any necessary major repair, wastewater fails to be automatically chlorinated. Maximum permissible discharge limits (MPL) for treated wastewater have been neither developed nor agreed upon. Due to the fact that the industrial effluents produced by both the Krasnogvardeysky Milk Factory and the canning factory of Domat LLC have considerably higher MAC (maximum allowable concentration) because of non-compliance with wastewater collection regulations, the treatment facilities located in the Krasnogvardeyskoye settlement discharge post-treatment substandard effluents into the Tikhaya Sosna River. The prevailing type of soils in the district are typical and ordinary Chernozem, which occupy 34% of the total cultivated area. The thickness of the humus layer reaches 60 to 70 cm in some places. They are most widespread in the northern half of the district (Geography, 2001).

Grey forest soils rank second after Chernozem. They are mostly occupied by woodlands. The thickness of the humus horizon is slightly higher than in chernozems. Their fertility is somewhat lower. There are narrow strips of meadow alluvial (riverside) and Chernozem meadow soils usually going along the floodplains. They occupy a little more than 8% of the total area of the district. On the floodplains of the Tikhaya Sosna and the Userdets rivers, there are swampy areas where meadow and floodplain soils with high organic matter content are formed. Near the villages of Maloalekseyevka, Kazatskoye, and Ezdotskoye and in some other places, there are sphagnum swamps where it is possible to extract peat for fertilizers. There are sparse spots of alkaline soils and exposed cretaceous (carbonate) outcrops.

The structure of the land fund of the district is as follows: lands intended for agriculture – 66.7%, areas covered by inhabited localities – 7.7%, industrial and transport lands – 0.5%, forest resources – 12.6% and free available land – 12.5%. The district's land is mainly characterized by the maximum degree of development of erosion processes. The territory of the district is located on slopes with various steepness, more gentle slopes of up to 8° are used for arable land, and pastures are on slopes of up to 20°. A significant area (more than 3,000 hectares) is occupied by gullies. The rugged topography and the transfer of arable farming systems from plains to slopes have led to the fact that nowadays 73% of the district's land is erosion-prone and erosion threatening and part of the land is withdrawn from agricultural use (Geography, 2001; Buryak and Marinina, 2020; Butkaliuk et al., 2021).

Because of the intensive use of arable land in the district, there are no Chernozem left with a very high content of humus, and those with high and increased humus content make up only 21.9%. Most of the land (77.8%) has a medium and low humus content. In the last 20 years, up to 20 cm of the humus horizon have been washed away. Nutrients are also washed out of the soil proportionally, which affects the agricultural yield. The Krasnogvardeysky District is geographically located at the junction of two natural areas: steppe and forest-steppe. Forests cover 24,245 hectares, which is 14% of the total area of the district. There are no purely oak forests. Usually, it is a three-tiered forest canopy consisting of various tree species. The first tier is occupied by oak and common ash, in some places — aspen. Below there are fruit, elm, and maple trees. One can sporadically see rowan tree, bird cherry, linden, and willow. In the third tier, there are shrubs: hazel, hawthorn,

blackthorn, rough spindle tree, rosehip and others. There are alders and thick willow along the floodplains and swampy areas. It is typical for the forb-feather grass steppe to have herbaceous vegetation.

The original steppe space is mostly ploughed. The herbage is lower and sparser there. It is predominated by xerophilous cereals with a deep root system. On the bottom deposits, the herbage is slightly higher. There you can find foxtail, meadow fescue, grasshopper, and others. There are thyme or meadow thyme, chalky hyssop growing on the blackened cretaceous outcrops. There are cloven-hoofed animals such as elk, roe deer, and wild boars all over the forests of the district. Among the mammals, rodents are widely spread. One can also see a brown hair. A wolf represents predators. A red fox is of great value as a commercial animal. There are several species of martens. There are about 250 species of birds. One can see herons, wild geese, and sometimes cranes and storks. Some rare and endangered plants can be found on the territories of botanical reserves: Kozopolyansky rock jasmine, low iris, large-flowered hedysarum, bulbocodium, pasqueflower, Biberstein tulip, May lily of the valley, Russian fritillary, sandy caraway, wood anemone, and others (Lisetskii et al., 2014; Yee et al., 2021)

Currently there are two species reserves in the district:1) The Pokrovsky Nature Reserve with a total area of 15,000 hectares was formed in order to preserve the populations of elk and deer;2) The Mandrovsky Nature Reserve with an area of 25,000 hectares, aimed at the conservation of such species as elk and European deer (according to the data recorded in 2017 there are 7 elks in the reserve). There are no breeding stations in the district. Currently, the natural water reservoirs of the district are inhabited by about 12 fish species, 6 of them are commercial ones. Because of changing conditions, the number of many fish species in the water reservoirs of the district has decreased. This is primarily due to pollution and destruction of their habitat. It is allowed to plough water conservation zones and coastal strips. The district has some historical and cultural landscape components. In the village of Nikitovka, the Krasnogvardeysky District, there is "Life-Giving Spring" – a spring consecrated in honour of the icon of the Most Holy Theotokos. In the centre of the city of Biryuch, there is a building of the former district council. Its history and unique style make it an attractive venue for various events. Various regional cultural programs are organized there: anniversaries are celebrated, meetings with writers, notable countrymen and district guests are held. The district council building is a tourist attraction of the Krasnogvardeysky District (Hryniuk and Hryniuk, 2020; Butkaliuk et al., 2021)

The Epiphany Spring that is located in one of the picturesque places of the village of Veseloye has recently become the place of recreation for the residents of the village and the Krasnogvardeysky District. The spring carries latent inexhaustible energy and mysterious depth. The life-giving spring is surrounded by many secrets and legends. The spring water has the same temperature, + 8oC, in winter and summer; the water of this spring is valued for its healing and taste qualities. The holy spring of St. Nicholas the Wonderworker on the Userdets River is located in the northwestern part of the village of Sorokino. The spring flow is constant. The water in the spring is colourless, transparent, odourless, and has a pleasant taste. The temperature of the spring is constant, 5oC. In winter, the water in the spring does not freeze. The Krasnogvardeysky District is characterized by high rates of territory settlement and development (Lisetskii et al., 2014, Silchenko and Semeniuk, 2019, Butkaliuk et al., 2021).

The high attractiveness of the territory is primarily due to the favourable natural conditions for living and farming. The study was conducted in the Streletskoye rural settlement, which is located in the eastern part of the Krasnogvardeysky District and was established on December 20, 2004 in accordance with the Law of the Belgorod Region No. 159. The administrative centre of the rural settlement is the village of Streletskoye, which is located on the hill at the confluence of the Userdets and Tikhaya Sosna rivers in the forest-steppe zone in the southeastern part of the Belgorod Region, 158 km away from the city of Belgorod. The rural settlement includes the village of Kazatsko, the village of Maloalekseyevka, the village of Malobykovo, the village of Streletskoye that is an administrative centre, and the hamlet of Yampi.

The total population of the Streletskoye rural settlement is 2,642 people as of January 01, 2020. The settlement's economy relies on agricultural production. There are deposits of sand, white and red (red – pottery) clay and chalk on the lands of this area. The social and cultural sector of the Streletskoye rural settlement includes the following community centres: Kazatsky, Malobykovsky, Streletsky. The educational sector of the settlement includes three secondary schools and three kindergartens located in the villages of Streletskoye, Malobykovo and Kazatskoye. The main rural sightseeing places, which predetermine the development of rural tourism, include the following: – A collective grave of 36 Soviet soldiers who died in the battles against Fascist invaders in 1943 (the village of Streletskoye).

– An ensemble of the 19th century: The Church of Our Savior with the interior dated back to 1845, the house of clergy (the second half of the 19th century), a fence with a gate (the second half of the 19th century) (the village of Streletskoye).– On the left bank of the Tikhaya Sosna River, there are some remains of the ancient settlement of Kamenny Brod (the village of Streletskoye) preserved.– Liman isolated terrain feature (near the village of Malobykovo) — a nature reserve for conservation of rare plant species.Religious sites: The Church of the Demetrius of Thessalonica, the Great Martyr (the village of Kazatskoye), the Church of Our Savior (the village of Streletskoye), the Church of St. Sergius of Radonezh (the village of Malobykovo).

As part of the study of socio-geographical parameters of regional natural resource management, in addition to the development of brand identity for the district and thus the rural territory, we conducted a sociological survey (questionnaire survey) in which 813 respondents took

part (the confidence probability was 0.683%).The analysis results for the spatial and temporal characteristics of the natural resource management areas are presented below: – most frequently visited territories: the average distance varies from 0.3 to 0.7 km; the average range radius is 0.5 km; the average frequency of visits is 3 times per week;– frequently visited territories: the average distance varies from 0.8 to 2.0 km; the average range radius is 1.4 km; the average frequency of visits is 2 times per week;– rarely visited territories: the average distance varies from 2.0 to 15 km; the average range radius is 8.5 km; the average frequency of visits is one time or less per week. The number of going-outs by local residents for the purpose of "pure recreation" is 28%, the remaining number of respondents (72%) prefer to combine recreation with other types of natural resource management.

Figure 1: Attendance of the territories of the Streletsky rural settlement

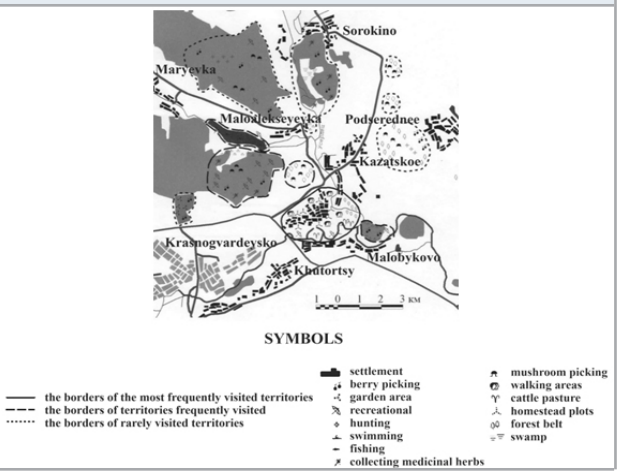
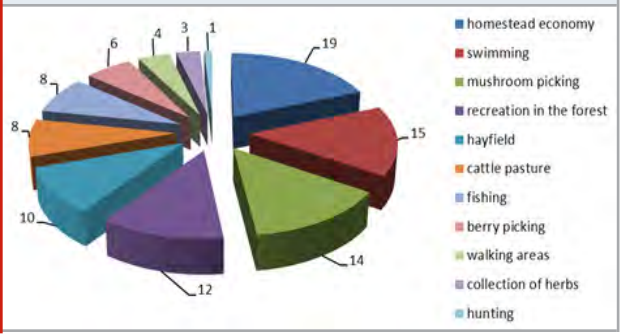


Figure 2: Types of public nature management used by residents The Streltsy of the rural settlement of Krasnogvardeysky district



The frequency of population-nature contacts in spring and summer varies from daily visits to the surrounding nature to one time a week per person near the settlement in autumn and winter. The frequency of visits by local residents to natural and cultural sites is decreasing as they move away from their place of residence. Most local people go more frequently outside the rural territory in summer; the population leaves the territory of the settlement because they need to visit the regional centre. This is mainly the city of Belgorod and the surrounding area. Using the obtained data, we compiled a sketch map titled "Attendance of the Streletskoye Rural Settlement Sites" (Fig. 1) with highlighted areas: the most frequently, frequently and rarely visited territories as well as the prevalence of different types of public natural resource management among the respondents. The sketch map clearly shows the most valuable territories for the residents of the Streletskoye rural settlement, which have a great recreational and consumer appeal. The closest area is most frequently visited (the average distance is 0.5 km). It is characterized by the greatest variety of types of natural resource management. The people living in the Streletskoye rural settlement actively visit the centre of their residential place. On holidays and weekends, they prefer to go to the administrative centre of the rural settlement.

In rarely visited natural landscapes, which are located at a distance of 0.8 to 2 km, there is nothing to do but have a rest in the forest, near water and sometimes hunting. Going farther away from the settlement there are some "detached areas" with their landscapes being used rarely. Moreover, an analysis of the questionnaire data made it possible for us to identify the dominant types of natural resource management.

The landscapes of the Streletskoye rural settlement have changed significantly and they are not dissimilar to the urban ones. This point can be noted not only by visiting the rural settlement but also by analysing the responses of the local residents. However, one particular feature should be noted: as a rule, respondents are satisfied with what they observe in the environment of their place of residence. They do not want to contemplate wild landscape pictures, which are as close as possible to natural ones. They prefer cultural, well-developed landscapes (gardens, parks, mini-parks). More than 70% of the respondents have contacts with nature 6–8 times a year. Nature is the main place for diverse recreation. The study materials can be used as local natural history material, as well as in the formation of students' environmentally oriented approach to their future professional activities (Lisetskii et al., 2015; Kudryavtseva et al., 2020; Butkaliuk et al., 2021).

CONCLUSION

Thus, the inhabitants of the Streletskoye rural settlement are certainly characterized by their own specifics in the aesthetic perception of landscapes. However, even among the local residents, there are significant differences in perception particular features, which is associated with a number of reasons. The main reason is the origin and duration of residence of respondents in the study area since the aesthetic appearance of the native landscape which is established in childhood and adolescence during the period of long-term residence in this territory forms the psychological self-identification of the respondent with these landscapes, which makes it possible to state that they are of great value. We visually examined the sites of natural resource management in general and recreation in particular which the residents of the Streletskoye rural settlement prefer. Based on the survey we have proposed some recommendations to improve recreational conditions

within the rural settlement, for example: to improve the territory adjacent to the Monastyrsky Spring (the village of Streletskoye).

For this purpose, you can install a pergola near the spring so that people could have an opportunity to relax after a long road to the spring; you can set up some recreation grounds in forest areas and improve places for recreation and safe bathing on the rivers Userdets and Tikhaya Sosna. Thus, we can draw the following conclusions: the population of the Streletskoye rural settlement is definitely characterized by its own specific socio-geographical parameters of natural resource management. The obtained significant results can be used in the territorial nature protection scheme, in settlements master plan and environmental impact assessment; in the future, they can also serve as a basis for the implementation of landscape planning activities.

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Biomedical Communication

Protective Effects of *Helianthus annuus* Seeds on Renal and Liver Function of Healthy Mice

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ABSTRACT

The present study showed the dose beneficial of *Helianthus annuus* L. on 5-7 month old Balb/c mice, using different important parameters like physiological, biochemical, haematological and histopathological as indicators. The effects of *Helianthus annuus* seeds were investigated with the dose concentrations of 25% and 50% for 21 day supplementation, which were compared with mice fed on normal standard pelleted food. The results of the study suggested that the physiological parameters considering body weight gain was reduced with a dose-dependent concentration of *Helianthus annuus* seed as compared to the controls, with the order of 50% (-1.10±0.22) < 25% (-1.05±0.43) < control (1.88±0.63). The other parameters; fluid intake, urinary output, food intake, faecal weight, and dry weight did not show much differences with increasing dose concentration of 50%, with increasing supplementation in the treatment days. The effect of the drug did not show much differences in all parameters including liver function test and histopathological aspects. It is concluded that the seed of *Helianthus annuus* showed a beneficial effect in weight loss, the high amount of seed may be beneficial to reduce weight, is good for diabetic conditions, and results in accordance with physiological, biochemical haematological parameters supporting the kidney histopathological data as well. Hence, the use of *Helianthus annuus* seed extract as a drug among young people should be encouraged for obesity and its management.

KEY WORDS: HELIANTHUS ANNUUS L, PHYSIOLOGICAL, BIOCHEMICAL, HAEMATOLOGICAL, HISTOPATHOLOGICAL, DOSE TOXICITY.

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INTRODUCTION

Obesity, which is an excessive fat accumulation in the body, significantly impairs health, leads to the onset of other diseases such as diabetes, hypertension, arthritis, atherosclerosis, and cardiovascular diseases (Akil & Ahmad 2011). Moreover, the prevalence of cancer due to less physical activity has been also reported for the last many decades, and it has been a serious issue globally. Major problem is the treatment of obesity, which depends on lifestyle related physical activity. Synthetic drugs cause severe side effects, hence natural herbs are of good choice, due to few or no harmful effects. Natural compounds such as plant extracts containing phytochemicals are leading medicines used from ancient to modern science due to less adverse effects as compared to synthetic drugs. Phytochemicals protect the plants and act as a defense mechanism against predators. Secondary metabolites such alkaloids, flavonoids, saponins, terpenoids, tannins have been used in treating different chronic diseases (Seca & Pinto 2019, Shirsath & Goswami 2020).

Free radical production in the body is the major area of concern as its progression leads to complications and forming of several pathological conditions, (Lobo, 2010). Antioxidant capacity of the plants leads to scavenging of the free radicals, (Engwa, 2018). In contrast, some studies have shown that phytochemicals are toxic to humans and animals, and some are lethal, (Halliwell, 2007 and Bode & Dong 2015). *Helianthus annuus* is a common sunflower, belonging to the genus *Helianthus,* which is widely grown for edible oil and fruits used for health and nutrition. Seed extract preparations have many nutritional values (Pal, 2011), recent research has exposed a high risk of aflatoxins in sunflower seeds (Mmongoyo, et al., 2017). Although the seeds of sunflower showed beneficial effects, but also have adverse effects, consuming large amounts of seeds can lead to many health problems like impairing the kidney function. Sunflower seeds possess a large amounts of phosphorus which consumed in large quantities and can impair kidney functions (Guo et al., 2017).

Consuming more of it can increase the weight gain, which can cause rashes on the skin due to the presence of selenium (Nordberg et al., 2014), can also cause chronic fatigue and mood swings. It elevates sodium in the blood, which can elevate blood pressure which in turn causes the risk of heart conditions (National Research Council. 1983). It has the property of dermatitis (Hausen & Spring 1989). Respiratory allergy was observed with pollen allergens (Ghosh0 et al., 2015). The other adverse effects were headache and constipation (Leverrier et al., 2019). The other findings state that oral toxicity of leaf extract in high doses elicits hepatic, testicular, and nephrotic disorders (Guo, et al., 2017, Onoja 2018, Puga et al., 2019).

The present research was initiated to observe the role of the seed extract of this plant in weight loss. By observing other parameters (physiological, biochemical, haematological, and histopathological) it has been attempted to ensure that the drug does not affect the other organs of the mice. Hence, the present study was aimed to study the weight loss by *Helianthus annuus* L. seed extract in balb/c mice.

MATERIAL AND METHODS

The seeds of *Helianthus annuus* L. were assessed in normal old wild-type balb/c, mice of (5-7) months old (n=18), pursued from King Fahd Center for Medical Research, KAU University, Jeddah, Saudi Arabia. All mice were housed under controlled environmental conditions (22-24°C, 50-70% humidity, and a 12-h light/dark cycle). *Helianthus annuus* L. seeds were purchased from local market of Mecca, Saudi Arabia, and identified by nutritional speciation. The outer layers of seeds *Helianthus annuus* L. were removed, grained very fine weight of different concentration, one group 25%, the other group 50% powder of *Helianthus annuus* L seeds, and mixed with normal standard pelleted food (C1310, Altromin, Heidenau, Germany), for the comparison with control group which had access to normal standard pelleted food (C1310, Altromin, Heidenau, Germany) throughout the study period. The mixed food was dried and then given to the mice. All animal experiments were conducted according to the guidelines of the local and international law for the care and welfare of animals. The effects of *Helianthus annuus* L seeds were investigated during the first 7 ,21 days after supplementation, all mice were put in metallic cages for 4 day (adaption one days and 3 days for sample collection) for determination of food, fluid intake and urine output, the body weight of all mice were measured daily.

On the last day of experiment, blood samples from all animals were taken by puncturing the retro-orbital plexus using di ethyl ether (Roth, Karlsruhe, Germany) and blood was withdrawn into the blood collecting tubes as required for different biochemicals.For the biochemistry of plasma and urine, the concentrations of Na+, and K+ were measured by flame photometry (AFM 5051, Eppendorf, Germany). Plasma and urinary creatinine concentrations were measured using (kinetic method), urinary urea concentrations were measured by (UREA, colormetric method), blood cholesterol (CHOD PAP method), AST GOT, ALT GPT, HDL and LDL Cholesterol (direct method) and Triglycerides (GOPt method) were measured using kits from BIOLABO, (Les Hautes Rivers, 02160, Maizy, France), www.biolabo.fr and all measurements were carried according to manufacturing requirements. Measurements of plasma cholesterol were measured using Erba Cholesterol Kit (CHOD-PAP Method), both with the help of Chem 5 Plus-V2 Auto-analyser (Erba Mannhein, Germany). Fasting and non-fasting blood glucose concentrations were measured using a glucometer (Accutrend, Roche, Mannheim, Germany), after fasting the mice for 8 hour.

The complete blood picture (CBC), analysis, packed cell volume, blood hemoglobin concentration, and white blood cell count were determined using an electronic hematology particle counter (MDM 905 from Medical

Diagnostics 140 Marx; Butzbach, Germany) equipped with a photometric unit for determination of hemoglobin. The histological analysis were done on the last days after taking the blood samples from all mice, then sacrificed and kidney and liver organs were removed and processed for further histological analysis by the method of John & Alan (1999). The stained sections were viewed and evaluated for pathological changes using a light microscope (Nikon, Eclipse i80). The required images were taken in different magnifications with Nikon mounted digital camera (OXM 1200C, Nikon, Japan). To see the significant difference between the control group and mice who had food supplementation of *Helianthus annuus* L seeds, all values were expressed as mean ± S.E.M. and statistical analysis was performed by one-way analysis of variance (ANOVA) using GraphPad Prism 8 Software, version 8-4-3(686), San Diego California USA. The results with a probability factor of $P < 0.05$ were significantly considered (Khan et al., 2019).

RESULTS AND DISCUSSION

Results of the present study, the physiological parameters considering body weight are shown in Table 1 and Fig. 1, with dose-dependent concentrations of *Helianthus annuus* seed extract, the weight gain was less comparable to control. the gradual increase in weight was observed in controls from the start with (25.65±0.33to 26.72±0.42) on 7th day, hence the body weight gain was (1.07±0.17), whereas on 21st day, the increased weight were (27.53±0.70), from the baseline (25.65±0.33), hence the weight increased is (1.88±0.63), similarly when treated with different concentrations of *Helianthus annuus* seed extract, there were t slight reduction in body weight after 7 days from baseline (24.96±0.22), to 24.55±0.20) after 25% of *Helianthus annuus* seed extract supplementation, but on day 21st there were reduction in body weight (24.96±0.22 to 23.90±0.53) compared to control. Moreover the same was noticed after 50% of *Helianthus annuus* seed extract supplementation on day 7th (24.39±0.43) and day 21st (23.82±0.53) compared to baseline body weight (24.92±0.34).There were significant reduction in body weight gain after 25% and 50% *Helianthus annuus* seed extract supplementation with (-1.05±0.43) and (-1.10±0.22), respectively as compared to body weight gain in control group after 21 days (1.88±0.63), showing the beneficial effect of *Helianthus annuus* seed extract supplementation on body weight reduction.

The metabolic cage results show the effects of *Helianthus annuus* seed supplementation (25%,50%), on food, fluid intake, urinary output, faecal wet and dry weight as compared to the control group (Table 2 for 7 days, and 21 days in Table 3). Observation shows no statistical differences in all groups of mice, and only urinary outputs were little reduced after *Helianthus annuus* seed treatment, (25%, 50%) but did not reach a significant difference. Table.3 Effect of (25 and 50%) *Helianthus annuus* seed supplementation on food intake, fecal wet, dry weight, fluid intake, urinary output, as compared to the control group after 21 days.

Table 1. Effect 25% of *Helianthus annuus* seeds supplementation on body weight after 7 and 21 days compared to control group.

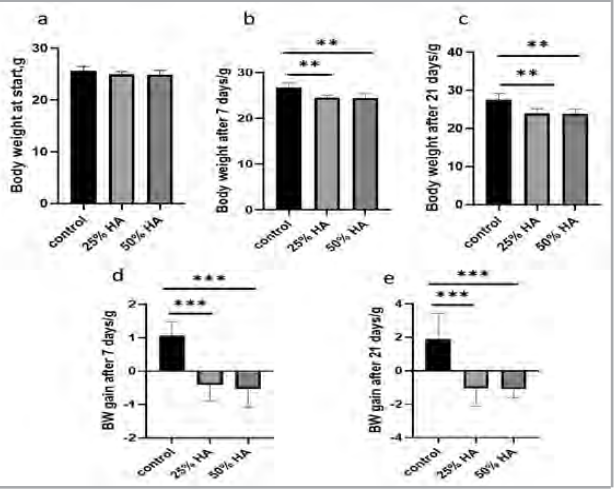
	Control	25% of *Helianthus annuus* L seeds	50% of *Helianthus annuus* L seeds
Body weight,			
at start/g. Body weight,	25.65±0.33	24.96±0.22	24.92±0.34
after 7 day/g. Body weight,	26.72±0.42	24.55±0.20**	24.39±0.43**
after 21 day/g. Body weight, gain	27.53±0.70	23.90±0.53**	23.82±0.53**
after 7 days/g. Body weight, gain	1.07±0.17	-0.41±20***	-0.53±0.23***
after 21 days/g.	1.88±0.63	-1.05±0.43**	-1.10±0.22***

annuus seeds after 7 and 21 days of supplementation compared to control.**indicates highly significant (P<0.0001), **indicates extremely significant (P<0.00001), between control and (25 and 50%) of *Helianthus annuus* seed and after 7 ,21 days ,.

Table 4 results showing the Effect of different concentrations of *Helianthus annuus* L seeds supplementation on urinary Na+, K^+, and Ca^{2+} concentrations compared to control group. Observing the above, results mentioned in Tables 1 and 2 acts in accordance with Table 4, showing that the biochemical results of urine comply with the above results, with normal levels of urine Na^+, Urine K^+ and calcium. Blood examination also showed normal levels of Na^+, K^+, and Ca^{2+}. The effect of different concentration of *Helianthus annuus* L. seed extract supplementation on 21st day as compared with control group, Table 5, shows that normalized levels after *Helianthus annuus* L. seed

extract supplementation of 25% and 50% respectively compared to control group.

Figure 1: Effect (25 and 50%) of *Helianthus annuus* seeds supplementation on body weight at start (a), after 7days (b), after 21 days (c), body weight gain after 7 days (d) and body weight gain after 21 days (e) days compared to control group.



The biochemical results in Table.6, show the effect of different concentrations of 25% and 50% of *Helianthus annuus* seed supplementation as compared to control group. Fasting blood glucose was little reduced in 25% as compared with 50% of *Helianthus annuus* seeds supplementation. The liver function of Alkaline Phosphatase, Alanine Aminotransferase (ALT), Aspartate Aminotransferase, Bilirubin, Cholesterol, High-density lipoproteins, and Triglycerides, were all in normal range. However, that the high HDL- cholesterol indicates to lower the risk of heart stroke, it is generally visualized that it reduces ischemic stroke in elderly people. Table 7, shows the effect of *Helianthus annuus* L seed supplementation on complete blood count (CBC) as compared with control group (WBC, LYM, GRA, MID, and MPV) were in normal range only there were slight reductions in hemoglobin level compared to control group but it's not significant. Table 7. Effect of (25 and 50%) *Helianthus annuus* L seeds supplementation on Complete Blood Count (CBC), as compared with control group after 21 days of supplementation. Supportive effect of 25% and 50% *Helianthus annuus* L. seed extract in BALB/c mice of 5-7 months old with different parameters like physiological, biochemical, haematological and histopathological studies was investigated.

Table 2. Shows the effect of (25 and 50%) *Helianthus annuus* seed supplementation on food intake, fecal wet, dry weight, fluid intake, urinary output, as compared to the control group after 7 days.

	Control	25% of *Helianthus annuus* L seeds	50% of *Helianthus annuus* L seeds
		7th day	
Food Intake, g/24 h.	1.43±0.20	1.41±0.04	1.45±0.15
Fecal wet weight, g/24h.	0.50±0.10	0.48±0.09	0.47±0.01
Fecal dry weight, g/24h.	0.37±0.01	0.35±0.01	0.33±0.02
Fluid Intake, ml/24h.	2.34±0.06	2.15±0.16	2.09±0.03
Urine output, ml/24h.	1.02±0.08	0.85±0.13	0.72±0.19

Arithmetic means ± standard error of (25 and 50%) of *Helianthus annuus* seeds after 7 days supplementation compared to control.

Physiological parameters in Table .1 and Fig.1 considering body weight showed the reduction of weight with the increasing dose concentration of *Helianthus annuus* L. seed extract with increasing days of supplementation (Leverrier et al., 2019). Table.2 with 25% *Helianthus annuus* L. seed extract showed no difference on food and fluid intake was observed, but there were reduced urine output (ml/24h) Fecal wet weight (g/24h) and Fecal dry weight (g/24h) was reduced with increasing supplementation of days, the same parameters in Table.3 the Effect of *Helianthus annuus* L seed supplementation (50%) for 7th and 21st day; Food intake, fecal wet and dry weight was also reduced with increasing the days of dose supplementation (Blicharska, et al., 2014).Due to the less fluid intake, the animal could be in a dehydrated state (Puga et al., 2019).

Table 3. Effect of (25 and 50%) *Helianthus annuus* seed supplementation on food intake, fecal wet, dry weight, fluid intake, urinary output, as compared to the control group after 21 days.

	Control	25% of *Helianthus annuus* L seeds	50% of *Helianthus annuus* L seeds
		21st day	
Food intake, g/24 h.	1.50±0.06	1.40±0.02	1.37±0.07
Fecal wet weight, g/24 h.	0.45±0.05	0.38±0.02	0.36±0.02
Fecal dry weight, g/24 h.	0.34±0.01	0.32±0.02	0.30±0.01
Fluid intake, (ml/24h.	2.25±0.20	2.09±0.22	2.05±0.14
Urine output. ml/24 h.	0.82±0.09	0.74±0.19	0.69±0.04

Arithmetic means ± standard error of (25 and 50%) of Helianthus annuus seeds after 7 days supplementation compared to control.

Table 4. Effect of (25 and 50) *Helianthus annuus* seed supplementation on urinary Na^+, K+ and Ca^{2+} as compared to the control group after 21 days of treatment.

	Control	25% of *Helianthus annuus* seeds	50% of *Helianthus annuus* seeds
Urine [Na^+], mmol/24 h.	220.75±5.04	226.87±8.43	237.17±8.23
Urine [K^+], mmol/24 h.	697.29±23.09	701.60±2.79	717.20±10.75
Calcium [Ca^{2+}], mmol/24 h.	7.54±0.19	7.72±0.17	7.89±0.34
[Na^+]plasma , mM.	139.00±1.39	142.64±1.69	141.80±1.49
[K^+]plasma, mM.	4.36±0.12	4.32±0.22	4.29±0.21
[Ca^{2+}]plasma , mM.	2.23±0.05	2.11±0.03	2.21±0.16

Arithmetic means ± standard error of (25 and 50%) of *Helianthus annuus* L seeds after 7 days supplementation compared to control

Table 5. Effect of (25 and 50%) Helianthus annuus L seed supplementation on blood urea nitrogen, urinary creatinine, plasma creatinine concentration, glomerular filtration rate and normalization 24-h creatinine clearance as compared with control group after 21 days of treatment.

	Control	25% of *Helianthus annuus* seeds	50% of *Helianthus annuus* seeds
Blood Urea Nitrogen,(mg/dl.	17.85±1.43	17.99±1.35	17.38±1.32
Urinary creatinine, mg/dl.	36.34±2.24	35.42±3.66	35.18±2.08
[creatinine]plasma , mg/dl.	0.29±0.02	0.28±0.02	0.28±0.03
Glomerular filtration rate	3.66±0.22	3.84±0.38	3.95±0.15
(GFR), µl/min.			
Normalized 24-h creatinine clearance, mL/min/g body weight.	11.15±2.40	11.96±2.03	12.21±2.41

Arithmetic means ± standard error of (25 and 50%) of *Helianthus annuus* L seeds after 7 days supplementation compared to control.

Table 6. Showing the effect of (25 and 50%) of *Helianthus annuus* seed supplementation on fasting blood glucose, Alkaline Phosphatase, Alanine Aminotransferase, Aspartate Aminotransferase, Bilirubin, Cholesterol, High-density lipoproteins, Triglycerides and Uric Acid as compared with control group after 21 days of treatment.

	Control	25% of Helianthus annuus seeds	50% of Helianthus annuus seeds
Fasting blood glucose, mg/dl.	97.60±4.09	91.33±14.11	95.67±9.83
Alkaline Phosphatase (AP), U/L.	96.67±2.06	76.48±13.43	68.57±17.27
Alanine Aminotransferase (ALT), U/L.	39.35±4.69	42.37±13.04	38.97±6.50
Aspartate Aminotransferase (AST), U/L.	47.18±3.03	39.55±7.78	38.00±5.75
Bilirubin, mg/dl.	0.26±0.02	0.23±0.01	0.26±0.03
Cholesterol, mg/dl.	31.77±7.06	28.97±4.11	30.83±11.72
High-density lipoproteins (HDL), mg/dl.	24.83±0.98	21.30±3.14	28.17±8.58
Triglycerides, mg/dl.	46.57±4.49	47.50±14.51	43.67±9.31

Arithmetic means ± standard error of (25 and 50%) of *Helianthus annuus* L seeds after 7 days supplementation compared to control.

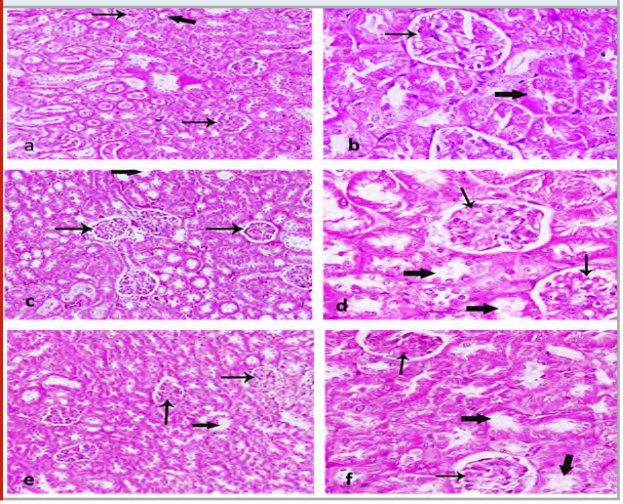
Table 7. Effect of (25 and 50%) *Helianthus annuus* L seeds supplementation on Complete Blood Count (CBC), as compared with control group after 21 days of supplementation

	Control 21st	25% of *Helianthus annuus* L seeds	50% of *Helianthus annuus* L seeds
White Blood Cells (WBC), 10^3/ml.	7.25±0.32	7.68±0.36	7.89±0.53
Lymphocyte (LYM),10^3/ml.	5.23±0.27	5.85±0.37	5.68±0.24
Lymphocyte (LYM), %.	91.32±0.83	90.90±2.27	89.48±1.19
Granulocytes (GRA),10^3/ml.	0.40±0.03	0.47±0.06	0.43±0.06
Granulocytes (GRA), %.	2.93±0.22	3.02±0.32	3.13±0.37
Monocytes (MID), 10^3/ml.	0.57±0.09	0.47±0.05	0.58±0.09
Monocytes (MID), %.	5.68±0.71	6.35±0.57	5.45±0.28
Hemoglobin (Hb), g/l.	13.92±0.27	13.33±0.29	13.35±0.24
Mean Corpuscular Volume (MPV), fl.	7.42±0.29	7.61±0.41	7.93±0.54

Arithmetic means ± standard error of (25 and 50%) of *Helianthus annuus* L seeds after 7 days supplementation compared to control.

Table 4. results showing mild elevated results of Urine Na^+ (µmol/24h), Calcium (mg/dl), Urine K^+ (µmol/24h), Uric acid (mg/dL) with the high dose Effect of different concentrations of *Helianthus annuus* L. seed extract supplementation compared to control group. Mild increase in WBC in accordance with Granulocytes (GRA) % is associated with the stress response of the animal with little output of fluids, which could also be due to inflammatory results (Nishitani & Sakakibara 2014). Effect of different concentrations of *Helianthus annuus* L seed supplementation on liver function test (ALT, AST, and ALT) and fasting and random blood glucose concentrations as compared with control group after 21 days of treatment (Table.6) showing normal results. Bilirubin was reduced in 25% conc., with not much difference compared to control. Cholesterol was reduced in both conc. High-density lipoproteins were improved in 50% dose conc., with the decline in Triglycerides showing blood lipid profile improvement (Leverrier et al., 2019). Table.7 showing Increase levels of WBC, LYM, GRA, and MID suggest the increase due to the response of infection. The Mean Corpuscular Volume increase can also be the cause of anemia with the deficiency of B vitamins, namely B-12 and foliate. Hemoglobin was not much significantly reduced, whereas large inflamed cells were observed after 25% and 50% of *Helianthus annuus* L. seed supplementation. Free radicals are generated in many ways of mechanism, which reacts in damaging cellular substances such as nucleic acids, protein, and lipids. Studies have shown the scavenging activity of *Helianthus annuus* L., this plant showed scavenging and anti- inflammatory activities (Guo et al., 2017).

Figure 2: Section of kidney showing Glomerulus (thin arrow), Renal tubules (thick arrow). (a, b) photo of the control group. (c, d) after 25% of *Helianthus annuus* L seed supplementation and (e, f) after 50% of *Helianthus annuus* L seed supplementation.



The effects shown in this research could be caused due to phytoconstituents present in the plant, the secondary metabolites such as alkaloids, tannins, flavonoids, terpenoids, saponins which were also reported (Dwivedi & Sharma 2014). These secondary metabolites are well known for its scavenging of free radicals and anti-inflammatory function. Treatment of 25% and 50% *Helianthus annuus* L. seed extract has resulted in weight loss in BALB/c mice of 5-7 months old without affecting the others parameters.

CONCLUSION

The study of this research concluded that *Helianthus annuus* L seed extract observed in this investigation on BALB/c mice has shown beneficial effects on animals. Can be used for a long period of time for weight management with well diagnostic condition, hence will be effective for diabetic patients. Can be used for weight loss management and can be considered for obese patients should be explored for clinical efficacy and safety of this plant extract as anti-obesity.

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Ethical Clearance Statement: The Current Research Work Was Ethically Approved by the Intuitional Review Board (IRB) of Turabah University Saudi Arabia.

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Pathological Communication

In vitro Evaluation of Biocontrol Agents Against *Fusarium oxysporum* to Eliminate Wilting of Cumin

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ABSTRACT

Cumin (*Cuminum cyminum* L.) is an essential herb of dryland. Although cumin is an economic cash crop, limited efforts have been made for varietal developments. In India, Rajasthan, Gujarat is central cumin cultivar states, the zone under cumin development is around 841940 hectares with yearly productivity of 546750 tones, which contributes around 8200 crore rupees yearly. Gujarat, it is covering a territory of around 3, 37007 hectares. Several biotic stresses can confine productivity. Cumin is seriously affected by the Fusarium wilt disease caused by the soil-borne pathogen *Fusarium oxysporum* and *Fusarium equiseti*, resulting in yield losses of up to 80% depending on severity of infestation. Biological control has emerged as one of the most promising alternatives to chemical fungicides. The experiment was carried out to assess their possible use as bio-agents for several antagonistic fungi and bacteria under in vitro conditions using the food poison technique with selected biological control agents. *Trichoderma harzianum, T. konngii, T. viride, Pseudomonas fluorescens, P. putida, A. niger, A. flavus, Penicillium citrinum, Serratia marcescens, Bacillus subtilis, Bacillus cereus, Bacillus amyloliquefaciens, Saccharomyces cerevisiae* were used for this study. In-vitro test showed that selected microbes were showing antagonistic activity towards *F. oxysporum*. Trichoderma spp. was the best bio-agent, who had 66 to 81% inhibition zone. Aspergillus spp and Pseudomonas spp. emerged as effective bio-agents with 63-67% and 68 to 76% zone inhibition. The aim of this study is to become a suitable milestone for future studies and provide with updated information on ways to eliminate wilting in cumin.

KEY WORDS: BIO AGENT, CUMIN WILT, FUSARIUM OXYSPORUM.

INTRODUCTION

Cumin (*Cuminum cyminum* L.) is widely grown as an important spice crop in arid and semi-arid regions of the Indian subcontinent. Although cumin is an economic cash crop, limited efforts have been made for varietal developments (Bhatnagar et al. 2013; Meena 2015). The fungal disease wilt, caused by the soil-borne phytopathogens, *Fusarium oxysporum* f. sp. cumini (Foc), is one of the major threats to cumin production in India and worldwide. It is a small, slender annual herbaceous plant, with a glabrous, branched stem 20 to 30 cm tall with a diameter of 3 to 5 cm diameter tending to hang down under its weight (Özer and Bayraktar 2015; Pandey et al. 2016; Didwania 2019).

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It is also used in food for flavor, it has many important medicinal. It is also a necessary part of the Indian system of medicine called Ayurveda (Singh et al. 2017). It is native to the Mediterranean and Near Eastern regions, mainly cultivated in India, Egypt, Libya, Iran, Pakistan and Mexico. It is extensively cultivated in India, China, South Russia, Japan, Indonesia, Iran, Morocco, Turkey, Egypt and Argentina. India is the largest producer, consumer and exporter of cumin in the world. In India, Rajasthan, Gujarat, Madhya Pradesh, Haryana, Punjab, Uttar Pradesh and Bihar (Khalequzzaman et al. 2018). India exports cumin seed to Bangladesh, Japan, Malaysia, Nepal, Pakistan, Singapore, South Africa, UAE, UK, USA and many other countries (Didwania 2019).

The demand for cumin is moderately increasing in the domestic and international market, which plays a vital role in the national economy. However, the production and productivity of cumin are decreasing year after year due to non-availability of good quality seed, slow and

uneven germination, and low adoption of seed production technologies being some significant obstacles. Seed production potential of cumin is also greatly affected due to degradation of seed quality, microbial load, a heavy infestation of diseases and pests, traditional harvesting & processing, unscientific and unhygienic handling post-harvest and storage (Trivedi et al. 2019). Because of the great economic importance of cumin, it is essential to study wilt and blight diseases in cumin to control them in best possible manner. This can be achieved by paving the way for devising management strategies through bio-control.

MATERIAL AND METHODS

To study the isolation and identification of pathogen, the sample of wilted plants were taken from the cumin field. A small part of the infected stem was taken and sterilized with 0.01% sodium hypochlorite, followed by three washes by sterile distilled water. The tissue was then placed on Potato dextrose agar medium plates. The stem was allowed to grow on Potato Dextrose Agar medium plate for 2 – 5 days at room temperature in dark condition and further sub-cultured to obtain pure isolates. The culture was maintained on PDA for immediate use and for long term use, the culture was stored at 4°C (Yadav et al. 2020; Aziz et al. 2021).

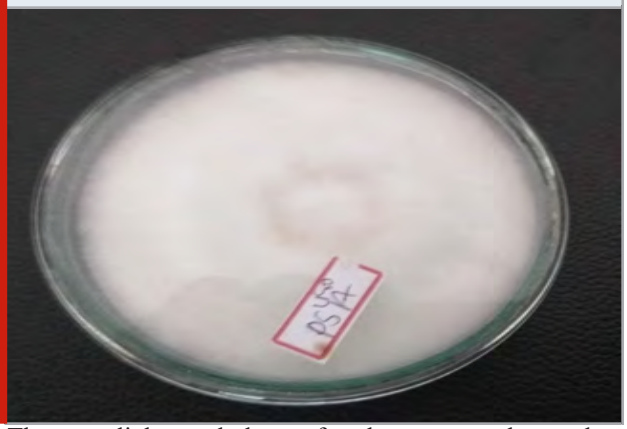
To study the characteristics and spore morphology, the measurements of conidia and conidiophores were made with the help of ocular micrometer (Booth1971; Vevre et al. 2021). To study the culture collection, three isolates of Trichoderma spp. was collected from Junagadh Agriculture University. Aspergillus spp., *Serratia marcescens, Bacillus spp., Saccharomyces cerevisiae, Pseudomonas fluorescens, P. putida* were obtained from the Microbiology Department of Sir Purshottamdas Thakordas Science College. Penicillium citrinum were isolated from soil using the specialized medium (Vevre et al. 2021).

To study antagonism of bacteria and fungi, T*richoderma harzianum, T. konngii, T. viride, Pseudomonas fluorescens, P. putida, A. niger, A. flavus, Penicillium citrinum, Serratia marcescens, Bacillus subtilis, Bacillus cereus, Bacillus amyloliquefaciens*, and *Saccharomyces cerevisiae* were tested for their antagonistic activity against *F. oxysporum* in dual culture technique. Culture disc of each potential antagonist and test pathogen were taken from 7-days old culture and transferred aseptically to 90 mm petri plates on opposite sides. The disc of *F. oxysporum* was placed 2-days earlier than the antagonist to compensate for the slow growth of the pathogen. The distance between the inoculum points of test pathogen and antagonist was kept as 5 cm. The disc of test the pathogen placed at the centre in separate Petri dishes served as a control. The inoculated plates were incubated in a Biological Oxygen Demand incubator at 28±1°C. Observations on growth of antagonists and F. oxysporum from the centre of the disc towards the centre of the plate was recorded after seven days of inoculation. The growth inhibition of pathogen over control was calculated using the formula given by (Vincent 1947; Yadav et al. 2020).

RESULTS AND DISCUSSION

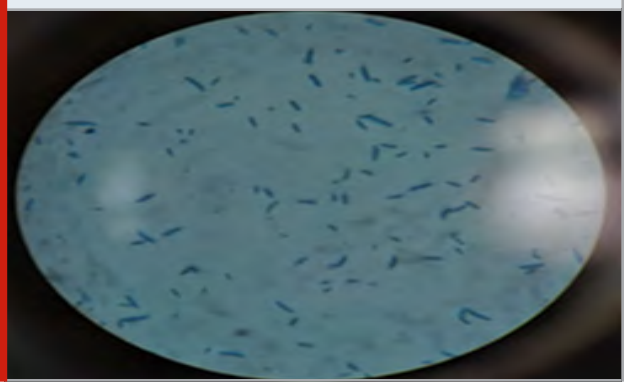
Isolation and identification: Sample were taken from infected plant parts confirmed *Fusarium* was the causative pathogen in the cumin field belonging to the geographical location of Ravi Pura Kampa, Aravalli district, North East Gujarat. After surface sterilization, the same were inoculated in PDA plates and incubated at room temperature in dark condition for four days (Figure 1).

Figure 1: Image of Pathogen culturing on PDA medium



The mycelial morphology of pathogen was observed as white mycelia, with presence of grayish spores. Microscopic observation was done by LABOMED microscope (Figure 2). The fungus forms studied were sickle shaped macroconidia, ovoid microconidia and chlamydospores (Figure 3). The microconidia of isolate were small, 1/2 celled and hyaline with oval to Reni form and oval to oblong with slightly curved shape. Length of spore ranged from 3.48 to 17.38 µm, the width ranged from 2.30 to 4.55 µm. Macroconidia were long, slightly curved or boat shaped, variable in size and smoothly rounded or pointed at the tip, mostly with 2 to 5 septa and hyaline in colour. Length ranged from 16.89 to 59.30 µm, while the width ranged from 4.08 to 6.98 µm. Chlamydospores were thick walled, hyaline in colour, varied in shape, produced intercalary and terminal, single and in pair or in chain identification (Table 1).

Figure 2: Microscopic observations of spore



Antagonism of bacteria and fungi: Bio-agents used effectively inhibited mycelial growth of *Fusarium*

oxysporum under *in vitro* conditions. The maximum inhibition (81 %) of *F. oxysporum* was observed with the treatment of *Trichoderma harzianum, T. konngii* 80%, T. viride 70%, *Pseudomonas fluorescens* 76 %, *P. putida* 70 %, *A. niger* 68 %, *A. flavus* 67%, *Bacillus subtilis* 44 %, *Bacillus cereus* 43%, *Bacillus amyloliquefaciens* 44 %, *Penicillium citrinum* 83.8 %, *Serratia marcescens* 50 % and *Saccharomyces cerevisiae* 49% inhibit. The data is presented in (Table 2).

Table 1. Morphological characters of pathogen

Length of Conidia		Septation		Shape
Macroconidia	Micro	Macro	Micro	Macro
16.89 to 44.18	0 to 1	3 to 4 septa	Oval to oblong	Slightly curved

Figure 3: Microscopic observations of mycelia and spore

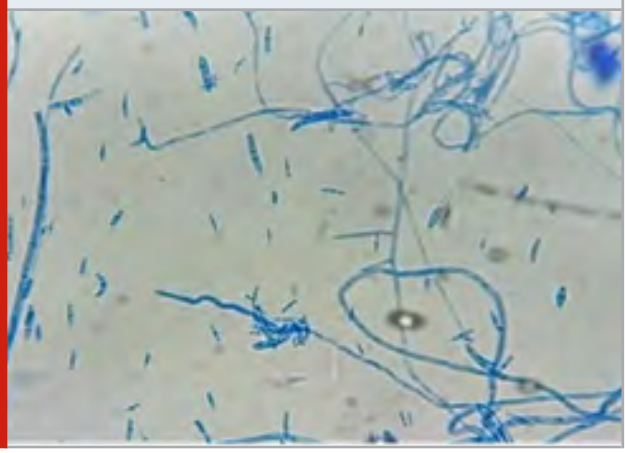


Table 2. *In-vitro* antagonistic effect of microorganism on pathogen

No.	Name of microorganism	Zone of Inhibition of isolate (mm)
1	*Trichoderma harzianum*	81
2	*T. konngii*	80
3	*T. viride*	70
4	*Pseudomonas fluorescens*	76
5	*P. putida*	70
6	*A. niger,*	68
7	*A. flavus*	67
8	*Penicillium citrinum*	83.8
9	*Serratia marcescens*	50
10	*Bacillus subtilis*	44
11	*Bacillus cereus*	43
12	*Bacillus amyloliquefaciens*	44
13	*Saccharomyces cerevisiae*	49

The present findings corroborate with the reports of Cook and Baker (1983), who reported that Trichoderma and *P. citrinum* effectively inhibited growth of the *Fusarium oxysporum* which may be due to the fungi static effect. In the present investigation, maximum disease control was observed in *Trichoderma* spp. and *Pseudomonas* spp. It was also reported by Abou-Zeid et al. (2003) *Pieta* and *Pastucha* (2004) Abo- Sedera (2005) El-Khair et al. (2010) and Otadoh et al. (2011) that treatment of *Trichoderma album, T. hamatum, T. harzianum, T. koningii, Trichoderma reesei* and *T. viride* provides adequate protection against *Fusarium* spp. In alignment with Deepak et al. (2008) research, results of the present study revealed that the maximum inhibition of radial growth of *F. oxysporum* f. sp. cumini was 81% in the treatment of *Trichoderma harzianum* and 68 % in A. niger (Deepak et al. 2008; Rathore et al. 2020).

Chawla and Gangopadhyay (2009) have successfully used *Trichoderma harzianum, T. viride, P. fluorescens*, and *B. subtilis* to reduce wilt incidence of cumin plants, and this could support the present study. Others have previously reported suppression of disease by *Bacillus* spp (Dukare and Paul 2021). (Yu G et al. (2010) and Wang et al. (2011) observed 44.4 % to 73.9 % inhibition of *F. oxysporum.* Kumar and Dubey (2012) reported that *T. harzianum* and *T. viride* also gave satisfactory control of *Fusarium* wilt in chickpea and coriander. Tan (2015) reported that *Serratia marcescens* showed 78.7 % inhibition of pathogen in banana wilt (Tan 2015; Dukare and Paul 2021). Hussain et al. (2016) observed that the 95.24 % inhibition zone was observed by Penicillium spp. against the pathogens similar to present study. Kumar et al. (2016) tested the efficacy of different fungal and bacterial antagonists against the wilt of cumin and found that *P. fluorescens* was highly inhibitory to the pathogen in vitro condition. Dhar et al. (2018) checked out efficiency of a Serratia marcescens to manage root rot disease in tea. Bubici et al. (2019) reported at least 143 articles about disease management using biocontrol agents the findings of the current work are also matched with Rathore et al. (2020). Dukare and Paul (2021) observed that *Pseudomonas* sp. NS 1 and *Bacillus* sp. NS 22 displayed the potential as bio fungicide under in vitro condition which is similar to current finding (Rathore et al. 2020; Dukare and Paul 2021).

CONCLUSION

The findings of the present study showcased a significant value of microorganism; it has been widely used as a biocontrol agent against many fungal pathogens. Among the tested isolates, *Trichoderma harzianum, T. konngii, T. viride, Pseudomonas fluorescens, P. putida, A. niger, A. flavus, Penicillium citrinum, Serratia marcescens, Bacillus*

subtilis, Bacillus cereus, Bacillus amyloliquefaciens, Saccharomyces cerevisiae was the most suitable candidate as they exhibited the capacity to inhibit maximum growth of the fungal pathogen. This study may enhance the knowledge in identifying the potential isolates for future use as a biocontrol agent against wilt disease of cumin. In future, it would be of interest to further investigate the potential of *T. harzianum, T. konngii, T. viride, P. fluorescens, P. putida, A. niger, A. flavus, P. citrinum, S. marcescens, B. subtilis, B. cereus, B. amyloliquefaciens, S. cerevisiae* isolates under controlled and field conditions as a biocontrol agent against cumin wilt.

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

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Pharmaceutical Communication

Extracts of Medicinal Plants for Preventing Postnatal Complications in Cows

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ABSTRACT

It is obligatory for every farm to carry out a set of measures in order to detect obstetric and gynecological diseases in the breeding stock of cattle at an early stage, as they can be detected in more than 50% after pathological childbirth. We reported here on determining the effect on the extracts of such medicinal plants as Maral root and stinging nettle, when feeding recipient cows in a dry period, on the prevention of postpartum complications in animals and a reduction in the recovery period of the reproductive system. Dry extracts of the selected medicinal plants containing an increased concentration of the main active substances of the initial plant raw materials were obtained by water-ethanol and liquid extraction with subsequent vacuum drying processing. The effect of extracts on the natural resistance of cows was determined by generally accepted, standard and original research methods using modern laboratory equipment. It was found that on the 30th day in animals that received additional medicinal extracts in the diet, an increase in the content of neutrophils and platelets in the blood and protein fractions of blood serum was observed, which indicates an increase in the resistance of the animal's body. An increase in the bactericidal, lysozyme activity of the blood serum of recipient cows and the phagocytic activity of leukocytes also indicates an increase in the immune response of the animal's body in response to the penetration of foreign microflora into the body. According to the results of the studies, it was recommended to add a two-component Phyto biotic feed additive to the main diet based on extracts of medicinal plants Maral root and stinging nettle at a dose of 50 g per head per day in the ratio of components: 100 kg of compound feed, 0.5 kg of maral root extract, 2, 5 kg of stinging nettle extract.

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

INTRODUCTION

The development of pathological processes in cows during pregnancy and dry periods often has a negative impact on the resulting offspring, which may be born with various physiological abnormalities or be stillborn (Gilbert, 2019). In this regard, antibiotic therapy for dry cows continues to play an important role in the treatment of infectious diseases and the prevention of new animal diseases. As Animal Health Ireland and many large farms (Sok City (Wisconsin), Eko Niva Agro, etc.) note, the use of antibiotics to treat animals is no longer acceptable. This is due to the fact that there is a high likelihood of developing antibiotic resistance in fetus, which may impede its effective treatment in adulthood (Petushok and Malashko, 2018).

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In artificial insemination technology, it is very important to obtain healthy embryos for the use in highly productive animal husbandry. To carry out their qualitative analysis and to establish the effectiveness of embryo transplantation, it is significant to get “clean” embryos without residual antibiotics obtained with colostrum. Medicinal plant raw materials, as a full-fledged source of biologically active substances, are a popular component in animal diets (Ezzat et al., 2016) used to increase the body's natural resistance to infectious diseases that are often manifested in cows in the postpartum period, and can serve as a good substitute for antibacterial therapy. The high demand for medicinal plant materials is explained by their wide availability in almost all parts of the planet, low cost, lack of side effects on the animal's body, high pharmacological and functional action (Zubova et al., 2019; Ulrikh et al., 2019; Lombardi et al., 2020).

The natural resistance of the organism reflects a complex of specific and nonspecific factors caused by the interaction of innate and adaptive immune responses (De Pablo-Maiso et

al., 2018). With the industrial keeping of animals, stresses and immune deficiencies contribute to the development of pathologies of the birth and postpartum periods of varying severity, reducing the animal productivity. The introduction of pharmaceutical substances based on herbal extracts into the composition of animal diets in order to correct the birth process and to prevent postpartum complications in animals' body by improving the properties of nutrition is a promising direction for replacing antibiotics and increasing the ecological value of animal products.

Conducting our research, the choice was made in favor of Stinging Nettle, since the leaves of stinging nettle have a rich multivitamin composition. They contain Ascorbic acid (up to 269 mg%), vitamin K (42-45 μg/g), vitamin B2, Pantothenic acid, carotenoids (beta-carotene, xanthophyll, violaxanthin) - up to 50 mg%, urticin glycoside, tanning and protein substances, such organic acids as Formic, Coffeic, P-coumaric and Ferulic ones, nitrogenous substances, amino acids, including essential ones. Aerial part of nettle contains important essential trace elements (Zn, Cu, Mn, Fe) and lead (Pb), as well (El-Haouari and Rosado, 2019). Preparations of stinging nettle stimulate blood clotting increase the percentage of hemoglobin and the number of erythrocytes and have a pronounced tonic effect on the smooth muscles of the uterus and produce a vasoconstrictor effect (Belmaghraoui et al., 2018; Chernyavskikh, 2019; Smulski et al., 2020; Yu et al., 2021).

The validity of the Maral Root use is associated with the phyto-ecdysteroids (420 mg/kg) contained in it - biologically active substances. Ecdysteroids are the most widespread and numerous families of steroid compounds. Phyto-ecdysteroids have a prophylactic effect in the treatment of inflammatory and immunological processes. Ecdysterone and the direct analogs of ecdysteroids are the main active substances with an anabolic effect and are promising for use in agriculture (Głazowska et al. 2018). Rhizomes and roots contain alkaloids, ascorbic acid (0.1%), carotene, tannins (about 5%), the amount of phytoecdysones, essential oil (0.9%), phenolics (11.4%), gums, ascorbic acid (68,8 mg%), inulin, coumarins, organic acids. The above-ground mass contains ascorbic acid, protein, sugars, and organic acids (Alamgir, 2017; Yu et al., 2021).

The unique biological activity of the plant is determined by the combination of a complex of substances: mono- and polysaccharides, inulin, organic acids, phyto-ecdysones, triterpenic saponins (raponticosides), vitamins, phenol carboxylic acids and their derivatives, lignin, tannins, essential oil, alkaloids, flavonoids, anthocyanins, and gums, crystals of calcium oxalate, phosphoric acid salts, macro- and microelements (Skala et al., 2016; Głazowska et al. 2018). The effective biological activity of pharmaceutical substances based on extracts of medicinal plants obtained in the form of a powder is 50-100 times higher than the activity of other drugs or preparations. High activity is obtained due to a complex combination of the main active ingredients (Pat. RU 2739625; Abdel-Lattif et al., 2021).

The aim of the research was to study on the effect of extracts of such medicinal plants as Safflower leuzea and Stinging Nettle in feeding recipient cows during the dry period for the prevention of postpartum complications in animals. Research objectives are: to determine the extracts' effect on the hematological parameters of the blood of recipient cows, the content of total protein and its fractions in the blood serum of animals, indicators of natural resistance of the blood serum of recipient cows; as well as their impact on the postpartum period indicators of recipient cows and the viability of the embryos obtained; to determine the possible concentration of the introduction of extracts of medicinal plants in the main diet of cows.

MATERIAL AND METHODS

In this study the reproductive system of recipient cows was carried out in the conditions of LLC "Farm Enterprise Mikhailovskoye" (Prokopyevskiy District, Kemerovo Region), on selected healthy Black-and-White recipient cows. Cows were selected taking into account their age, live weight, clinical and physiological condition. For the study, three groups of cows were formed; there were 6 heads in each of them (control, first experimental, second experimental, third experimental). The control group consisted of animals kept in farm conditions and received a traditional diet: flaked wheat, sunflower cake, feed yeast, table salt, monocalcium phosphate, hayage silage of vetch, raw potatoes, meadow or pasture hay in the ratios established in the farm.

Experimental group I - animals were fed with a feed additive in the following ratio of components: 100 kg of compound feed, 0.5 kg of Maral Root extract, 1.0 kg of Stinging Nettle extract. Experimental group II - animals were fed with a feed additive in the following ratio of components: 100 kg of compound feed, 0.5 kg of Maral Root extract, 2.5 kg of Stinging Nettle extract. Experimental group III - animals were fed with a feed additive in the following ratio of the components: 100 kg of compound feed, 0.5 kg of Maral Root extract, 4.0 kg of Stinging Nettle extract. The selected recipient cows were fed with an introduction of the enriched feed additive with the first day of the dry period, the amount of the feed additive was 50 g per head per day. The content of the Maral Root extract in the experimental groups remained unchanged due to the high content of phyto-ecdysteroids in its composition.

The effectiveness of enrichment of the recipient cows' main ration with extracts of Maral Root and Stinging Nettle was determined by indicators of hematological and biochemical composition of blood samples taken from the jugular vein of cows in the morning, in the research laboratory "Biochemical, molecular genetic studies and breeding of farm animals" on the basis of the Federal State Budgetary Educational Institution of Higher Education "Kuzbass State Agricultural Academy". The blood samples were taken from recipient cows before the start of the experiment and after 30, 60 and 90 days of feeding them with feed additives of the Maral Root and Stinging Nettle extracts to the main diet. Hematological blood parameters were determined by using a hematological analyzer VetScan HM5 (ABAXIS, USA). When performing the research, the following research objects were observed:

- Maral Root in accordance with GOST 24027.2-80;
- Stinging Nettle in accordance with GOST 24027.2-80;
- Blood serum of recipient cows, samples were taken in accordance with GOST 34105-2017.

When performing the research there were used the generally accepted standard and original research methods. Hematological blood parameters were determined by using a hematological analyzer VetScan HM5 (ABAXIS, USA). The content of total protein, albumin and globulin fractions was determined by using a biochemical analyzer AU 480 (Beckman Coulter, USA). The natural (nonspecific) resistance of the blood serum of donor cows was determined by the bactericidal indicators according to the nephelometric method proposed by Smirnova and Kuzmin. The lysozyme activity of the blood serum of donor cows was determined by using an indicator in the form of a dry lyophilized form of Micrococcus lisodecticus according to the method of Dorofeychuk. The phagocytic activity of the blood serum of donor cows was determined by the method of Kost and Stenko. The viability of embryos obtained from recipient cows was determined according to GOST 28424-2014. The presence of mastitis in recipient cows was determined according to the "Manual on the diagnosis, therapy and prevention of mastitis in cows" No. 13-5-2/1948. The presence of postpartum subinvolution of the uterus and postpartum endometritis was determined rectally.

RESULTS AND DISCUSSION

Hematological blood parameters of the recipient cows are presented in Table 1 and Table 2.

Table 1. Hematological blood parameters of recipient cows. Part 1.

Indicator	Blood test	Group			
		Control	Experimental group I	Experimental group II	Experimental group III
Leukocytes, (WBC),10^9/l	before experiment	9,6±0,48	10,0±0,50	9,8±0,49	9,6±0,48
	30 days	10,8±0,54	10,0±0,50	8,5±0,42	8,6±0,42
	60 days	12,0±0,60	9,8±0,49	7,0±0,35	6,8±0,34
	90 days	12,6±0,63	9,5±0,47	6,8±0,34	6,8±0,34
Leukocyte formula, %	before experiment	-	-	-	-
Basophils		3,0	4,0	3,0	3,0
Eosinophils		39,0	38,0	39,0	40,0
Neutrophils		54,0	53,0	54,0	54,0
Lymphocytes		5,0	4,0	4,0	5,0
Monocytes	30 days	-	-	-	-
		3,0	4,0	4,0	3,0
		39,0	39,0	43,0	44,0
		54,0	52,0	50,0	50,0
		5,0	5,0	5,0	6,0
	60 days	-	-	-	-
		3,0	4,0	4,0	3,0
		38,0	41,0	43,0	45,0
		54,0	52,0	49,0	49,0
		5,0	5,0	5,0	5,0
	90 days	-	-	-	-
		3,0	3,0	4,0	3,0
		38,0	42,0	44,0	45,0
		54,0	50,0	49,0	49,0
		6,0	6,0	7,0	6,0

Changes in hematological blood parameters are associated with an increase in the immunobiological status of animals. As can be seen from Table 1, the additional introduction of Maral Root and Stinging Nettle extracts into the diet of recipient cows has a significant effect on the change in hematological blood parameters. Thus, the quantitative indicators of leukocytes in the control group increased from 9,6•109/l to 12,6•109/l (44%), while in experimental groups I, II and III this indicator decreased by 5%, 31 % and 30%, respectively. The content of lymphocytes in the control group remained unchanged throughout the experiment; in the experimental group I the number of leukocytes decreased by 6%, in the experimental groups II and III - by 10%. Since neutrophils have an ability to recognize any bacteria that enter the body, it is important to increase this indicator in the blood to help the body fighting with extraneous microflora. In the control group, at the end of the experiment, there was no increase in the content of neutrophils; while in the experimental group II, the number of neutrophils in the blood of recipient cows increased by 13%.

The normal development of the functional capabilities of the animal's body largely depends on the optimal functional activity of platelets, which determines a high level of organism's resistance to infectious diseases and a sufficient rate of metabolic processes. The introduction of extracts of Maral Root and Stinging Nettle into the diet of recipient cows allows increasing the platelet content of in the blood by 8%, 15% and 17% in the experimental groups I, II and III, respectively. The erythrocytes and hemoglobin parameters throughout the experiment period

were within the physiological norm in all the groups both at the beginning and at the end of the study. The blood serum was tested to determine the content of total protein, albumin and globulin fractions by using a biochemical analyzer AU 480 (Beckman Coulter, USA). The biochemical parameters of the blood of recipient cows are presented in Table 3.

Table 2. Hematological blood parameters of recipient cows. Part 2.

Indicator	Blood test	Group			
		Control	Experimental group I	Experimental group II	Experimental group III
Erythrocytes, (RBC), 10^{12}/l	before experiment	6,9±0,34	6,6±0,33	6,2±0,31	6,3±0,32
	30 days	6,6±0,33	6,4±0,32	5,9±0,29	6,8±0,34
	60 days	6,6±0,33	6,0±0,30	6,5±0,32	6,0±0,30
	90 days	6,2±0,33	6,4±0,32	6,8±0,34	6,8±0,34
Hemoglobin, (HGB), g/100ml	before experiment	11,0±0,55	11,2±0,56	10,7±0,53	10,5±0,54
	30 days	11,2±0,56	11,2±0,56	10,3±0,53	10,9±0,54
	60 days	11,6±0,60	11,2±0,56	9,9±0,49	11,3±0,56
	90 days	11,7±0,60	11,4±0,57	10,3±0,53	10,2±0,53
Platelets, x 10^9/l	before experiment	456±22,8	468±23,4	462±23,1	452±22,6
	30 days	450±22,5	488±24,4	517±25,8	486±24,3
	60 days	443±22,1	500±25,0	530±26,5	522±26,1
	90 days	448±22,4	506±25,3	533±26,6	530±26,5
Hematocrit, %	before experiment	40,50±2,03	40,48±2,03	41,43±2,07	40,52±2,03
	30 days	41,12±2,06	40,23±2,01	41,06±2,05	40,46±2,02
	60 days	41,56±2,07	40,44±2,02	40,72±2,04	40,46±2,02
	90 days	41,88±2,10	40,12±2,00	40,15±2,01	41,12±2,00
Cellular hemoglobin content, pg (picograms)	before experiment	14,4±0,72	14,2±0,72	14,0±0,70	13,8±0,70
	30 days	13,6±0,68	13,2±0,66	14,0±0,70	14,2±0,72
	60 days	14,0±0,70	13,4±0,67	13,8±0,70	13,7±0,67
	90 days	13,9±0,70	13,2±0,66	14,2±0,72	13,8±0,68
Corpuscular hemoglobin concentration, g/l	before experiment	56,4±2,82	56,1±2,80	56,2±2,81	55,7±2,78
	30 days	56,0±2,80	55,6±2,83	55,8±2,79	55,2±2,76
	60 days	56,6±2,83	55,6±2,83	55,2±2,76	56,2±2,81
	90 days	55,7±2,78	55,6±2,83	56,0±2,80	55,9±2,79

Table 2 presents data on studies of blood serum protein fractions. All the noted indicators were within the normal range. Blood proteins have a direct effect on the metabolism in the body of animals. The content of total protein in the blood and its fractions allows making a conclusion about the physiological state of recipient cows, as well as the body's resistance to unfavorable environmental factors. Extracts of Maral Root and Stinging Nettle, when added to the cow main diet, increase the total protein content in the blood by 10% compared to the control group, where this indicator practically does not change. Many scientists point out the connection between the content of albumin in the blood of animals with their productivity (Lacasse et al., 2018; Lombardi et al., 2020; Fallah et al., 2021).

The use of extracts of medicinal plants in the feeding of recipient cows makes it possible to increase the content of the albumin fraction in the blood by 2-3%. The carotene content in the blood of animals from the experimental groups increased almost 2 times compared with the control group and amounted to 0.50-0.52 mg%. A low level of carotene in the blood serum of cows is the cause of hypocarotinemia - insufficient supply of provitamin A in the diet, when there is a lack of protein and easily digestible carbohydrates, and B vitamins in the feed. Lack of carotene in the blood of recipient cows can lead to reproductive function abnormality of the animal, poor heat and prolonged ovulation (Petushok and Malashko, 2018; Smulski et al. 2020; Abdel-Lattif et al., 2021). During the experiment, the effect of Maral Root and Stinging Nettle extracts on the phagocytic activity of leukocytes, lysozyme and bactericidal activity of blood serum of recipient cows, which have a direct effect on the animal body natural resistance, was determined, as well (Table 4).

The bactericidal activity of blood serum is one of the important indicators of the body's natural resistance to viral diseases. This parameter shows the ability to suppress the growth of microorganisms and depends on the activity of all humoral resistance factors (Kirikovich et al., 2012; Tresnitsky, 2019). The bactericidal activity of blood serum in the recipient cows of the control group did not change during the experiment; in all experimental groups, it increased by 40%, 94% and 87%, respectively. The lysozyme activity of blood serum also, in turn, makes it possible to characterize the natural resistance of the organism (Islam et al., 2017; Eremenko and Rotmistrovskaya, 2021).

Table 3. The content of total protein and its fractions in blood serum

Indicator	Blood test	Group Control	Experimental group I	Experimental group II	Experimental group III
Total protein, г/л	before experiment	73,89±3,69	73,27±3,66	73,62±3,68	73,55±3,68
	30 days	74,15±3,70	74,33±3,72	76,16±3,81	78,71±3,93
	60 days	74,32±3,72	74,90±3,74	81,00±4,05	80,79±4,04
	90 days	74,43±3,72	75,00±3,75	80,98±4,05	81,24±4,06
Carotene, mg%	before experiment	0,25±0,01	0,26±0,01	0,25±0,01	0,25±0,01
	30 days	0,25±0,01	0,28±0,02	0,38±0,03	0,42±0,02
	60 days	0,28±0,02	0,28±0,02	0,50±0,03	0,49±0,03
	90 days	0,29±0,02	0,31±0,02	0,52±0,03	0,50±0,03
Albumin, %	before experiment	37,12±1,86	37,15±1,86	37,22±1,86	37,12±1,86
	30 days	37,00±1,86	37,34±1,87	38,00±1,90	37,88±1,89
	60 days	36,88±1,85	37,88±1,89	38,12±1,91	38,14±1,91
	90 days	36,92±1,85	37,87±1,89	38,10±1,91	38,14±1,91
α-globulins, %	before experiment	12,46±0,62	12,42±0,62	12,35±0,61	12,40±0,62
	30 days	12,46±0,62	12,46±0,62	12,40±0,62	12,42±0,62
	60 days	12,53±0,63	12,58±0,62	12,48±0,63	12,42±0,62
	90 days	12,50±0,62	12,56±0,62	12,50±0,63	12,50±0,63
ß-globulins, %	before experiment	13,87±0,69	14,15±0,71	14,33±0,72	14,60±0,73
	30 days	13,92±0,70	13,96±0,70	14,18±0,71	14,42±0,72
	60 days	13,84±0,69	14,00±0,70	14,18±0,71	13,96±0,70
	90 days	13,85±0,69	14,10±0,71	14,25±0,72	14,15±0,71
γ-globulins, %	before experiment	36,04±1,80	35,57±1,78	35,77±1,79	36,66±1,83
	30 days	36,16±1,81	35,85±1,79	35,26±1,76	36,21±1,81
	60 days	35,77±1,79	36,26±1,81	34,58±1,73	35,96±1,80
	90 days	35,59±1,78	36,14±1,81	34,32±1,72	35,96±1,80

Table 4. Indicators of natural resistance of blood serum of recipient cows

Indicator	Blood test	Group Control	Experimental group I	Experimental group II	Experimental group III
Serum bactericidal activity, %	before experiment	29,30±1,46	28,68±1,43	27,83±1,40	29,62±1,48
	30 days	28,53±1,43	34,16±1,71	38,33±1,92	39,42±1,97
	60 days	29,22±1,46	38,49±1,92	50,17±2,51	51,39±2,57
	90 days	29,61±1,47	40,06±2,00	53,93±2,70	55,38±2,77
Serum lysozyme activity, %	before experiment	13,82±0,69	13,68±0,69	13,90±0,69	14,01±0,70
	30 days	13,75±0,69	13,70±0,69	14,47±0,72	14,34±0,72
	60 days	13,00±0,65	13,96±0,70	15,00±0,74	14,73±0,74
	90 days	13,00±0,65	13,74±0,69	14,89±0,74	14,80±0,74
Phagocytic activity of leukocytes, %	before experiment	63,58±3,18	63,29±3,16	63,65±3,18	62,79±3,14
	30 days	63,38±3,17	63,57±3,17	64,65±3,23	63,80±3,19
	60 days	64,00±3,20	64,36±3,16	65,60±3,28	64,00±3,20
	90 days	63,72±3,19	64,36±3,22	65,72±3,29	64,00±3,20

Table 5. Indicators of the postpartum period of recipient cows

Indicator	Control group	Experimental group II
Duration of the placental stage, hour	8,21±0,41	5,30±0,26
Postpartum subinvolution of the uterus, heads	2	-
Postpartum endometritis, heads	1	-
Serous mastitis, heads	1	-
Number of animals for subsequent embryo transfer, heads	6	10

Table 6. Determination of the viability of the obtained embryos

Indicator	Control group	Experimental group II
Number of born calves, incl.	6	6
healthy	4	6
stillborn	1	-
with physiological abnormalities	1	-

Lysozyme activity in all experimental groups increased on average by 5-7%, while this indicator in the control group decreased by 6%. The addition of Maral Root and Stinging Nettle extracts to the main feeding ration of recipient cows also contributes to an increase in the phagocytic activity of leukocytes in the blood by 2-4% compared to the control group. The increase in the intensity of the phagocytic activity should be associated with an increase in the immune response from the body, in response to the penetration of foreign microflora inside the organism (Ulrikh et al., 2020; Eremenko and Rotmistrovskaya, 2021).

After analyzing the obtained data of morphological and biochemical parameters of blood got from recipient cows feeding with different concentrations of Maral Root and Stinging Nettle extracts, it was established that the optimal feed additive should be prepared in the following ratio of components: 100 kg of compound feed, 0.5 kg of extract of Maral Root, 2.5 kg of Stinging Nettle extract. An increase in the concentration of the extract of Stinging Nettle in the feed additive does not have a significant effect on the hematological parameters of blood and indicators of natural resistance of blood serum. At the same time, a decrease in the dose of the extract does not allow achieving the desired result; the morphological and biochemical parameters of the blood practically do not differ from the control group. In addition to studying the morphological and biochemical parameters of blood, recipient cows of the control and experimental group II were analyzed on the point of the duration placental stage, the timing of uterus involution, the period from calving to the embryo introduction (Table 5).

As can be seen from Table 4, in the experimental group II, the duration of the placental stage was reduced by 36%. In addition, in the control group, which received only the basic diet, postpartum complications were noted in 4 cows (40%). Out of 10 cows-recipients of the control group, 2 animals after calving were diagnosed with subinvolution of the uterus (20%), 1 cow (10%) had postpartum endometritis, and one animal had serous mastitis (10%). In animals of the experimental group, no signs of postpartum complications were revealed, this fact confirms the effectiveness of the introduction of the selected extracts of medicinal plants into the diet of recipient cows. In order to confirm the effectiveness of herbal extracts as a substitute for antibiotics in feeding dry cows and, as a consequence, to obtain healthy young animals, embryos obtained from recipient cows of the control and experimental group II were analyzed (Table 6).

According to the presented results, it was found that the introduction of extracts of medicinal plants into the main diet of recipient cows makes it possible to obtain healthy embryos without pathological changes. At the same time, 16% of embryos obtained from recipient cows of the control group had physiological abnormalities, in particular, they were hypotrophic, and 16% of embryos were stillborn.

CONCLUSION

After analyzing the obtained data of morphological and biochemical parameters of blood, depending on the concentration of the Maral Root and Stinging Nettle extracts in the feed additive for recipient cows, it was found that the feed additive is introduced to the animals in the ratio of the components: 100 kg of compound feed, 0.5 kg of Maral Root extract, 2, 5 kg of Stinging Nettle extract, which enhances the immune response from the animal's body, in response to the penetration of foreign microflora inside the organism.

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Parasitological Communication

Comparative Genomics of Aquatic and Fish Pathogenic *Flavobacterium* spp.

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ABSTRACT

The disease causing organisms of aquatic ecosystems negatively affects the aquaculture. The diseases caused by *Flavobacterium* is a common problem in commercially cultivated/ cultured fish species worldwide. The flavobacteira are common inhabitant of soil, plants and aquatic habitats belongs to phylum bacteroidetes. Comparative genomics studies help in generating valuable information about their diversity, and special adaptations. The present study gives a comparison of the genome of six pathogenic *Flavobacterium* spp. from different geo-locations using publically available genome data. The possible genomic similarities and distances were predicted using EDGAR. Further, the genome of *F. indicum* GPTSA100-9 was compared with five other genomes on the basis of genome genome distance (GGD), prediction of dDDH and MCI between the sequenced genomes. Among these six genome, the genome size varied from 2.71 Mb to 3.98 Mb. *F. psychrophilum* FPG3 has the smallest genome (2.71 Mb) followed by *F. indicum* (2.99 Mb). The functional annotation and phylogenetic studies based on orthology revealed that 51-60% genes are orthologous whereas, the paralogs ranged between 5 to 15% of the total genes. The DDH, AAI, ANI and POCP results indicate that these species are distinct and different, further on the basis of Pan and Core Genome analysis, 41% genes were recorded to contribute to core genome of *Flavobacterium*. Analysis of the core genome showed that the number of shared genes decreased with the addition of each new genome. The average gene content in six genomes are 2934 whereas, the core genome was estimated to contain 1210 genes, which is corresponding to the 41 % of the genome and might remain relatively constant. In conclusion, the comparative analysis exhibited that *F. indicum* GPTSA100-9 is closely related to *F. branchiophilum* FL-15 and strains from South Korea and China shares same clade and are phylogenetically similar.

KEY WORDS: COMPARATIVE GENOMICS, CORE GENOME, GGD, DDH, FLAVOBACTERIUM.

INTRODUCTION

The genus Flavobacterium is a pigmented, Gram -ve bacteria consisting about 130 species reported from aquatic and terrestrial habitats.Many of the species are reported as fish pathogen including *F. psychrophilum, F. columnare*, and *F. branchiophilum*, which can cause severe fish diseases called "cold water disease" (CWD) in freshwater aquaculture with a global distribution. Disease are the main cause of economic losses in aquaculture and constitutes a major constrain for rapid growth and intensification of aquaculture, the new virulent strains/pathogens of cultivated fishes are steadily increasing due to pollution, globalization, and transboundary movement of aquatic fauna it also poses challenge to the workers, vendors and associated researchers (Wahli and Madsen 2018; Silva 2019).

The CWD leads to increased predisposition of other infections and increased mortality. Antibiotics are normally used to control the disease which causes a bi economic burden and also contribute to anti-microbial resistance in bacteria, (Jia et al. 2017; Silva 2019). The major molecular methods used to distinguish between specific bacterial taxa include serotyping, multilocus sequence typing (MLST), pulsed field gel electro- phoresis (PFGE) plasmid profiling, genome restriction enzyme analysis (GREA) etc. (Madsen et al. 2000; Arai et al. 2007; Fujiwara et al. 2013; Castillo et al. 2014; Nilsen et al. 2014; Kumru et al. 2020).

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However, the unique adaptation towards pathogenic lifestyle, virulence and its evolutionary relationships among different species studied by genome sequencing approaches provides exciting information. Comparative genomic analyses gives insight into variations in the genomic rearrangements, presence of phage sequences, horizontal gene transfer (HGT) and changes in the gene repertory, structural features thus unravels on the evolution in the organisms (Land et al. 2015). The results of comparative study leads to division of genome in, conserved "core" shared among nearly all members and "accessory" genomic elements which might be present in one or few and absent in other strains (Tettelin et al. 2008). The flexible part of genome might be the results of the acquisition of genetic information specific to the strain and represented by mobile genetic elements, such as plasmids, phage sequences, genomic islands, pathogenicity islands, transposons, IS elements etc (Srivastava et al. 2020).

The flexible and mobile genes scattered in the genome (mobilome) provide an additional repertoire of arsenal to the microbes viz: antimicrobial resistance, virulence factors, heavy metal/stress related modifications and toxic compounds. The comparative study among different species of same genera offers the possibility of defining their phylogenetic closeness (Srivastava et al. 2020). However, very little information is available about comparative genomics of *Flavobacterium* from different geographical regions. Therefore, the present study is focused on comparing the genome of six pathogenic *Flavobacterium* spp. using publically available genome data, to derive the insights about their similarity, conserved core genomic and pan-genomic elements. The analysis was executed with the genomic datasets available on NCBI and tools like EDGAR, TYGS and GGDC were used in study.

MATERIAL AND METHODS

The whole genome sequence of six species of *Flavobacerium* viz: *F. album* HYN0059, *F. branchiophilum* FL-15, *F. columnare* Pf1, *F. crassostreae* LPB0076, *F. indicum* GPTSA100-9, and F. psychrophilum FPG3 downloaded from microbial genome and microbiome datasets sequenced at Joint Genome Institute (JGI; IMG) (Chen et al.2017). The genome statistics and annotation values were obtained through genome field search for selected genomes with the metadata and Data statistics following the instructions given on the IMG/M server (https://img.jgi.doe.gov/cgi-bin/m/main.cgi). The possible genomic similarities and distances were predicted using EDGAR was used to predict pan genome of all 6isolates and calculate the accessory (specific genes, present in one) and core genome (common genes, conserved across). The iterative pairwise comparison of a set of genomes was calculated for Pan-genome development (Dieckmann et al. 2021).

Table 1. Comparison of genome assembly of different species of *Flavobacterium*

Genome Name / Sample Name	***Flavobacterium columnare* Pf1**	***Flavobacterium album* HYN0059**	***Flavobacterium branchiophilum* FL-15**	***Flavobacterium crassostreae* LPB0076**	***Flavobacterium indicum* GPTSA100-9**	***Flavobacterium psychrophilum* FPG3**
Host Name/Habitat	***Pelteobagrus fulvidraco***	**Fresh water**	***Silurus glanis***	***Crassostrea gigas***	**Aquatic (hot water string)**	***Oncorhynchus kisutch***
Isolation Country	China	South Korea	Hungary	South Korea	India	USA
Genome Size	3171081	3983546	3563292	3027315	2993089	2715909
Gene Count	2816	3715	2925	2863	2738	2548
Scaffold Count	1	1	2	1	1	1
GC %	31.58	44.54	32.86	35.98	31.38	32.67
CDS Count	2710	3633	2872	2656	2671	2349
RNA Count	106	77	53	207	67	199
RNA %	3.76	2.07	1.81	7.23	2.45	7.81
rRNA Count	19	9	9	21	12	18
tRNA Count	81	47	44	61	55	49
Other RNA Count	6	21	0	125	0	132

The genome of *F. indicum* GPTSA100-9 was compared with five other genomes of *Flavobacterium* (*F. album* HYN0059, *F. branchiophilum* FL-15, *F. columnare* Pf1, *F. crassostreae* LPB0076, and *F. psychrophilum* FPG3) on the basis of genome genome distance (GGD), prediction of dDDH and MCI between the sequenced genomes using the tool GGDC on web server http://ggdc.gbdp.org (Meier-Kolthoff et al. 2014). The phylogenetic relationship among Flavobacterium isolates based on genomic data was determined using Average Amino acid Identity (AAI), Average Nucleotide Identity (ANI) and Pairwise Percentage of Conserved Proteins (POCP) analysis. The Flavobacterium spp. genome sequence data were retrieved from IMG/M and uploaded to the Type (Strain) Genome Server (TYGS), available under https://tygs.dsmz.de, for a whole genome-based taxonomic analysis (Meier-Kolthoff and Göker 2019). The determination of closely related

type strains genome was performed by using two different complementary means.

RESULTS AND DISCUSSION

The assembled genomes of *Flavobacterium album* HYN0059, *F. branchiophilum* FL-15, *F. columnare* Pf1, *F. crassostreae* LPB0076, *F. indicum* GPTSA100-9, and *F. psychrophilum* FPG3 was obtained from the database and used for comparative study. These organisms were originally isolated from; fresh water (*F. album*), *Pelteobagrus fulvidraco* (*F. columnare*), Silurus glanis (*F. branchiophilum*), *Crassostrea gigas* (*F. crassostreae*), hot water spring (*F. indicum*) from different continents Asia, Europe and North America (Table 1).

Among these six genomes, the genome size varied from 2.71 Mb to 3.98Mb. *F. psychrophilum* FPG3 had the smallest genome (2.71 Mb) followed by *F. indicum* (2.99 Mb). The assembly statistics revealed that the all the genomes are nearly complete and possess the genes in a range of 2548 to 3715 genes. 92-98% of the sequences represent the coding sequences (CDs). High variability was observed in number and percentage of total RNA count with a variation in tRNA and other RNA (Table 1). This variation in tRNA gene loss and/or gain could be explained by repeat-driven expansion of pseudo-tRNAs and genome assembly artifacts (Rogers et al. 2010). The vast numbers of differences between the selected species were observed. The results are corroboratory to the findings of Kumru et al. (2020).

The functional annotation and phylogenetic studies based on orthology revealed that 51-60% genes were orthologous whereas, the paralogs ranged between 5 to 15% of the total genes. The Pfam database was used to decipher protein families at different domains. Not much variation in number and percentage of proteins were recorded, however, minimum 1874 P fam count was recorded in *F. psychrophilum* and maximum in 2549 P fam families in *F. album*. Corresponding to the number of genes 73.55% of the genes in *F. psychrophilum* represents for protein, whereas, it was only 68.61% in *F. psychrophilum*, other falls in between (Table 2). The data presented for each entry was based on the UniProt Reference Proteomes related by similarity of sequence, structure or profile following hidden Markov models (HMMs). The KEGG resource for understanding high-level functions and utilities of the biological system gave more or less similar range of function orthologs (KO values). The signal peptides and transmembrane count was much higher in *F. album* (904 and 655 respectively).

Table 2. Comparative genome analysis of different *Flavobacterium* species

Genome Name / Sample Name	*Flavobacterium columnare* Pf1	*Flavobacterium album* HYN0059	*Flavobacterium branchiophilum* FL-15	*Flavobacterium crassostreae* LPB0076	*Flavobacterium indicum* GPTSA100-9	*Flavobacterium psychrophilum* FPG3
Paralogs Count	416	559	206	349	143	248
Paralogs %	14.77	15.05	7.04	12.19	5.22	9.73
COG Count	1452	2284	1512	1486	1460	1354
COG %	51.56	61.48	51.69	51.9	53.32	53.14
KOG Count	483	0	511	505	465	470
KOG %	17.15	0	17.47	17.64	16.98	18.45
Enzyme Count	671	702	730	671	651	643
Enzyme %	23.83	18.9	24.96	23.44	23.78	25.24
Pfam Count	2043	2549	2130	2029	2023	1874
Pfam %	72.55	68.61	72.82	70.87	73.89	73.55
KEGG Count	675	691	722	681	652	639
KEGG %	23.97	18.6	24.68	23.79	23.81	25.08
KO Count	1150	1250	1190	1143	1137	1065
KO %	40.84	33.65	40.68	39.92	41.53	41.8
Signal Peptide	192	665	297	223	314	235
Count Signal Peptide %	6.82	17.9	10.15	7.79	11.47	9.22

The genomes undergo both large-scale and local mutational processes during the course of evolution. Large scale mutations were occurring mainly due to duplication of large segments, gain and loss or generated by unequal recombination events. Whereas, the local mutations include insertion or deletion of nucleotides, nucleotide substitution and affected only a small number of nucleotides. Local evolutionary factors influence individual genes while a large-scale evolutionary process poses direct influence on genomes. Genome wise comparison reveals the similarity and differences among the different organisms (Bernardet et al. 1989;).

Table 3. Comparison of different *Flavobacterium* species for Horizontal Gene Transfer and Biosynthetic Gene Cluster

Genome Name / Sample Name	*Flavobacterium columnare* Pf1	*Flavobacterium album* HYN0059	*Flavobacterium branchiophilum* FL-15	*Flavobacterium crassostreae* LPB0076	*Flavobacterium indicum* GPTSA100-9	*Flavobacterium psychrophilum* FPG3
Horizontally Transferred Count	0	156	3	58	123	1
Horizontally Transferred %	0	4.2	0.1	2.03	4.49	0.04
Biosynthetic	124	0	32	136	105	79
Biosynthetic Cluster Gene %	4.4	0	1.09	4.75	3.83	3.1
Biosynthetic Cluster Count	4	0	2	5	4	2

Figure 1: Core genome based AAI, POCP and ANI matrix of six *Flavobactrium* genomes generated with EDGAR 3.0, Pan-genome comparison using correlation of DDH and GGD values for 5 different *Flavobacterium* spp. in reference to the F. indicum GPTSA100-9

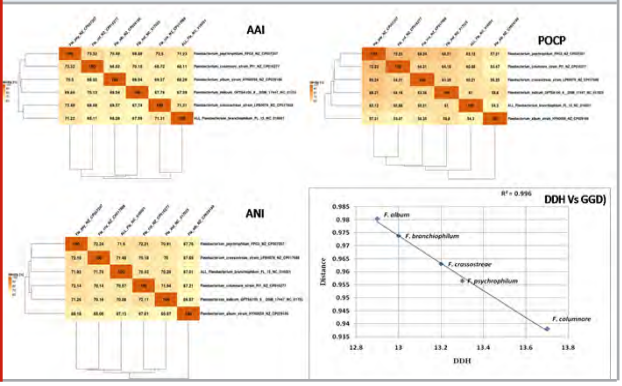
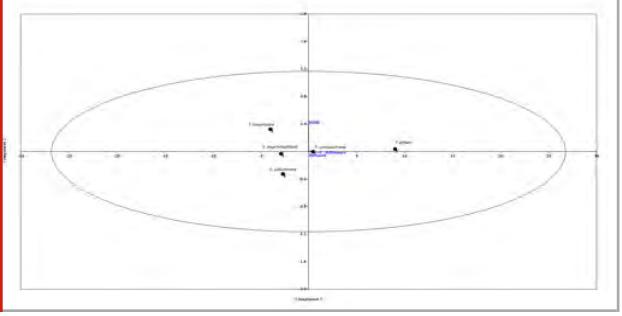


Figure 2: Principal component analysis (PCA) by PAST 2.0, Considering the DDH, GGD and C+G difference parameters a PCA plot was generated by putting all the values of the compared *Flavobacterium* spp. into three different components.



During genome comparison Analysis of cluster of orthologous groups revealed that *F. album* has significantly higher gene abundance than average level found in other genome sequences under study. A very small fraction of genes (1-09 to 4.75%) contributes to Biosynthetic Gene clusters. BGCs are very important in the process of identifying novel biosynthetic pathways; tens of thousands of biosynthetic gene clusters (BGCs) have been identified in other microbial genomes, most of which encode unknown compounds (Tracanna et al. 2017; Lebedeva et al. 2021). Horizontal gene transfer events to 0.04, 0.1, 2.03, 4.2 and 4.9% were predicted in *F. psychrophilum, F. branchiophilum, F. crassostreae, F. album* and *F. indicum,* respectively (Table 3).

Current innovations in species delineation (ANIb, ANIm, and dDDH, etc.) based on computational algorithms have made dDDH as one of the highly correlative approach to overcome the pitfalls of traditional DDH based bacterial taxonomy (Auch et al. 2010; Meier-Kolthoff et al. 2013; Meier-Kolthoff et al. 2014; Meier-Kolthoff and Göker 2019). In order to measure the differences between the genome of six *Flavobacterium* species the Genome-to-Genome distance (GGD) was calculated using GGCD server. Similarly, the DDH estimate (GLM-based), Model confidence interval (Model CI) was also calculated on the basis of HSPs length/total length using formula 1 The comparison on the basis of GGD, DDH (Fig.1) showed the clear distinctness between the species. The DDH for species are less than 14 % DDH, because of this the probability of >= 70 is very low and insignificant. Similarly, the GGD is also more than 0.9. The graph between the GGD and DDH shows the clear difference of these strains (Fig. 4A). In order to further validation of the finding the ANI, AAI and POCP values were also calculated with reference to the *F. indicum* GPTSA100-9. The matrix for ANI, AAI and POCP is given in Fig. which again confirms the distinctness of the selected species (Fig. 5).

This much high distinctness and the orthology information among these species were also represented in the ring analysis (Fig.3). The outer rings of the circular plot represent the genes of one selected reference genome (*F. indicum*). The further rings of the circular plot show the core genome as well as the orthologs of each individual genome in comparison to the reference. The phylogenetic tree on the basis of the genomic similarity indicates shows that there are two distinct clades separating *Flavobacterium album*

HYN0059, *F. columnare* Pf1, *F. crassostreae* LPB0076, from *F. psychrophilum* FPG3 *F. indicum* GPTSA100-9, and *F. branchiophilum* FL-15. (Fig.4) (Meier-Kolthoff and Göker 2019).

Figure 3: Comparative genome mapping using EDGAR 3.0, the inner circle represents the size of the *F. indicum* GPTSA100 genome.

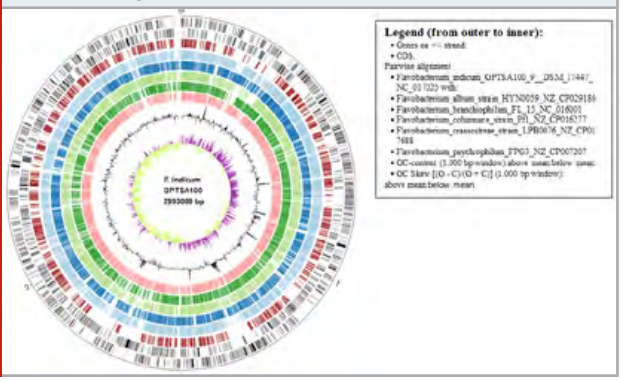


Figure 4: Whole genome sequence based phylogenetic tree analysis using Type (Strain) Genome Server (TYGS). The species clusters represented in different colours

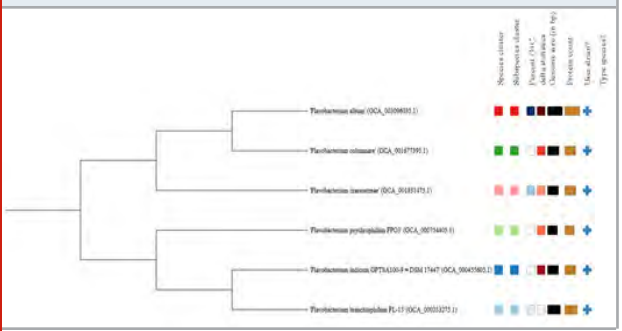
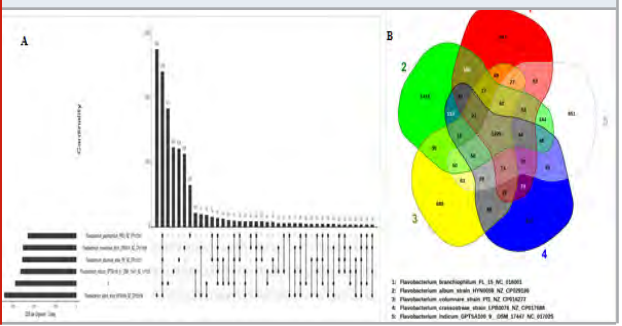
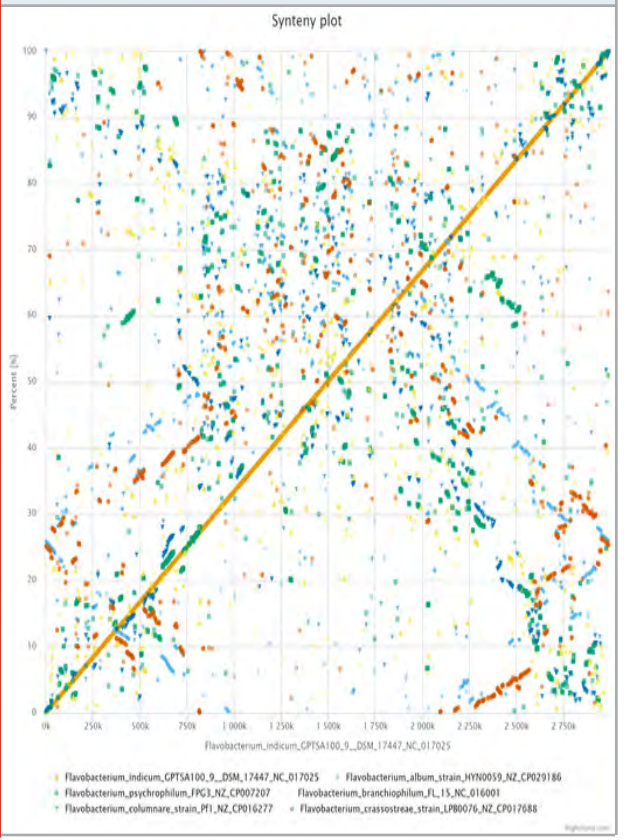


Figure 5: The UpSet plot (A) showing intersection between the sets of genes from various comparisons. The dot plot reports the set participation in the intersection, vertical bar plot reports the intersection size, and the horizontal bar plot reports the set sizes. (B) Venn diagram based on the prediction of orthologous proteins annotated from the pan-genome of the *Flavobacterium*. Each structure shows in sum the total number of coding sequences of one strain. Intersections indicate predicted shared content.



The intersections of five genome datasets are given in Venn diagram (Fig 5B) to represent the genes intersecting within the genome for various sets. Further the new visualization features of the UpSet plots of platform EDGAR 3.0 were used for visual inspection of shared and differential gene content of genome sets (Fig 5A). The matrix layout in Fig5B shows the dark grey circles represent the genomes included in a set, while missing genomes are visualized as light grey circles. The UpSet visualization makes it easy to quickly get insights into the distribution of genes among the set of genomes (Meier-Kolthoff and Göker 2019). Genome rearrangement and synteny provide evolutionary relationships between genomes. We used EDGAR 3.0 to determine the synteny among six genomes (Fig. 6). Majority of the genome regions were not in the form of syntenic blocks. These findings suggest extreme low level of synteny and divergence, might be because of selective aggregation of genes under evolutionary pressures or incomplete dislocation of gene.

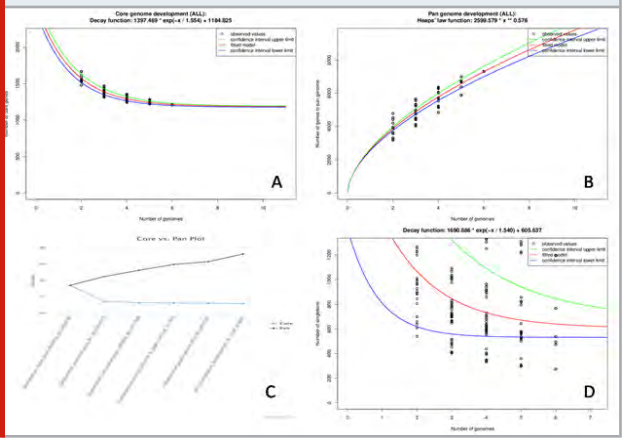
Figure 6: Synteny analysis using EDGAR 3.0, Dot-plot representing whole genome comparison between six *Flavobacteriaum* sp., the Dots closest to the diagonal line represent co-linearity between the genomes.



EDGAR platform is one of the most established web servers providing databases of precomputed orthology data for phylogenomics and comparative genomics used for quick identification of the differential gene content, viz: the core and pan genome, or singleton. Examination of the pan-genome indicated that with the addition of new genome the gene repertoire is increased (Castillo et al. 2016). Further as expected, analysis of the core genome showed that the number of shared genes decreased with the addition of each new genome(Fig 7A). The average gene content is 2934

whereas, the core genome was estimated to contain 1210 genes, which is corresponding to the 41 % of the genome and might remain relatively constant. (Fig 7C) shows the plot Pan verses Core genome, where the number of genes in core is almost stable but increasing in the pan genome with addition of the genomes, the model also predicts 535 singeltons out of which 314 represents for hypothetical proteins. The graphic based on the median value for the accessory genes shows the exponential decay model.

Figure 7: *Flavobacterium* core and accessory genome evolution analysis (A) Number of shared genes (core genome) as a function of the number of genomes sequentially added. B). Total number of genes (pan-genome) for a given number of genomes sequentially added c. Plot showing Pan verses Core genome, D) Number of unique genes (accessory genome) for a given number of genomes sequentially added. The lower (blue) lines indicate third (75th percentile), upper (green) lines indicate first (25th percentile) of the data and central (red) line refers the sample median (50th percentile) of random input order of the *Flavobacterium* genomes.



CONCLUSION

The findings of the present study exhibited that Flavobacterium indicum GPTSA100-9is closely related to *F. branchiophilum* FL-15 on the basis of genome genome distance (GGD) and DDH and also the genome wise phylogeny also confirms that they both are quite closer to each other. Further, the strains from South Korea and China shares same clade and are phylogenetically similar and overall, all the strains having 44% common genepool as core genome.

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

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Dental Communication

Psychological Effects of Covid-19 Pandemic on Dental Students: A Cross-Sectional Study

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ABSTRACT

The COVID-19 pandemic has had a psychological effect on the general population and healthcare workers. Dentists were not excluded from this and were placed in a high-exposure risk category for infection. This cross-sectional study used the DASS-21 scale to evaluate the level of depression, anxiety, and stress among dental students in Riyadh, Saudi Arabia, during the COVID-19 pandemic and the effects of other variables on their responses. Continuous variables were expressed as mean ± standard deviation, and categorical variables were expressed as percentages. The student's t-test and one-way ANOVA were used for continuous variables. Multiple linear regressions were also utilized. A p-value <0.05 was considered statistically significant. A total of 138 students contributed to the study. All participants (100%) were female, and 99.28% were single. The majority of the students were enrolled at King Saud University (81.88%), with 42.75% of the participants in the third year. High levels of depression, anxiety, and stress were reported among dental students during the pandemic: 73.2% of respondents had depression, 84.8% had anxiety, and 75.4% had stress. In addition to the stress of dental education itself, the contagious nature of this disease and those dentists were at a higher risk of contracting the infection affected the student's psychological health.

KEY WORDS: PANDEMIC, COVID-19, DENTAL STUDENT, DEPRESSION, PSYCHOLOGICAL IMPACT.

INTRODUCTION

Since late 2019 and early 2020, a new threatening virus, COVID-19, has been spreading worldwide. The World Health Organization (WHO), on 30 January 2020, stated that COVID-19 was a pneumonia-like disease that was highly infectious and displayed severe symptoms. It was officially classified as a pandemic in 2020 (Meng et al., 2020). The causative virus is the *coronavirus*, SARS-CoV-2 virus, discovered in Wuhan, China, in December 2019, then spread worldwide (Zhu et al., 2019). The major route of transmission of the disease is through the respiratory droplets of an infected person or close contact with an infected person (Huang et al., 2020). To date, the confirmed global COVID-19 cases equal 161,513,458; Saudi Arabia has 431,432 cases (WHO, 2019).

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The pandemic has had a psychological effect on the general population, and healthcare workers have experienced additional burdens as they are exposed to infected patients, are at risk of getting infected, and fear transmitting the infection to their loved ones and being rejected by society as a possible source of infection (Abdelhafiz et al., 2020; Cawcutt et al., 2020; Nguyen et al., 2020). The activities of dentists, as healthcare workers, have been adversely affected during the pandemic (Consolo et al., 2020; Attia and Howaldt, 2021; Ammar et al., 2021).

Dental care professionals were placed in the high-exposure risk category for COVID-19 by the Occupational Safety and Health Administration (OSHA; OSHA, 2020). This exposure could happen during specific dental procedures that produce bioaerosols (Harrel and Molinari, 2004; Peng et al., 2020). This places dental practitioners and students under pressure, possibly leading to stress, anxiety, and depression. Worldwide, different studies have shown that dental students have various psychological problems, including stress, anxiety, depression, and obsessive-compulsive disorders

(Loyd and Musser, 1989; Humphris et al., 2002; Montero-Marin et al., 2014; Harris et al., 2020).

The lockdown and online learning have negatively affected students in general and dental students specifically since they face additional stress to finish their clinical requirements. This study uses the DASS-21 scale to assess the level of depression, anxiety, and stress among dental students in Riyadh, Saudi Arabia, during the pandemic, as well as the effects of other variables on their responses.

MATERIAL AND METHODS

A cross-sectional study evaluating the psychological well-being during the COVID-19 pandemic of dental students living in Riyadh using the DASS-21 scale. After ethical approval was obtained from the IRB committee (E-20-4489), an online questionnaire was established using Google Forms Questionnaire and sent via email to the students. The survey consisted of three parts; the first part was consent, where a short explanation of the study was provided, and voluntary participation was requested; the second part consisted of six demographic questions, including age, gender, marital status, number of children, dental academic year level, and psychological status; the last part included "the Depression, Anxiety, and Stress Scale-21 Items (DASS-21), which is a set of three self-report scales designed to measure the emotional states of depression, anxiety, and stress." (Lovibond and Lovibond, 1995). Inclusion criteria included any dental student living in Riyadh that was not receiving any psychological management (cognitive or behavioral therapy, medication, or a combination).

In contrast, the exclusion criteria included non-dental students living outside Riyadh who had a history or were receiving any psychological management that would make their participation biased. Data were analyzed using the Statistical Package for Social Studies (SPSS 22; IBM Corp., New York, NY, USA). Continuous variables were expressed as mean ± standard deviation, and categorical variables were expressed as percentages. The student's t-test and one-way ANOVA were used for continuous variables. Cronbach's alpha was used to assess the reliability and internal consistency of the items in the questionnaire. Multiple linear regression was used. A p-value <0.05 was considered statistically significant. The required sample size was 148.

The sample size was calculated using the following formula

$$n = \frac{Z^2 * P(1-P)}{d^2},$$

where n = sample size

Z = level of confidence (2-sided 95% CI = 1.96)

P = The percentage of abnormal depression levels in previous studies (p = 55.9%) (Basudan et al., 2017).
d = precision (8%).

Therefore, we distributed the questionnaire to 200 dental students to compensate for non-response.

RESULTS AND DISCUSSION

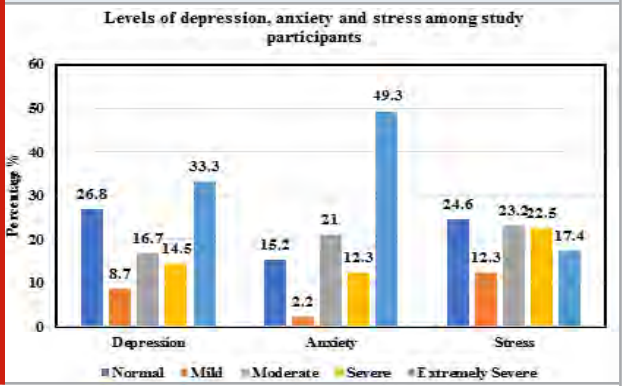
The Cronbach's alpha value of the questionnaires was 0.942, which reflects the excellent reliability of the questionnaire. One hundred thirty-eight students contributed to the study. All the participants (100%) were female, and 99.28% were single. The majority (81.88%) attended King Saud University, and 42.75% of the participants were in their third year (Table 1).

Table 1. Demographic Characteristics of the participants

		Number	%
Gender	Female	138	100.00
Educational institutes	King Saud university	113	81.88
	Riyadh elm University	14	10.14
	Prince norah Bint	11	7.97
Academic year	Internship	14	10.14
	Year 5	16	11.59
	Year 4	27	19.57
	Year 3	59	42.75
	Year 2	13	9.42
	Year 1	9	6.52
Marital status	Single	137	99.28
	Married	1	0.72
Having children	I do not have any children	138	100.00

The levels of depression, anxiety, and stress among study participants were evidently abnormal; it showed that 73.2% of respondents had depression, 84.8% had anxiety, and 75.4% had stress (Figure 1). Multiple linear regression showed that none of the tested variables was a predictor of depression, anxiety, and stress (Table 2).

Figure 1: Levels of depression, anxiety and stress among study participants



The association of demographic characteristics and depression, anxiety, and stress was investigated, and it showed that the academic year was associated with increased depression, anxiety, and stress; p-value 0.008 (Table 3).

The DASS-21 scale, a standardized self-reported scale that measures depression, stress, and anxiety, was used in this study to measure the psychological effects of the COVID-19 pandemic on dental students. This scale is highly reliable and valid; it is a short-version of DASS-42, proven to be more dependable and less time-consuming than the full version (Westerman et al., 1993; Lovibond and Lovibond, 1995). In the current study, 73.2% of respondents had depression, 84.8% had anxiety, and 75.4% had stress. This was higher than previous studies on dental students in the Riyadh and Mecca regions of Saudi Arabia. This is because those studies were conducted before the pandemic, revealing that the pandemic significantly impacted dental students' psychological health (Aboalshamat et al., 2015; Basudan et al., 2017; Hakami et al., 2021).

Table 2. Multiple linear regression model for the prediction of depression, anxiety and stress

Predictor	Subscale	Unstandardized Coefficients		Standardized Coefficients	t	P value	Collinearity Statistics	
		B	SE	Beta			Tolerance	VIF
(Constant)	D	18.325	2.597		7.058	<0.001*		
	A	17.811	2.224		8.010	<0.001*		
	S	21.007	2.162		9.714	<0.001*		
Marital status (1 = Married, 0 = Not Married)	D	-6.325	12.432	-0.042	-0.509	0.612	0.963	1.038
	A	6.189	10.646	0.049	0.581	0.562	0.963	1.038
	S	-3.007	10.354	-0.025	-0.290	0.772	0.963	1.038
Academic year (1. Internship=1, from year 1 to year 5 = 0)	D	-6.443	3.486	-0.154	-1.848	0.067	0.967	1.034
	A	-1.001	2.985	-0.028	-0.335	0.738	0.967	1.034
	S	-2.599	2.903	-0.076	-0.895	0.372	0.967	1.034
Educational institutes (KSU = 1, other = 0)	D	1.738	2.769	0.053	0.628	0.531	0.941	1.062
	A	0.740	2.372	0.027	0.312	0.756	0.941	1.062
	S	0.476	2.306	0.018	0.206	0.837	0.941	1.062

Another study, conducted during the pandemic, reported 60.64%, 37.02%, and 34.92%, respectively (Hakami et al.,2021). This might be due to differences in the timing of data collection, as we collected data during the peak of confirmed COVID-19 cases, which might attribute to the high level of depression, anxiety, and stress among dental students. Comparing our study results to studies worldwide, the current study reported higher levels of depression, anxiety, and stress; this might be due to that some of these studies were conducted before the pandemic, the difference in sample size, the scale used, differences in the curriculum, and requirements of each university (Abu-Ghazaleh et al., 2011; Radeef and Faisal, 2018; Saravani et al.; 2018; Moore et al., 2020; Ammar et al., 2021).

The association of demographic characteristics and depression, anxiety, and stress showed that the academic year was associated with increased depression, anxiety, and stress (p-value = 0.008), where the 4th-year students reported the highest level of depression, anxiety, and stress. This might be due to the fact that the 4th-year curriculum is filled with several didactic and clinical requirements. Also, previous studies reported that senior students experience more stress than junior students (Shamsuddin et al., 2013). In previous studies, there was an association between gender and marital status with depression and stress, as females and married students were more depressed, anxious, and stressed than males and unmarried students (Takayama et al., 2011; Al-Sowygh et al., 2013; Takayama et al., 2013; Aboalshamat et al., 2015; Radeef and Faisal, 2018; Saravani et al.; 2018; Moore et al., 2020). However, there were exceptions, as some studies reported that male students and students who are single reported higher levels of depression, anxiety, and stress (Basudan et al., 2017; Hakami et al., 2021).

In our study, all participants were female, and only one student was married; this explains why no association was detected. The high levels of depression, anxiety, and stress among dental students in this study might be related to the fear of the unknown nature of COVID-19 and the

high-risk cross-infection transmission among dentists. A study assessed the mental health among dentists from 30 countries during the pandemic and revealed that most of them were at high levels of stress and fear (Ahmed et al., 2020). Another study reported that dental caregivers had high levels of fear, distress, and pressure during the pandemic outbreak (Shacham et al., 2020). The current study's limitations include being an online survey; as such, reporting bias should be accounted for. We do not have any background check of the past students' psychological status other than the psychological medication, which might affect the results.

Table 3. Mean and standard deviation for score of Depression by Demographic Characteristics of the participants

		Mean$	SD	P value
Educational institutes	King Saud university	10.22	6.51	0.381
	Riyadh elm University	7.71	4.91	
	Prince Norah Bint	9.91	6.27	
Academic year	Internship	6.50	4.74	0.008*
	Year 5	7.88	5.78	
	Year 4	12.59	6.60	
	Year 3	10.97	6.13	
	Year 2	7.92	5.88	
	Year 1	7.22	7.03	
Marital status	Single	9.97	6.36	0.535
	Married	6.00		
	Yes	17.13	3.44	

* Significant p value
$ out of 21

CONCLUSION

High levels of depression, anxiety, and stress were reported among dental students during the pandemic. In addition to the stress of dental education itself, the contagious nature of this disease and those dentists were at a higher risk of getting the infection affected the student's psychological health. Universities should take this into consideration by developing psychological programs to help students face stress and depression.

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Microbiological Communication

Identification and Growth Characterization of Native Microalgae Isolated from Different Environments of Saudi Arabia

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ABSTRACT

Selection of appropriate strains of microalgae that work well in local conditions is important for a establish algae-based production system. The general aim of this study was to isolate and identify native microalgae species for exploring its further potential applications. To achieve this aim, 25 samples were collected from different locations of western region of Saudi Arabia. Standard isolation and purification techniques were applied *In vitro* to obtain axenic cultures. Among the best performing isolates, seven predominant strains were chosen for characterization based on morphological and molecular features. Morphological observations and molecular markers analysis using internal transcribed spacer sequence (ITS) were performed. Moreover, phylogenetic relationship of these strains was constructed. According to the DNA sequence analysis of the seven isolates, they were belonged to six genera of *Chlamydomonas, Dunaliella, Chlorococcum, Graesiella, Coelastrella* and *Chlorella*. Screening the growth rates of all strains showed that *Chlorella sorokiniana* had the highest growth rate (0.180 day^{-1}) and biomass productivity (150 mg. $L^{-1}.day^{-1}$). Whereas other strains showed comparable growth rates under same growth conditions. This study found that *Chlorella sorokiniana* UJ as robust species which holds a great potential to be used in different commercial and environmental applications. In conclusion the identification of microalgae is considered key step in microalgae-based industry. This work screened microalgae strains isolated from the local environment of Saudi Arabia. The best performing algal strains were selected to be identified and characterized to discover strains that can be utilized for mass cultivation. This study successfully isolated and identified seven local strains, most of them are already known with high biomass productivity (fast growers) and are considered as good candidate to serve as platform for many further applications.

KEY WORDS: CHARACTERIZATION, GROWTH RATE, IDENTIFICATION, MICROALGAE SAUDI ARABIA.

INTRODUCTION

Algae constitute a diverse group of photosynthetic organisms, which ranging from single cellular bodies to multicellular seaweeds, with a broad diversity in morphological, physiological and biochemical characteristics, distributed in almost all environments (Buijks, 2012). Using microalgae have attracted much attention in various industrial and environmental sectors such as human food, animal and aquaculture feed, pharmaceuticals, cosmetics, wastewater treatment, and bio-fertilizers (Olaizola, 2003; Draaisma et al., 2013). Moreover, microalgae are considered as promising alternative biofuel feedstocks due to their rapid growth, high biomass productivity and their capability to grow under divers conditions (Wijffels and Barbosa, 2010; Elliott et al., 2012; Ratha and Prasanna, 2012; Ratha et al., 2012; Markou and Nerantzis, 2013). Over the last decade, a number of microalgae have been cultivated on large scale to be used in industry because of their ability to produce valuable products (Olaizola, 2003; Markou and Nerantzis, 2013; Wijffels et al., 2013; Kaspar et al., 2014; Mulders et al., 2014; Ciurli et al., 2021). The common genera used are *Spirulina* and *Chlorella* as nutritional supplement, *Haematococcus*, to produce the antioxidant astaxanthin (Guerin et al., 2003; Yuan et al., 2011), and *Dunaliella salina* to produce carotenoids (Borowitzka, 1999; Hosseini

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Tafreshi and Shariati, 2009; Knothe, 2010; Camacho et al., 2019; Ciurli et al., 2021).

Due to these successful achievements of the using algae, the unexploited and undiscovered microalgae offer great possibilities for future. Until now, the actual number of microalgae species are still unknown, it is estimated that there are between 200,000 and 800,000 species (De Clerck et al., 2013; Lizzul et al., 2018; Camacho et al., 2019; Yin et al., 2020; Ciurli et al., 2021). Only 50,000 species have been defined and characterized (Yin et al., 2020). New genera and species are being discovered very consistently indicating the presences of large portion of undescribed species that exist (De Clerck et al., 2013). Saudi Arabia could be a good source of algal biodiversity due to variation of geographical and environmental nature. The capability of microalgae to grow in the local environmental conditions is an important prerequisite toward successful cultivation of microalgae-based for industrial production. Therefore, there is a need for a proper identification and characterization of local species. Microalgae are usually identified based on their morphological features.

However, environmental factors may cause some of the phenotypic plasticity, therefore morphological identification could be insufficient and misleading (Hoshina et al., 2010; Gour et al., 2016). A new classification based on the ultra-structure of the basal body in the flagellation cells and cytokinesis during mitosis has been suggested. Nevertheless, these principles are difficult to practice especially by non-taxonomists (Gour et al., 2016). The presence of molecular-based techniques such as polymerase chain reaction (PCR) and sequencing have improved such studies and helped in demonstrating evolutional relationships between different organisms and species. Molecular based techniques are usually recommended to confirm morphological classifications (Chung et al., 2018). Nowadays, internal transcribed spacer sequence (ITS) is considered a very powerful and helpful tool to discriminate between microalgae at the genus and species level (An et al., 1999; Van Hannen et al., 2002; Coleman, 2003, 2009; Hegewald et al., 2005, 2010; Jeon and Hegewald, 2006; Schultz et al., 2006; Schultz and Wolf, 2009; Hegewald et al., 2013; Lizzul et al., 2018; Wang et al., 2019; Goecke et al., 2020; Karm and Dwaish, 2021).

Thus, in current study, amplification and sequencing of internal transcribed spacer sequence (ITS 1 and ITS 4) was chosen as molecular markers to confirm the primary morphological identification of isolated strains, compared with other known sequences of species from public databases. The results of these comparisons were represented in a phylogenetic tree. This sort of studies is limited in Saudi Arabia and therefore this work will contribute in the field of discovering and exploiting microalgae from the local environment.

MATERIAL AND METHODS

Sampling, isolation and purification of microalgae: Twenty-five samples were collected through duration (Jan-April, 2018) from different environments of western region of Saudi Arabia (Table 1). Once the samples were collected and transferred to the laboratory, they were exposed to light, enriched with BG-11 medium and incubated for few days (Rippka et al., 1979). In order to obtain an axenic culture, the basic microbiological techniques for isolation and purification were used (serial dilution in liquid media and streak plate method).

In the serial dilution method, a series of test tubes with 9 ml of sterilized distilled water were prepared. One ml of the mixed enriched sample was taken, diluted in the first test tube (10^{-1}) and mixed. Next, 1 ml was taken from the first dilution and transferred to the second test tube (10^{-2}), this process was repeated until reaching to dilution of (10^{-6}). Serial dilution increases the chance of getting individual colonies. Then, each diluted tube was cultured in both liquid and semi-solid agar plates media, incubated in controlled conditions at 22 ± 1°C, exposed to continues light using LED fluorescent tube of intensity 2000 LUX 28 $\mu mol.m^{-2}. s^{-1}$ (Figure 1a). Further purification was achieved by consecutive streaking on semi-solid BG-11 agar (Figure 1b). This process was repeated several times until axenic culture was obtained. To ensure purity, the cultures were regularly monitored using light microscope. Subculturing was performed every 2 weeks. Axenic culture was preserved in cell culture flasks (Figure 1c).

Figure 1: Isolation and purification of algae. a) Algae stock cultures, b) Streaking purification of the microalgae on semi-solid BG-11 agar plates, c) Axenic culture preserved in cell culture flasks.

Growth of microalgae isolates in different media: Algae isolates were cultivated on different growth media, Kuhl (SAG Göttingen, 2013), BG-11 (Rippka et al., 1979), F2 (Guillard and Ryther, 1962) and Johnson's (Johnson et al., 1968). The cultures were aerated and incubated under controlled condition mentioned previously. The

growth of algae was estimated and determined as shown in (Table1).

Morphological identification: To identify morphological characterization of isolated strain, cell shape and arrangement were documented using light microscope (BX51; Olympus, Tokyo, Japan) equipped with a built-in digital camera, and microphotographs were processed with cell Sens Standard program. Morphological identification was determined according to Sime (2004) and Serediak and Huynh (2011).

Genomic DNA Isolation and PCR amplification of ITS Regions: For DNA isolation, 50 ml of each culture were harvested using centrifuge at 5000x g for 15 minutes, the genomic DNA (gDNA) then extracted using Qiagen kit following the manufacturer's instructions. The extracted gDNA was confirmed by agarose gel electrophoresis (1%) stained with ethidium bromide and visualized under ultraviolet light (UV). The extracted DNA was kept at –20 °C till using as PCR template. To amplify the ITS gene, the universal oligonucleotide primer set described in Van Hannen et al., (2002) was used. The sequences of these primers are: FW primer ITS1: (5′- TCCGTAGGTGAACCTGCGG -3′) RE primer ITS4: (5′- TCCTCCGCTTATTGATATGC -3′).

The PCR reaction was performed in a total volume of 25 ml by mixing up the following reagents: 12.5 µL master mix (2x), 8.5 µL dH_2O, 1 ml from each forward primer (FW) and reverse primer (RE) (10 pmoles) and 2 ml of genomic DNA. The PCR reaction was carried on at the thermocycler (Bibby Scientific, UK) using the following conditions: 5 minutes at 94 °C for the initial denaturation followed by 35 cycles of denaturation at 94 °C for 1 minute, annealing at 60 °C for 1 minute, extension at 72 °C for 2 minutes, followed by a final extension step at 72 °C of 10 minutes. Then the PCR products (860 bp) were resolved in 1.5 % agarose gel stained with ethidium bromide and visualized using UV light detector system. To perform DNA sequencing, the PCR products were shipped to (Macrogen, Seoul, Korea) to perform DNA sequencing reaction using Sanger sequencing methods.

Sequence analyses of ITS region: In order to identify the isolated strains, the obtained sequences were analyzed by searching for homology in the National Center for Biotechnology Information (NCBI) database using BLAST tool. All sequences were submitted to GenBank under the number SUB9546968. The phylogenetic tree of identified microalgae was constructed using the neighbor-joining (NJ) method (Ratha et al., 2012) of Mega 5 software (Draaisma et al., 2013). A bootstrap analysis of 1000 replication was used to test the degree of support of the branches produced by NJ analysis (Figure 2).

Growth characteristics: To study algal growth, four liters of BG-11 medium (except for S2 and S6 Jonson medium) was inoculated with active 7 days old inocula and incubated under controlled conditions as described above. Culture's densities were adjusted to be (OD_{680} = 0.25) in zero day for all strains. Optical densities were measured on regular basis using spectrophotometer (T60 UV–visible spectrophotometer, PG instruments, UK). To determine the dry weight of the biomass, 60 ml of each culture were centrifuged at 4000×g for 10 minutes and the washed several times with distilled water to remove residual salts. Then, the pellets dried at 60 °C oven for 48 h, weighed. The specific growth rate (µ) of each strain was calculated using equation below (Gill et al., 2016):

$$\mu = ln\frac{x_2 / x_1}{t_2 - t_1}$$

where x_1 is weight of dry biomass at the beginning of the selected time interval, x_2 is weight of dry biomass at the end of the selected time interval, (t_2-t_1) is the selected time for the determination of dry biomass. Biomass productivity (Pdwt) was expressed as dry biomass produced per litter per day (mg. L^{-1}.day^{-1}) during the exponential growth phase (Song et al., 2013) according to the following equation:

$$Pdwt = dwt \cdot \mu$$

RESULTS AND DISCUSSION

The pre-enrichment step allowed strains with high growth performance to compete with other weak strains. Therefore, this step is important to screen the best candidate strain for further scale up cultivation. After two weeks of initial incubation, most isolates were showed growth in liquid media. The growth performance was noticed and reported as ++++, +++, ++, + or – (Table 1). It was clear that the maximum growth was mostly observed in BG-11 medium except for (S2 and S6) were they had the highest growth (++++) in Johnson's medium. Samples with good growth were examined under light microscope to determine the type of microalgae present in them. Even though sub-culturing was done with extreme care, in some samples, especially when they were grown in BG-11 medium, protozoa and rotifers prey on microalgae rapidly and could not be processed or rescued, because BG-11 medium was improved microscopic grazer growth along with microalgae. In case of marine-origin samples, the growth was very weak, thus, those cultures were discarded.

Microscopic investigation of several samples during the screening process revealed the presence of flagellated, coccoid and filamentous algae. In some cases of mixed culture with filamentous algae, the growth of filamentous algae were very dense and no single cell strains could be purified. In addition, filamentous algae have sticky nature and very hard to handled, hence, they were excluded from selected collection. Also, it was noticed that S11 (obtained from 70 °C hot spring) requires at least 40°C incubation temperature to grow, therefore it is excluded too. Based on the growth performance and microscopic examination, seven isolates (S1, S2, S3, S4, S5, S6, S7) were chosen for morphological and molecular identification. For subsequent sub-culturing rounds, most microalgae were showed growth after seven days in the agar surface, probably due to acclimatization in the new conditions. Whereas in case of S2 and S6 (which later defined as *Dunaliella salina*), they

did not show growth on agar plates, however, they grew well in liquid media.

Morphological and molecular identification and characterization: In this study, seven strains were chosen to be identified and characterized. The selection criteria were focused on strains that could be easily cultivated. Based on morphological and reproductive features, all chosen strains were belonging to the phylum of Chlorophyta under the genera of *Chlamydomonas, Dunaliella, Chlorococcum, Graesiella, Coelastrella*, and *Chlorella*. To verify their taxonomical positions, the sequences of internal transcribed spacer (ITS) obtained from our samples were compared with the sequences available in the NCBI database, the results are summarized in (Table 2). Similar sequences were used to construct independent molecular phylogenetic trees. The reliability of the phylogenetic tree was evaluated using neighbor-joining analysis.

Table 1. Growth of the isolated microalgae on different media after 14 days. Excellent growth (++++), moderate growth (+++), poor growth (++), very poor growth (+), no growth (-).

Isolate ID	Source	Habitat	Isolation location	Kuhl	Medium		
					BG-11	F2	Jonson
S1	Fresh	Agriculture soil	Al-Madina	+	++++	-	-
S2	Fresh	Anthropogenic silt soil	Al-Baqi cemetery	-	++	-	++++
S3	Fresh	Agriculture soil	Al-Madina	++	++++	-	-
S4	Fresh	Stagnant water (effluent from air conditioner).	Jeddah	+	++++	-	-
S5	Fresh	Agriculture soil	Al-Madina	+	++++	-	++++
S6	Marine	Al Khumra salt marshes	Jeddah	+	+	++	++++
S7	Fresh	Agriculture soil	Jeddah	+	++++	-	-
S8	Marine	Sharm beach	Yanbu	-	-	+	-
S9	Fresh	Asphalt surface	Jeddah	++	++++	+	-
S10	Fresh	Agriculture soil from public walkway	Jeddah	-	++++	-	-
S11	Fresh	Gomygah hot spring	Al-Lith	+	++	-	-
S12	Fresh	Damp walls	Jeddah	-	++++	-	-
S13	Fresh	Garden irrigation dripper	Jeddah	+	++++	-	-
S14	Fresh	Birdbath	Jeddah	+	++++	-	-
S15	Fresh	Water barrel	Jeddah	+	++++	-	-
S16	Fresh	Planter	Jeddah	+	++++	-	-
S17	Fresh	Fountain	Jeddah	+	+++	-	-
S18	Fresh	Roadside mud	Al-Lith	+	++++	-	-
S19	Marine	Waterfront Corniche	Jeddah	-	-	+	-
S20	Fresh	Agriculture soil	Jeddah	+	+	-	-
S21	Fresh	Agriculture soil	Yanbu	+	++++	-	-
S22	Fresh	Agriculture soil	Al-Taif	+	+++	-	-
S23	Fresh	Roadside soil	Al-Madina	+	++++	-	-
S24	Fresh	Greenhouse hydroponics	Thuwal	+	++++	-	-
S25	Marine	Thuwal beach	Thuwal	+	+	-	-

Strain (S1) *Chlamydomonas zebra*: Microscopic examination of S1 demonstrated green, motile, unicellular; oval shaped cells (4-5.5 µm long, 4-3 µm wide) showed morphology consistent with *Chlamydomonas* sp. features (Figure 3). Previous research described *Chlamydomonas* species as an ovoid-shaped unicellular, of (9-16 µm long, 5-12 µm wide) with two anterior isokont flagella and single cup-shaped chloroplast, single nucleus; two anterior contractile vacuoles. Reproduction by producing 2-8 zoospores; or by isogamous (Serediak and Huynh, 2011; Zhang et al., 2014). The phylogenetic tree revealed that the sequence of S1 strain aligned with other strains of *Chlamydomonas* genus described in previous studies (Luo et al., 2010; Hoshina, 2014). According to the BLAST analysis results, the S1 is homologous and is closely related to the *Chlamydomonas zebra*. Therefore, the isolated S1 strain was given the name *Chlamydomonas zebra* UJ (Figure 2 a).

Strain (S3) *Chlorococcum pamirum*: Microscopic observation of S3 showed green spherical cells, vary in size (4.5-6 µm in diameter), solitary and form temporary groups (Figure 4). This morphology was similar to *Chlorococcum* sp. as mentioned in Feng et al., (2014), vegetative cells range in diameter from 5-16 µm. The cells have a distinct pyrenoid surrounded by a sheath of biplate starch and are uninucleate. Reproduced asexually by zoospores and aplanospores or sexually by isogametes (Blackwell, Cox and Gilmour, 1991). Therefore, S3 was preliminarily hypothesized to belong to genus *Chlorococcum* sp. The BLAST analysis result

supported our hypothesis, as shown in phylogenetic tree (Figure 2 a). The sequence of S3 was located in the same clade with other *Chlorococcum* species, and therefore, it was given the name of *Chlorococcum pamirum* UJ.

Table 2. Identification results of isolated stains according to the BLAST hits.

E-value	Identity (%)	Nucleotide length	Coverage (%)	Name and accession of number of the most related strain in NCBI GenBank		Isolates
2E-114	90%	333	98 %	AF033294.1	*Chlamydomonas zebra*	S1
2E-99	98%	221	99 %	MF360000.1	*Dunaliella salina*	S2
7E-129	99%	269	98 %	KX147379.1	*Chlorococcum pamirum*	S3
3E-72	91%	221	96 %	JX456465.1	*Graesiella emersonii*	S4
0	100%	493	100 %	MH176127.1	*Coelastrella* sp.	S5
0	96%	442	99 %	MF360000.1	*Dunaliella salina*	S6
4E-75	95%	221	85 %	LR215790.1	*Chlorella sorokiniana*	S7

Figure 2: Neighbor-joining phylogenetic tree of ITS region sequences of isolated algae.

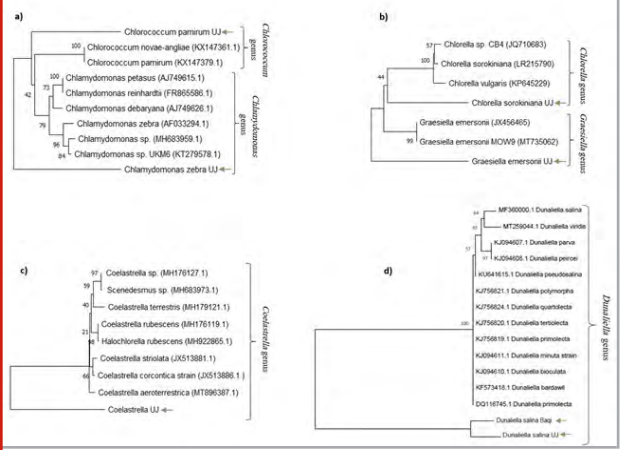
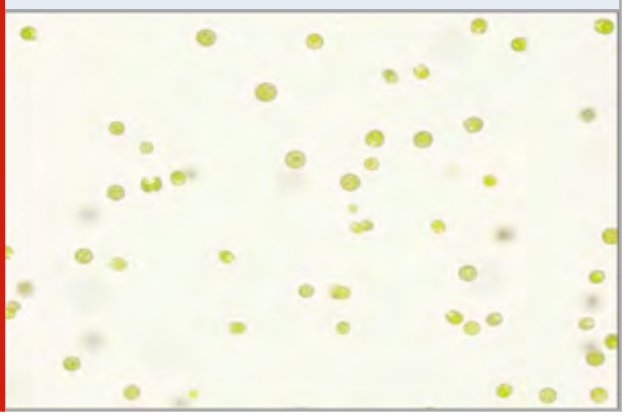


Figure 3: Microscopic photograph of *Chlamydomonas zebra* cells at a magnification of 400x.



Strain (S4) *Graesiella emersonii*: Under light microscope, the cells of S4 strain were giant (diameter ranging 9-22 µm), non- motile, unicellular, nearly globose to ellipsoidal shape with different sizes, enclosed by thick transparent sheath, and form auto-spores as a mean of asexual reproduction (Figure 5). These observations were similar to *Graesiella emersonii* features described by Nozaki et al., (1995) as a large globose to ellipsoidal cells (up to 5-17 µm in diameter), surrounded by double-layered cell wall. *Graesiella emersonii* also had a massive chloroplast containing a single pyrenoid and exhibiting several vacuoles and reproduce asexually by autospores. The molecular analysis of ITS regions of S4 showed a high similarity to *Graesiella emersonii* (Table 2) which supports the morphological characteristics; therefore, it was given the name of *Graesiella emersonii* UJ (Figure 2 b).

Strain (S5) *Coelastrella* sp.: Microscopic observation of isolated strain S5 shows there was similarity in morphological characteristic described in literature of *Coelastrella* sp. which is unicellular spherical cells, tend to aggerate in temporary groups (Figure 6 a,c). These characteristics were previously described in *Coelastrella* species (Wang et al., 2019; Goecke et al., 2020). Another important characteristic that has been reported in several *Coelastrella* species is the production of secondary pigments of carotenoids (Punčochářová and Kalina, 1981; Abe et al., 2007; Hu et al., 2013a; Kawasaki et al., 2020).

Changing of cell color in old and stressed cultures was noticed in S5 which indicating carotenoids production (Figure 6 b). Based on these finding, it is suggested that S5 to is a member of genus *Coelastrella*, and the molecular analysis supported this suggestion. Thus, the isolate S5 was given the name *Coelastrella* sp. UJ (Figure 2 c). *Coelastrella* species are widely distributed worldwide. It has been reported to be found in temporary waterbodies, birdbaths, and fountains (Neofotis et al., 2016). They found as single cell or in aggregations of few cells, these species are characterized by double layered cell wall, with distinct sculpture. The inner layer composed of cellulose and an outer one composed from (sporopollenin) which is acetolysis-resistant material (Tschaikner and Kofler, 2008). Previous research noticed the presence of small thickenings at the poles of the cells a citriforme in addition of longitudinal ribs considered as an important character

of *Coelastrella* species (Punčochářová and Kalina, 1981; Abe et al., 2007; Hu et al., 2013b; Kawasaki et al., 2020; Ciurli et al., 2021).

Coelastroideae subfamily members have been previously placed under family of Oocystaceae, *Chlorella*ceae, and Sctielloideae regarding to morphology and cellular structure (Kalina and Punčochářová, 1987). Later, the phylogenetic molecular studies suggested that *Coelastroideae* should be placed within the family Scenedesmaceae, order Sphaeropleales (Hanagata, 1998; Hegewald et al., 2010; Kaufnerová and Eliáš, 2013; Lee et al., 2016; Ancona-Canché et al., 2017). Nowadays, many species of this genus attract attention from researchers due to its ability of accumulation carotenoids and fatty acids, as well as for a potential use for bioremediation (Abe et al., 2007; Hu et al., 2013b; Kawasaki et al., 2013; Luo et al., 2016; Dimitrova et al., 2017; Thao et al., 2017; Goecke et al., 2020; Karm and Dwaish, 2021).

Figure 4: Microscopic photograph of *Chlorococcum pamirum* cells at a magnification of 400x.

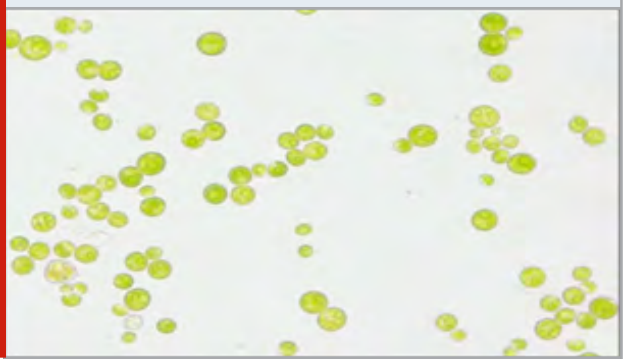
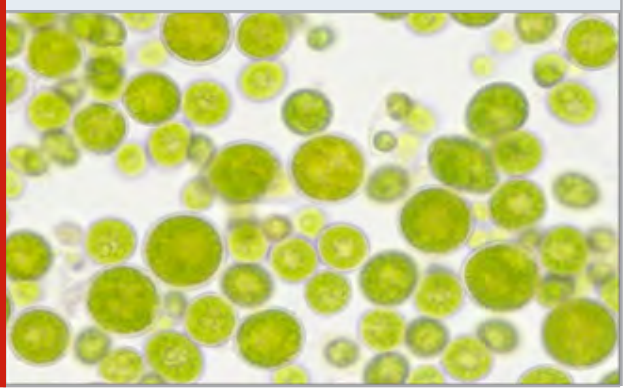


Figure 5: Microscopic photograph of Graesiella emersonii cells at a magnification of 400x.



Strains (S2 and S6) *Dunaliella salina*: Although S2 and S6 obtained from different environments (anthropogenic silt soil and saltmarshes), respectively, both of them were shows similarity in morphological and physiological characteristic of *Dunaliella* species. Under microscope, ovoid motile cells with cup shaped chloroplast were observed (Figure 7 a,b). It was also noticed that both strains change their cell color from green into orang under high light and salt stress (Figure 7 c). Previous works described *Dunaliella* sp. characteristic as following: ovoid to spherical motile cell with two equal flagella and cup-shaped chloroplast. Depending on different environmental conditions, the size and shape of the cell can vary within a species (between 2 to 28 µm and in width between 1 to 15 µm) (Hosseini et al., 2009). Although, *Dunaliella* cells are naked, they are surrounded by mucilaginous substance (Ben Amtoz et al., 2009).

Furthermore, it is well known that many species of *Dunaliella* are halotolerant and capable to grow in environment of high salinities, they were previously isolated from Dead Sea, and the Great Salt Lake, USA (Oren, 2014). *Dunaliella* response to such environmental stress through over-accumulation of beta-carotene pigment which is responsible for turn cell color into orange (Polle et al., 2017). Until now, 26 saltwater species and five freshwater species have been described for the genus *Dunaliella*. All freshwater species considered rare and its classification is still uncertain (Ben Amtoz et al., 2009; Gonzalez et al., 2001; Melkonian and Preisig, 1984). Molecular analysis of both isolated strains (S2 and S6) was confirmed the morphological and physiological findings. As our isolates were closely related to *Dunaliella salina*, they have been given the following names *Dunaliella salina* Baqi and *Dunaliella salina* UJ to distinguish its unique origin for further studies (Figure 2 d).

Figure 6: Microscopic photograph of *Coelastrella* sp. cells at a magnification of 400x. a) Cell's aggregation, b) Orange cells indicating carotenoids production, c) Single spherical cell.

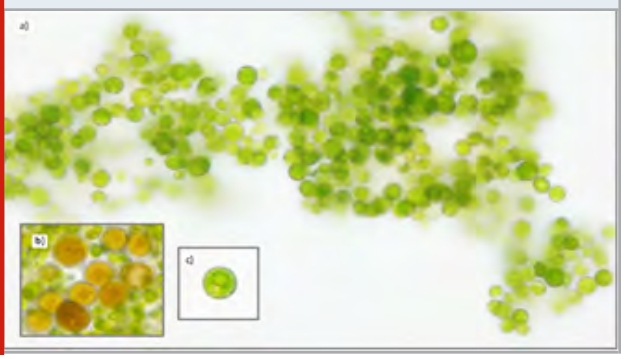
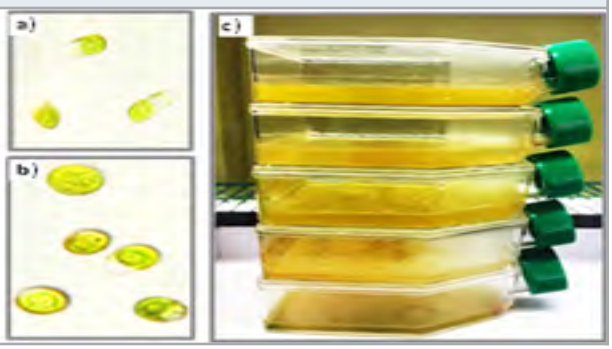


Figure 7: Light microscopic images (magnification of 400x) of *Dunaliella salina*. a) Strain isolated from saltmarshes, b) Strain isolated from anthropogenic silt soil, c) Orange color of *Dunaliella salina* stressed cultures.



Strain (S7) *Chlorella sorokiniana*: Microscopic observation of S7 shows a small (diameter 2-2.5 µm) spherical unicellular, emerald-green color alga (Figure 8). These morphological characteristics are similar to the morphological characteristics of *Chlorella* genus (Krienitz and Bock, 2012; Lizzul et al., 2018). The molecular identification confirmed this finding, therefore, S7 was designated as *Chlorella sorokiniana* UJ (Figure 2 b). *Chlorella* genus members are widely distributed in different habitat due to their rapid growth (Lizzul et al., 2018), *Chlorella* species have been used as model organisms for photosynthesis studies and biotechnological applications for decades (Béchet et al., 2013; Lizzul et al., 2018).

Figure 8: Microscopic photograph of *Chlorella sorokiniana* cells at a magnification of 400x.

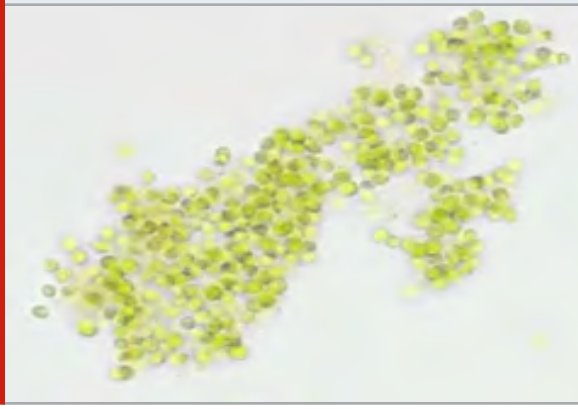


Figure 9: Growth curves of a) *Chlamydomonas zebra* UJ, b) *Dunaliella salina* Baqi, c) *Chlorococcum pamirum* UJ, d) *Graesiella emersonii* UJ, e) *Coelastrella* sp. UJ, f) *Dunaliella salina* UJ, g) *Chlorella sorokiniana* UJ, every point on the graph is showing the mean of three OD reading.

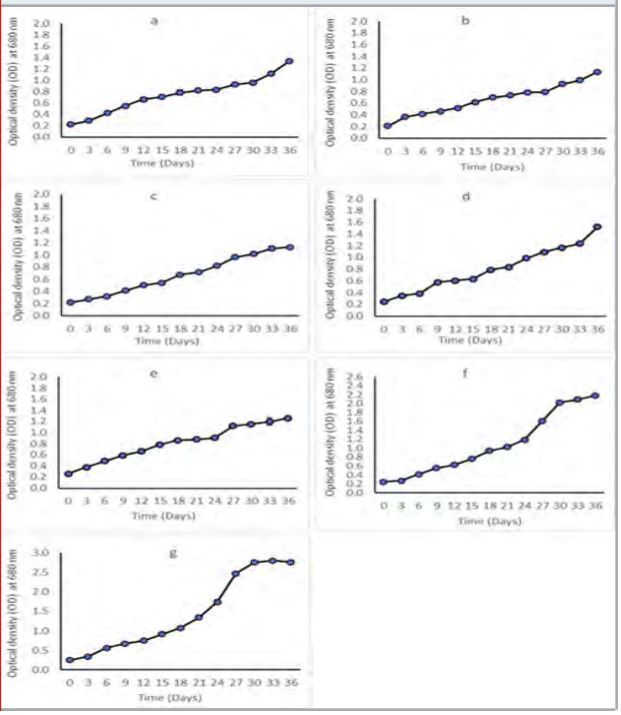


Table 3. Algae species, culture medium, specific growth rate, and biomass productivity

Isolates	Algal species	Culture medium	Specific ($mg.\ L^{-1}.day^{-1}$)	Biomass productivity growth rate (day^{-1})
S1	*Chlamydomonas zebra*	BG-11	0.158	68.3
S2	*Dunaliella salina Baqi*	Johnson	0.087	14.703
S3	*Chlorococcum pamirum UJ*	BG-11	0.169	113.9
S4	*Graesiella emersonii UJ*	BG-11	0.171	123.8
S5	*Coelastrella* sp. UJ	BG-11	0.169	128.9
S6	*Dunaliella salina UJ*	Johnson	0.173	111
S7	*Chlorella sorokiniana UJ*	BG-11	0.180	150.2

To date there are more than 20 characterized *Chlorella* species (Furnas, 1990; Krienitz et al., 2015). In the past few years, the classification of the genus of *Chlorella* has received a lot of attention and many species within the genus were re-classified (Luo et al., 2010; Lemieux et al., 2014; Krienitz et al., 2015). *Chlorella sorokiniana* is a sub-species first isolated in 1953 by Sorokin, and believed to be a thermotolerant mutant of *Chlorella pyrenoidosa* (Sorokin and Myers, 1953; Kunz, 1972; Lizzul et al., 2018). Later, this taxonomy was changed and re-classified *Chlorella sorokiniana* as a separate species (Kessler, 1985; Dörr and Huss, 1990; Kessler and Huss, 1992). Furthermore, it is worth to mention that this sub-species is unique and robust alga due to its ability to thrive under harsh conditions such as high salinities and temperature up to 40 °C. Therefore, *Chlorella sorokiniana* consider the subject of research in several major laboratories (de-Bashan et al., 2008; Lizzul et al., 2014, 2018; Krienitz et al., 2015; Neofotis et al., 2016; Jiang and Pei, 2021).

Growth and biomass productivity: In general, algal growth can be monitored by determining the changes in biomass directly using cell count, or using other parameters such as chlorophyll a, optical density and dry weight (Richmond, 2003). The growth response of our isolates was measured using optical density at 680 nm on regular basis throughout the span of cultivation. The data obtained from OD showed similar trend for all species under same

growth condition as presented in (Figure 9). The growth rate defined as the increasing of biomass over specific period of time (Richmond, 2003).

In this study, *Chlorella sorokiniana* had the highest growth rate followed by *Dunaliella salina* UJ and *Chlamydomonas zebra*. Whereas, *Chlorococcum pamirum, Coelastrella* sp. and *Graesiella emersonii* showed a comparable growth rate. The slowest growth rate was noted in *Dunaliella salina* Baqi. The growth parameters of all strains are shown in (Table 3).

As growth rate measures the cellular response to nutrients and growth conditions, different algal strains grown under a variety of culture conditions gives a variable response depending on cultivation mode, types of media, temperature, light intensity, photoperiod, and supplying of CO_2 (Enamala et al., 2018). Several studies performed previously to investigate the growth potential of different microalgae strains, however, these studies indicated that there is no standard species can be used to make precise comparisons, as well as the differences of cultivation conditions and methods (Richmond, 2003). Feng et al. (2014) found that growth rate of *Chlorococcum pamirum* was 1.88 day $^{-1}$ which is slightly higher than our result. The growth rates of *Dunaliella salina* and *Chlorella sorokiniana* were found to be 0.16 -0.20 day^{-1} and 0.19 - 0.20 day $^{-1}$ respectively (Pertumbuhan et al., 2017; Sajjadi et al., 2018; Khatoon et al., 2020; Karm and Dwaish, 2021).

Which are close to the growth rate obtained from our study (Table 3). Specific growth rate reflects the time required for cells to divide, however, some microalgae species grow by increasing their size rather than increasing their cell number (Zachleder et al., 2016) therefore, high growth rate does not necessary reflect high productivity. Thus, biomass productivity is usually used as a more reliable method to evaluate strain efficiency. Biomass productivity is generally calculated as the increase in biomass over a period of time. In this study, we compared the biomass productivity of all strains and found that *Chlorella sorokiniana* is the most productive strain, whereas *Dunaliella salina* Baqi was the least. In general, the productivity values obtained from this study (shown in Table 3) were approximately similar to the values mentioned in previous works (Khan et al., 2009; Rodolfi et al., 2009; Mata, Martins and Caetano, 2010; Park et al., 2012; Enamala et al., 2018; Sajjadi et al., 2018; Khatoon et al., 2020; Ciurli et al., 2021).

CONCLUSION

Identification of microalgae is considered a key step in microalgae-based industry. This work screened microalgae strains isolated from the local environment of Saudi Arabia. The best performing algal strains were selected to be identified and characterized to discover strains that can be utilized for mass cultivation. This study successfully isolated and identified seven local strains, most of them are already known with high biomass productivity (fast growers) and are considered as good candidate to serve as platform for many further applications. Among these strains are *Dunaliella* and *Coelastrella genera*, these two genera were reported to be capable to accumulate carotenoids and thus they could be exploited for carotenoid production. We also identified strain of Chlorella sorokiniana which is currently one of the most promising algal species that can be used as biofuel feedstocks. The results of this study can greatly enrich our knowledge of microalgae biodiversity in Saudi Arabia. To the best of our knowledge, this study is one of few reports focus on exploring the local microalgae isolates. Integrating phyco-prospecting and characterizing native isolates could contribute in supporting algae-based industry for future.

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Medical Communication

Role of Environment and Contact-Pattern Factors in Pulmonary Tuberculosis Patients from Bandung City, Indonesia

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ABSTRACT

Pulmonary Tuberculosis is one of the the major infectious diseases and is among diseases with the highest mortality and morbidity in the world. Although many interventions had been done to prevent this disease, its spread of infection is still high. One of the factors that play an important role in the spread of this disease are environmental factors and contact patterns. There are only a few articles that discuss this issue in Indonesia. So, this study aims to explore the association between environmental, social contact factors, and pulmonary tuberculosis (PTB) in Bandung, Indonesia. An unmatch case-control with a 1:2 ratio was designed for the purpose of this study. Cases were defined as smear-positive pulmonary TB cases that received treatment during 2015-2016 at the four selected Public Health Centers (PHCs) in Bandung. Controls were selected from healthy neighbours of the cases. Data were collected through home visits, interviews, and observations using a structured-questionnaire. Multivariable logistic regression was used to determine the risk factors associated with PTB. Findings from analyses on 330 respondents consisting of 113 cases and 217 controls demonstrated that the absence of Cross-ventilation inside the house was associated with PTB (AOR: 1.91; 95 % CI: 1.03-3.57) as the environment factor while family history of pulmonary TB (AOR: 4.90; 95% CI: 2.30–10.75), number of household member (AOR: 2.73; 95% CI: 1.33–5.65) and time spent inside the house (AOR: 1.12; 95% CI: 1.08–1.27) were found to be the social contact factors associated with PTB. Thus, the environment and social contact-pattern are essential factors in TB transmission. Regulations regarding this factor need to be strengthened so that this disease can be controlled.

KEY WORDS: PULMONARY, RISK FACTOR; SOCIAL CONTACT FACTOR, TUBERCULOSIS.

INTRODUCTION

Pulmonary tuberculosis (PTB) is world's top infectious disease killer (World Health Organization, 2020). This disease has infected a quarter of the world's population and caused 60 million cumulative death since 2000 (World Health Organization, 2020). Although this disease was treatable and preventable with 85 % successful treated rate, but this disease is still difficult to eradicate. Indonesia, as developing country, ranks second among countries with the highest incidence of PTB (World Health Organization, 2015). It is estimated in the latest data available on TB incidence that 1 million TB cases occur annually with an incidence rate of 395 per 100,000 population per year (World Health Organization, 2017). Bandung, as one of the major cities in Indonesia, is also facing the same problem. Bandung ranks third among cities with the highest TB incidence in West Java Province and recent PTB notification rate shows 477 cases per 100,000 population (West java Provincial Health Office, 2020; Yang et al., 2021).

This condition will affect city health and productivity. Despite the enormous efforts to control this disease, the PTB in Indonesia is still rampant. In 2016, the Minister of Health of the Republic of Indonesia issued a regulation

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to update and complement TB management efforts in Indonesia (Ministry of Health of the Republic of Indonesia, 2016). This regulation has already included the promotive, preventive, and curative aspects for fighting PTB. However, the social contact or contact pattern is not among them despite the fact that it plays major role in TB transmission thus becomes a potential point for intervention (Lönnroth et al., 2010, 2009; Ortblad et al., 2015). How people interact with each other indeed influences the risk for being infected by PTB, with number of contacts, duration of contacts, and a history of PTB among the contacts as the most prominent risk variables. Therefore, contact pattern needs to be evaluated when dealing with PTB infections (Mossong et al., 2008; Dodd et al., 2016; Horton et al., 2020; Yang et al., 2021).

Various literature have asessed the relationship between environmental risk factors and PTB (Alisjahbana et al., 2006; Baker et al., 2008; Bam et al., 2015). However, only few studies explores the relationship between contact factors and PTB (Dodd et al., 2016; Rahayu et al., 2015; Gelaw et al., 2019; Abreu et al., 2020; Yang et al., 2021). Therefore, this study focuses on exploring and identifying the social contact factors associated with PTB. In addition, the household environmental factors such as household density, indoor and outdoor hygiene, and availability of cross-ventilation in the house are also explored.

MATERIAL AND METHODS

Study Location: Bandung, as one of the major cities in Indonesia, is a busy and highly populated city with 2,5 million population living in an area of 167 kilometer squares. The city consists of 30 districts and 151 sub-districts. It also has 73 Public Health Centers (PHCs) to cater for the healthcare-related needs of the citizen. Each PHCs is responsible for 2 to 6 sub districts.

Sample and procedure: We conducted an unmatched case-control study in late 2016 in four selected PHCs in Bandung: Arcamanik, Babakansari, Padasuka, and Ujungberung Indah PHCs. The criteria for selecting PHCs were having a broad working area that covers two or more subdistricts; having a laboratory capability to diagnose PTB, and having a good performance in recruiting TB patients (i.e. having a high number of PTB patients in the last two years). We use the 2015-2016 TB register data for recruiting respondents. A 1: 2 ratio was used for case and control selection. Controls were selected systematically by recruiting healthy adult respondents who lived nextdoor to the case.

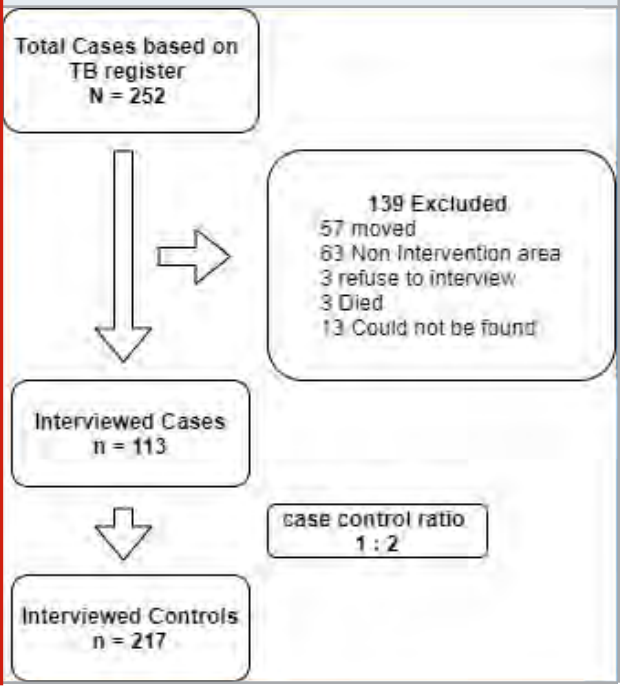
Social Contact Pattern: We collected data on respondents' social contact pattern using a structured questionnaire based on their social interaction habit in the community. We used four variables to describe the social contact pattern: number of household member (i.e., total individuals living in the house excluding respondent), time spent inside the house (i.e., the average time spent in the house each day), family history of TB (i.e., a history of family member who received treatment or were currently under treatment for PTB)(Cohen, 2000; Volz et al., 2011).

Household Environmental Factors: The household environment factors consist of four variables, namely household density, indoor household hygiene, outdoor household hygiene, and availability of cross-ventilation. We measured the household density as the total household area divided by total household members. Good indoor and outdoor hygiene was confirmed when there were no dust, waste, rats, or cockroaches seen in the household (Ministry of Health of the Republic of Indonesia, 2013). Cross-ventilation availability was confirmed if there were at least two different wall-windows in the house.

Definition and Measures: We defined cases as individuals with a history of PTB or were receiving treatment due to smear-positive result for PTB and recorded in the "TB-register" at the selected PHCss. Control was defined as healthy individual neighbours to the index case who had never been diagnosed with any TB disease.

Individual characteristics: We collected subject demographic data such as age, gender, level of education (i.e., time spent in formal education measured in years), marital status (married/widowed/single), employment (government officer/private employee/unemployed), insurance ownership (yes/no). We also characterized respondents by their economic status (described by the total monthly family income in Indonesian Rupiah), daily cigarette consumption, history of Diabetes Mellitus, and environment factors that influence TB transmission (e.g. people density/person/m2, Indoor and outdoor home hygiene, and presence of cross-ventilation in the house) as the confounding factors.

Figure 1: Study Population



Ethical Approval: All procedures performed in studies involving human participants are in accordance with the ethical standards of the institutional and/or national research

committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Statistical analysis: We explored the association between social contact factor, environmental factor, and PTB using multiple regression analyses. We also include potential confounders, i.e. age, sex, marital status, cigarette consumption, total monthly family income, and history of diabetes, in the analyses. All statistical analyses were performed with "R" version 3.2.3 and "R studio" as the user interface (Team Rstudio, 2015). We employed the AIC (Akaike Information Criterion) as the tool to assess best fit models.

Table 1. PTB-Associated Sociodemographic, Environmental, and Social Contact Factors in Bandung City

Variable	Case (n= 113)	Control (n= 217)	p- value
Sociodemographic			
Age (years), median (IQR)*	38(25 – 48)	45 (36 – 56)	<0.001‖
Sex , n (%)*			
•Female	55(48.7)	145(66.8)	<0.001 δ
•Male	58(51.3)	72(33.2)	
Duration of education (years)*	9.39(3.02)	9.54(3.41)	<0.001‖
Marital status n(%)*			
•Single	30(26.5)	11(5.1)	<0.001 δ
•Married	74(65.5)	174(80.2)	
•Divorced/widowed	9(8)	32(14.7)	
Job Qualification n(%)			
•Government officer	1(0.9)	14(6.5)	0.06 δ
•Private employee	54(47.8)	91(41.9)	
•Unemployed	58(51.3)	112(51.6)	
Insurance Ownership, n(%)*			
•Yes	79(69.9)	154(71)	0.84 δ
•No	34(30.1)	63(29)	
Total monthly family income (Thousand Rupiah) , median (IQR)*	1,200 (500-6,800)	1,500 (1,000-10,000)	<0.01‖
Environmental Factors			
Household density (person/m2)	9.09(10.05)	10.34(8.82)	0.25‖
Poor Household Indoor Hygiene*			<0.001δ
•Clean	86(76.1)	196(90.3)	
•Dirty	27(23.9)	21(9.7)	
Poor Outdoor Household Hygiene*			<0.001δ
•Clean	84(74.3)	188(86.6)	
•Dirty	29(25.7)	29(13.4)	
Cross-ventilation inside house *			<0.001δ
•No	44(39)	114(52)	
•Yes	69(61)	103(48)	
Social Contact Factors			
Number of household members (IQR)*	4(0-11)	3(0-11)	<0.001‖
Time spent inside house (hours) (SD)*	18.06 (±4.04)	16.49(±4.57)	<0.001‖
Family history of pulmonary TB n(%)*			
•No	78(69)	197(90.8)	<0.001 δ
•Yes	35(31)	20(9.2)	

Abbreviation : IQR = interquartile range ‖= Independent t test δ = chi square test
*indicates that the finding is statistically significant at the level of the confidence of 5 % (P-value < 0.05)

RESULTS AND DISCUSSION

Based on the 2015-2016 TB register in four PHCs, there were 252 potential respondents as cases. About 139 eligible cases were excluded due to several reasons such as moving out of town (n=57), living outside the work area of the PHC (n=63), refused to be interviewed (n=3), died (n=3), and could not be traced (n=13). Thus, the total number of cases in this study was 113 respondents. We invited 217 respondents to participate as controls but 9 refused to be interviewed (Figure 1).

Cases were younger than controls (38 ± 15.2 vs. 45.6 ± 13.5) and more likely to be male (51%). Compared to controls, cases were more likely to have lower duration of formal education (9.39 ± 3.02 vs. 9.54 ± 3.41). There were also statistical differences between cases and controls in terms of marital status, job qualification, and total monthly family income, albeit no statistical difference in Insurance Ownership variable was identified. On household density, both cases and controls had a similar household density (9.09 ± 10.05 vs. 10.34 ± 8.82) but differences were seen in Indoor household hygiene, outdoor household hygiene, and availability of cross-ventilation variables. Respondents from the case group had higher proportions for poor indoor household hygiene , poor outdoor household hygiene , and absence of cross-ventilation inside the house. Respondents from the case and control groups had slight difference in the number of household members but the difference was statistically significant ($p < 0.05$). Respondents from the case group also spent longer time inside the house than those in the control group (18.06 ± 4.04 vs. 16.49 ± 4.57). A higher proportion of respondents in the case group had a family history of PTB compared to those in the control group (n=35, 31% vs. n=20, 9.2%) (Table 1).

Table 2 presents the model of multivariable logistic regression of the study. This model has been adjusted to socioeconomic factors (such as: age, sex, marital status, total monthly family income in a month), history of cigarette smoking, and history of diabetes. We found that PTB was associated with several social contact factors including family history of pulmonary TB (AOR: 4.90; 95% CI: 2.30–10.75), number of household members (AOR: 2.73; 95% CI: 1.33–5.65), and time spent inside the house (AOR: 1.12; 95% CI: 1.08–1.27). In contrast, the only household environment risk factor associated with pulmonary TB was the absence of cross-ventilation in the house (AOR: 1.91; 95 % CI: 1.03-3.57). The model also demonstrated that history of diabetes, cigarette consumption, age and marital status are potential confounders for PTB (Table 2).

Table 2. Univariable and multivariable odds ratio's (OR) and 95% confidence interval (95% CI) for social contact factors and environmental factors associated with PTB in Bandung City

Variable	Crude OR			Adjusted OR		
	OR	95 % CI		OR	95 % CI	
(Constant)				0.190	0.029	1.144
Demographics						
Age	0.962*	0.945	0.978	0.948*	0.924	0.971
Sex (Male)	2.123*	1.336	3.389	3.221*	1.479	7.160
Marital Status (Married)	0.156*	0.071	0.318	0.214*	0.073	0.588
Marital Status (Divorced/Widowed)	0.103*	0.035	0.272	0.217*	0.048	0.931
Cigarette consumption	1.056*	1.019	1.098	1.061*	1.006	1.128
Total monthly family income (IDR)	1.000	1.000	1.000	1.000	1.000	1.000
History of Diabetes (Yes)						
environmental Risk	4.973*	1.933	14.397	8.976*	2.724	32.402
People density (person/m2)	0.984	0.955	1.058	1.023	0.987	1.060
Poor Indoor Household Hygiene	2.930*	1.575	5.521	1.880	0.629	5.652
Poor Outdoor Household Hygiene	2.238*	1.257	3.991	1.883	0.699	4.970
No Cross-ventilation in house						
Social Contact factors	1.735*	1.096	2.768	1.911*	1.038	3.573
Time spent inside house (hours)	1.085*	1.029	1.146	1.172*	1.084	1.274
Number of household members	2.351*	1.378	4.017	2.728*	1.332	5.658
Family history of PTB						
AIC	4.419*	2.427	9.246	4.901*	2.301	10.753
	-	315				

Abbreviation : IDR = Indonesian rupiah OR = Odds Ratio CI = Confident Interval AIC = Akaike Information Criterion

*indicates that the finding is statistically significant at the level of the confidence of 5 % (P-value < 0.05)

Our study shows that social contact pattern, i.e. family histroy of PTB, number of household members, and time spent inside the house, is associated with PTB in Bandung City. Family history of TB has been proven and shown to be associated with PTB in many papers. Previous studies stated that a positive PTB case can infect about 30 -40 % of theirs

contacts (Gaur et al., 2017; Jindal, 2017; Lienhardt et al., 2005; Rathi et al., 2002; Sabri et al., 2019). Therefore, the risk for getting PTB increases with the increasing number of family members who are positive for PTB as this means that there are more contacts in the house (AOR: 2.73; 95% CI: 1.33–5.65). One of the possible reasons may be related to the intensity of contact. We know that household members share the same air space and living activities, which increases the chance of disease transmission especially when there is a family member with PTB (Baker et al., 2008; Dodd et al., 2016; Qian et al., 2006).

With every additional hour spent inside the house, a 10% increase is seen in the odds of acquiring PTB (AOR = 1.172 , 95 % CI : 1.084 – 1.274). This study shows that respondents in the case group are more likely to spend more time inside the house than those in the control group. The longer people stay in their house, the more likely they will be infected with PTB bacilli. *Mycobacterium tuberculosisis* more likely to live in a condition with a high humidity and low light (Jindal, 2017; Sornboot et al., 2019; Taye et al., 2021). This theory could become the reason why this finding is significant. Other biological theory that can support this finding relates to the presence of cross-ventilation and history of PTB in the family (Dodd et al., 2016; Jindal, 2017; Sornboot et al., 2019; Taye et al., 2021).

There is also significant association between the absence of cross-ventilation inside the house and PTB (AOR: 1.91; 95 % CI : 1.04 – 3.57). The absence of cross-ventilation in the house almost doubles the probability to get PTB. Many studies suggested that poor ventilation is a risk factor for PTB. This study justifies and strengthen this notion (Chan and Fang, 2020; Escombe et al., 2019; Muchsin et al., 2019; Rahayu et al., 2015). Individual characteristics such as age, gender, marital status, cigarette smoking, total monthly income, and history of diabetes have been analyzed in earlier studies and are considered to be associated with PTB. Our findings also support this association (Alisjahbana et al., 2006; Andrade et al., 2019; Bam et al., 2015; Gelaw et al., 2019; Abreu et al., 2020; Yang et al., 2021). The selection of PHCs was performed by purposive method based on several criteria, i.e : cover two or more sub districts and have laboratory facilities. In order to get normally distributed data, we use big sample and a higher ratio for case and control (1 :2). We did not assess interactions for our model because our experience is limited. However, a multicollinearity testing was performed on our model. Since our study shows that the absence of cross-ventilation is a potential factor for PTB. This emphasizes the need for the local government to implement interventions or regulations regarding healthy homes.

CONCLUSION

Based on our study, social risk factors, such as family history of PTB, number of contacts at home, time spent inside the house, and household family risk factor are associated with PTB in Bandung City. These findings show that social and environmental factors are important parts of the solution to eradicate pulmonary TB. We, therefore, recommend that the government should invest more in social interventions to eradicate PTB. Since our study demonstrated that PTB is associated with the higher number of family members in the house or a high total household contacts, it is our suggestion that the interventions should target areas with dense population as a priority area for PTB prevention.

Conflict of Interest: The authors declared no conflict of interest in this study.

Ethical Approval Number : The Current Research Work Was Ethically Approved by the Institutional Review Board (IRB) Reference No **1164/UN6.C1.3.2/KEPK/PN/2016 Date : 01 -12 -2016**

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Biotechnological Communication

Molecular Identification of Newly Isolated Foodborne Bacterial Strains from Chevon Meat

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ABSTRACT

Red meat consumption (chevon, beef, and lamb) is one of the most traditional favorable dishes and remain in the modern lifestyle. Chevon is the meet of adult goats and it is considered one of the beef and sheep meat competitor. It is characterized by its lower calories content in comparison to beef content, total fat; "cholesterol and saturated fat" which maintain consumers health. Studies on chevon-microbial interactions are not fulfilled globally, therefore, we aimed to achieve microbial survey and molecular identification of chevon meat food contaminants from different markets of Makkah region, Saudi Arabia. A total of 50 chevon samples were purchased from different retail markets within Makah from September 2019 until January 2020. Samples were transported to the laboratory in a cooler. They were macerated in peptone water and then cultured on selective media of some indicator's microorganisms. About; 2.1×105, 2.1×104, 2.1×105, 1.2×10, 1.5×105, 2.5×105, 1.6×105, 3.7×105 CFU/g were the mean of total aerobic counts, anaerobic count, Enterobacteriacea spp. and *Staphylococcus* spp. counts respectively. All tested chevon meat samples were within permissible limit and fit for human consumption. Molecular Identification of isolated microorganism were as following; *Staphylococcus sciuri* were 2/9 (22.2), while; for all, *Aeromonas enteropelogenes, Escherichia fergusonii, Proteus mirabilis, Pseudomonas aeruginosa* and *Staphylococcus lentus* were 1/9 (11.1) respectively. This is the first report isolated of Aeromonas enteropelogenes from chevon meat. All isolated microorganisms had public health concern as food poisoning microorganisms. Further investigation needed to study the chevon meat and its microbial quality.

KEYWORDS: AEROMONAS ENTEROPELOGENES, FOODBORNE MICROORGANISMS, GOAT MEAT, 16S RDNA, PROTEUS MIRABILIS, PSEUDOMONAS AERUGINOSA.

INTRODUCTION

Red meat consumption (chevon, beef, and lamb) is one of the most traditional favorable dishes and remain in the modern lifestyle (Jiang and Xiong, 2016). The goat meat (Chevon) is very common and widely flavored by the consumers worldwide. Chevon is a type of red meat but better in their nutritional vales when compared with beef and other red meat types. Unlike with beef chevon is lower in calories, cholesterol and saturated fat which protect the consumers health especially for consumers which suffering from heart disease that affected by the lower sodium and higher potassium content in addition to their essential amino acids which differ than other red meats, the chevon cutlets consider as a better option for consumers (Singh et al., 2014).

Recently, chevon consumption become more attractive to the consumers due to the risen of the health conscious as this type of meat characterized by its lower fat contents than other different red meat types which make it the most excellent low fat meat sources (Mazhangara et al., 2019 and Sial, et al., 2021).

Three mechanisms in different meat products spoilage occur during processing and/or storage: lipid oxidation, enzymatic autolysis, and microbial spoilage. Microbial population affected by microflora of the animals, skin and intestinal tract, in addition to other sources of microbial contamination handling, from environment, storage conditions. Growth of microorganisms in meat appeared as slime formation, off odors, texture, changes in appearance, degradation of components and change the

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water holding capacity. Oxidation of lipid affected by the composition of different fatty acids, vitamin E content, and free iron content in muscles. Many enzymes degrade different nutrient component such as proteins, fats and carbohydrates of the tissues which resulting in greenish discoloration and softening of meat which resulting in microbial decomposition. The proteolytic enzymes remain active even at low temperatures (5^0C) which leading to loss of water holding capacity, microbial growth, and biogenic amines production (Heifa'a et al., 2018 and Vita, et al., 2020; Priya et al., 2021).

Food borne pathogens considers the main problems, especially in developing countries. Food considered as the most important sources that causes the microorganisms to human, these microorganisms still a major cause of food-borne human disease in most parts of the world (Aseel et al., 2011). The presence of food pathogens in food items mainly leading to undesirable gastrointestinal symptoms. It has been mentioned that hazards such as bacteria, fungi, allergens, chemicals, and foreign matter can be present in different meat types of bacterial pathogens (Pal et al., 2018 and Abebe, et al., 2020). There was a big shortage in chevon microbial evaluation studies globally that is the reason of our choice and aim of this study which conducted to survey the microbial contamination and serological identification of chevon meat from different markets in Makkah region, Saudi Arabia.

MATERIAL AND METHODS

A total of 50 chevon samples, about 450g/each were purchased from different retail markets within Makah from September 2019 until January 2020. Samples were collected and shipped without delay in pre-cooled insulated containers with frozen packs to laboratory. All samples were prepared according to the technique recommended by APHA (2002) as following, one gram from each sample were homogenized under aseptic condition with 9 ml of sterile buffered peptone water 0.1%, and then homogenized to have a dilution (1/10) further serial dilution up to 107 were prepared. About 0.1 ml of each prepared serial dilution were inoculated on standard plate count agar then incubated at 30±1oC/72±3 hrs. the obtained colonies were recorded.

The same procedures were incorporated for total anaerobic counts using Reinforced Clostridial medium agar (Oxoid; CM151), then were incubated in an anaerobic jar (Gaspak plus anaerobic system) at 37 °C/48 hours. Countable plates were recorded. *Staphylococcus* sp. Count (ISO, 1999) using mannitol salt agar which incubated at 30±1 °C/ 24 – 48 ±4 hrs. Suspected yellow colonies with yellow zone were counted and then 5 typical colonies were picked up on nutrient agar slant for further confirmation biochemically by Coagulase test using rabbit plasma, suspected colonies were transferred to Brain heart infusion (BHI) broth tube at 35 – 37 °C/24 hrs then 0.1 ml of the culture was aseptically mixed to the sterile test tube containing 0.3 ml rabbit plasma (Difco, BD) at 35–37 °C/4hrs, if test was – ve was re-examined each 2 hrs until 24 hrs.

Total Enterobacteriaceae Count (ISO, 2004) by violet red bile glucose agar (VRBGA) at 30-35 °C/24 hrs. DNA extraction: suspend a single microbial cell in 20 µl of lysis buffer containing 0.25% (vol/vol) sodium dodecyl sulfate and 0.05 N NaOH. Then heating at 95°C/15 min., addition of chromatography-grade H_2O (Fisher), and stored the lysis suspension at −20°C (Spilker et al., 2004). The extraction of DNA done according to manufactured of commercial DNA extraction kit (Presto Mini-DNA Bacteria Kit. Geneaid Biotech Ltd. USA) instructions. Which followed by DNA extracted using nanodrop device at wave length of 260/280 nm (Al-Azawi et al., 2018).

Primer design: 16S rDNA relevant sequences which were presented in the database of the GenBank. Based on this alignment, species-specific primers, and putative genus-primers were designed and shown on table (1) (Spilker et al., 2004). PCR master mix preparation from AccuPower®PCR-PreMix-Kit according to the company directions as shown in (Table 2), then all the PCR tubes were vortexed for 3 min and were transferred in the thermocycler apparatus (MyGene, Bioneer. Korea) (Al-Azawi et al., 2018).

Amplification of targeted DNA was added in 25-µl volume, that containing; 50 mM Trizma (St. Louis, Mo., pH 8.3; Sigma), 2 mM MgCl2, 0.4 µM for each primer, 1U of Taq polymerase (Invitrogen, Carlsbad, Calif.), 250 µM for each deoxynucleoside triphosphates (Promega, Madison, Wis.) and 2 µl whole-cell bacterial lysate, added to 25 µl of high-performance liquid chromatography-grade H_2O. Amplification by a Rapid Cycler (Idaho Technology Inc., Salt Lake City, Utah) thermo controller (Spilker et al., 2004). The determination 16S rDNA sequence: to ensure identification PCR-based results, analyzing 16S rDNA comparative sequence performed. The complete 16S rRNA genes (positions between 9-1500 by numbering system) which PCR amplified using Pfu DNA polymerase (Stratagene, La Jolla, Calif.) with conserved primers UFPL and URPL as described. DNA purification using QIAquick PCR purification kit (Qiagen Inc., Valencia, Calif.). Sequencing DNA performed using Applied Biosystems ABI model 3700 sequencer (PE Applied Biosystems, Foster City, Calif.) with BigDye Terminator cycle sequencing ready reaction kit. Resultant sequences visualized as chromatograms then manually edited using Chromas version 2.22 (Technelysium Pty. Ltd., Helensvale, Australia). Sequences assembled by EditSeq (DNASTAR Inc.) and identified by BLASTN compared with the NCBI database available sequences (www.ncbi.nlm.nih.gov/BLAST) (Spilker et al., 2004).

RESULTS AND DISCUSSION

Bacteriological Profile of Chevon Samples revealed on table (2) as following; the positive aerobic bacteria were about 50/50 (100%) of the total chevon sample same result detected in case of Anaerobic, *Enterobacteriacea* spp. count while, only about 25/50 (50%) of tested chevon sample have *Staphylococcus* spp.

Statistical analysis for Different Types of Micro-organisms (CFU/g) in the chevon samples discussed on table (3)

were the total aerobic counts reported about; 90×103, 38×104, 2.1×10^5 $\pm2.1\times10^4$ as minimum, maximum, mean ±SE values respectively while, about; 16.2×10^4, 32.1×10^4, 2.1×10^5 ±1.2×10 CFU/g. were detected as minimum, maximum, mean ±SE values respectively in case of total anaerobic count. *Enterobacteriacea* spp. showed about; 20×10^3, 6.0×10^5, 1.5×10^5 $\pm2.5\times10^5$ CFU/g. as minimum, maximum, mean ±SE values respectively while, about; 10×103, 1.2×106, 1.6×105 ±3.7×105 CFU/g. were detected as minimum, maximum, mean ±SE values respectively in case of *Staphylococcus* spp. counts.

Table 1. Molecular identification

Description	Sequencing primer name primer sequences	PCR primer name primer sequences
Aeromonas enteropelogenes	785F 5' (GGA TTA GAT ACC CTG GTA) 3' 907R 5' (CCG TCA ATT CMT TTR AGT TT) 3'	27F 5' (AGA GTT TGA TCM TGG CTC AG) 3 1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'
Escherichia fergusonii	785F 5' (GGA TTA GAT ACC CTG GTA) 3' 907R 5' (CCG TCA ATT CMT TTR AGT TT) 3'	27F 5' (AGA GTT TGA TCM TGG CTC AG) 3 1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'
Proteus mirabilis	785F 5' (GGA TTA GAT ACC CTG GTA) 3' 907R 5' (CCG TCA ATT CMT TTR AGT TT) 3'	27F 5' (AGA GTT TGA TCM TGG CTC AG) 3' 1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'
Pseudomonas aeruginosa	785F 5' (GGA TTA GAT ACC CTG GTA) 3' 907R 5' (CCG TCA ATT CMT TTR AGT TT) 3'	27F 5' (AGA GTT TGA TCM TGG CTC AG) 3 1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'
Staphylococcus Lentus	785F 5' (GGA TTA GAT ACC CTG GTA) 3' 907R 5' (CCG TCA ATT CMT TTR AGT TT) 3'	27F 5' (AGA GTT TGA TCM TGG CTC AG) 3 1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'
Staphylococcus Sciuri	785F 5' (GGA TTA GAT ACC CTG GTA) 3' 907R 5' (CCG TCA ATT CMT TTR AGT TT) 3'	27F 5' (AGA GTT TGA TCM TGG CTC AG) 3 1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'

Table 2. Bacteriological profile of tested chevon samples

(Bacteriological profile)	Positive samples		Negative samples	
	No.	Percent %	No.	Percent %
Aerobic bacteria	50	100	0	00
Anaerobic bacteria	50	100	0	00
Enterobacteriacae spp. counts	50	100	0	00
Staphylococcus spp.	25	50	25	50

Comparison with microbiological criteria for foodstuffs (GSO 1016/ 2015): According to Saudi Arabia microbiological criteria for foodstuffs (GSO 1016/2015) the permissible limit of different meat products average between; 5×10^5-5×10^6 CFU/gm. In case of total aerobic count and anaerobic, 10^2-10^3 CFU/gm in case Enterobacteriacae, all samples should be free from foodborne pathogens, while, in case of *Staphylococcus* the acceptable limit ranged between 10^2 -10^3 CFU/gm. Comparison with microbiological criteria for foodstuffs (GSO 1016/ 2015) viewed in table (4) which declared that; all tested chevon meat samples were within permissible limit and fit for human consumption.

Molecular Identification of isolated microorganism: PCR identification mentioned in table (5) and figures (1-6) were as following; *Staphylococcus sciuri* were 2/9 (22.2), while; for all of, *Aeromonas enteropelogenes, Escherichia fergusonii, Proteus mirabilis, Pseudomonas aeruginosa* and *Staphylococcus lentus* were 1/9 (11.1) respectively.

Bacteriological profile of the minced chevon samples were as following; the positive aerobic bacteria were about 50/50 (100%) of the total minced chevon sample same result detected in case of Anaerobic, Enterobacteriacea spp. count while, only about 25/50 (50%) of tested minced chevon sample have *Staphylococcus* spp. Similar results of the total aerobic count was detected by (Ragab, et al. 2016; Shaltout, et al. 2016 and Mardziah, et al., 2019) during their assessment of the bacteriological quality of some meat products in the Egyptian retail markets. Also, nearly similar results observed by Bantawa, et al. (2018) during their bacteriological evaluation of different meat samples of Dharan markets as; (54%) in case of staphylococcus sp. Lower results reported by Erdem, et al. (2014) during their microbiological quality survallaince in Istanbul as (96.67%). While, only about (24%) of *Enterobacteracea* sp. and (8%) of *staphylococcus* sp. was estimated by Salem, et al. (2018) in different meat sold in Menofia markets, Egypt. According to Ragab, et al. (2016) staphylococcus sp. was (20%). Aerobic Plate Count play an important role in judging of the hygienic conditions under which it has been produced, handled and stored as well as unsuitable condition during storage (Shaltout, et. al., 2016 and Zelalem, et al., 2019; Sial, et al., 2021).

Statistical analysis for Different Types of Micro-organisms (CFU/g) in the minced chevon samples were; the total aerobic counts reported about; 90×10^3, 38×10^4, 2.1×10^5

± 2.1×10^4 as minimum, maximum, mean ±SE values respectively while, about; 16.2×10^4, 32.1×10^4, 2.1×10^5 ±1.2×10 CUF/g were detected as minimum, maximum, mean ±SE values respectively in case of total anaerobic count. *Enterobacteriacea* spp. showed about; 20×10^3, 6.0×10^5, 1.5×10^5 ±2.5×10^5 CUF/g as minimum, maximum, mean ±SE values respectively while, about; 10×10^3, 1.2×10^6, 1.6×10^5 ±3.7×10^5 CUF/g were detected as minimum, maximum, mean ±SE values respectively in case of Staphylococcus spp. counts. The Comparison with microbiological criteria for foodstuffs (GSO 1016/ 2015) viewed that; all tested chevon meat samples were within permissible limit and fit for human consumption. Nearly similar results found by Salem, et al. (2010) whome reocrded about 5.61×10^5 CFU/g of total aerobic counts from grand total of thirty random different meat samples were collected from different butcher shops in Kaluobyia governorate, Egypt.

Table 3. Statistical analysis for Different Types of Micro-organisms (CFU/g) in Chevon samples

Micro-organisms	Minimum	Maximum	Mean	SE±
Total Aerobic counts	90 X 10^3	38 X 10^4	2.1X 10^5	2.1 X 10^4
Total Anaerobic count	16 X 10^4	32 X 10^4	2.1X 10^5	1.2 X 10
Enterobacteriacae spp.	20 X 10^3	6.0 X 10^5	1.5 X 10^5	2.5 X 10^5
Total *Staphylococcus* spp. counts	10 X 10^3	1.2 X 10^6	1.6 X 10^5	3.7 X 10^5

Table 4. Comparison with microbiological criteria for foodstuffs (GSO 1016/ 2015)

(Bacteriological profile)	Within permissible		Over permissible	
	No.	Percent %	No.	Percent %
Aerobic bacteria	50	100	0	Zero %
Anaerobic bacteria	50	100	0	Zero %
Enterobacteriacae spp. counts	50	100	0	Zero %
Staphylococcus spp.	50	100	0	Zero %

Table 5. Different Incidence of Molecular Identification of Chevon samples

(Bacteriological profile)	Positive samples		Negative samples	
	No.	Percent %	No.	Percent %
Aeromonas enteropelogenes	1	11.1	8	88.8
Escherichia fergusonii	1	11.1	8	88.8
Proteus mirabilis	1	11.1	8	88.8
Pseudomonas aeruginosa	1	11.1	8	88.8
Staphylococcus lentus	1	11.1	8	88.8
Staphylococcus sciuri	2	22.2	7	77.7

Higher result reported by Erdem, et al. (2014) were total aerobic counts were (9×10^6 CFU/g in minced meat) and by Ragab, et al. (2016) who detected about (6.6×10^8 CFU/g) of total aerobic counts in minced meat). Salem, et al. (2018) recorded about (7.35×10^4 CFU/g) of *Enterobacteracea* sp.in meat. While Tefera, et al. (2019) also recorded about (4.27×10^3 CUF/g) of Enterobacteracea sp. in minced meat. While about 5.6×10^5 CUF/g of staphylococcus sp. was detected by Haileselassie, et. al., (2013). Gonulalan and Kose, (2003) recorded about (6.7×10^6 CUF/g) Staphylococcus spp. from Turkey minced meat samples. Lower result observed by Shaltout, et al. (2016) who obtained about 8.03×10^4 CUF/g of total aerobic count while, in case of *Enterobacteracea* sp. they recorded about (2.02×10^2 CUF/g) and isolated about (2.67×10^2 CFU/g) of staphylococcus in Egyptian meat. Hazaa, and El-Shater, (2019) who recoded about 1.21×10^3 staphylococcus sp. in meat. The results recorded by (Salem et al., 2018) in case of *Enterobacteracea* sp. was (7.35×10^4 CFU/g). while, in another study performed by Hassanien et al., (2018) about (4.27×10^3 CUF/g) of *Enterobacteracea* sp. was obtained.

Enterobacteriaceae group consider one of the most challenging bacterial contaminants to meat globally. *E. coli, Salmonella, klebsiella* species and Proteus species, are the most common food poisoning that associated with meat (Al-Mutairi, 2011). The presence of *Staphylococcus* aureus a indicated improper hygienic practice and posed a risk to consumer safety (Abdelrahman et al., 2016). Quick, sensitive, specific, and easy techniques for detection of the foodborne microorganisms needed for effective implementation of food safety. Polymerase chain reaction (PCR) became advent from 1980s and become one of the basic tool in molecular diagnostics and can be very efficiently used in rapid detection of food-borne pathogens (Armany et al., 2016). PCR identification mentioned in table (5) and figure (5) were as following; *Staphylococcus sciuri* were 2/9 (22.2), while; *Aeromonas* enteropelogenes, *Escherichia fergusonii, Proteus mirabilis, Pseudomonas aeruginosa* and *Staphylococcus lentus* were 1/9 (11.1) respectively.

Figure 1: phylogenetic molecular tree of *Aeromonas* spp. isolate and most relate genera

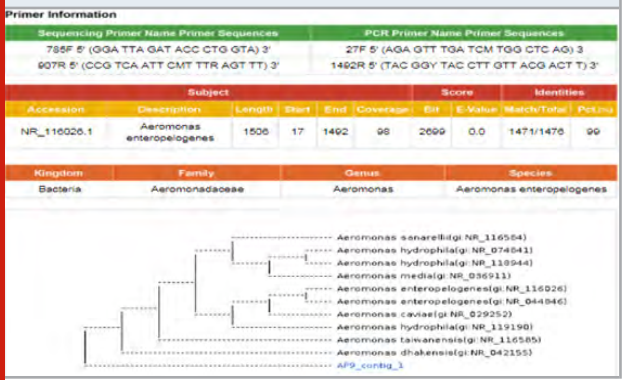
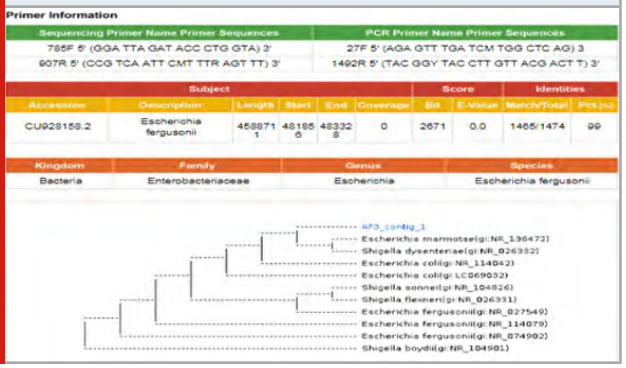


Figure 2: phylogenetic molecular tree of *Escherichia* spp. isolate and most relate genera



Aeromonas is one of facultative anaerobic gram-negative, non-spore forming, rod-shaped, bacterium morphologically resembles members of the family *Enterobacteriaceae.* The most important pathogens are; *A. enteropelogenes, A. caviae, A. hydrophila,* and *A. veronii* biovarsobria. The organisms that are widely distributed in mainly among aquatic habitats. *A. enteropelogenes* is virulent pathogenic bacterium but its pathogenicity is still under investigation. This is the first report isolated of *Aeromonas enteropelogenes* from chevon meat (Aberoum & Jooyandeh 2010 and Silva, et al., 2019; Sial, et al., 2021).

Aeromonas spp. are pathogenic for man. Gastroenteritis, septicemia, muscle infections, soft-tissue and skin diseases are one of the most common illnesses caused by pathogenic *Aeromonas* spp. (Igbinosa et al. 2012). *Aeromonas* species has virulence activity on the cell structural including lipopolysaccharides (LPS), haemolysis, outer membrane proteins (OMPs), pili, flagella, toxins, that have a vital pathogenic role to the host (Matys, et al, 2020). *Aeromonas enteropelogenes* considered one of sever pathogenic bacterium (Ramesh and Souissi, 2018). The pathogenicity depends on the microbial hemolytic toxins which lysis of neutrophils and erythrocytes. *A. enteropelogenes* had β_ haemolytic action (Mogrovejo et al., 2020). The microorganisms mainly produce haemolysin which help them to adhere in the mucosal gut epithelial cells before starts its multiplication (Gudeta et al., 2016; Matys, et al, 2020; Sial, et al., 2021).

Figure 3: phylogenetic molecular tree of Proteus spp. isolate and most relate genera

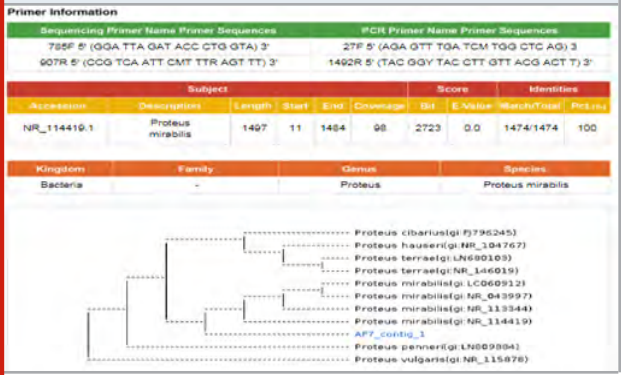


Figure 4: phylogenetic molecular tree of *Pseudomonas* spp. isolate and most relate genera

Escherichia fergusonii is a rod-shaped Gram-negative species, usually motile, and catalase positive, ferment D-glucose of bacterium, reduce nitrate to nitrite, they are positive for methyl red, acetate utilization, indole production & motility, and negative for the H2S production on triple sugar-iron agar, Voges-Proskauer reaction, urea hydrolysis, phenylalanine deaminase. This isolates highly related to; *Escherichia coli,* while *E. fergusonii* usually isolated from human blood samples. This microorganism

considering as opportunistic pathogens of humans and were reported from human clinical specimens of an outbreak of food poisoning (Mogrovejo-Arias et al., 2020). The study concluded that four types of *Escherichia* species were isolated from raw meat in Khartoum State, Sudan: *E. coli, E. vulneris, E. albertii* and *E. fergusonii*. were existed in meat samples. The presence of foodborne microorganisms including for example, *E. coli* samples reflects the role of meat as major reservoir for causative pathogenic agents (Ahmed and Al Sanosi, 2018). Similar results recorded by Mahapatra, and Mahapatra, (2005) who recognized that *Escherchia fergusonii* as a pathogen member of family Enterobacteriaceae. *Escherichia fergusonii* may be found in humans or animals as pathogens or commensals. On the other hand, *E. coli* considering one of the most public foodborne illnesses which has significant public health concern (Luna-Gierke et al., 2014).

Proteus mirabilis, is one of the Gram-negative *Enterobacteriaceae* family; facultative anaerobic, bacilli rod-shaped bacterium and resides in normal flora of man intestine. *Proteus bacilli* are widely distributed in nature as saprophytes, about one from each four persons of the population suffering from *P. mirabilis* in their fecal matters in addition to animal matter, sewage, manure soil, the mammalian intestine and animal feces. This opportunistic nosocomial pathogen may cause urinary septic infections. *Proteus mirabilis* causes 90% of humans Proteus infections. Pathogenicity of *P. mirabilis* pathogenesis by two steps; firstly, by colonization of the microorganisms in the urinary tract followed by complete evade of the body defense (Schaffer & Pearson, 2017 and Armbruster et al., 2018; Mogrovejo-Arias et al., 2020; Milton et al., 2021).

P. mirabilis is one of the seldom food borne microorganisms which transmitted from seafood, vegetables, and meat (Wang et al., 2019). *P. mirabilis* reported one of the most food poisoning microorganism in China. The clinical symptoms of *P. mirabilis* infection including; fever, dizziness, abdominal pain, nausea, diarrhea and vomiting after 0.67–9 h incubation period (Huo et al., 2014). About 3.61% *P. mirabilis* food poisoning incidents recorded in Datong from 2016 to 2017 (Shanxi Province, China) (Gong et al., 2019). *P. mirabilis* play an important role in food spoilage and considering as enteropathogens (Kushwaha et al., 2014). *Pseudomonadaceae* family containing 191 species, *Pseudomonas* is gram-negative, encapsulated, rod-shaped bacterium. *P. aeruginosa* is considered all over the world as one of the most dangerous organisms causing different diseases and capable of secreting many extra cellular products which play a role in the virulence of pathogenic strains of *P. aeruginosa* (Pang et al., 2019; Milton et al., 2021).

P. aeruginosa may infect animal, plant, and commonly be opportunistic to the human as it mainly affecting the immunocompromised persons through cystic fibrosis or through burned tissues and traumatic tissues (Bassetti, et al., 2018). *P. aeruginosa* has antibiotic resistance, and considered nosocomial infection including various sepsis syndromes and ventilator-associated pneumonia (Ruffin, M. and Brochiero, 2019). *P. aeruginosa* infections hardly treated due to its natural antibiotics' resistance. *P. aeruginosa* is present in the intestinal tract of both man and animals and its presence in food could be taken as an index of fecal contamination (Mostafa et al., 2018). These pathogenic strains play an important role in bloodstream infection and respiratory tract infections, mastitis, endometritis, chronic pulmonary disease, urogenital tract infection, cystic fibrosis and sever form of gastroenteritis among man, animals and sometimes may cause fatal infections specially with the immunodeficient persons (Rocha, et al., 2019).

Figure 5: phylogenetic molecular tree of *Staphylococcus lentus* isolate and most relate genera

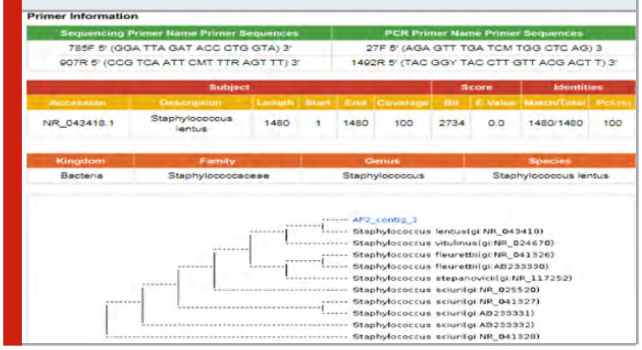
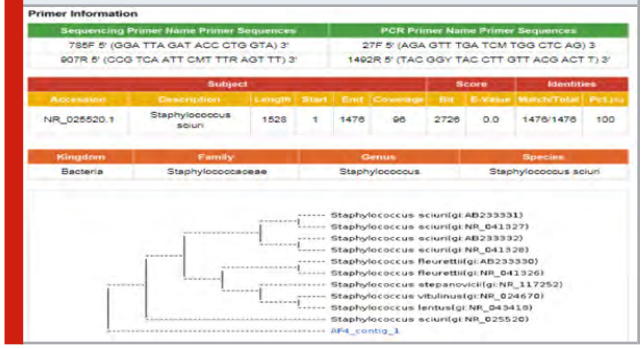


Figure 6: phylogenetic molecular tree of *Staphylococcus sciuri* isolate and most relate genera



Pseudomonas aeruginosa was detected in many food items. Although it did not identify as one of food poisoning microorganisms, but it mainly spoil food. The infection mainly transmitted through food sold in open air which exposed to dust and flies (Alayande, et al., 2018). *Staphylococcus aureus* cause food intoxication (Chikwanha, et al., 2018). *Staphylococcus* lentus is oxidase-positive, coagulase-negative, gram positive member of *Staphylococcus* genus. These microorganisms are related originally to *Staphylococcus sciuri* derived from subspecies "lentus" (Shaker et al., 2018). *Staphylococcus* lentus is colonize on the skin of human and animals and reported as commensal bacterium. It has commonly isolated from food-producing animals, including dairy animals, poultry, and their food products. Animals man workers recorded as carriers of *S. lentus* (Schwendener and Perreten, 2012). *Staphylococcus lentus* forming biofilm that resist antibiotics which increase mortality rate as a result of the difficulty to controlling the infections (Al-Azawi et al., 2018; Mogrovejo-Arias et al., 2020; Milton et al., 2021).

Consumption of foods contaminated *Staphylococcus* lentus have been described as able to produce enterotoxins (Zabrodskii, 2020; Milton et al., 2021). *Staphylococcus sciuri* is kwon as animal-associated microorganisms in addition to its presence on mucosal and skin surfaces of farm, wild animals, and pets and in animal origin food items, its clinical importance for man is increasing. *Staphylococcus sciuri* is novobiocin-resistant, oxidase-positive, coagulase-negative staphylococcal species. It is widely distributed in environmental as reservoirs including water, soil, sand, and marsh grass (Lu, et al., 2020). S. sciuri is widely found in environment and from several animals and animals' products (Heilmann et al., 2019) as well as from human, this microorganism considers as animals' pathogens (Koli et al., 2018). Their signs containing; septic shock, endocarditis, pelvic inflammation, peritonitis, endophthalmitis, and wound infections and urinary tract infection (Kentzi et al., 2016). *S. sciuri* may causing ruminants mastitis especially in goats and cow. There was a big shortage on information about S. sciuri pathogenicity in animals (Romanò et al., 2020; Milton et al., 2021).

CONCLUSION

Chevon can be considered best replacement of beef meat due to its lower unhygienic total fat; "cholesterol and saturated fat" and its lower calories content in comparison to beef content, which protect the consumers health. All tested chevon meat samples were within permissible limit and fit for human consumption. Molecular Identification of isolated microorganism declared the following: *Staphylococcus sciuri, Aeromonas enteropelogenes, Escherichia fergusonii, Proteus mirabilis, Pseudomonas aeruginosa* and *Staphylococcus lentus*. This is the first report isolated of *Aeromonas enteropelogenes* from chevon meat. All isolated microorganisms had public health concern as food poisoning microorganisms. Further investigation needed to study the chevon meat and its microbial quality.

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Biomedical Communication

Biofilm-Mediated Drug Resistance in *Candida* Species Isolated from Vulvovaginal Infections: A Descriptive Cross-Sectional Study

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ABSTRACT

Candidal vulvovaginitis is accountable for a third of all cases of vulvovaginitis in reproductive-aged women, and 70% of women report having or had candidal vulvovaginitis at certain point in their lifespans. Vulvovaginal Candidiasis (VVC) is a recurrent, multifaceted and unwieldy illness that can cause corporeal and mental distress to the individual. *Candida albicans* was stated as the greatest common cause of VVC yet it appears that we are newly facing changes in the configuration of Candida species in VVC. In the present study we measured diverse species of *Candida* isolated from patients with VVC. This research was a descriptive analytical cross-sectional study. *Candida* Sps were isolated from females aged 20 - 60 years, who existing erythema and itching of vulva, vagina, or both and unpleasant vaginal exoneration. Biofilm formation of the isolates were evaluated using crystal violet staining and their drug resistance pattern were evaluated using standard antifungal antibiotic discs. Biofilm production could act as one of the factors in reducing the penetrability of the antifungal agents and also increasing the virulence nature of invasive candidiasis. Biofilm development test was done on all the 85 samples. All sample was prepared in triplicate and the average was determined. Light microscopic biofilm imaging showed the biofilm formation of the budding yeast cells from tiny micro-colonies (2-4 h). After four hours, the budding cells started to divide, and formed pseudo hyphae and ultimately true hyphae. We report that this extremely contagious yeast has the capability to form antifungal resistant biofilms sensitive to the antifungal agent *in vitro*.

KEY WORDS: BIOFILMS, CANDIDA ALBICANS, VULVOVAGINAL CANDIDIASIS.

INTRODUCTION

In the last decade, fungal infections were presented as a serious problem in hospitals, especially in ICUs that are epicentres in *Candidemia* and invasive *Candida infections* (ICI). *Candida* infections are the leading opportunistic fungal pathogen, with *C. albicans* responsible for most of this infection significantly increasing world-wide. These diseases are highly morbid and deadly, which also affect healthcare costs. (i.e., increased hospital length of stay, high costs for antifungal therapy). They are generally associated with many therapies, such as parenteral feeding, pre-exposure to antibacterial therapy, chemotherapy, and dialysis, and intravascular equipment (mainly CVC and urinary catheters) (Pristov and Ghannoum 2019; Fakhim et al. 2020).

A septic epidemiology study found that, between (1979) and (2000), the rate of fungal sepsis was triple. In 80 percent of cases, Candida is the liable agent in about 8% of all nosocomial infections. Different *Candida* species may cause these challenging infections. *Candida albicans*, preceded by *Candida glabrata*, is the most common pathogen responsible for *Candida*'s infections. In urinary tract infections, *Candida* tropicalis is especially relevant, while *Candida parapsilosis* is also present in the skin of healthy hosts, and is the causative substance of catheter infections. A number of factors such as phenotype flipping, dimorphic transitions between the hyphae and yeast, and the secretion of proteases and phospholipases may be caused by virulence of *C. albicans* (Blostein et al. 2017; Fakhim et al. 2020).

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C. albicans are capable of dimorphating between two principal types, a circular budding yeast and a long, parallel, real hypha (with an intermediary, pseudohyphae form consisting of stretched ellipsoid cells), is the source of the germ tube test used in most hospitals to recognise *C. albicans* from other *Candida* species, *C. dubliniensis* being the exception as it can form true hyphae. Another key feature of *C. albicans* pathogenesis is biofilm production (Shukla et al. 2019; Willems et al. 2020). Biofilm is a group of cells that are attached to a surface, are surrounded by an extracellular matrix and have characteristics which differ from its free-floating counterparts. A biofilm is an extracellular matrix (Kalaiarasan et al. 2017; Kannan et al. 2020).

This phenomenon lets *Candida* to attach to mucosal cells and to plastic surfaces of medical devices such as catheters and dentures leading to device associated infections and eventually spreading nosocomial infections (Rosati et al. 2020). The cells forming biofilms are distinct from that of floating cells, since they are embedded in a 3D matrix and will proliferate inside the host's immune system in stable individuals that are more resistant to antimicrobial medicines. The connection between drug resistance and *C. albicans* virulence is, however, still little understood (Rosati et al. 2020). This study obtained 85 isolates in two major hospitals between March and December 2019 in patients with vulvovaginal candidiasis. API, germ tunnel, CHROM agar and ITS sequencing were used to identify isolates. Furthermore, antifungal susceptibility testing against four antifungal drugs was performed, and the isolates were additionally tested for biofilm formation to determine the adherence mediated virulence of the isolates.

MATERIAL AND METHODS

This research was a descriptive analytical cross-sectional study. The members were married females aged 20 - 60 years, who existing erythema and itching of vulva, vagina, or both and unpleasant vaginal exoneration. The patients filled out a consent form to participate in the research. Concomitant with each obtained specimen, a questionnaire was completed for each patient enquiring about their age, marital status, and duration of symptoms, comorbidities, signs and symptoms of current condition, methods of pregnancy prevention, prior parturitions, and history of antibiotic consumption (Hosseini et al. 2020).

Sterilised speculum moistened with sterile water was positioned in the vagina (in case of being married), and posterior fornix where excretions are hoarded was experimented by introducing two swabs concomitantly. The swabs were located in a tube comprising 1 ml of sterile PBS (Phosphate buffer saline) and then the samples were transported to laboratory. Then the swabs were inoculated on HI Chrome Candida agar plates, incubated at 35°C for 4 days, aerobically. All dishes were assessed for the fungal growth and colony shade on a daily basis. Direct microscopy slides were prepared from each colony for yeast confirmation. All yeast isolates were cautiously sub cultured on Sabraud dextrose agar (SDA, Merck, Germany) plates and 'incubated at room temperature for future mycological analyses (Hefzy et al. 2021).

Biofilm development test was done on all the 85 samples. All sample was prepared in triplicate and the average was determined. Three to four colonies were suspended in YNB (Yeast Nitrogen Base, Fluka, Switzerland) and incubated overnight with moderate pulsating. The optical density of each of the suspensions was adjusted to 0.1. 0.5 mL of the suspension was added to a flat-bottomed 96-well microtiter plates at 4°C and placed in a shaker at 37°C for 3 h to allow for preliminary grip. Plates were then eroded with 0.5 mL PBS and another 0.5 mL of the cell suspension was added. Following 48 h incubation at 37°C, cells were washed with 1 mL PBS and fixed using 0.2 mL of 99 % methanol for 15 min. Plates were then allowed to air-dry for 20 min. Staining was performed by adding 0.2 % crystal violet, removed after 20 min, and followed by 0.75 mL of 33% acetic acid. The absorbance was immediately measured using a spectrometer (Thermo Spectronic) at 590 nm. *C. albicans* strain SC5314 was used as a reference strain (Yassin et al. 2021).

Penetration of antifungal agents over biofilms was measured by an alteration of the strainer disk technique defined before for bacterial biofilms. After biofilm development on film sieves, smaller polycarbonate membrane filters (diameter, 13 mm; pore size, 0.2 µm; Whatman) existed sterilized by disclosure to UV radiation for 15 min on both sides and were then cautiously located on top of the 48-h-old biofilms. Paper concentration disks (diameter, 6 mm; Becton Dickinson) were also decontaminated by introduction to UV radiation for 15 min per side and then moisturised with growth medium before settlement on top of the membranes. Often a slightly higher or lower medium volume was essential for saturating the disks because of a difference in the disk thickness. Biofilms crammed amongst the membranes and moisturized disks were transported to antifungal agent-containing agar medium (Said et al. , 2020). To regulate statistical significance of the biofilm experiment, both a -test and a post hoc ANOVA test were conceded. For the ANOVA test isolates were assembled into 3 clusters: those with biofilm competences below the reference strain, those comparable to the reference strain, and those overhead the reference strain. Statistical significance with the reference strain group was observed for both groups containing isolates above and below the reference strain (data not shown). p value below 0.05 was deemed significant.

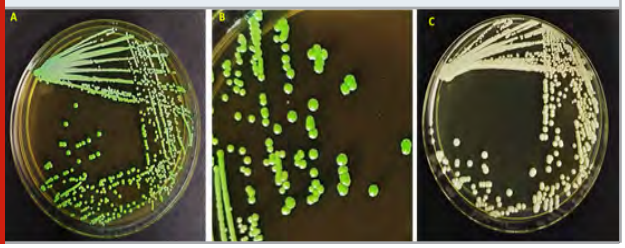
RESULTS AND DISCUSSION

Sample collection and Laboratory diagnosis of *Candida* Sps: Candidiasis is among the most common fungal diseases that can contribute to systemic and life-threatening diseases, such as vaginitis. *C. albicans* is an opportunistic pathogen that is now one of the main causes. Candida is one of the most common presentations of genital involvement in women (Tits et al. 2020).

Thirty three out of 97 patients were identified with Candida infection, among whom 32 isolates of Candida were

obtained. The mean patients' age was 36.5 years. The most commonly described symptoms were vaginal discharge (99%), vulvovaginal itching (52.3%), vulvovaginal burning sensation (32.7%) and dysuria (3.2 %). The risk factors were including previous VVC infections (n = 14), consumption of antibiotics (n = 7) and antifungal agents (n = 3), pregnancy (n = 9), diabetes mellitus (n = 13), RVVC (n = 4), and intrauterine contraceptive device (IUCD) usage (n = 2). No patients were positive for the human immunodeficiency virus (HIV). Of these isolates, 28 were germ tube positive and presented a green colony colour on the chromogenic medium that were classified as *C. albicans* complex species. Vulvovaginal candidiasis consequences to irregular progress of *Candida* in the genital tract mucosa and has augmented intensely in the latest existences (Tits et al. 2020).

Figure 1: Morphology of *Candida albicans* strains (A, B) in HiChrome Candida agar, (C) In SDA agar medium

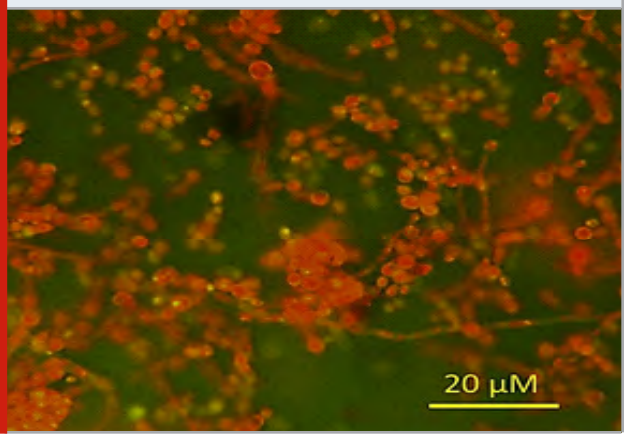


This contamination is a universal health problem and disturbs a lot of women, yearly. *Candida albicans* was stated as the most mutual agent of VVC yet it appears that we are recently encountering changes in the pattern of Candida species in VVC (Ramage et al. 2006; Tits et al. 2020). The microscopic study of the genital specimens revealed the presence of yeasts and a small number of lactobacilli. A culture of the vaginal specimen on Sabraud Dextrose agar (Difco, Becton, Dickinson and Co., Sparks, MD) yielded a single type of yeast colony (Figure 1). The latter was morphologically compatible with Candida spp. showing a light creamy dull colour on SDA. The yeast isolate grew better at 30°C than at 37°C and was unable to grow at 40°C. In some cases, the organism developed slowly settled turquoise blue colonies, resembling those of *Candida dubliniensis* (Paiva et al. 2020).

Microscopically, the fungal isolate showed ovoid to elongate cells, singly or in pairs and the development of pseudohyphae (Figure 2). It formed germ tubes in horse serum after 3 h of incubation at 37°C, but it did not produce chlamydospores on Corn-meal agar after 10 days of incubation at 30°C.

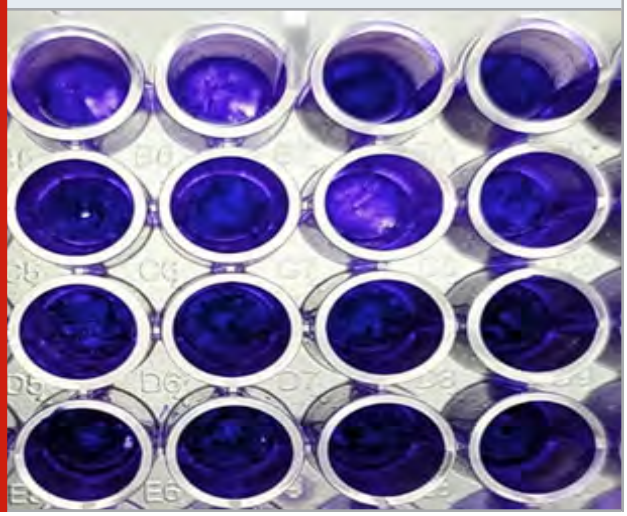
***In vitro* biofilm formation by *C. albicans*:** The fact that this organism has a high tendency to bind to a rank of surfaces such as tissues living substratum and biomaterials is partly linked to these results. Subsequently, they form spatially ordered populations of phenotypically diverse sessile cells that are significantly different from their planktonic components (Bandara et al. 2020). Cells like this are known to be micro-biofilm communities and are normally known to have anti-microbial and immune resistant (Kuhn et al. 2002). Maximum illnesses instigated by *C. albicans* are related with the development of biofilms on abiotic or host surfaces (Bandara et al. 2020). More considerably, *C. albicans* is proficient at stick to catheters and numerous medical implants, and is presently classified by the Centres for Disease Control and Prevention, United States, as the third most commonly isolated bloodstream pathogen in hospitalized patients with a mortality rate of up to 50% (NikkyGoel et al. 2021).

Figure 2: Microscopic examination of *C. albicans* after acridine orange staining.



Light microscopic biofilm formation parallel analyses showed how the biofilm existed most of the budding yeast cells from tiny micro-colonies (2-4 h). After four hours, the budding cells started to divide, and formed pseudo hyphae and ultimately true hyphae. After 8 hours hyphae from the surrounding micro-colonies, made up mainly of formation cells of yeast, fused in an inventive network of filamentous spatially scattered forms which formed a cohesive monolayer of woven frameworks. As biofilm maturing happened (24 and 48 h growth), the difficulty of the biofilm augmented into a multi-layered biofilm matrix with all fungal morphologies being present in the ending biofilm construction (Kannan et al. 2021).

Figure 3: Biofilm formation by the strains isolated



The kinetics of adherence and subsequent biofilm formation by *C. albicans* on the surface of polystyrene wells over 48 h, as determined by the crystal violet assay, are showed in Figure 3. The production of the soluble colour crystal violet from sessile cells, a direct reflection of cellular metabolic activity, increased over time with the increased sessile cellular density. The biofilms were highly metabolically active in the first 8 h, but as the biofilm matured and the complexity increased (24 to 48 h) the metabolic activity reached a plateau, but remained high probably reflecting the increased number of cells that constituted the mature biofilm. Experiments were performed in sets of eight replicates on three separate occasions, with similar results obtained in all experiment (Perira et al. 2020).

Susceptibility testing of *C. albicans* biofilms against clinically used antifungal agents: There exist a problem with antimycotic treatment, which may be due to several factors, leading to clinical resistance (Kannan et al. 2020). The *in vitro* activity of clinically used fluconazole and amphotericin B against pre-formed *C. albicans* biofilms was assessed using the modified biofilm penetration assay. Experiments revealed the increased resistance of sessile *C. albicans* cells compared to their planktonic counterparts. The antifungal agents tested showed less activity against 48 h biofilms compared to planktonic MIC's, generally much greater than the concentration of antifungal required to inhibit planktonic cells. Data revealed that *C. albicans* biofilms were intrinsically resistant to fluconazole (MICs >1700 µg/ml), and the activity of this azole derivative against biofilms was reduced up to 325 times compared with its activity against planktonic cultures. Previous reports were stated the high resistance rate for *C. albicans* and *C. tropicalis* from animal origin and the fact that the antifungal against *C. parapsilosis* sensu lato from animals (Paiva et al. 2020).

Amphotericin B demonstrated certain activity against *C. albicans* biofilms, as indicated by 18 µg/ml, but this concentration is generally regarded as resistance already, due to the high toxicity displayed by this drug. Importantly, complete killing of cells within the biofilms was never achieved, as reflected by residual metabolic activity of biofilms at concentrations up to 25µg/ml. In few latest published reports, we observed this lack of inhibitory properties of and fluconazole resistance when delivered to *C. albicans* biofilms rather than the free-floating cells (Tummanapalli et al. 2021).

Few literatures indicate fungal biofilm science and antimicrobial resistance. An understanding of the complexities and phenotypic properties of *C. albicans* biofilm will enable one to improve antifungal agents and treatment methods to eradicate and avoid Candida biofilm, which reduce the occurrence of *C. albicans* infection. In line with this outcomes, recent studies have also shown an increase in *C. albicans* antifungal resistance in *C. albicans*, *P. aeruginosa* mixed species biofilms via upregulated *C. albicans* proteins associated with drug resistance and virulence (Tita et al. 2020). Studies on increasing resistance of vaginal candida isolates to mainstay antibiotics are a worry, and there is sign that for vaginal disease this resistance translates into worse clinical outcomes (Pereira et al. 2020). Novel antibiotics are being established, but not by large pharmaceutical companies and mostly in university research laboratories and smaller biotech companies (Castelo et al. 2020).

CONCLUSION

The findings of the present study show the occurrence of biofilms may trait to the development of drug resistance in the clinical isolates. Henceforth, testing for the analysis of biofilm development is suggested in the routine laboratory diagnostic practices. In addition, the contour of treatment in recurring and drug resistant VVC cases should aim both treating the planktonic cells as well as extinguishing the biofilms formation of the *Candida* sp. Advanced study of biofilm mediated molecular genetic markers and procurement of evidence on the association of the genetic and phenotypic properties of *Candida* spp., as well as features distressing gene expression, will make it possible to improve diagnostics for the timely recognition of resistant strains and the rational selection of therapy.

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

Ethical Clearance Statement: The Current Research Work Was Ethically Approved by the Institutional Review Board (IRB) of Madurai Medical College, Madurai Tamil Nadu 625020, India.

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Nutritional Communication

Antioxidant and Antimicrobial Potential of *Moringa oleifera* Extract Against Food Pathogens

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ABSTRACT

Moringa oleifera, commonly known as Moringa, is an extraordinarily nutritious vegetable tree with a variety of potentially medicinal benefits, also referred to as the Miracle tree due to its multiple uses. The leaves of *Moringa oleifera* are high in phenolic compounds, which act as antimicrobials and antioxidants. Antioxidant phenolic compounds may stabilize free radicals by compensating for their electron deficiency. Consumption of polyphenol-rich plants as a dietary component provides protection against such cell damage. The present study explores the antimicrobial, antioxidant ability, total phenolic content (TP) and total flavonoid content (TF) of different extracts prepared from leaves of *Moringa oleifera* grown locally Saudi Arabia. Higher TP, TF and antioxidant activity have been demonstrated by methanol extracts followed by di ethyl ether solvents. The present study indicates that all extracts may, to some degree; act as radical scavengers due to the existence of polyphenolic compounds. Additionally, Methanol extracts showed significant inhibitory activity against food poisoning bacteria *Shigella sonnei* 19 ± 1.73, *Klebsiella pneumonia*17.33 ± 0.57 and *Pseudomonas aeruginosa* 17.00 ±6.93. The di ethyl ether extracts showed lower activity. Data provided in this study show that *Moringa oleifera* leaves have great potential for the development of food preservatives and antibiotic drugs. In conclusion the Methanolic solvent could be reasonable choice for antioxidant compounds extraction and the potential uses of M. oleifera as alternative natural preservatives in food products.

KEY WORDS: ANTIOXIDANT, FREE RADICALS, FOODBORNE DISEASE, *MORINGA OLEIFERA*, PATHOGENIC MICROORGANISM.

INTRODUCTION

Foodborne diseases considered one of the worldwide health concern especially in developing countries (Sapkota et al., 2012; Kirk et al., 2017), which may occur at any point during the preparation, distribution, and/or consumption of food. Gram-negative and Gram-positive bacteria which have been identified as the causal agents of food spoilage and food borne diseases; *Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, Bacillus cereus* and *Staphylococcus aureus* (Braga et al., 2005; Pandey and Singh, 2011). While uses of chemical preservatives were thought to be effective against food poisoning outbreaks, their accumulation in the feed and food chain resulted in microbial resistance, which had negative implications for human life (Akinyemi et al., 2006, Bialonska et al., 2010). As a result, eco-friendly techniques are now needed to not only minimize pathogenic bacteria growth but also to reduce the use of chemical preservatives and to extend the shelf life of food (Clarke et al., 2017). Among these contexts is the use of plant extracts as antimicrobials for food safety, several researchers have demonstrated the antimicrobial activity of plant extracts against food poisoning bacteria as natural sources of antimicrobials and are considered healthy in nutrition and easily consumable (Akinpelu et al., 2015 and Suppakul et al., 2016; Saleem et al., 2020; Ali et al., 2021).

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One alternative treatment for bacteria-infected infections is by using natural ingredients, such as *Moringa oleifera* L. Plant. This plant is called the most important multipurpose and miracle tree in the world, since all parts of the plant are useful for fruit, medicine, cosmetics or purified water (Fahey, 2005). *Moringa oleifera* (Moringaceae), also known as the "tree of life," has made a breakthrough in this field. *M. oleifera*, which is native to India and Africa, appears promising due to its safety for animal and human consumption (Makkar, and Becker, 1996). *Moringa oleifera* L. leaf has many active components such as triterpenoids, flavonoids, tannins saponins and alkaloids so pharmacologically has benefits as antimicrobial, antifungal, antihypertensive, antihyper-glycemic, antitumor, anticancer, anti-inflammatory (Mahmood et al., 2010; Sharma et al., 2011). Antimicrobial activity of *Moringa oleifera* against human pathogens was proved by several investigators (Singh and Tafida, 2014; Morgan et al., 2019; Das et al., 2020; Naseer et al., 2021).

However, the potential of *M. oleifera* as alternative natural preservatives in food products has not been thoroughly studied. Therefore, the goal of the present study was to evaluate the antioxidant and antibacterial activity of *Moringa oleifera* leaves extract *in vitro* against food poisoning diseases caused by *E. coli, S. aureus, P. aeruginosa, S. typhi* and *B. cereus*.

MATERIAL AND METHODS

Plants extraction preparation: Fresh leaves of *Moringa oleifera* were washed, then air dried, powdered. The *Moringa* leaves powder (5 g) are successively extracted using with methanol and di ethyl ether solvents. the dry extracts of methanol and di ethyl ether completely re-dissolved in methanol.

Total phenolic content: using the spectrophotometric method; a solution was made with 0.5ml of the sample and distilled water, raising the total volume of the sample to 3 ml, then 0.5 ml of Folin-phenol Ciocalteu's reagent was added, followed by 2 ml of 2 % Na_2CO_3 solution after 5 minutes, thoroughly mixed. The total phenolic (TP) was calculated using the extrapolation of the calibration curve when the mixture's absorbance reached 650 nm after 60 minutes in the dark at 30° C. The gallic acid solution was used to establish the curve. The TP was determined as milligrams of gallic acid equivalents (GAE) per gram of dried sample after the phenolic compounds were estimated in triplicate.

Total flavonoids content (Chang, et al., (2002): The total solution was increased to 1 ml by adding methanol to a 0.5 ml sample. The resulting mixture was left unchanged for 5 minutes after adding 4 mL of distilled water and 0.3 mL of a 5% NaNO2 solution. After adding 0.3 ml of ALCL3 solution 10 % the solution could sit for another 6 minutes before being increased to a volume of 10 ml by adding two ml of NaOH solution (1 M) and distilled water. The concentrations of total flavonoid were determined once the absorption read 510 nm after being thoroughly shaken and left for 15 minutes. For the analysis, Quercetin equivalents (QUE mg/g of dry weight) were used.

Antioxidant's assay: The radical scavenging activity of methanolic extracts was calculated quantitatively. In a nutshell, using 1,1-diphenyl-2-picryl hydrazyl (DPPH) a 0.1 mM DPPH solution was prepared using methanol. At various concentration (100 - 300 g/ml), 1 ml of DPPH stock solution was combined with 3 ml of each methanolic and di ethyl ether extract. As a positive regulation, butyl-4-hydroxyanisole (BHA) was used. After 30 minutes of incubation, discoloration was estimated at 517 nm. At the very least, three measurements were taken. The following equation was used to measure the capacity to scavenge the DPPH• radical: DPPH• scavenging impact (%) = Abs Control/Abs Control 100.

Antimicrobial's activity of the *Moringa* extracts: Bacterial strains and growth conditions the reference strains used in this analysis were bacterial isolates selected for their historical relevance to pathological effects on humans and food product degradation. Among the eight food-borne pathogenic bacteria, obtained from the culture collection of Microbiology Dept. King Abdulaziz University, Jeddah, K.S.A, two were gram-positive bacteria, namely, Bacillus cereus DSM 4312, *Staphylococcus aureus* (ATCC 25923) and six gram-negative; *Enterococcus faecalis* ATCC (29212), *Escherichia coli* O157:H7 (ATCC 43889), *Klebsiella pneumonia* ATCC (700603), *Pseudomonas aeruginosa* ATCC (27853), *Salmonella typhimurium* (ATCC 14028), *Shigella sonnei*, ATCC (25931). 10 mL nutrient agar media was sterilized as part of the preparation. The test organisms were added to this sterilized mixture and shaken vigorously before being transferred to a sterile petri dish via sterile loop and held aseptic. The test species were kept in a nutrient broth after an overnight incubation time at 37°C. They were then standardized at 560 nm to achieve a concentration of 106 colony-forming units per milliliter (CFU/mL).

Agar well diffusion assay: The antimicrobial activity of *Moringa oleifera* was investigated using the agar-well diffusion method. Fresh overnight cultures of bacteria (100L) were obtained for this purpose. These cultures were uniformly spread on a sterile surface using cotton swabs, and 50 l methanolic plant extracts were mixed in the agar-wells (7 mm). Aseptic conditions were maintained for the latter part of the process. Additionally, an equal volume of methanol (50 l) was added to one of the wells to serve as a negative control. At 37 °C, all the plates were incubated for 24 hours. The average zone of inhibition was calculated using CLSI guidelines, and the experiment was repeated three times.

Minimum bactericidal and minimal inhibitory concentrations (MICs): To evaluate the minimum bactericidal concentration and minimal inhibitory concentrations (MICs) The standard micro-dilution method in 96-well microtiter plates given by the Clinical Laboratory Standards Institute (CLSI) were carried out. The *Moringa oleifera* active metabolites were serial diluted two

folds with Muller-Hinton broth medium then the selected pathogenic bacteria inoculated at 0.5 on the MacFarland scale then, final density be 6 x 106 CFU/well. The plates were incubated for 24hr/35°C and the bacterial growth was measured using a Bio-Rad Microplate Reader at 600 nm. The lowest concentration that inhibiting the bacterial growth was determined as the MIC values. All experiments were carried in duplicate. The minimal bactericidal concentration (MBC) was performed after the above experiment, after incubation a 5 µL from each well without growth were be inoculated onto Muller-Hinton agar plates. The inoculated plates were incubated overnight at 30°C.

Cytotoxicity Assay: According to the manufacturer's instructions, the cytotoxic effects of methanolic and Di ethyl ether extract of *Moringa oleifera* leaves Annexin FITC are used to assess the apoptotic activity of the ovarian cancer cell line (SKOV-3) in relation to tested compounds (BD Biosciences, USA). Cultivated at a density of 3 × 105 cells/well, both treated and untreated SKOV-3 cells were used for the induction of apoptosis for 48 hours. The FACS flow cytometer (BD FACSAria™ II - BD Biosciences) and BD FACSDiva™ Software (BD Biosciences, USA) were used for cell apoptosis analysis.

Statistical Analysis: Variations between the values of selected plant extract samples and controls was performed using a one-way analysis of variance (ANOVA). Statistical significance was described as a P value of less than 0.05.

RESULTS AND DISCUSSION

Total phenolic (TP) and total flavonoid (TF) contents: as shown in Table 1, extraction with methanol was found to provide the highest values of total phenolic and total flavonoid contents (14.70 ±0.28 mg of GAE per g of dried sample and 37.88±0.18 mg of QUE per g of dried sample respectively) in the leaves of *Moringa oleifera* as compared with extraction by Di ethyl ether (8.95 ±0.59 mg of GAE per g of dried sample and 29.30 ± 1.23mg of QUE per g of dried sample respectively).

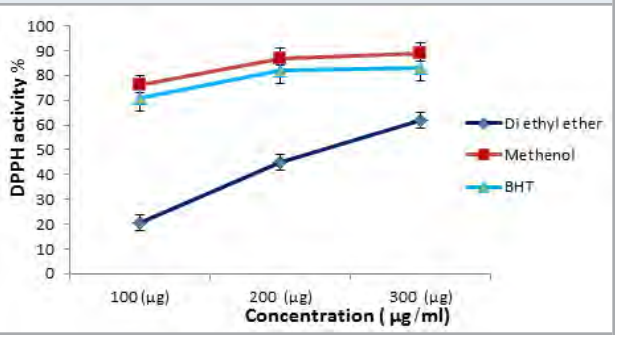
Table 1. Total phenolic and flavonoid contents of different extracts of Moringa oleifera leaves

Solvent extracts	Methanol	Di ethyl ether
Total phenolic (mg GAE/g d.w.)	14.70 ±0.28	8.95 ±0.59
Total flavonoid (mg QUE/g d.w.)	37.88 ±0.18	29.30 ± 1.23
Ratio (TP)/(TF)	0.39	0.31

Total phenolic and total flavonoid contents are expressed as mean ± S.D (n = 3).

Antioxidant activities: Antioxidants react with DPPH•, reducing a number of DPPH• molecules equal to the number of their available hydroxyl groups. The methanolic extract exhibited the most potent DPPH• scavenging activity (76.9±0.41%) at concentration 100 µg/ml as compared to the Di ethyl ether extract which exhibited (20.7±0.12%) activity at the same concentration. The same pattern of DPPH• scavenging activity was found in the methanolic and Di ethyl ether extracts at concentration 200 µg/ml and 300 µg/ml. The values were in the ascending order methanolic < BHT extract < Di ethyl ether extract These results indicated that methanolic extract exhibited the highest DPPH radical scavenging activity compared to BHT and the Di ethyl ether extract.

Figure 1: DPPH• scavenging activity (%) of methanolic and di ethyl ether crude extracts of *Moringa oleifera*. Vertical bars on the columns represent mean ± SD (n = 3).



Antimicrobial activity: of the Methanol extracted *Moringa* leaves and the Di ethyl ether extracted *Moringa* leaves residue was determined *in vitro*, using disc diffusion and MIC method against selected eight pathogenic bacteria including two gram positive: *Bacillus cereus* DSM 4312, *Staphylococcus aureus* (ATCC 25923) and six gram negative: *Enterococcus faecalis* ATCC (29212), *Escherichia coli* O157:H7 (ATCC 43889), *Klebsiella pneumonia* ATCC (700603), *Pseudomonas aeruginosa* ATCC (27853), *Salmonella typhimurium* (ATCC 14028), *Shigella sonnei*, ATCC (25931). The bacterial growth inhibition of various *Moringa* leaves extracts was firstly tested and the result was shown in Table 2 &3. In this study, Moringa leaves extracts were considered active against tested bacterial strains when the zone of inhibition was greater than 6 mm in according to the general rule for the antimicrobial activities of plant extracts (Eilert et al., 1981). The result showed that all *Moringa* leaves extracts inhibit the growth of Gram-positive bacteria as well as the Gram-negative bacteria. Methanol extracts showed varying degrees of antimicrobial activity on the microorganism tested. The maximum zone of inhibition was seen in *Shigella sonnei* 19mg/ml, and the lowest was seen in *Escherichia coli* O157:H7 10mg/ml. It is thus established that *Moringa* leaves extracts by methanol contains compound that has antimicrobial property. While *Moringa* leaves extracted by Di ethyl ether did not show detectable suppression in growth of *Escherichia coli* O157:H7. The Moringa leaves extracted by Di ethyl ether were less active against all bacterial strains tested.

Quantitative analysis of cell apoptosis by flow cytometry: The various *Moringa* leaves extract used in this study all had varying ability to induce SKOV-3 cell apoptosis. This ability was measured using Annexin FITC staining (Fig 2). The findings indicate that all Moringa leaf extract induces

apoptosis in the SKOV-3 ovarian cancer cell line when compared to untreated cells. Moreover, the apoptotic rate is significantly higher after 48 hours when compared to untreated cells. Methanol-treated cells showed the highest percentage of apoptosis, followed by Di ethyl ether extracts (i.e., 39.2 and 34.0 respectively).

The methanol extracts had higher TPC more than di ethyl ether extract, one possible explanation for this is that the fact that phenolic extraction is greater in more polar solvents, such as methanol, than in non-polar di ethyl extract. This extraction method is the first step in recovering and purifying bioactive compounds from plant materials. The enhanced recovery of antioxidant compounds with methanol is consistent with previous studies (Razali et al., 2012). The polarity of solvents played a crucial role in the extraction process as it would increase the solubility of antioxidant compounds. While antioxidant activity has been observed in *M. oleifera* leaf extracts in both *in vitro* and *in vivo* conditions as a result of abundant phenolic acids and flavonoids (Verma et al., 2009). However, there are several variables that could influence the composition of *M. oleifera* tissues linked to their antioxidant function. For example, the season and location of development (Iqbal and Bhanger, 2006) and maturity (Sreelatha and Padma, 2009) have been shown to influence the antioxidant activity. Free radicals (included within lipid peroxidation) play a key role in a variety of chronic diseases, including cancer and cardiovascular disease (Dorman et al., 2003; Saleem et al., 2020; Ali et al., 2021).

Table 2. The antimicrobial activities of *Moringa* leaves extract

Food-borne pathogens	Zone of inhibition (mm)	
	Methanol	Di ethyl ether
Bacillus cereus DSM 4312	13 ± 6.9	10 ± 1.7
Staphylococcus aureus (ATCC 25923)	16.00±2.00	11 ±1.7
Enterococcus faecalis ATCC (29212)	14 ± 1.53	11 ± 0.0
Escherichia coli O157:H7 (ATCC 43889)	10 ± 1.73	00± 00
Klebsiella pneumonia ATCC (700603)	17.33 ± 0.57	14 ±0.0
Pseudomonas aeruginosa ATCC (27853)	17.00 ±6.93	11± 1.73
Salmonella typhimurium (ATCC 14028)	14 ± 3.0	10.33±0.58
Shigella sonnei ATCC (25931)	19 ± 1.73	12.00±1.00

Table 3. The minimum Inhibition concentration of Moringa leaves extract.

Organisms species	Methanol	Di ethyl ether		
	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
Bacillus cereus DSM 4312	100 ± 6.9	100±1.7	100± 3.46	200±0.0
Staphylococcus aureus (ATCC 25923)	100 ± 3.0	200±1.7	200±0.0	<200± 1.73
Enterococcus faecalis ATCC (29212)	100± 1.53	200± 0.0	200±0.0	200±0.0
Klebsiella pneumonia ATCC (700603)	100± 3.46	200±0.0	100±0.0	200±0.0
Pseudomonas aeruginosa ATCC (27853)	50 ±3.00	100±1.73	100± 1.73	<200± 1.73
Salmonella typhimurium (ATCC 14028)	50 ± 6.93	200± 0.0	100±0.0	200±0.0
Shigella sonnei ATCC (25931)	100± 1.73	100± 1.7	200±0.0	<200±1.0

At a concentration of 100 g/ml, the methanolic extract had the most potent DPPH, the same pattern of DPPH• scavenging activity was found in the methanolic and Di ethyl ether at concentrations of 200 g/ml and 300 g/ml, the methanolic and Di ethyl ether extracts displayed a similar pattern of DPPH• scavenging activity. Methanolic < BHT extract < Di ethyl ether extract, this ascending order was observed. In comparison to BHT and Di ethyl ether extract, these findings showed that methanolic extract had the highest DPPH radical scavenging activity. *Moringa oleifera* methanolic extracts have a higher DPPH• scavenging activity, which may be attributed to their higher total phenolic and total flavonoid contents, as shown in table 1. These hydroxyl phenolic compounds can scavenge DPPH• by donating hydrogen atoms to it. Lu and Foo provided such a clarification (2001). The DPPH scavenging method is now widely used to investigate the antioxidant function of herb extracts (Chatha et al., 2006; Khor et al. 2018; Saleem et al., 2020; Ali et al., 2021).

It is well known that the solvents used for antioxidant extraction have a major effect on the DPPH scavenging capability determination. Indeed, because of their superior structural chemistry, free radical scavenging methods (DPPH) exhibit reduced alcoholic DPPH solutions in the presence of a hydrogen donating antioxidant (Koleva et

al., 2002). Phenolic compounds, on the other hand, have been documented and given to be potent hydrogen donors to the DPPH radical (Mohamed et al., 2003). The bacterial growth inhibition of various *Moringa* leaves extracts was firstly tested and the result was shown in Table 2. In this study, *Moringa* leaves extracts were considered active against tested bacterial strains when the zone of inhibition was greater than 6 mm in according to the general rule for the antimicrobial activities of plant extracts (Eilert et al., 1981). The result showed that all *Moringa* leaves extracts inhibit the growth of Gram-positive bacteria as well as the Gram-negative bacteria. Methanol extracts showed varying degrees of antimicrobial activity on the microorganism tested.

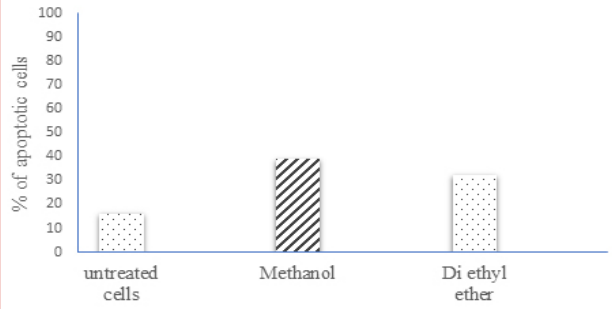
The maximum zone of inhibition was seen in *Shigella sonnei* 19mg/ml, and the lowest was seen in *Escherichia coli* O157:H7 10mg/ml. It is thus established that Moringa leaves extracts by methanol contains compound that has antimicrobial property. While *Moringa* leaves extracted by Di ethyl ether did not show detectable suppression in growth of Escherichia coli O157:H7. The *Moringa* leaves extracted by Di ethyl ether were less active against all bacterial strains tested. Similarly, Spiliotis et al. (1997) studied the antimicrobial activity of MO oil on various microorganisms and found that the oil was not effective against the microbial activity. However, Lalas et al. (2012), (Adeyinka et al., 2018; Das et al., 2020; Milla et al., 2021) assessed the antimicrobial activity of the oil of *Moringa peregrina* on various bacterial strains and the extracts proved effective against all microorganisms studied.

The variation in antimicrobial activity of MO extracts reported by different studies could be attributed to the differences in *Moringa* species and the different extraction method they used. As shown in Table 2, The MIC of the two extracts against test organisms ranged from 50 to 100 mg/mL with methanol having the highest activity at 50 mg/mL against *Pseudomonas aeruginosa* ATCC (27853) and *Salmonella typhimurium* (ATCC 14028). which demonstrated a significantly higher activity against the tested bacteria compared to the MO Di ethyl ether extract. For both extracts, the maximum MBC was found at 200 mg/mL. (Table 2).

The extract's inhibitory activity, MIC, and MBC against the food pathogenic bacteria suggested that Moringa leaves could be used as antimicrobial agent. Adeyinka et al. (2018) reported *Staphylococcus aureus, Salmonella typhi or Escherichia coli* were sensitive to MO methanol extracts. Similarly, Saadabi and Abu Zaid (2011) also indicated that aqueous extract of MO showed a superior antibacterial activity against gram positive bacteria including *Staphylococcus aureus* and *Bacillus subtilis*. It is interesting to note that the presence of types of phytochemicals and their contents in the MO extracts dominates the antimicrobial activity of the extracts (Bukar et al., 2010; Das et al., 2020; Milla et al., 2021).

The type of the solvent used for extraction plays a dominant role on antimicrobial activity of the extract. Seleshe and Kang (2019) reported that MO extract from Methanol and chloroform showed significant antimicrobial activity against *Klebsiella pneumoniae* and *Bacillus cereus*, while Water extract showed the lowest inhibition against these microorganisms. In contrary, Ajaiyeoba (2002) and Bukar et al. (2010) indicated that MO extracts from polar solvent (ethanol and water) extraction were more active than the extracts from non- or less polar solvents such as chloroform. In relation to the extracts' phytochemical content, the presence of saponins, alkaloids, tannins and flavonoids was confirmed to enhance the antimicrobial activity of the plants (Bukar et al., 2010; Sing and Bhat, 2003; Saleem et al., 2020; Ali et al., 2021).

Figure 2: Effect of *Moringa oleifera* on SKOV-3 cell apoptosis. Flow cytometry analysis of apoptosis in SKOV-3 cells either untreated or treated with 10 µg/ml of every compound for 48h. After the treatment period, the cells were stained with Annexin FITC and subsequently analyzed by flow cytometry.



This could possibly be attributed to the difference in the extraction method. This study evaluated the cytotoxic effect of crude extracts (Methanol and Di ethyl ether). The results of the study revealed a higher percentage of apoptosis in the Methanolic MO extract. Second in place was the Di ethyl ether extract. (Fernandes et al. 2016; Khor et al. 2018) confirmed that the cytotoxic effect of MO extracts was found to be selective to cancer cell lines and not to normal cell lines, which were found to be immune. The phytochemicals present in MO flower extract may be responsible for the high cytotoxic activity. Previous study revealed that presence of quinic acid in MO is chemo-preventive in nature (Padmini et al., 2013; Saleem et al., 2020; Ali et al., 2021).

CONCLUSION

Moringa oleifera extracts contain potent antioxidant compounds that have the potential to be applied in the pharmaceutical and food industries. The first step in obtaining these compounds is finding the best extraction solvent for subsequent biological applications. In comparison to Di ethyl ether extract, methanolic extracts of MO have higher antioxidant activity and antimicrobial capacity against foodborne pathogen, Moreover, they show anticancer capacity when tested over ovarian cancer cells. We conclude that the Methanolic solvent could be reasonable choice for antioxidant compounds extraction and the potential uses of *M. oleifera* as alternative natural preservatives in food products.

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Conflict of Interest: The authors declared that present study was performed in absence of any conflict of interest.

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Biotechnological Communication

Comparison on the Effectiveness of *matK* and *rbcL* Barcode Loci to Authenticate Jackfruit *Artocarpus heterophyllus* grown in Vietnam

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ABSTRACT

Jackfruit (*Artocarpus heterophyllus*) is one of the popular fruit trees grown in tropical countries. This tree is grown popularly since it has many uses in cuisine, aesthetics as well as in medicine. Although Vietnam is located in an area suitable for the growth of jackfruit trees, the cultivation of this fruit tree is mainly spontaneous on a small scale and the recent studies carried out on jackfruit mostly focused on the evaluation of agronomical criteria, describing the botanical characteristics and using traditional breeding techniques that are low accuracy leading to low quality of jackfruit, uneven fruit, and low economic efficiency. Currently, DNA barcoding techniques are a highly reliable method for genetic diversity assessment and plant taxonomy, in which the *matK* and *rbcL* regions are commonly used in the classification of different crops. In this study, *matK* and *rbcL* loci from jackfruit accessions of Vietnam were sequenced and searched for homology in NCBI Genbank to identify Latin names. The obtained sequences from Vietnam jackfruit were then combined with published corresponding sequences of jackfruit from different countries and targeted for alignment and phylogenetic analysis. The obtained data present the significant variation in both examined DNA barcode loci among jackfruit accessions. The sequence alignment also reveals the distinct variation in *rbcL* regions of jackfruit accessions in Vietnam in the comparison to *rbcL* sequences of jackfruit accession from other countries. The obtained results show high effectiveness of using rbcL in classifying jackfruit. Furthermore, it could be helpful tool for scientists in managing, conserving and developing genetic resources for breeding programs, it also has the potential to promote the building of an authentication process of jackfruit in Vietnam.

KEY WORDS: DNA BARCODE, JACKFRUIT, MATK, RBCL.

INTRODUCTION

Jackfruit (*Artocarpus heterophyllus*) is a perennial tree commonly found in several countries such as India, Bangladesh, Myanmar, Sri Lanka, South China, Nepal, Laos, Vietnam, Cambodia, Malaysia, Philippines, Indonesia and throughout Africa, Australia. Presently, Bangladesh, India, Myanmar, Thailand, Vietnam, China, the Philippines, Indonesia, Malaysia and Sri Lanka are major jackfruit producers (Sidhu 2012). Jackfruit is widely grown since it has many uses from cuisine, aesthetics, medicine as well as in construction. Jackfruit wood is hard, durable and easy to saw or carve. It is used to build homes, manufacture high-quality furniture, and make musical instruments such as violins. Jackfruit contain high nutrition contents such as carbohydrates, proteins, vitamins, minerals and phytochemical so it is considered as importance food and feed. Jackfruit seeds can be prepared in many ways such as boiled, roasted or soaked in syrup. The leaves and pods of the fruit are an excellent source of food for livestock such as cows, goats, and sheep (Ranasinghe et al. 2019; Srivastava and Singh 2020).

Mature jackfruit is used in salads or used as a vegetable. Ripe jackfruit can be used as a dessert. In addition, pureed jackfruit is also produced into baby food, juice, jam, and jelly. Freeze-drying, vacuum frying and freezing are new preservation methods for modern jackfruit products (Swami

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et al. 2012). Furthermore, jackfruit is also traditionally applied to treat several diseases such as asthma, ulcers, wound healing, cough, hypertension (Srivastava and Singh 2020). Usually, jackfruit species are distinguished from each other by the stems, leaves and flowers on the mature tree (Chandrashekar et al. 2018; Dey and Baruah 2019; Dhakar et al. 2020). However, this method is often affected by external factors, the stage of tree development or lack of sufficient morphological variations. The morphological characteristics of many types of jackfruit are very similar in the non-flowering stage. However, when the plants are young, the morphological characteristics have many similarities leading to difficulties in distinguishing varieties (Bogale et al. 2020).

In addition, jackfruit is a cross-pollination plant, seed-based propagation will not guarantee the genetic characteristics as well as the desired characteristics in the offspring compared to the mother plant, so the common method is through asexual propagation. However, if the first parent plant is not correctly identified, all subsequent seedlings will be affected (Sherif et al. 2020). This puts the importance of accurately identifying plants at the molecular level to ensure seedling quality. Recently, molecular markers have been applied to identify plant and animal varieties that are accurate to the molecular level and are not affected by environmental influences and the developmental stage of the organism. Today, molecular markers are increasingly applied for classifying and analyzing the genetic diversity and characterization of plant species. Compared with traditional markers, molecular markers are easier to perform under laboratory conditions, giving fast results and high accuracy (Chesnokov et al. 2020; Hariharan and Prasannath 2021).

Therefore, molecular markers become effective support tools allowing accurate assessment of the genetic diversity of medicinal plants for conservation, selection and correct identification of plant species, serving for further studies on breeding and breeding of important fruit tree species (Kreuzer et al. 2019; Wu et al. 2019). DNA barcoding is one of molecular markers developed recently, it has begun to be applied intensively in plants. In (2009) the International DNA Barcoding Organization (CBOL) recommended the use of DNA barcoding from the *rbcL* and *matK* genes in plant research. This method is presently used to serve the classification, biodiversity assessment and genetic resource conservation. Several gene regions have been utilized as barcodes for plant classification such as ITS, *matK, rbcL, atpF-atpH, psbK-psbI* and *trnH-psbA*. Among them, *matK* and *rbcL* have been chosen as standard plant barcoding loci by The Consortium for the Barcode of Life (CBOL Plant Working Group 2009; Swami et al. 2012).

These two loci have been sucessfully applied to classified several plant species such as *Cymodocea* seagrass genus, jewel orchid, *Pseuderanthemum palatiferum* (Bchir et al. 2019; Ho and Bui 2021; Ho et al. 2021). The aim of present study was to investigate the discrimination ability in classification of jackfruit accessions collected in Vietnam and from other countries based one of DNA sequences of *matK* and *rbcL* loci. The archived results in this study would be applicable for authentication, genetic conservation and breeding purposes of jackfruit in Vietnam.

MATERIAL AND METHODS

DNA from jackfruit leaves was extracted by Cetyltrimethyl Ammonium Bromide (CTAB) method followed Allen et al (Allen et al. 2006). After extraction, DNA quality was examined by electrophoresis on 1% agarose then spectrophotometer (Optima SP 3000 nano UV-VIS, Japan) was used to determine DNA concentrations. The DNA samples were then kept at -20 °C freezer until use for PCR reactions.

MatK and *rbcL* regions were amplified using the PCR method with composition reactions as follows: 12.5 µL 2X Mytaq Red Mix (Bioline, UK), 20 ng DNA, 0.2 µM of each primer (either *matK* 390F: 5'-CGATCTATTCATTCAATATTTC-3'; and 1326R: 5'-TCTAGCACACGAAAGTCGAAGT-3' or *rbcL*: cF: 5'- TGAAAACGTGAATTCCCAACCGTTTATGCG-3'; cR: 5'- GCAGCAGCTAGTTCCGGGCTCCA-3' and PCR water for a final volume of 25 µl (Hasebe et al. 1994; Cuénoud et al. 2002). The PCR cocktails were run in thermal cyclers SureCycler 8800 Thermal Cycler (Agilent, USA) with following conditions: initial denaturation at 94 °C for 2 minutes; then repeated by 35 cycles of 30 seconds at 94 °C, 30 seconds at 55 °C, 50 seconds at 72 °C, and finally one minute at 72 °C to complete the reaction. The PCR products were then stained with 6X GelRed (Biotum, UK) and visualized on gel electrophoresed and 1 kb ladder (Bioline, UK) was used to determine amplification length. Correct PCR products were sequenced by Sanger methods at Nam Khoa Company (Ho Chi Minh City, Vietnam).

Each sample were sequenced for both sense and antisense directions. Antisense sequences were reversed and aligned with sense sequences to ensure accuracy. Furthermore, the all published sequences of *matK* and *rbcL* sequences of *Artocarpus heterophyllus* available on NCBI Genbank (http: //www.ncbi.nlm.nih.gov) were downloaded and evaluated as criteria proposed by Suesatpanit and colleagues (2017) (1) sequences are not 'unverified' without a species name (2) contain <3% ambiguous base 'N'. All sequences were then included for alignment and phylogeny analysis (Suesatpanit et al. 2017). The DNA sequences from Vietnam jackfruit accessions was checked for homology by using Basic Local Alignment Tools (BLAST) (NCBI, USA) database. These sequences will be combined with NCBI-archived sequences then aligned using the Clustal method in Molecular Evolutionary Genetics Analysis (MEGA) 6 software (https://www.megasoftware.net). Neighbour Joining (NJ) and Maximum Likelihood (ML) phylogeneny analysis were then performed and compared since they represent for distance methods and discrete character methods, respectively. To increase the accuracy in phylogenetic construction, 1000 replicates was applied for bootstrap analysis (Kress et al. 2005).

RESULTS AND DISCUSSION

Sequence retrieve: After *matK* and *rbcL* loci were successfully amplified and sequenced, the sequences were targeted to find homology using BLAST. However, both *matK* and *rbcL* sequences are not applicable for identification of jackfruit due to the returned BLAST results showing the presence of several species in Artocarpus genus such as *Artocarpus hypargyreus*, *Artocarpus altilis* or *Artocarpus camansi*. This result is in agreement with previous study in Indian when 6 jackfruit varieties were analyzed by matK marker and the result showing the high sequence variation among this locus (Vazhacharickal et al. 2017).

Table 1. Estimates of Evolutionary Divergence over Sequence Pairs between Groups for *matK*

matK_MH748883.1_USA		0.08	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
matK_MK264372.1_Sri_Lanka	0.99		0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
matK_KU856361.1_USA	0.00	0.99		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
matK_KU856360.1_USA	0.01	1.00	0.00		0.00	0.00	0.00	0.00	0.00	0.00	0.00
matK_KU856359.1_USA	0.00	0.99	0.00	0.01		0.00	0.00	0.00	0.00	0.00	0.00
matK_KU856358.1_USA	0.00	0.99	0.00	0.01	0.00		0.00	0.00	0.00	0.00	0.00
matK_KU856357.1_USA	0.01	1.02	0.01	0.02	0.01	0.01		0.01	0.00	0.01	0.00
matK_LC461814.1_Thailand	0.01	0.99	0.01	0.00	0.01	0.01	0.02		0.00	0.00	0.00
matK_Vietnam_1	0.00	0.99	0.00	0.01	0.00	0.00	0.01	0.01		0.00	0.00
matK_Vietnam_2	0.01	0.99	0.01	0.00	0.01	0.01	0.02	0.01	0.01		0.00
matK_Vietnam_3	0.00	0.99	0.00	0.01	0.00	0.00	0.01	0.01	0.00	0.01	

(Standard error of comparison is presented in italics upper diagonal).

Table 2. Estimates of Evolutionary Divergence over Sequence Pairs between Groups for *rbcL*

rbcL_MK264364.1_Sri_Lanka		0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.25	0.25	0.25	0.25	0.25	0.24	0.24	0.26	0.25	0.83	0.83	0.80
rbcL_MH748842.1_USA	1.14		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.00	0.00	0.01	0.01	0.74	0.74	0.69
rbcL_KU856240.1_USA	1.14	0.00		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.00	0.00	0.01	0.01	0.74	0.74	0.69
rbcL_KU856239.1_USA	1.14	0.00	0.00		0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.00	0.00	0.01	0.01	0.74	0.74	0.69
rbcL_KU856238.1_USA	1.14	0.00	0.00	0.00		0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.00	0.00	0.01	0.01	0.74	0.74	0.69
rbcL_KU856237.1_USA	1.14	0.00	0.00	0.00	0.00		0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.00	0.00	0.01	0.01	0.74	0.74	0.69
rbcL_KU856236.1_USA	1.14	0.00	0.00	0.00	0.00	0.00		0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.00	0.00	0.01	0.01	0.74	0.74	0.69
rbcL_KF724291.1_USA	1.14	0.00	0.00	0.00	0.00	0.00	0.00		0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.00	0.00	0.01	0.01	0.74	0.74	0.69
rbcL_JX856635.1_India	1.14	0.00	0.00	0.00	0.00	0.00	0.00	0.00		0.00	0.01	0.01	0.01	0.01	0.01	0.00	0.00	0.01	0.01	0.74	0.74	0.69
rbcL_LC461815.1_Thailand	1.14	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		0.01	0.01	0.01	0.01	0.01	0.00	0.00	0.01	0.01	0.74	0.74	0.69
rbcL_MG873185.1_Sri_Lanka	1.17	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01		0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.69	0.69	0.74
rbcL_MG873184.1_Sri_Lanka	1.17	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00		0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.69	0.69	0.74
rbcL_MG873183.1_Sri_Lanka	1.17	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.00		0.00	0.00	0.01	0.01	0.01	0.01	0.69	0.69	0.74
rbcL_MG873182.1_Sri_Lanka	1.17	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.00	0.00		0.00	0.01	0.01	0.01	0.01	0.69	0.69	0.74
rbcL_MG873181.1_Sri_Lanka	1.17	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.00	0.00	0.00		0.01	0.01	0.01	0.01	0.69	0.69	0.74
rbcL_MN082731.1_Sri_Lanka	1.14	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01		0.00	0.01	0.01	0.74	0.74	0.69
rbcL_MN082730.1_Sri_Lanka	1.14	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.00		0.01	0.01	0.74	0.74	0.69
rbcL_AB981777.1_Japan	1.18	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02		0.01	0.80	0.80	0.83
rbcL_AB981765.1_Japan	1.17	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01		0.77	0.77	0.80
rbcL_Vietnam_1	1.85	1.64	1.64	1.64	1.64	1.64	1.64	1.64	1.64	1.64	1.58	1.58	1.58	1.58	1.58	1.64	1.64	1.75	1.68		0.00	0.01
rbcL_Vietnam_2	1.85	1.64	1.64	1.64	1.64	1.64	1.64	1.64	1.64	1.64	1.58	1.58	1.58	1.58	1.58	1.64	1.64	1.75	1.68	0.00		0.01
rbcL_Vietnam_3	1.77	1.58	1.58	1.58	1.58	1.58	1.58	1.58	1.58	1.58	1.64	1.64	1.64	1.64	1.64	1.58	1.58	1.81	1.75	0.01	0.01	

By using keyword "species names + *matK/rbcL*" to find the sequences deposited in NCBI GenBank, after removal of unrealizable sequences as Suesatpanit e al. (2017), total of 27 sequences were obtained, there are 8, and 19 DNA sequences of *matK* and *rbcL* regions, respectively (Table 1). In general, *rbcL* locus was more intensively studied with up to 19 sequences acquired these sequences mostly come from USA, Sri Lanka and Japan. Whereas, *matK* are mostly from USA, Thailand and Sri Lanka have one accession for each country (Suesatpanit et al. 2017; Ho et al. 2021).

Estimation of sequence divergence: The number of base substitutions per site from averaging over all sequence pairs within each group are shown in Table 1 and Table 2. The variation among *matK* and *rbcL* regions is from 0.00 - 0.99, 0 - 0.06, and 0.00 – 1.85, respectively.

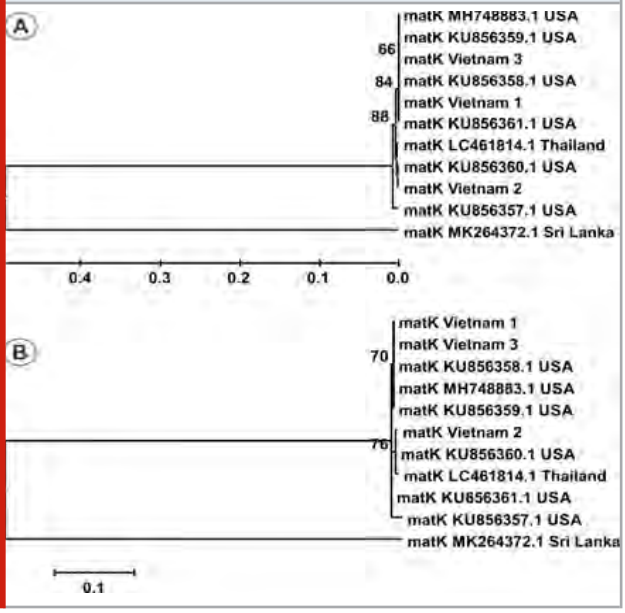
Substitution bias consisting of transition and transversion at codon position for each luster could reveal the trend of evolution. In this study, the substitution of different bases in analyzed regions is evaluated on entire codon positions (1st+ 2nd + 3rd nucleotide) and shown in Table 3. In general, transitional substitution is higher than transversional substitution in all loci. *rbcL* show a higher transversionsal substitution than *matK*.

Table 3. Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution (in percentage)

	matK				*rbcL*			
	A	T	C	G	A	T	C	G
A	-	*12.15*	*5.67*	**1.02**	-	*11.87*	*10.52*	**3.31**
T	*9.89*	-	**9.88**	*5.29*	*12.82*	-	**0.12**	*11.09*
C	*9.89*	**21.18**	-	*5.29*	*12.82*	**0.14**	-	*11.09*
G	**1.9**	*12.15*	*5.67*	-	**3.83**	*11.87*	*10.52*	-

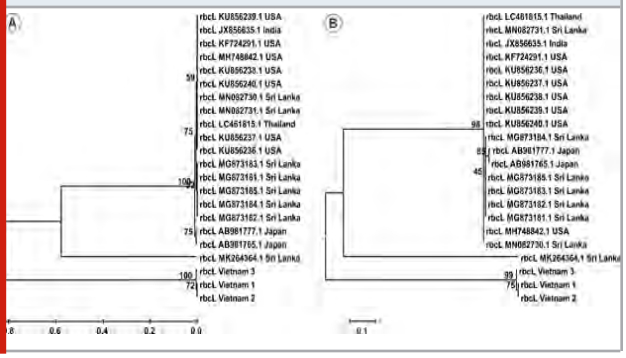
(Note: Each entry shows the probability of substitution from one base (row) to another base (column). For simplicity, the sum of r values is made equal to 100. Rates of different transitional substitutions are shown in bold and those of transversionsal substitutions are shown in italics).

Figure 1. Phylogenetic tree of 11 matK sequences by UPGMA (A) and Maximum- Likelihood (B) methods with 1000 bootstrap replicates.



Estimation of species resolution: Based on phylogenetic analysis, the accession resolution is variable between two DNA barcode loci (Figure 1, and 2). Accession from Sri Lanka is branched into a separate group and remaining accessions are grouped together (Figure 1). Nevertheless, the sequence number should be increased for analysis to enhance the accuracy of study (Sikdar et al. 2018). Whereas *rbcL* locus is able to distinguish jackfruit accessions from Vietnam with those from other countries (Figure 2). The high discrimination power of *rbcL* found in this study is in line with previous reported as a good marker to differentiate species in several plant species such as suaeda, jewel orchid. After surveying over 10,000 *rbcL* sequences from Genbank, Newmaster and colleagues also reported that this region is effective for plant classification (Newmaster et al. 2006; Ho et al. 2021).

Figure 2: Phylogenetic tree of 22 rbcL sequences by UPGMA (A) and Maximum- Likelihood (B) methods with 1000 bootstrap replicates.



CONCLUSION

The findings of the present study suggest that the discrimination capacity of *matK* and *rbcL* DNA barcode loci are variable, in which *rbcL* locus reveals more potential for classification of jackfruit. In the future, higher sequence number should be included in the analysis to give more reliable result. The information from this study could be useful in conservation and development programs of jackfruit plants. The usefulness of two main DNA barcode loci in classify different jackfruit accessions was investigated.

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Technological Communication

A Novel Patent Assessment Criterion for Carbon Dioxide Capture Technologies

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ABSTRACT

In the past two decades, global warming has increased abruptly due to human-induced activities. The contribution of CO^2 implies 74.4% and adheres to the most significant impact on observed global warming. In contrast, 66% of total CO^2 emission is from fossil-fuel combustion. Carbon Capture and Sequestration (CCS) is an innovative solution to curb the rising level of CO^2 from point emitting sources. The current work proposes a novel search criterion for patent extraction on CCS-based technologies, as well as an assessment of the leading patent technologies related with CCS. The patent retrieval was carried out using the commercial database 'curly' of Relecura Technologies Pvt. Ltd. in order to measure global technological growth via patent life, claims, forward citations, patent strength, top assignees, and ultimate parent. During the implication of search filter and manual screening, 3376 patents were found globally. China and the United States (U.S.) contribute to 2099 patents, with a share of 62% globally. In context to technology, most research and innovation focus on post-combustion capture. A low number of patents publications were observed on oxy-fuel, algal, and Cryogenic Carbon Capture (CCC) technology. The study also revealed that General Electric Company ranks highest in filing patents compared with other industries. Amine-based post-combustion capture was found to be the most mature and globally available technology. However, ILs (Ionic Liquids), MOF's (metal-organic framework), membrane and CCC tends to be emerging technology. The current article provides readers an insight about the recent developments, technological drift, major patent filing organizations, and the status of CCS globally.

KEY WORDS: CARBON DIOXIDE CAPTURE, CPC CODES, GREENHOUSE GAS, IPC CODES, PATENT ASSESSMENT.

INTRODUCTION

In recent years, the consumption of fossil fuels has raised the level of CO_2 at an extreme level of 419.13 ppm, whereas the risen level of CO_2 is way above the permissible level of 350 ppm (Lab 2021). Until the year 2100, it is estimated to reach around 750 ppm if no such steps for abatement in CO_2 emission were considered. In the current scenario of 2019, China tops the Greenhouse gases (GHG) emissions followed by U.S., India, and Russia; these top emitters produce approx. 55% of global CO_2 emissions. However, CCS technologies are promising and significantly affect emission reduction targets. A brief history was studied to find the initiation of CCS technology. The beginning of capturing CO_2 starts in the late '70s where this technology was considered emerging in abatement of GHG emissions (Kurihara and Shirayama 2004; Petroleum 2020).

Furthermore, in the chemical and natural gas sectors, CO_2 separation was done using chemical and natural gas, in which CO_2 was regarded as an impurity. In 1991, the Norwegian government was the first to introduce the carbon tax, which became a milestone in policy planning (Kaarstad 2002). The IPCC conducted a workshop on Carbon Capture and Storage with WMO and UNEP at Regina, Canada in 2002, intending to produce scoping paper on possible ways to assess CCS (Davidson and Metz 2002). The first decision to apply underground storage of CO_2 captured from natural gas as a climate change mitigation effort - was taken by Statoil and partners in the Sleipner North Sea license in 1990 (Kaarstad 2002; Sood and Vyas 2017; Sharma et al. 2020).

The IPCC produced a Special Report on CCS in (2005), which elaborates the role of CCS to climate policy expert community (Kheshgi et al. 2012). In 2006 Carbon Capture

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& Storage Association (CCSA) was formed to promote CCS business; after COP/MOP-11, the rapid growth in CCS was noted (Qiu and Yang 2018). In recent years only a few patent assessment articles on CCS were published (Li et al. 2013; Qiu and Yang 2018; Míguez et al. 2020). Nonetheless, not one of them offers a detailed explanation of the patent search criteria. This article sets out to design a search query that extracts relevant patents and ranks them depending on how much the parent organization influences its related entities. The article is divided into five sections, beginning from section 1 as the introduction, which briefs the requirement and past developments. However, section 2 explains the methodology and patent retrieval task. Then, the analysis of patent information was done in section 3 whereas, section 4 states the policy implications and future perspectives of the current article, and section 5 refers to the conclusion.

METHODOLOGY

A large majority of patents pertain to little value and only a few pertain high impact value in terms of finance, innovation and research implementation on the ground. Further, the significant impact in value was observed by only the top 10% of patents of the total sample value. The technological capabilities and strategies of an assignee could be effectively determined by analyzing the quality of its patent based on indicators that have been used to assess the value and quality of the patent, like the number of patents cited, citation of the particular patent, patent family size, technological strength, renewal trend and others. However, the extraction of such data in bulk is not available on the open platform. Therefore, an artificial intelligence-based platform named "Relecura" was used (Og et al. 2020).

However, during the patent search, it was observed that the majority of patents found through keywords were misleading and generate garbage value. Therefore, accurate indicators related to the concerned field were used to remove irrelevant data from the search result. Furthermore, during the literature survey, it was observed that the patent classification system follows a hierarchy, and the common classification systems around the world are: (a) International Patent Classification (IPC) and (b) Cooperative Patent Classification (CPC). Thus, we considered both CPC and IPC codes to incorporate all at once in the current article. The patent retrieval work has been divided into four phases: Phase-I (a) Determination of initial keywords, (b) Determination of CPC and IPC sections and subsections of relevancy. Phase-II: Determination of relevant keywords and finalizing query. Phase-III: Query-based search with filters. Phase-IV: Extraction of data (Shalaby and Zadrozny 2019; Og et al. 2020).

Table 1(a). Technology-based keywords inclusive of thefinal query

S. No.	Technology Based Keywords	Synonyms	Search domain	Refined Keyword	Final Query
1	CO_2	Carbon, Carbon dioxide, Carbonic Acid Gas, Carbonic Acid, CO_2	Title, Abstract and Claims	CO_2, Carbon*, Carbon dioxide	(CO_2 OR Carbon* OR (Carbon NEAR2 dioxide))
2	Capture	Capture, Capturer, Captured, Capturing, Seize, Seizure	Title, Abstract and Claims	Capture*, Seiz*	(Capture* OR Seiz*)
3	Storage	Storage, Storing	Full text	Storag*	(Storag*)
4	Absorption	Absorb, Absorbing, Absorption, Absorbent	Full text	Absor*	(Absor*)
5	Transport	Transport, Transporting, Transportation	Full text	Transport*	(Transport*)
6	Sorbent	Sorbent	Full text	Sorbent	(Sorbent)
7	Delivery	Deliver, Delivering, Delivery, Delivered	Full text	Deliver*	(Deliver*)
8	Adsorption	Adsorb, Adsorbing, Adsorption, Adsorbent	Full text	Adsor*	(Adsor*)
9	Separation	Separate, Separation, Separating	Full text	Separat*	(Separat*)
10	Sequestering	Sequester, Sequestering	Full text	Sequest*	(Sequest*)
11	Acid Gas	Acid Gas, Acidic Gas, Acidic Gases, Acid Gases	Full text	Acid*, Gas*	(Acid* NEAR2 Gas*)

In phase-I, the patents on CCS were studied and keywords were extracted. Later, the CPC and IPC libraries, including their sub-sections, were deeply studied and the relevant codes were extracted. The finalised IPC codes are "B01D19/00, B01D47/00, B01D53/00, B01D53/02, B01D53/04, B01D53/06, B01D53/14, B01D53/18, B01D53/22, B01D53/26, B01D53/32, B01D53/34, B01D53/40, B01D53/46, B01D53/47, B01D53/48, B01D53/50, B01D53/52, B01D53/56, B01D53/60, B01D53/62, B01D53/72, B01D53/73, B01D53/74, B01D53/75, B01D53/77, B01D53/78, B01D53/81, B01D53/83, B01D53/84, B01D53/86, B01D53/92, B01D53/96, B01D61/00, B01D63/02, B01D67/00, B01D69/00, B01D69/02, B01D69/08, B01D69/10, B01D69/12, B01D69/14, B01D71/02, B01D71/06, B01D71/64, B01D71/70, B01J19/00, B01J20/02, B01J20/04, B01J20/06, B01J20/08, B01J20/10, B01J20/18, B01J20/20, B01J20/22, B01J20/26, B01J20/28, B01J20/30, B01J20/32, B01J20/34, C01B13/02, C01B17/16, C01B21/04, C01B23/00, C01B3/02, C01B3/34, C01B3/38, C01B3/48, C01B3/50, C01B3/52, C01B3/56, C01B31/20, C01B32/40, C01B32/50, C01B32/60, C01F11/18, C07C7/00, C07C7/11, C07C7/12,

C07C9/04, C09K3/00, C10K1/00, C10K1/12, C10K1/14, C10K1/16, C10L3/10, C12M1/00, F01N3/08, F17C11/00, F23J15/00, F23J15/02, F23J15/04, F25J1/00, F25J1/02, F25J3/00, F25J3/02, F25J3/04, F25J3/06, F25J3/08, H01M8/06 OR Y02C-010/02+ OR Y02C-010/04+ OR Y02C-010/06+ OR Y02C-010/08+ OR Y02C-010/10+ OR Y02C-010/12+ OR Y02C-010/14+)"

To decrease the possibility of overlapping, finalized codes were also co-related with their appropriate CPC. Later, a literature review was conducted in second phase to choose acceptable keywords for further minimising junk data. As indicated in Table 1(a), the completed keywords containing approximately all technologies linked to CCS were included in the search query, which comprised technological terms and their probable synonyms (Abbas et al. 2014; Moullec et al. 2014; Liu et al. 2018; Norhasyima and Mahlia 2018; Qiu and Yang 2018).

While study it was discovered that merely technological synonyms were incapable of extracting the entire CCS technological domain patents. As a result, as stated in Table 1(b), the general keywords of CCS technologies were also incorporated in the current syntax. A search for "OR", "AND", and "NEAR" operators, together with "all" in-field operator, was conducted. At first, the search string was limited to keywords from which 22,676 families were discovered out of 47,111 documents. The categorization search was done with a 100 IPC and CPC filter, and the result was a list of 3,680 families of 9,542 patent documents. Phase IV entails reviewing data gathered from patents to ensure their relevance.

Table 1(b). General keywords inclusive of the final query

S. No.	General Keywords	Synonyms	Search domain	Refined Keyword	Final Query
1	Pre-Combustion Capture	Pre-Combustion Capture, PCC, Pre-Combustion	Full text	Pre, Combustion, PCC	(Pre NEAR2 Combustion)
2	Post-Combustion Capture	Post-Combustion Capture, PCC, Post-Combustion	Full text	Post, Combustion, PCC	(Post NEAR2 Combustion)
3 4	Oxy-Fuel Combustion Membrane Separation	Oxy-Fuel Combustion, Oxy/Fuel Membrane Separation	Full text Full text	Oxy, Fuel Membrane, Separation	(Oxy NEAR2 Fuel) (Membrane NEAR2 Separation)
5	Cryogenic Carbon Capture	Cryogenic Carbon Capture, CCC, Cryogenic Capture	Full text	Cryogenic, Capture, CCC	(Cryogenic NEAR2 Capture)

Table 2. Comparative analysis

S. No.	Search domain	Year	Patents	Search engine	Ref.
1	Global	2015	2325	Innography	(Qiu and Yang 2018)
2	Global	2015	2546	Relecura	Current paper (priority date)
3	Global	2020	3376	Relecura	Current paper (priority country code)
4	U.S. and China	2015	1171	Innography	(Qiu and Yang 2018)
5	U.S. and China	2015	1295	Relecura	Current paper (priority date)
6	US and China	2020	2099	Relecura	Current paper (priority country code)

Validation of patent search: A comparative analysis of patent review has been carried out to verify the search string as described in Table 2. The data of patents published in the U.S. and China till 2015 was compared with current search filters and keywords. The results show a slightly higher value than previously published data due to the low selectivity of keywords. Later, while the deep study of patent documents it was observed that no keyword search is perfect as the total relevant patents found were 3376, which concludes to the accuracy of keywords to 91.76% (Qiu and Yang 2018).

Statistical Summary: In most nations, patents can be awarded for a term of 20 years; however, in Australia and Japan, the time can be extended. Each nation charges a unique fee to keep the patent valid. Table 3 shows the annual fees for the various patent offices. The annual distribution of patents and their applications elucidates the evolution of R&D in CCS across its numerous technological fields. Figure 1 depicts the granted patents in CCS from the top ten nations in order to visualise the efforts and maturity in CCS-based technologies. It was also discovered that the

U.S. and China accounted for 62% of all patents published worldwide (Wang and Song 2020).

China's ability to leverage CCS will accelerate in the coming years due to present trends in patents. However, Figure 2 illustrates the expiration year in which both the U.S. and China imprint significant peaks of patents in large. During critical analysis, it was observed that some of the patents were in a unique field. Still, most patents do not belong to a single domain of technology comprising two or more IPC technological fields of expertise. The top five technologies according to IPC classification shown in Figure 3. The chemical or physical (inclusive of separation and catalysis) process-based patents were on the top and comprised around 84% share. A global patent publishing analysis was also done to analyze the patent filing per year to evaluate the growth in technology worldwide. A plot was generated, as illustrated in Figure 4. The data revealed that a rise in levels was observed from 2008. However, the maximum numbers of publications were noted in 2019.

Table 3. Annual fee and structure of renewal of major patent offices

Year	EPO (Europe)	USPTO (United States)	IPO (UK.)	I.P. (Australia)	CNIPA (China)	JPO (Japan)	INPI (France)	DPMA (Germany)	CIPO (Canada)	KIPO (Korea)	CGPDTM (India)
	Euro	USD	GBP	AUD	CNY	JPY	Euro	Euro	CAD	KRW	INR
1	-	-	-	-	-	2300	-	-	-	-	-
2	-	-	-	-	900	2300	38	-	-	-	-
3	468	-	-	-	900	2300	38	70	50	-	4000
4	580	1600	-	-	1200	6400	38	70	50	40000	4000
5	810	1600	70	-	1200	6400	38	90	50	40000	4000
6	1040	1600	90	300	1200	6400	76	130	100	40000	4000
7	1155	1600	110	300	2000	19300	96	180	100	100000	12000
8	1265	3600	130	300	2000	19300	136	240	100	100000	12000
9	1380	3600	150	300	2000	19300	180	290	100	100000	12000
10	1560	3600	170	300	4000	55400	220	350	100	240000	12000
11	1560	3600	190	550	4000	55400	260	470	125	240000	24000
12	1560	7400	220	550	4000	55400	300	620	125	240000	24000
13	1560	7400	260	550	6000	55400	350	760	125	360000	24000
14	1560	7400	300	550	6000	55400	400	910	125	360000	24000
15	1560	7400	360	550	6000	55400	450	1060	125	360000	24000
16	1560	7400	420	1250	8000	55400	510	1230	225	360000	40000
17	1560	7400	470	1250	8000	55400	570	1410	225	360000	40000
18	1560	7400	520	1250	8000	55400	640	1590	225	360000	40000
19	1560	7400	570	1250	8000	55400	720	1760	225	360000	40000
20	1560	7400	610	1250	8000	55400	790	1940	225	360000	40000
21	-	-	-	2550	-	55400	-	-	-	-	-
22	-	-	-	2550	-	55400	-	-	-	-	-
23	-	-	-	2550	-	55400	-	-	-	-	-
24	-	-	-	2550	-	55400	-	-	-	-	-
25	-	-	-	2550	-	55400	-	-	-	-	-

Note:- The abbreviations used for patent office's in above are EPO (European Patent Office), USPTO (United States Patent and Trademark Office), IPO (Intellectual Property Office), IP Australia, CNIPA (China National Intellectual Property Administration), JPO (Japanese Patent Office), INPI (France's Institut National de la Propriété Industrielle | National Industrial Property Institute), Deutsches Patent- und Markenamt (DPMA) – the German Patent and Trade Mark Office, CIPO (Canadian Intellectual Property Office), KIPO (Korean Intellectual Property Office), CGPDTM – The Office of the Controller General of Patents, Designs and Trade Marks | Indian Patent Office.

Figure 1: Top 10 countries V/s Priority year of patents

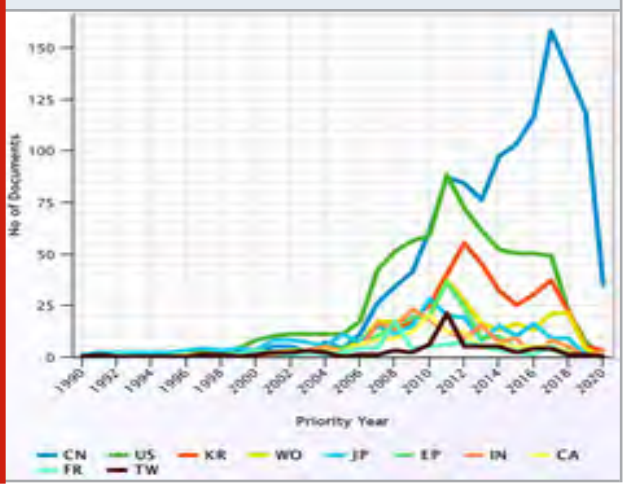


Figure 2: Top 10 countries V/s expiry year of patent

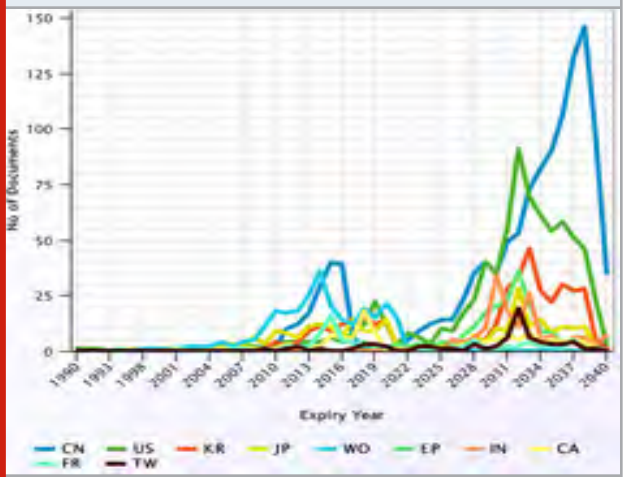


Figure 3: Technological trend per year

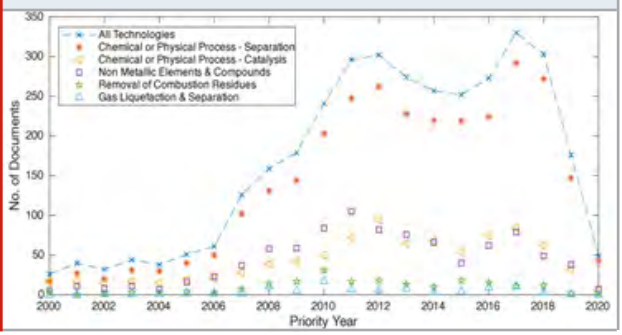
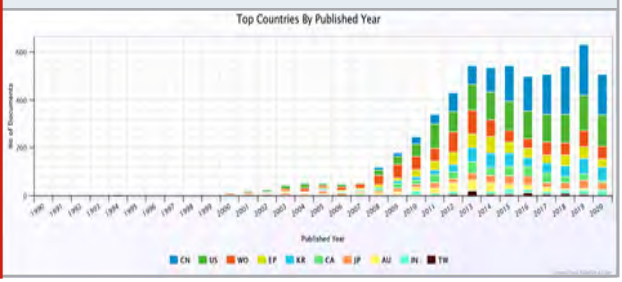


Figure 4: Technological trend per year



Technological assessment: Most review articles only review research publications; however, the patented technologies were left untold. Therefore, to emphasize the significant development scenario of the patent status of CCS, the article is sub structured into eight subsections where recent and key technological benchmarks has been reviewed. Two approaches were implemented for the selection of patents. First, based on the back citation, the patents with the least referring documents and the maximum number of citations were assumed impactful.

$$\text{Selection index} = \frac{\text{Number of citations} - \text{Number of references}}{\text{Current year} - \text{Year of publication}}$$

However, it was also observed that the latest patents might not achieve many citations. Therefore, another method was also used considering technological breakthroughs in industrial applications, and patents with the maximum number of families were treated to be the best among all while keeping the constraint of low citing documents in the particular patent (Wang and Song 2020).

Pre-Combustion capture: Pre-combustion capture involves the reaction of a fuel with oxygen or an air stream to produce a synthesis gas (syngas) which is composed of CO and H_2. Whereas, produced CO is made to react with steam to give CO_2 and additional H_2. The CO_2 is then separated using physical or chemical absorption. Pre-combustion capture provides high purity rates and a high-efficiency potential. However, the major drawback of pre-combustion is the high capital investment, which leads to a low number of IGCC plants globally. Generally, three commercial pre-combustion technologies were used: (a) Rectisol-based, (b) MDEA-based, and (c) Selexol-based. Pre-combustion envisaged to capture CO_2 before combustion or, in other words, can be described as the process of treatment of synthetic gas principally composed of CO_2 and hydrogen. In recent years, pre-combustion technology has risen and is commonly employed in IGCC or NGCC plants (Wang and Song 2020).

Di-substituted siloxane solvents with 2 to 4 Si atoms were introduced in US10589228B2, which was covalently modified with polyethylene glycol; however, the solvent inherits the capability to replace glycol-based solvents due to their high-temperature workability and low foaming characteristics (Hopkinson et al. 2020).

Post Combustion capture: The post-combustion carbon capture technique is usually used in coal plants. The carbon is captured after the fuel combustion in conjunction with the purification of the flue gases. After combustion, the methods for carbon separation include absorption in a solvent, adsorption, membrane filtration, and cryogenic separation (Chiang and Pan 2017; Rackley 2017; Subramanian et al. 2017). The most significant advantage of post-combustion is getting the maximum degree of purity ($\geq$ 99.99%). When integrating post-combustion with power plant processes, no significant adjustments were necessary for the power plant processes. However, high investment costs and reduced operational flexibility of power plants equipped with post-combustion units are downsides of the post-combustion process (Rackley 2017; Hopkinson et al. 2020).

Water washing was employed in the early days to remove CO_2 from the gas stream. Later, after the advent of amines as a substitute for ammonia, technical drift was observed US1783901A (Bottoms 1930). However, unlike other solvents, amines have a low energy regeneration capacity and great selectivity; this patent started the CO_2 capture trend as a foundation for CCS in post-combustion capture systems. The technology was surpassed by US1897725A, which described the first extraction of CO_2 using a succession of scrubbing towers using aqueous ammonia (Wilhelm and Walter 1933; Jovanovic and Krishnamurthy 2020).

In US20200147544A1, a method for producing a CO_2-containing flue gas was devised by combusting a carbonaceous fuel in a high pressure steam generating unit with combustion air and capturing the CO_2 in the flue gas, which was at least partially captured and compressed into CO_2 (Clerveaux and Lefebvre 2019). In CN108295802A potassium based CO_2 absorbents granules posing low temperature decarburization properties along with good mechanical and fluidization characteristics was synthesized however, the developed absorbent includes active component as potassium carbonate with activated alumina, aluminous cement, kaolin or aluminium hydroxide as carrier (Yafei et al. 2018; Jovanovic and Krishnamurthy 2020).

Oxy-fuel combustion capture: In oxy-fuel combustion, pure oxygen or oxygen-enriched air is used for combustion purposes. However, combustion products are CO_2, water vapour and oxygen. The plant process must include an air separation process, flue gas processing unit and a CO_2 processing unit. Whereas, lack in the commercial

application was for two major reasons, (a) requirement of a specialized oxy-fuel boiler and (b) NOx production (Yoro and Sekoai 2016; Jovanovic and Krishnamurthy 2020).

However, the initial development of recirculation power production technology was started with the burning of fuel and high-concentration oxygen. EP1592867B1 was subsequently introduced and oxygen fuel technology was demonstrated for improved efficiency and cost-effective CO_2 collection from enhanced flue gas (Lynghjem et al. 2016). CN108729965A invented a novel extraction process to help combustion in the power plant using oxygen-enriched flue gas, resulting in increased boiler outlet concentration to 30%-60% and posing CO_2 capture efficiency ≥ 95% (Xiaoqian et al. 2018). KR102048844B1 was designed by combining a liquified air re-gasification system with a coal fired plant and a CCS unit, which considerably enhances CO_2 separation and removal efficiency as well as overall power generation (Nam et al. 2019).

Membrane: In application to CCS the separation of CO_2 focuses on flue gas stream before the subsequent transportation and storage/utilization of captured CO_2. The prime focus towards membrane separation was its applicability in a continuous system, preferred by industrial and power generation sectors. Membrane separation is one of the few technologies that demonstrates its applicability in all three capture technologies. For post-combustion capture: CO_2/N_2 separation from flue gas, pre-combustion capture: CO_2/H_2 separation for IGCC processes, natural gas refining: CO_2/CH_4, and similarly in oxy-fuel combustion capture: O_2/N_2 separation for air separation (Lee et al. 2020).

In KR20200015664A, an apparatus was constructed to collect high-concentration CO_2 using a low-temperature membrane separation technique, which dramatically minimizes cooling energy demand. CN111111464A developed composite membrane technology involving electrospun polyacrylonitrile fibre film layer and cyclodextrin MOF layer posing enhanced gas selectivity. A CO_2 capture system and pre-treatment technique has been developed by CN110813047A utilizing sodium-based weak alkaline absorbent as a pre-treatment (Xiaofu et al. 2019a; Lee et al. 2020). CN211358301 invented a utility model to pre-treat the exhaust of a coal-fired plant. The proposed embodiment includes the usage of a membrane electrolyzer to regenerate the pre-washing liquid, which significantly reduces the amount of absorbent required for the regeneration (Xiaofu et al. 2019b). Three-step membrane technology for capturing CO_2 is described in CN109731437A, providing exceptional gas separation performance for CO_2/N_2 when used with MOF-ZIF-716-8 (Qianqian et al. 2019). A composite membrane composed of a polyether-based copolymer by dissolving in one or more alcohol and water solutions was developed US20190366277A1 to remove CO_2 from the fluid composition (Akhtar et al. 2019; Lee et al. 2020).

Algae-based carbon capture technology: In recent years, the algal route of capturing CO_2 gained more interest for commercializing, whereas algae belong to large and diverse groups of simple aquatic organisms. They may be unicellular or multicellular forms and mainly cultivate on the photosynthesis mechanism, like the plants. Algae play an essential role in the global ecosystem as these are spread globally as they pertain capability to utilize CO_2 as a carbon source. They are likely to possess higher efficiency against CO_2 fixation capability and in optimal culture conditions (Beal et al. 2018; Anguselvi et al. 2019). These can give higher growth rates than conventional crop plants. The biomass produced can be utilized as a feedstock for other value-added products such as biofuel and chemicals (Pires 2017; Beal et al. 2018; Norhasyima and Mahlia 2018; Yang et al. 2020). A microalgae carbon fixation based energy utilization system for supercritical water treatment was developed in which the waste gas and water generated from supercritical wastewater treatment were used to cultivate microalgae resulting in low-cost energy production with CCS (Yang et al. 2020).

CN111151119A developed a biomass production method that is inexpensive and convenient which utilizes a culture media that is more efficient for removing CO_2 from the dilute source stream (Chi and Zhu 2020). In order to lower the energy use while concurrently increasing carbon fixation and bio-oil product yield, especially for coal-fired power plant containing CO_2 component in flue gas. A novel approach by using food grade *Pseudochlorococcum* microalgae for the treatment of flue gas containing $CO_2$was developed CN111266000A. CN109621699A CO_2 capture using three-step chemical absorption and biological transformation coupled with waste water culture was developed (Na et al. 2019). An alternative of conventional CCS system was developed in CN109126361A, the system aimed to facilitate the low power consumption and reduced capital cost using a flue gas separation system and microalgae cultivation resulting in increased recovery rate of waste gas (Yongliang and Liang 2019; Shujun et al. 2020).

Ionic liquids: ILs belong to a category of compounds with ions entirely and pertain to be liquid at or below the process temperature. In most cases, even low-temperature ILs are in the liquid phase, concluding they do not crystallize at low temperatures or below 0°C. Moreover, they show low corrosivity and are non-volatile in prominent working conditions. ILs also pertains low desorption temperature and enthalpies (Brennecke and Gurkan 2010; Shujun et al. 2020).

Therefore, ILs can also be used in pre, post and oxy-fuel combustion. Moreover, ILs are less hazardous in the environment and are less prone to energy losses, which are vital reasons to attract more attention. CN111715031A disclosed method and medium for efficient absorption of CO_2 using 1-aminopropyl-3-methylimidazolium bromide. However, despite these benefits, the created IL is stable, possesses a high CO_2 absorption speed, and has a high capture rate. Moreover, applications of IL in these various areas attract interest across a wide variety of science and engineering domains, including chemistry, chemical engineering, energy, resources and environment (Pingquan et al. 2020).

The Taiyuan University of Technology disclosed a simple preparation method to modify MOF using IL, resulting

in excellent CO_2/O_2 selectivity, high stability, and recyclability. CN110743326A invented a high-efficiency energy-saving non-water-absorbent for capturing CO_2 in which low-viscosity functional treatment on the molecular design level was performed to reduce the rise in viscosity during absorption providing the effect of controlling the viscosity with improved flow and mass transfer capacity with significant improvement in lowering the energy consumption and capture efficacy (Jiejie et al. 2020; Lili et al. 2020). To reduce energy consumption and lower the solvent cost, CN109200760A developed a kind of eutectic solvent wherein the hydrogen bond acceptor can be ammonium chloride or hydrochloric acid-ammonium chloride and the hydrogen bond donor can be composed of an organic amine and a polyol. However, the organic amine may be MEA, DEA, TEA, DETA and N,N-dimethyl or any of the ethylenediamines; and the polyol may be any one of pentaerythritol, ethylene glycol, glycerine and butylene glycol (Yingying et al. 2019; Jiejie et al. 2020).

Major assignees in CCS: Analysis of patent application and granted patents in the field of CCS elaborates that majority of patents published on absorption followed by adsorption, MOF, ILs, algal and CCC technology. Top assignees in filing patents were General Electric, Air Liquide, Exxon Mobil, CO2 Solutions Inc., Alstom, IFP Energies Nouvelles and Mitsubishi Heavy Industries.

CONCLUSION

The findings of the present study reveals that most patents in recent years originated from research institutes and universities globally. It was also noticed that the United States was leading the way with new patents initially. However, Chinese research and innovation rapidly moved on leaving behind the U.S. after 2015. In recent years, many technologies has been developed for separating, transportation, and utilization against CCS. A systematic keyword search query incorporating relevant CPC & IPC codes, and operators to conduct a comprehensive patent search using paid database Relecura.

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Biotechnological Communication

Germicidal Properties of Biosynthesised Gold Nanoparticles from *Streptomyces* sp. PRO 15

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ABSTRACT

Nanotechnology involves the utilization and creation of nanomaterials that range up to 1 to 100 nm in size. The synthesis of nanoparticles using biological roots involves the usage of bacteria, fungi, actinomycetes, algae, enzymes, and plants. These synthesized nanoparticles show various activities which are of medical importance (i.e., Antimicrobial, Anticancer, Antioxidant, etc.). In the present investigation, the synthesis, characterization, and application of Gold Nanoparticles (Bio-GNPs) Streptomyces sp.PRO-15 was reported. The Cell-free extract of Streptomyces isolate was used as the reducing agent for facile, cost-effective, and eco-friendly synthesis of GNPs. The synthesis of gold nanoparticles was carried out by treating 1mM Gold chloride (HAuCl4) solution with the Cell-free extract of Streptomyces isolate. Using UV-visible spectroscopy, the formation of GNPs was confirmed by measuring the peak ranging between 400-700 nm. The synthesized GNPs showed a sharp peak at 520 nm. The involvement of biomolecule in the stabilization of nanoparticles was confirmed with Fourier transform infrared spectroscopy (FTIR). The size of GNPs present in the colloidal solution was determined using Dynamic light scattering (DLS), with an average size ranging from 24. 97nm. The Zeta potential of GNPs was noted to be -23.2 mV. The shape of the nanoparticles was determined using Scanning Electron Microscope (SEM). The germicidal activity of the synthesized gold nanoparticles was tested against pathogens like *Bacillus cereus* NCIM 2217, *Enterococcus faecalis* NCIM 5253, *Vibrio cholerae* MTCC 2906, *Salmonella typhi* NCIM 2501, and *Candida albicans* NCIM 3628, *Cryptococcus neoformans* NCIM 3541, and consequently, it may be used as a potential germicide in the treatment of the infection caused by germs.

KEY WORDS: ACTINOMYCETES, BIOSYNTHESIS, BIO-GOLD NANOPARTICLES (BIO GNPS), GERMICIDAL ACTIVITY.

INTRODUCTION

Nanotechnology is a field of science that is rapidly growing in current scientific research, which involves the production and usage of nanoscale materials for different purposes of human life, like energy storage, cosmetics, clothes, optical devices, bactericidal, biological labeling, biosensors, and treatment of cancer. The term nanotechnology was first used in 1974 by Norio Taniguchi of Tokyo Science University (Vadlapudi and Kaladhar 2014; Hasan 2015). Nanoparticles are materials with sizes ranging from 1 to 100nm (Hasan 2015). Nanoparticles have distinctive chemical and physical properties, and the nanoscale (1-100 nm) plays a crucial role in various aspects of pharmaceutical, medicine, and environmental technologies. Different physical and chemical methods are employed for the synthesis of nanoparticles with a particular size, shape (Natesan et al. 2020).

Physical method yields product with the complication of consuming high energy and generation of heat, chemical methods use the toxic chemical releasing hazardous chemicals as bi-products and are expensive (Babu et al. 2009; Natesan et al. 2020). To overcome this biological method of synthesizing nanoparticles provides a decent solution, which involves using microorganisms like bacteria, fungi, actinomycetes, enzymes, amino acids, and plants. The nanoparticles synthesized using biological roots are eco-friendly and cost-effective (Keshavamurthy and Rai 2021).

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Actinomycetes are Gram-positive aerobic filamentous bacteria with high GC content. They are Saprophytic and readily break down complex biopolymers. About 100 genera of Actinomycetes exist in soil and produce over 10,000 bioactive compounds, out of which 7600 are from Streptomyces genera (Salimath and Onkarappa 2016; Mesta and Onkarappa 2017). These bioactive compounds are of high clinical importance and are applicable as germicidal, anti-parasite, antitumor, anti-viral agents, and other pharmaceutically beneficial compounds. Multidrug resistance of bacteria has become a significant threat to public health. The emerging drug resistance in bacteria and the increase in the production cost of new antimicrobial compounds to tackle them leads to the need for the development of new technology which is economically cheap and effective to combat Multidrug resistance bacteria (Al-Ansari et al. 2019).

Nanoparticles synthesized by microorganisms are among the best candidates for producing new antimicrobial material (Buszewski et al. 2018; Al-Ansari et al. 2019). The present study is focused on the biosynthesis of gold nanoparticles (GNPs) using the cell-free extract of Streptomyces sp. (PRO15) as stabilizing and reducing agent. The synthesized GNPs were characterized using UV-visible spectroscopy, FTIR, SEM, Dynamic light scattering, and Zeta Potential Analysis (Składanowsk et al. 2017; Hamed and Abdelftah 2019; Hamid et al. 2020). The present study also focused on the germicidal activity of synthesized GNPs against bacterial and fungal pathogens.

MATERIAL AND METHODS

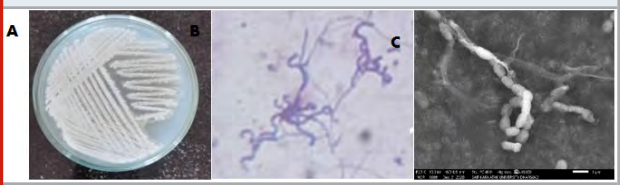
The Streptomyces sp. (PRO15) was isolated from the coffee plantation soil of Kodagu Karnataka, India. The obtained pure strain was then streaked onto Starch Casein Nitrate (SCN) Agar slants and was stored at 4^0 C for future use. Morphological studies were carried out using the coverslip technique. Grams staining and acid-fast staining were performed. Biochemical tests viz., Starch hydrolysis, Casein hydrolysis, gelatine hydrolysis Catalase test, H2S production test, and carbohydrate fermentation test were carried out (Aneja 1996; Cappuccino and Sherman 2014).

Following the methodology by Skladanowski et al. (2017), the synthesis of nanoparticles was carried out with slight modification. Streptomyces sp. (PRO 15) was inoculated into sterile SCN Broth and incubated at 30±2ºC on a closed rotary shaker at 150 rpm for 96hrs. After incubation, the broth was centrifuged at 7500 rpm for 15 min. The supernatant was collected, and 1mM gold chloride nitrate ($HAuCl_4$–$3H_2O$) was added at a ratio of 1:1 (V/V). Sterile SCN broth and culture supernatant without $HAuCl_4$ was kept as control. During incubation, the flasks were observed for the development of pinkish-purple color, which indicated the production of nanoparticles. Bioreduction of gold ions was recorded using a UV-Vis spectrophotometer. A small aliquot sample was taken in a quartz cuvette and observed for wavelength scanning between 380-800 nm. The UV-Vis spectrum of gold nanoparticles was obtained using an ELICO SL159 UV-Visible spectrophotometer (Shah et al. 2020).

Fourier transform infrared spectroscopy PerkinElmer FT-IR C94012 was used to detect the functional groups present on the synthesized gold nanoparticles. The colloidal gold nanoparticle solution was used for FT-IR measurements. The wavelength for spectral reading was recorded between 4000and 400cm-1 (Hamed and Abdelftah 2019; Shah et al. 2020). The powdered nanoparticle was placed on a carbon-coated plate. Then the plate was gold-coated by a sputter-coating instrument to enhance the conductivity and accuracy of the picture. A scanning electron microscope (Hitachi, S-3400N, Japan) was used to analyze the morphology and size of the particles (Sadhasivam et al. 2012). Size distribution and Zeta potential of the synthesized Gold Nanoparticle were determined using Zetasizer Ver. 7.12 (MAL1169468 Malvern Instrument Ltd., UK) (Kulshrestha and Patel 2021).

The germicidal property of the synthesized nanoparticle was carried out by agar well diffusion method against *Bacillus cereus* NCIM 2217, *Enterococcus faecalis* NCIM 5253, *Vibrio cholerae* MTCC 2906, *Salmonella typhi* NCIM 2501, *Candida albicans* NCIM 3628, and *Cryptococcus neoformans* NCIM 3541. 24hrs old cultures of these pathogens were prepared in Luria-Bertani broth and were used for the test. Muller Hinton Agar was inoculated with the pathogens, and 8mm wells were punched using a cork borer, and 100µl nanoparticle was pipetted into each well. Ampicillin and Nystatin were used as standard antibiotics for bacteria and fungi, respectively. Gold chloride was used as a control. After incubation at 37^0C, the radius of inhibition around the well was measured in mm (Hamed and Abdelftah 2019; Kulshrestha and Patel 2021).

Figure 1: (A) Actinomycetes isolate PRO15 (B) Spore chain arrangement of isolate



RESULTS AND DISCUSSION

Isolation and Identification of Actinomycetes strain: A total of 36 isolates were obtained which were subjected for the biosynthesis of nanoparticles. Out of all, two isolates (Represented as strain PRO15 and PRO 16) showed promising results in the synthesis of gold nanoparticles. In the present investigation, one of the isolates (PRO15) was used for further studies. The isolate was identified as Streptomyces sp. by referring to Bergey's Manual of Determinative Bacteriology, Systematic bacteriology, and the International Streptomyces Project guidelines (Shirling and Gottlieb 1966; Holt et al. 2000; Hamed and Abdelftah 2019). The microscopic studies revealed the spore chain arrangement of the isolate was rectus. And the electron microscopic studies showed the spore surface ornamentation was smooth (Fig: 1).

Streptomyces sp. PRO15 (C) Scanning Electron Microscopy image of Streptomyces sp. PRO15: The isolate was Gram-positive and non-acid-fast. Different biochemical tests were performed, and positive results were observed for catalase, starch hydrolysis, casein hydrolysis, gelatine hydrolysis. A negative result was observed for H_2S production (Table 1). The carbohydrate fermentation studies showed alkali production in sucrose, fructose, maltose, and starch, acid Production in Dextrose, and No gas production was observed (Table 2).

Table 1. Morphological and biochemical characterization of Streptomyces sp. PRO 15

Sl. No	Test	Result
1	Aerial mycelium	White
2	Substrate Mycelium	Cream
3	Diffusible pigment	None
4	Starch Hydrolysis	+
5	Gelatine Hydrolysis	+
6	Casein Hydrolysis	+
7	Catalase	+
8	H_2S Production	−
9	Grams Staining	Gram-positive
10	Acid-Fast staining	Non-acid fast

+: Positive, −: Negative

Table 2. Carbohydrate utilization Test of Streptomyces sp. PRO 15

Isolate	Dextrose			Sucrose			Fructose			Maltose			Starch		
PRO 15	G	A	Al	G	A	Al	G	A	Al	G	A	Al	G	A	Al
	-	+	-	-	-	+	-	-	+	-	-	+	-	-	+

*G: Gas, A: Acid, Al: Alkali

Synthesis and characterization of Bio-Gold nanoparticle: Actinomycetes are known to produce more than 10,000 bioactive metabolites (Berdy 2005). Only three of the actinomycetes genera viz. Streptomyces, Rhodococcus, Thermomonospora are the potent producers of nanoparticles (Ahamad et al. 2003; Sadhasivam et al. 2010; Ranjitha and Rai 2017). Applications of Nanoparticles have attained much importance in biomedical applications. But the development is still in its initial stage (Skladanowski et al. 2017; Hamed and Abdelftah 2019).

The change in the reaction mixture from yellow to pinkish-purple indicated the production of nanoparticles (Figure: 2). The difference in color in the reaction was due to the reduction of $HAuCl_4$ and the surface plasmon resonance (SPRs) effect (Philip 2008; Manivasagan et al. 2013). Using the results of Skladanowski et al. (2017), the synthesis of GNPs by actinomycetes cell-free extract was confirmed. Synthesis of GNPs by different methods like biomass starving was also reported. The UV-Vis spectroscopy study revealed the absorbance peak of the Bio GNPs at 520nm (Figure: 3), which represented the formation of GNPs. The surface plasmon resonance (SPR) of GNPs ranges from 520 to 560nm.

Figure 2: Biosynthesised gold nanoparticle from Streptomyces sp. PRO 15. (A) Reaction mixture before incubation (B) Reaction mixture after incubation

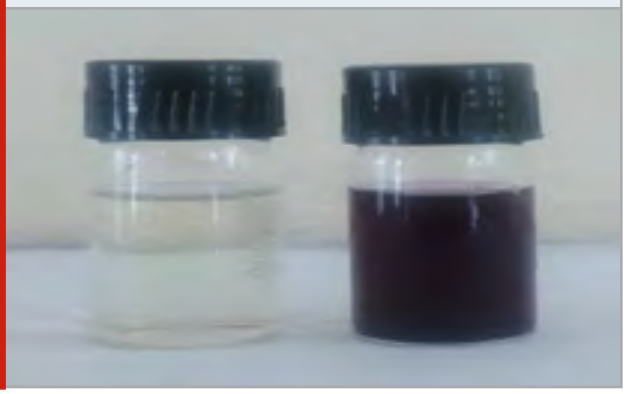


Figure 3: UV-Vis absorption of Bio GNPs synthesized from Streptomyces sp PRO15

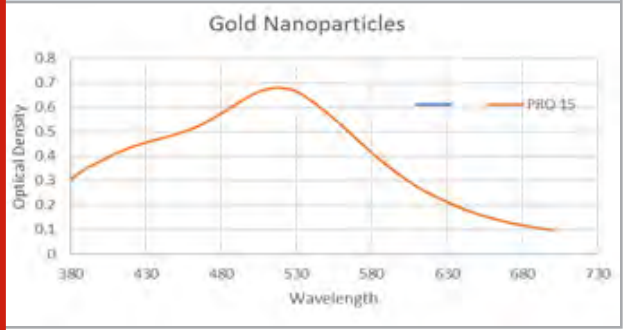
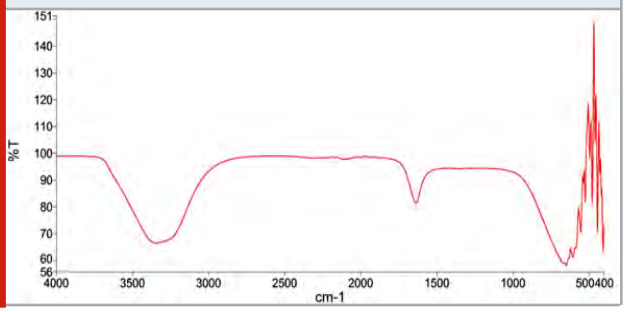


Figure 4: FTIR spectra of Bio GNPs Synthesised from Streptomyces sp. PRO 15.



The peak height indicated the concentration of GNPs, and the shift of the peak towards a higher wavelength indicated an increase in size. The free electrons present in the metal nanoparticles gave the SPR absorption band due to the combined vibration from the electrons of nanoparticles with the light wave (Noginov et al. 2007). FTIR spectroscopy was carried out to determine the biomolecules present on the nanoparticle, which were responsible for formation and stabilization. The IR spectral readings of GNPs showed a strong band at 3340.85cm^{-1},1637.9 cm^{-1}, and 648.27 m^{-1}. The

peak that appeared at 3340 cm^{-1} showed Carbon -Hydrogen bond(=-C-H) stretching, representing the alkyne group. The band seen at 1637.9 cm-1 had characteristics of -C=C- stretching that represented the alkene group. The band seen at 648.27 cm^{-1} also represented C-I stretching represented halo compounds (Figure: 4). Sivalingam et al. (2012) and Tamuly et al. (2013) reported the presence of biomolecules on biosynthesized nanoparticles that act as capping agents and are responsible for stabilizing nanoparticles.

SEM analysis: The scanning electron images of synthesized Bio GNPs showed that the nanoparticles were irregular in shape. (Figure: 5). Similar results were reported by Sadhasivam et al. (2012).

Figure 5: Scanning electron microscopic imaging of Bio GNPs synthesized from Streptomyces sp. PRO 15.

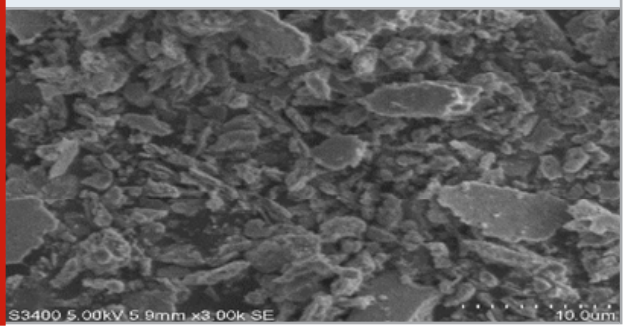


Figure 6: Size distribution of GNPs synthesized from Streptomyces sp. PRO 15

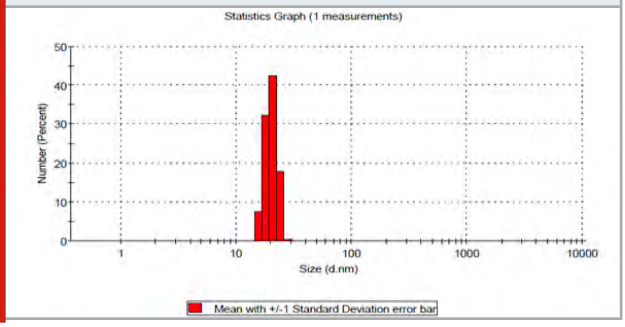
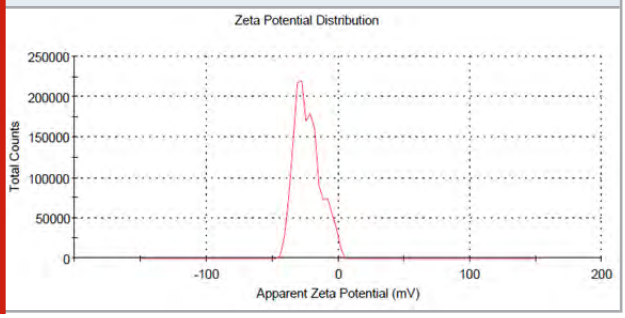


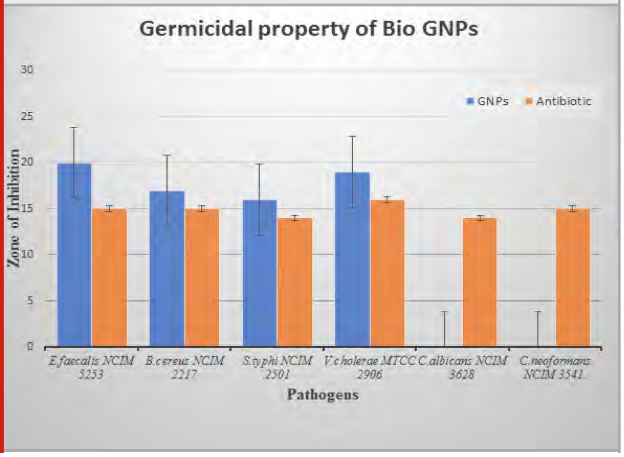
Figure 7: Zeta potential distribution of GNPs synthesized from Streptomyces sp. PRO 15.



Dynamic light scattering and Zeta Potential Analysis: Dynamic light scattering (DLS) was used to determine the partial size of GNPs present in the colloidal solution. The average size of GNPs synthesized using Streptomyces sp. PRO 15 was found to be 24.97 nm (Figure:6). The Zeta potential of the bio-GNPs was noted to be -23.2 mV (Figure: 7). Results indicated the stability and monodispersed of the synthesized GNPs in the colloidal solution. Gade et al. (2010) and Rai et al. (2015) reported that the Zeta potential analysis of Bio GNPs provides information about the stability of synthesized nanoparticles. Zeta potential valve closer to -30mV indicates the metal nanoparticles to be highly stable.

Germicidal effect: Germicidal effect of Bio-Gold nanoparticles synthesized from *Streptomyces* sp. PRO 15 is shown in Figure:8. Germicidal property of Bio-GNPs against microbial pathogens *E. faecalis* showed the highest inhibition zone of 20mm followed by *V. cholerae* 19mm, *B. cereus* 17mm, *S. typhi* 16mm. The synthesized Bio-GNPs showed no inhibition against fungal pathogens *C. albicans* and *C. neoformans*. Standard antibiotics Ampicillin and Nystatin showed a zone of inhibition around 14mm to 16mm. Similar results were found by GNPs synthesized from *Streptomyces* sp. against pathogenic microbes (Kumar et al. 2016; Składanowski et al. 2017; Hamed and Abdelftah 2019).

Figure 8: Germicidal property of Bio GNPS synthesized from Streptomyces sp. PRO 15



CONCLUSION

The findings of the present study highlight the use of Actinomycetes as a biological source to synthesize Gold Nanoparticles (GNPs). The study was focused on developing an effective, low-cost protocol for the production of GNPs. This method does not employ toxic agents during the process, making it environmentally friendly, and the GNPs can be used in clinical applications. This study also Investigate the effect of GNPs as a germicidal agent against microbial pathogens, which may lead to the development of a new strategy to tackle antimicrobial resistance.

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Biomedical Communication

Effects of Regular Jogging on Functional Capabilities of the Cardiovascular System in Students

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ABSTRACT

Physiological alternation of work and rest, systematic physical training, a balanced diet are necessary to maintain the health of the body and especially its cardiovascular system. Prolonged abstinence from regular physical activity causes a pronounced weakening of health and leads to the development of pathology of the heart and blood vessels. This study examined 48 boys. Of these, two observation groups were collected. The experimental group consisted of 25 young men with vegetative-vascular dystonia of the hypertensive type. The second observation group was the control group. It consisted of 23 healthy youths. Initially, both observation groups had a comparable low level of physical fitness. In the course of the study, the same physical activity was used in both observation groups for three months in the form of athletics jogging for 5 days a week for half an hour a day. Methods for assessing physical condition and methods of statistical processing were used. Systematic athletics jogging in young people with disorders of the cardiovascular system leads to an optimization of the heart rate and a stable optimization of the blood pressure level. At the same time, their complete normalization was possible after 3 months of jogging. As a result of regular jogging, the surveyed experienced an increase in the volume of the vital capacity of their lungs. Against this background, there was an increase in the general level of physical fitness, speed-strength characteristics and general endurance. It can be considered that regular jogging is a very effective means of health improvement in adolescence in conditions of beginning functional disorders of the cardiovascular system.

KEY WORDS: ADOLESCENCE, ATHLETICS, CARDIOVASCULAR SYSTEM, HEALTH IMPROVEMENT, PHYSICAL ACTIVITY.

INTRODUCTION

The way of life of a person in modern society is very different from the way of life of all previous generations. Previously, human life and work were associated with great muscular efforts of the entire human community. Currently, the situation has changed and the lack of muscle activity is growing (Fayzullina et al. 2020; Karpov et al. 2020). This situation has a very detrimental effect on the entire organism of our contemporaries. Currently, a massive deficit of physical activity is becoming evident in society for about 80-90% of the working-age population. This position of the lice weakens the adaptive capabilities, primarily of the cardiovascular system (Kotova et al. 2017; Makurina et al. 2020). In many economically developed countries, this is accompanied by the appearance of an asthenic state in a large part of the population, which ultimately leads to the appearance of diseases of the cardiovascular system (Skoryatina and Zavalishina 2017; Karpov et al. 2021a).

Low physical activity detrains the heart and blood vessels, leading to the development of their dysfunctions at a young age. The situation is further aggravated by the fact that most of the population lives in cities, sometimes with a difficult environmental situation, which further weakens the human body (Vorobyeva et al. 2018; Vorobyeva et al. 2020). There is an opinion that in order to level this difficult situation, it is necessary to consistently apply recreational running, which,

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being an aerobic load, strengthens the entire human body. It is recognized that with regular jogging, you can achieve greater productivity of physical and mental work and greater resistance to fatigue (Zavalishina et al. 2021a).

A very effective option for jogging is regular jogging in a free mode, which has previously shown its great potential for optimizing the work of individual internal organs. Another serious advantage is the absence of any material costs when using them (Zavalishina 2018a). The health-improving effect of jogging is associated with an increase in its background, the stimulation of the muscles of the lower extremities, which stimulates the return of venous blood to the heart in a strictly aerobic mode (Zavalishina 2018b; Zavalishina et al. 2021b). It is possible to engage in regular jogging not only at home, but also in the conditions of a sanatorium or resort stay, complementing any types of treatment (Lantsberg 1988; Karpov et al. 2021b).

The most rational way to strengthen the muscles of the heart is to run with a gradual increase in distance and speed, shortening and shortening the periods of stopping or moving in steps (Zavalishina 2018c). Serious health-improving potential of running is also associated with a clear positive effect on the conjugation of the work of the heart, blood vessels and the nervous system (Zavalishina 2018d; Zavalishina 2018e). In view of the strong health-improving effect of jogging, it seemed important to assess their influence on the functional parameters of the cardiovascular system in young men with emerging dysfunction in its work (Karpov et al. 2021c). The aim of the study is to determine the level of effectiveness of regular athletics jogging in relation to the improvement of the cardiovascular system in adolescence.

MATERIAL AND METHODS

The study was approved by the local ethics committee of the Russian State Social University on September 15, 2017 (Protocol No. 9). 48 people of adolescence who are university students were examined. Of these, two observation groups were collected, on which the entire study was carried out. The experimental group consisted of 25 young men (18.7±1.2 years) with a diagnosis of hypertensive vegetative-vascular dystonia confirmed by doctors. These examinees had a burdened heredity of hypertension - both or one of their parents had a diagnosis of hypertension. The second observation group was called the control group.

It consisted of 23 healthy boys (19.0±1.0 years). This group of surveyed did not have a hereditary burden of cardiovascular diseases. Initially, both observation groups had a comparable low level of physical fitness. All of them experienced regular, but very moderate physical activity during academic physical education classes at the university. During the study, both observation groups used the same physical activity in the form of athletics jogging. Jogging sessions were carried out at a free pace for 5 days a week for half an hour a day on a horizontal plane or on a surface with an upward or downward slope of no more than 15º. The study was carried out during the warm season.

The general condition of the subjects who formed both observation groups was assessed daily. The registration of the indicators taken into account was carried out using traditional methods twice - when included in the observation groups and after 3 months of jogging, that is, at the end of the study. In all cases, the value of the vital capacity of the lungs, the value of the pulse, and the level of blood pressure were recorded (Skoryatina and Zavalishina 2017; Makurina et al. 2020). In the subjects of both observation groups, the state of physical capabilities was determined using a number of dosed physical loads. We took into account the results of a 12-minute run test (called the Cooper test), the results of a run at a distance of 20 meters, the results of a test for flexion and extension of the arms while staying in an emphasis on a bench for 10 seconds and the result of a test for throwing a ball weighing 1 kg from vertical position. Statistical processing of the results found during the study was carried out by the Student's t-test.

RESULTS AND DISCUSSION

The observed young men, who made up both study groups, in the course of regular jogging, demonstrated an improvement in their general well-being. In all cases, their feeling of fatigue decreased by the end of the day, headaches stopped, emotional instability subsided, and sleep normalized. Frequent episodes of destabilization of the blood pressure level in the form of its increase, which initially existed in all representatives of the experimental group, ceased to arise by the end of the study. In both groups, by the end of the observation, a feeling of cheerfulness and high performance throughout the day became habitual. The results obtained during the study are presented in Table 1.

Systematic physical activity in the form of jogging in both groups of young men led to the normalization of their pulse values. During the observation period, this indicator for the representatives of the control group decreased by 10.9%, and for the young men of the experimental group by 40.7%. The level of systolic blood pressure decreased in boys of the control group by 2.6%, in representatives of the experimental group by 15.1%. The value of diastolic blood pressure also decreased in both groups by 3.4% and 19.6%, respectively. The obtained dynamics of these indicators indicates the elimination of the phenomena of vegetative-vascular dystonia in the young men of the experimental group as a result of regular three-month runs. Systematic jogging for three months led to an increase in the volume of vital lung capacity in young men. In the control group, this indicator increased by 25.7%, in the experimental group, it increased by 34.9%. At the same time, an increase in the degree of physical fitness was noted in all those observed.

In both groups of young men, speed-power characteristics significantly increased, and endurance increased. In the control group, there was an acceleration of running at a distance of 20 m by 32.3%, the number of flexions and extensions of arms in a position with an emphasis lying during 10 seconds increased by 54.2%, the distance at which it was possible to throw the ball weighing 1 kg from an upright position increased by 39.7%, the value of the

Cooper's test result increased by 43.8%.The indicators of the physical capabilities of the young men in the experimental group improved more significantly and by the end of the observation they tended to be higher than in the control group. For young men in the experimental group, the acceleration of running at a distance of 20 m was 43.7%, the number of flexions and extensions of the arms in a prone position for 10 seconds increased by 52.9%, the distance of throwing the ball from a vertical position increased by 34, 3% with an increase in the Cooper test by 44.4%.

Table 1. The results of the observation in both study groups

Indicators of physical condition	At the start of the study, M±m		At the end of the study, M±m	
	Control group, n=23	Experimental group, n=25	Control group, n=23	Experimental group, n=25
Pulse rate, beats / minute	80.1±0.28	88.9±0.22 p<0.05	66.3±0.32 p_1<0.001	63.2±0.29 p_1< 0.001
The value of systolic blood pressure, mm Hg.	125.6±0.86	138.1±0.60 p<0.05	128.4±0.76	120.0±0.94 p_1<0.05
Diastolic blood pressure, mm Hg	83.0±0.32	92.6±0.78 p<0.05	80.2±0.43	77.4±0.34 p1<0.05
Lung vital capacity, l	2.88±0.24	2.81±0.19	3.62±0.17 p1<0.001	3.79±0.14 p1< 0.01
Running time for a distance of 20 m, s	4.5±0.05	4.6±0.07	3.4±0.06 p1<0.001	3.0±0.09 p1< 0,01
The number of flexions and extensions of the arms in the lying position for 10 s, the number of times	4.8±0.39	5.7±0.36	7.4±0.27 p1<0.001	7.8±0.37 p1< 0.001
Distance of throwing a ball weighing 1 kg from a vertical position, cm	342.7±1.75	344.4±2.16	458.1±1.92	462.6±2.16
Cooper's test value, m	1360.1±2.62	1372.2±2.46	1956.7±3.02	1981.0±21.1

Legend: p - reliability of the initial differences between the indicators of both observation groups; p1-reliability of differences in the dynamics of observation.

The results obtained indicated a clear healing effect of regular jogging in a free mode in adolescents suffering from vegetative-vascular dystonia. Such training successfully normalizes the functioning of the heart, vascular tone, and increases the level of physical fitness (Zavalishina 2018g). Despite the presence of initial dysfunction in the experimental group, by the end of the observation, a slightly more preferable level of indicators recorded in the study was achieved. Apparently, this is largely due to the more responsible approach of young men in this group to jogging because of the desire to overcome their existing disorders in the work of the cardiovascular system (Zavalishina 2018h; Zavalishina 2018i). According to the results of the study, regular jogging has reason to be considered as one of the most effective means of overcoming dysfunction of the cardiovascular system in adolescence.

CONCLUSION

The study suggests that the systematic muscle activity in the form of jogging among young people is always accompanied by general recovery. With dysfunctions of the cardiovascular system in adolescence, they contribute to the normalization of heart rate and blood pressure levels. As a result of regular and feasible running loads in young men who previously had dysfunction of the cardiovascular system, an increase in the volume of the pulmonary vital capacity occurs. This is accompanied by a significant increase in the observed level of physical fitness, an increase in speed-power properties and the level of general endurance. The achieved effects allow us to speak about the presence of great health-improving opportunities for regular jogging. Especially for persons with vegetative-vascular dystonia. Regular jogging exercises stabilize heart activity and optimize vascular tone. Increases the level of physical fitness. It becomes clear that regular jogging is a very effective means of optimizing the work of the cardiovascular system in young men with functional disorders (Karpov et al. 2021c).

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Biotechnological Communication

Structural Characterization of (E)-10-Hydroxy-4,6,8,10-Tetramethyldodec-4-en-3-one Bioactive Molecule from Marine *Paenibacillus macerans* isolate

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ABSTRACT

In our going research on identifying interesting bioactive molecules from various natural resources, marine microbes represent an interesting category because of their large biodiversity and unexploited background to the scientific world. Many of the marine natural products for those particularly procured from macroorganisms, have already undergone clinical trials. So, the research on natural products derived from marine microbes have tremendously increased in the recent years due to the demand of bioactive molecules having pharmaceutical potential for various target applications. Hence, the present study was undertaken with an aim to purify and chemically characterize a potential bioactive molecule from a symbiotic marine bacterium, *Paenibacillus macerans* SAM 9 isolated from a sea anemone, Heteractis aurora. After microbial fermentation under standardized conditions, the bioactive molecule was ethyl acetate extracted and purified using a Reverse Phase-HPLC method. The purified fraction revealed promising antimicrobial activity against a clinical human pathogen, *Proteus mirabilis* which was extensively studied for the chemical and structural characterization. Using FT-IR, ^{1}H and ^{13}C NMR, GC and MS/MS spectral studies, the bioactive molecule was identified as (E)-10-hydroxy-4,6,8,10-tetramethyldodec-4-en-3-one. This is the first report that the (E)-10-hydroxy-4,6,8,10-tetramethyldodec-4-en-3-one is identified from a bacterium and from this study, this bioactive molecule can easily be procured using the standardized conditions explored in this investigation. Moreover, this study acts as the baseline data for the purification and future exploration of this bioactive molecule regarding its extensive biomedical significance. The aim of this study was to become a suitable milestone and act as a credible source for future studies.

KEY WORDS: BIOACTIVE, (E)-10-HYDROXY-4,6,8,10-TETRAMETHYLDODEC-4-EN-3-ONE, MARINE SYMBIOTIC BACTERIUM, RP-HPLC, SPECTRAL STUDIES.

INTRODUCTION

Presently many bioactive compounds have been derived from various terrestrial resources. Hence, the exploration of interesting, novel and needful biochemical metabolites to the biomedical applications is becoming low (Jensen and Fenical 2000). So, the need of new sources for the exploration of bioactive substances is much required nowadays. In this condition, marine ecosystem offer enormous biodiversity and have potential resources to find interesting bioactive molecules with numerous worthwhile applications. As per the Global Biodiversity Assessment by the United Nations Environment Programme, there are 178,000 marine species belonging to 34 phyla have been documented which reveals that the ocean's biodiversity represents 50% of the globe's whole biodiversity and making marine microbes a sustainable and appreciable source of interesting bioactive molecules discovery (Jimeno et al. 2004; Balan et al. 2012; Mitra and Zaman 2016; Balan et al. 2019b; Yadav 2021). Many of the marine natural products for those particularly procured from macroorganisms, have already undergone clinical trials (Newman and Cragg 2004; Yadav 2021).

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So, research on natural products derived from marine microbes have tremendously increased in the recent years due to the demand of bioactive molecules having pharmaceutical potential for various target applications (Andersen and Williams 2000; Balan 2014). In contrast to macroorganisms, microorganisms represent appreciable natural product resources having the advantage of feasible and sustainable production of secondary metabolites under large quantities at reasonable cost as well as the fermentation of the whole microbial species (Waites et al. 2009; Balan et al. 2013). Further, adaptive species of the marine ecosystem characterize very special and unique conditions which differ them from those found in other habitats. Furthermore, marine microbes sometimes accumulate structurally unique bioactive molecules which are not relatively present in terrestrial sources (Bhakuni and Rawat 2006; Balan and Jayalakshmi 2013; Balan et al. 2019a; Varijakzhan et al. 2021).

Considering the above facts, the present study aims to identify the bioactive molecule produced by a marine symbiotic bacterium, *Paenibacillus macerans* SAM 9. Further, this strain has already been reported for its potential antimicrobial activity against a panel of clinical human pathogens in our previous study and this present study structurally characterize the molecule responsible for the bioactivity through a systematic scheme of spectral characterization after purification (Bharathi et al. 2021). To the best of our knowledge, *Paenibacillus macerans* have never been reported earlier for the identification and its detailed structural characterization of a bioactive molecule. Hence, exploring the chemical nature of this bioactive molecule makes sense in regards with the novelty and this warrants the present investigation.

MATERIAL AND METHODS

For the symbiotic marine bacterium and culture conditions, *Paenibacillus macerans* SAM 9 is the bioactive molecule producing symbiotic marine bacterium used in this study which was previously isolated from the sea anemone, *Heteractis aurora* and the 16S rRNA partial sequence of this bacterium was submitted in the NCBI GenBank with the accession no. MT941031.1 (Bharathi 2021). In our earlier study, this marine bacterium was reported to produce a bioactive molecule which exhibited promising antimicrobial activities against many clinically isolated human pathogens. The fermentation production of this bioactive molecule was carried out here for the purification and detailed structural characterization using a previously optimized microbiological medium intended for the maximum production of this bioactive compound with the following conditions: 1% maltose, 0.5% ammonium nitrate, 34 ppt salinity, pH 8, 35°C temperature with an incubation period of 96 hrs, respectively. Further, the inoculum was prepared using 1% exponential phase culture in the same fermentation medium as described above, where the optical density (OD620 nm) of the inoculum was adjusted to 0.1 based on McFarland turbidity standard of 0.5, equivalent to the bacterial concentration of 1×10^8 cfu ml^{-1}. The fermentation was carried out in one litre conical flask with 400 ml working volume.

The extraction and HPLC purification of the bioactive molecule was done using optimized conditions. Production of bioactive molecule was carried out freshly and the bioactive molecule was extracted from the cell free supernatant using ethyl acetate after centrifugation at 3000 rpm for 30 min. After an overnight incubation, the organic phase containing the bioactive molecules was separated and rotary vacuum evaporated at 50ºC to a dried form. The dried form was subjected to purification of this bioactive molecule using a high-pressure liquid chromatography (HPLC). The dried crude form was initially dissolved in 3:2 ratio of 5 ml acetonitrile and methanol and passed through a 0.2 µm syringe filter. The purification was done by Reverse Phase (RP)—C18 silica gel (230–400 mesh) column at 30 °C temperature. The solvent system consisted of acetonitrile (solvent A) and methanol (solvent B) and the elutions were made at 0.5 ml/min flow rate using the stepwise gradient initiated from the ratio of 60:40, vol/vol (A:B) to the finalized ratio of 100:0, vol/vol (A:B). The absorbance of the eluate was measured at 210 nm. All the collected fractions were dried individually using rotatory vacuum evaporation and studied for the antimicrobial activity.

For antimicrobial assay, all the dried fractions of the HPLC purification were individually studied for antimicrobial activity using the most susceptible pathogen, Proteus mirabilis of this bioactive molecule as reported in our earlier study. The human clinical pathogen, *P. mirabilis* was kindly gifted by Rajah Muthiah Medical College Hospital, Annamalai University, Tamil Nadu, India. The antimicrobial activity of the purified fractions was evaluated using microtiter plate-based assay method (Casey et al. 2004). The assay was conducted in a 24-well flat bottom polystyrene microtitre plates with lids (Tarsons, India). Briefly, the well plates were filled with 100 µL of sterile tryptone soy broth followed by the addition of 10 µL purified (dried) fraction dissolved in phosphate buffer saline (PBS: 10 mM KH2PO4/K2HPO4 and 150mM NaCl with pH adjusted to 7.0) and 10 µL of the pathogenic *P. mirabilis* culture.

The growth control well plates was done by the addition of 100 µL of tryptone soy broth, 10 µL pathogenic *P. mirabilis* culture and 10 µL phosphate buffer saline (without any addition of purified fraction). The susceptibility control well plate was carried out with 100 µL of tryptone soy broth, 10 µL pathogenic *P. mirabilis* culture and 10 µL of 4mg/ml streptomycin (commercial antibiotic). After inoculation, the well plates were covered with lids and incubated at 37 °C for 48 hrs. After incubation, the absorbance of the well plates was recorded at 600 nm using a microplate reader (Biotek Elx808, WI, USA), where the assays were carried out in triplicate. The growth inhibition percentages of the individual purified fractions were calculated as follows:

$$\% \text{ Growth inhibition} = [(1 - (As/Ac)] \times 100$$

Where As represents the absorbance of the well with purified fractions and Ac represents the absorbance of the growth control well (without any added bioactive sample). The fraction showed the appreciable antimicrobial activity was studied for further detailed chemical and structural characterization of the bioactive molecule. For the chemical

and structural characterization of purified bioactive molecule, the purified fraction showing antimicrobial activity against the clinical human pathogen *P. mirabilis* was identified for the bioactive molecule present in it. The compound was chemical analyzed and structurally characterized as per the following instrumentation procedures.

Fourier Transform Infrared Spectroscopy (FT-IR) was used to identify the characteristic functional groups of the bioactive molecule present in the purified bioactive fraction (Mani et al. 2016a). Five milligram of the purified molecule was dispersed in dry powdery potassium bromide (KBr), the resultant was thoroughly mixed using a mortar and pressed at 6 bars pressure within 2 min to form a KBr thin disc. The resulting disc was placed in a sample cup of a diffuse reflectance accessory. Infrared absorption spectrum was analyzed on an IR affinity, FT-IR system (Shimadzu, Japan) at a spectral resolution of 4 cm-1 with an average of 10scans in the wavenumber range between 400–4000 cm^{-1}.

^{1}H & ^{13}C Nuclear Magnetic Resonance Spectroscopy (NMR) spectrum of the purified bioactive molecule was analyzed using a Bruker AV600NMR spectrometer (Germany) in which the deuterated $CDCl_3$ was used as the solvent. Chemical shifts were expressed in parts per million (ppm) downfield from an internal standard of tetramethylsilane (TMS). The purified fraction containing the bioactive molecule was characterized using a Gas Chromatography and Mass Spectroscopy (GC-MS). GC-MS was performed 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. 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 – 300 m/z with an Ion trap EI^{+}.

RESULTS AND DISCUSSION

Extraction and purification of bioactive compounds are important steps as it determines the level of significance and use as well as identification of the particular molecule (Thangaraj 2016). Initially, the production of bioactive molecule by the marine symbiotic bacterium, *P. macerans* SAM 9 was done as per the optimized fermentation conditions from our earlier study. Further, the ethyl acetate solvent was used for the extraction of this bioactive molecule (Fig. 1) in which organic solvent was pooled and concentrated using rotary vacuum evaporation. The concentrated dried form was further used for the purified with a HPLC and there were seven fractions obtained (Fig. 2). All the fractions were individually collected and dried with rotary vacuum evaporation and studied for the antimicrobial activity against the human clinical pathogen *P. mirabilis* (Thangaraj 2016).

Among the fractions, fifth fraction alone showed antimicrobial activity with 85.5 ± 2.3 % which confirmed the presence of bioactive molecule present in it and rest of the fractions revealed no antimicrobial activity. The fifth fraction evidenced antimicrobial activity was subjected to further detailed chemical and structural characterization studies. Similar to this study, earlier investigations have noted that the significance of non-polar solvents followed by reverse phase column chromatography for the purification of bioactive compounds (Mani et al. 2016b; Balan et al. 2017; Balan et al. 2019b).

Figure 1: Solvent extraction of the bioactive molecule produced by *P. macerans* SAM 9

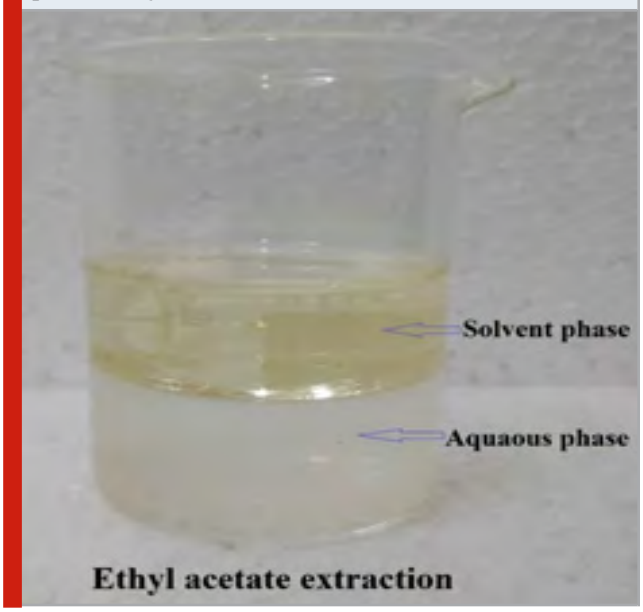


Figure 2: HPLC chromatogram of the crude ethyl acetate extract from the cell free supernatant of *P. macerans* SAM 9

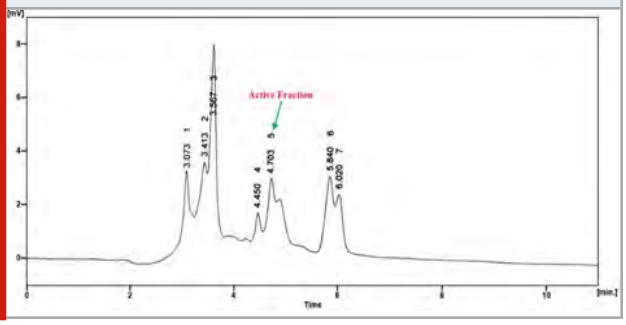
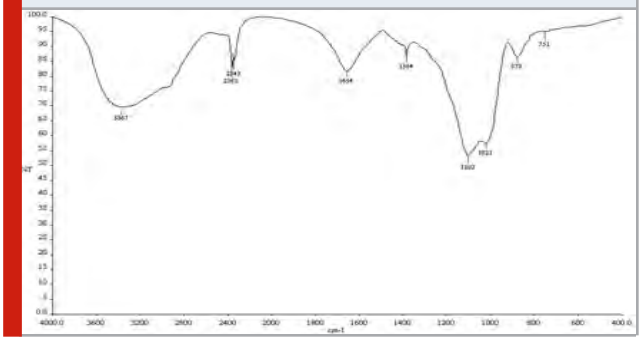


Figure 3: FT-IR Spectrum of the purified bioactive molecule, (E)-10-hydroxy-4,6,8,10-tetramethyldodec-4-en-3-one



FT-IR spectrum of the purified bioactive molecule revealed all the functional groups of (E)-10-hydroxy-4,6,8,10-tetramethyldodec-4-en-3-one as shown in the Figure 3. The terminal alkane group (CH_3) present at the wavelengths of 1384cm^{-1}, similarly, intermittent alkane groups (CH_2 and CH) of the bioactive molecule were observed at 751 and 1021cm^{-1} wavelengths. Further, the alkene group (C=CH) was depicted at wavelength of 1654 and 879cm^{-1}. Likewise, the most significant groups of ether (C=O-CH_2) were predicted at 2343 and 2361cm^{-1} and the aldehyde (C-OH) was evidenced at 1102 and 3367cm^{-1} wavelengths, respectively.

^{1}H and ^{13}C NMR spectrum of the purified bioactive molecule represented all the functional groups of (E)-10-hydroxy-4,6,8,10-tetramethyldodec-4-en-3-one as shown in the figures 4 and 5. ^{1}H NMR spectrum revealed the existence of terminal alkane hydrogen (CH_3) within chemical shifts of 0.8821 – 0.9674ppm, similarly, the chemical shifts between 1.0296 – 1.3532ppm and 1.4147 – 1.5908ppm responsible for the presence of aliphatic alkane hydrogen atoms (CH_2 and CH). The presence of protons (hydrogen atom) in the alkene group (C=CH) evidenced from the chemical shifts between 5.5038 to 5.7548ppm. Further, the protons within the chemical shifts, 4.0532 – 4.1310ppm and 9.8021 – 9.9802ppm corresponds to the existence of aldehyde hydrogen (C-OH) and ether hydrogen (C=O-CH_2) which were the significant functional groups of (E)-10-hydroxy-4,6,8,10-tetramethyldodec-4-en-3-one.

Figure 4: 1H NMR spectrum of the purified bioactive compound, (E)-10-hydroxy-4,6,8,10-tetramethyldodec-4-en-3-one

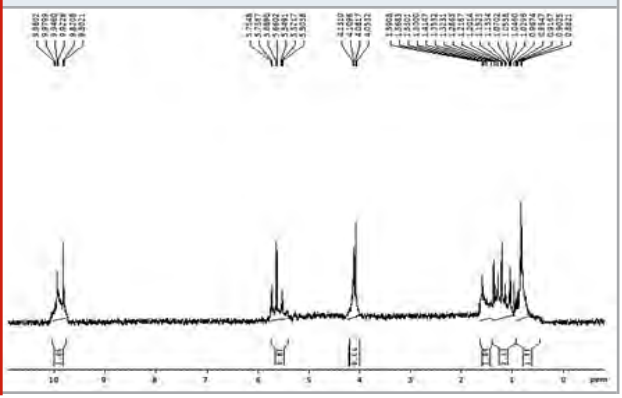
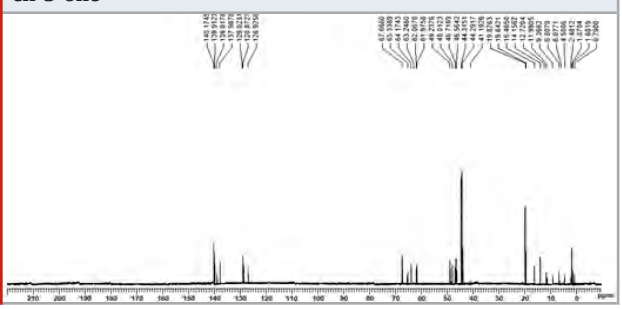


Figure 5: ^{13}C NMR Spectrum of the purified bioactive compound, (E)-10-hydroxy-4,6,8,10-tetramethyldodec-4-en-3-one



Furthermore, ^{13}C NMR spectrum of this bioactive molecule revealed the presence of same functional groups of (E)-10-hydroxy-4,6,8,10-tetramethyldodec-4-en-3-one as predicted in the ^{1}H NMR spectrum. The presence of terminal alkane carbon (CH_3) and alkane carbons (CH_2 and CH) were observed between the chemical shifts of 0.7980 – 4.5806ppm, 6.0771 – 9.3662ppm and 11.9905 – 19.8763ppm, similarly, the presence of alkene carbon (C=CH) was predicted within 126.9256 – 140.1745ppm. Moreover, the carbon atom from the significant functional groups of aldehydes (C-OH) and carbonyl (C=O) groups were represented within the chemical shifts of 61.9756 – 67.6680ppm and 41.1926 – 49.2376ppm.

GC-MS analysis of purified bioactive molecule was illustrated in the Figure 6. GC spectrum exhibited only one molecular ion peak at the retention time of 18.61 min which revealed the presence of single bioactive molecule in the purified fraction (Fig. 6a). Further, the mass spectral analysis evidenced that the peak was (E)-10-hydroxy-4,6,8,10-tetramethyldodec-4-en-3-one with a molecular mass of methylated and non-methylated form with 268.4 and 253.3 MW (Fig. 6b). Further, the interpretation of the mass spectrum showed exact sequential molecular weight pattern of (E)-10-hydroxy-4,6,8,10-tetramethyldodec-4-en-3-one under methylated form which were 29.1, 43.1, 71.1, 87.2, 98.2, 101.2, 111.2, 129.2, 139.2, 143.2, 153.3, 171.3, 181.3, 184.3, 195.3, 211.3, 239.4, 253.4 and 268.4 MW, respectively (Fig. 6c).

Furthermore, this molecular pattern revealed exact similarity of (E)-10-hydroxy-4,6,8,10-tetramethyldodec-4-en-3-one with the available NIST database used for the molecular identification of chemical compounds. All these above information using FT-IR, NMR and GC-MS analysis confirmed that the purified bioactive molecule was a (E)-10-hydroxy-4,6,8,10-tetramethyldodec-4-en-3-one and exact molecular structure was illustrated in the figure 7 based on the above spectral information. Similar to the present study, various earlier studies have used FT-IR, NMR and MS spectral analysis for the structural identification of different bioactive molecules.

Recently, a bioactive molecule purified from a marine yeast, *Cyberlindnera saturnus* SBPN named as Cybersan revealed a molecular structure of three galactose followed by a heptadecanoic acid which was active against several human clinical pathogen bacteria (Balan et al. 2019a). Likewise, Staphylosan, a bioactive glycolipid compound purified from a marine bacterium, *Staphylococcus saprophyticus* SBPS-15 showed a molecular structure of mannose-mannose-oleic acid which exhibited antimicrobial and biofilm activity against many marine biofilm forming bacteria and Pontifactin a bioactive lipopeptide compound identified from a marine *Pontibacter korlensis* SBK-47 evidenced the chemical structure of palmitic acid-serine-aspartic acid-valine-serine-serine which was biological active against many MTCC pathogenic bacterial strains (Balan et al. 2016; Balan et al. 2019b). The bioactive molecule identified in this study, (E)-10-hydroxy-4,6,8,10-tetramethyldodec-4-en-3-one was earlier isolated from an actinomycete, *Actinopolyspora erythraea* YIM 90600 which was named

as *Actinopolysporins* C. Till date, this metabolite is not reported from any bacterial species, henceforth, this is the first study reporting the production of this biomolecule from a bacterium originated from marine environment (Zhao et al. 2011; Balan et al. 2019b).

Figure 6: (a) GC, (b) MS and (c) Interpretation based on molecular weight pattern of the methylated (E)-10-hydroxy-4,6,8,10-tetramethyldodec-4-en-3-one

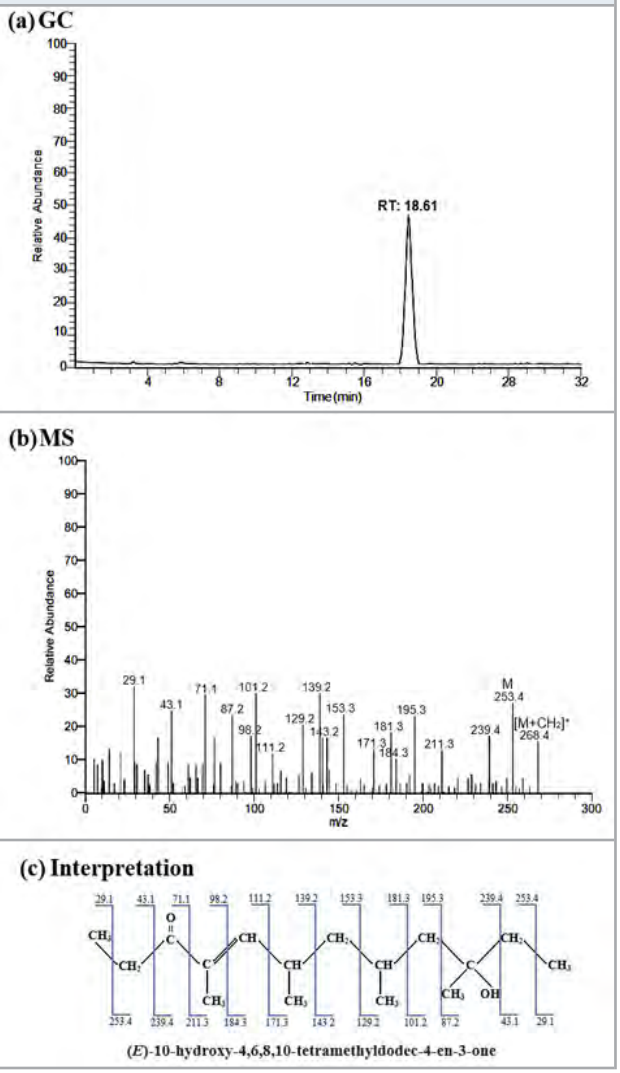
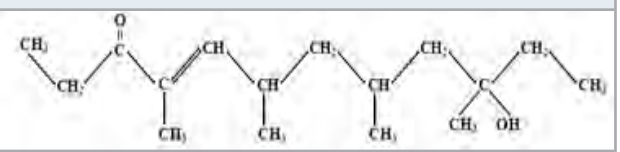


Figure 7: Structure of (E)-10-hydroxy-4,6,8,10-tetramethyldodec-4-en-3-one



CONCLUSION

The findings of the present study reveals that the bioactive molecule from the marine symbiotic bacterium, P. macerans SAM 9 was extracted with ethyl acetate and purified using a Reverse Phase C18 silica gel column. The purified bioactive molecules were identified as (E)-10-hydroxy-4,6,8,10-tetramethyldodec-4-en-3-one based on various spectral studies. The present investigation proved that this bacterium is the promising source for the appreciable production of this excellent bioactive compound, further, this study holds as the baseline data for the exploration of many biomedical properties of this bioactive molecule in near future.

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

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Biomedical Communication

Preventive Effects of Korean Red Ginseng and *Lepidium sativum* Seeds on Induced Osteoporosis in Female Rats

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ABSTRACT

Traditional therapies for osteoporosis have undesirable side effects so that new therapeutic agents have been improved to use natural products and herbal extracts for therapeutic purposes. Therefore, the aim of this work was to evaluate the combined effects of *Panax ginseng* and *Lepidum sativum* (LS) seeds on biochemical changes and histological alterations of induced osteoporosis in female rats. Fifty adult female *albino* rats (200-320 g) were divided into five groups (n=10 rats/group). Group I, control group included non-ovariectomized untreated rats. Group II, ovariectomized (OVX) rats. Group III, OVX rats administrated with KRG extract (100 mg\kg bw\day). Group IV, OVX rats administrated with LS seeds extract (20 mg\kg bw\day). Group V, OVX rats administrated with both extracts of KRG and LS seeds. In the present study, the ameliorative effects of *Ginseng* and LS seeds on bone remodeling against induced osteoporosis by ovariectomy were demonstrated by increasing serum levels of calcium (Ca), phosphorus (P), magnesium (Mg), vitamin D as well as decreasing serum alkaline phosphatase (ALP). The histological study of cortical and trabecular femur bone revealed that the bone resorption and trabecular bone loss were increased in the OVX group. The administration of *Ginseng*, LS and their combination showed a marked improvement in trabecular bone and a significant reduction in bone loss. *Ginseng* and LS seed extracts and their combination possess an ameliorative effect against ovariectomy-induced osteoporosis which may be attributed to presence of wide range phytochemicals in the studied extracts.

KEY WORDS: GINSENG; LEPIDIUM SATIVUM; OSTEOPOROSIS; OVARIECTOMY; RATS.

INTRODUCTION

Osteoporosis is a progressive skeletal disorder represented by reduced bone mineral density and microarchitectural bone deterioration (Qaseem et al., 2008), leading to increased bone fragility and fractures which is commonly occurred in the wrist, hip or spine (De Martinis et al., 2021). More than 200 million people have been affected by osteoporosis worldwide (Khan et al., 2019) and estimated at 21.4- 39.5% of Saudi population (Barzanji et al., 2013; Alwahhabi, 2015; Barake et al., 2021). Several types of therapies have been designed to manage postmenopausal osteoporosis on using medications that inhibit bone resorption (e.g.estrogen, calcitonin and bisphosphonates)or stimulate bone formation (e.g. growth hormone, sodium fluoride, and parathyroid hormone) (Elkomy and Elsaid, 2015; Hagino et al., 2021). However, these medications have side effects (such as malignant reproductive tissues, cancers, gastrointestinal issues, and reduced skeletal strength) (Cao et al., 2014; Albert and Wood, 2021).

Accordingly, to reduce the side effect of currently used medicines, new therapeutic agents have been improved for efficacy therapy. There is a growing attitude in recent studies to use natural products and herbal extracts that possess active components (Khalil et al., 2008; Andargie et al., 2021). Among the traditional herbs is Ginseng (*Panax ginseng* C. A. Meyer) which has been widely used in Eastern countries for thousands of years (Kim et al., 2015; Kim et al., 2016). Ginseng belongs to the Araliaceae family and has been widely grown in Korea (Siddiqi et al., 2013; Sharma and Goyal, 2015). Nowadays, all Ginseng products such as Korean Red Ginseng (KRG) have been used as alternative medicine in Europe as well as in Asian countries (Burden et al., 2021).

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Ginsenosides are active components of Ginseng, which include protopanaxatriol, oleanane and protopanaxadiol (Bai et al., 2018). Ginseng has been reportedly a source of many effective substances including proteins, polysaccharides, polyacetylenes, and phenols compounds (Sharma and Goyal, 2015). Ginseng presents different biological, medical and pharmaceutical functions such as anti-oxidant, anti-inflammatory, anti-allergic, anti-cancer, anti-fungal, anti-stress, memory enhancement; promoting liver function and protein synthesis, prevention of arteriosclerosis and hypertension (Sharma and Goyal, 2015; Karmazyn and Gan, 2021). In addition to its anti-osteoporosis activity by increasing bone formation in bone marrow cells and pre-osteoblast cells, amelioration of osteoporosis, as well as altering osteo-clastogenesis and bone resorption (Jung et al., 2021).

However, the mechanisms anti-osteoporotic effects of Ginseng are not clear. *Lepidium sativum* (LS) or Hab Al- Rashad "as locally termed in Saudi Arabia" belongs to the Brassicaceae family and have is widely grown in the Middle East (Elshal et al., 2013). LS seeds contain the most important phytochemicals and plant phytosterols have proved their potential anti-oxidant, anti-inflammatory and protection against certain diseases. It also contains a flavonoid compound that protects the human body from oxidative stress (Yadav et al., 2011; Mahassni and Al-Reemi, 2013). LS has been used as a traditional herbal healer to diabetes control, hypertension, renal disease, cancer prevention, cardiovascular diseases protection and Phytotherap (Mahassni and Al-Reemi, 2013; Juma et al., 2007). LS seeds are particularly used in healing bone fracture (Juma et al., 2007; Abdul-Jabbar and Mohammed, 2021). The aim of this work is to evaluate the combined effects of Ginseng and *Lepidium sativum* (LS) on biochemical changes and histological alterations of induced osteoporosis in female rats.

MATERIAL AND METHODS

Korean Red Ginseng (KRG) *Panax ginseng* was purchased from Korea Ginseng Corporation, Korea. KRG dose was prepared according to literature (Kim et al., 2015; Shin et al., 2021). The aqueous extract was given orally to experimental rats at a dose of 100 mg/kg bw/day for one month. *Lepidium sativum* (LS) seeds were obtained from a local herb shop in Jeddah, Saudi Arabia. The aqueous extract of LS seeds was prepared according to the traditional Moroccan method described earlier (Eddouks et al., 2005). The obtained extract was administrated orally at a dose of 20 mg/kg bw/day for one month to different groups of rats. A total of 50 healthy female albino rats (200-320 g, 3 months old) were obtained from the Animal Experimental Unit at KFMRC (King Fahd Medical Research Center), King Abdulaziz University, Jeddah, Saudi Arabia with approved ethical permission. The animals were fed with a standard diet manufactured by Grain Silos and Flour Mills Organization, Saudi Arabia. A bilateral ovariectomy was performed for 40 rats (called after as OVX rats) according to standardized procedures as previously described (Idris, 2012; Liu et al., 2015).

The animals were divided into 5 groups (n=10 rats/ group). Group I: untreated rats were fed on a standard diet and served as a negative control group. Group II (OVX group): OVX rats fed on a standard diet and served as a positive control group. Group III (Ginseng group): OVX rats administrated with the KRG extract (100 mg\kg bw\day). Group IV (LS group): OVX rats administrated with the extract of LS seeds (20 mg\kg bw\day). Group V (Ginseng+LS group): OVX rats administrated with a combination of KRG extract (100 mg\kg bw\day) and LS seeds extract (20 mg\kg bw\day).

Blood samples collected from rats were centrifuged at 3000 rpm for 15 min. The serum was separated and frozen at -20 °C until used for analysis. Serum phosphorus (P), calcium (Ca), magnesium (Mg) and alkaline phosphatase (ALP) were determined by commercial kits purchased from Siemens Healthcare Diagnostics Inc., Newark, USA. Serum Ca, P and Mg were measured as bone profile markers whereas ALP was measured as a bone turnover marker to monitor the bone metabolism. Serum levels of vitamin D were measured for the assessment of vitamin D sufficiency. [25(OH) D] kit was purchased from Roche Cobas Diagnostics International Ltd., Mannheim, Germany.

After blood collecting, rats were sacrificed, and the right and left femurs were harvested. Each femur was carefully cleaned, weight was recorded, and then stored in 10% neutral buffered formalin. The bone was decalcified with 5% aqueous nitric acid for one week. Once decalcified, the specimens were followed by routine histological processing and were embedded in paraffin. The paraffin sections (5 µm thick) were deparaffinized and stained by hematoxylin and eosin (H&E) for light microscopic examination as described in the literature (Mawhinney et al., 1984). Data was analyzed by using SPSS statistics (version 15). All variables were double-checked for their values and outliers. Data were presented as mean ± SD. The parametric tests were used with normally distributed variables. The variance analysis was conducted to explore relationships between subgroups.

RESULTS AND DISCUSSION

Osteoporosis, represented by the rapid reduction and deterioration of the micro-architecture of bone tissue, causing an increased bone susceptibility to bone fracture has been a serious health problem worldwide (Genant et al., 2007; Hamed et al., 2021). Bone remodeling is described by the speed of two opposite processes; bone formation and bone deterioration. Any imbalance in bone remodeling may lead to bone mass loss (Hamed et al., 2021). With all their clinical and pharmacological advantages, the hormone replacement therapy has been widely applicable for osteoporosis. Recently, medicinal herbal extracts have been investigated for their therapeutic effects related to bone remodeling (Kurasawa, 2005; Guo et al., 2021).

Ginseng has been reported for its beneficial effects in the treatment of osteoporosis and increased bone formation although its effects and mechanism of action are not fully understood. Studies have shown that ginseng has an effective estrogenic activity on the OVX rats, due to

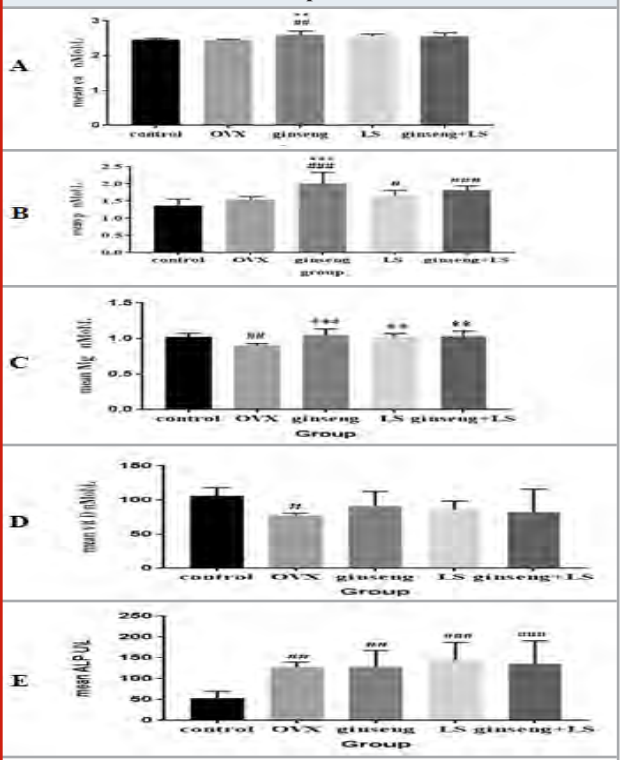
its ability to stimulate the biosynthesis of estrogen and increase the quantity of receptor in the reproductive target tissues. The aglycone part of ginsenosides has shown to be similar to several steroids in structure, especially female hormones (Lee et al., 2021). This encouraged us to explore the beneficial effects of Ginseng, LS seed and their combination against OVX-induced osteoporosis in rats. The results for the ameliorative effects of Ginseng, LS seed and their combined extract on some biochemical parameters of ovariectomized – induced bone loss were presentedin (Table 1). Serum Ca level was significantly increased with the treatment of Ginseng compared to control group and OVX group, (P< 0.01, Fig. 1 A). Level of serum P was significantly increased in Ginseng group (P< 0.001), LS group (P< 0.05), and their combination group (P< 0. 001) compared to the control group, Fig. (1 B). Furthermore, Ginseng administration increased the serum phosphorus concentration compared to the OVX group, P< 0.001.

Table 1. Serum biochemical parameters among different rat groups

Parameters	Control group	OVX group	Ginseng group	LS group	Ginseng + LS group
Serum Ca(nmol\L)	2.46± 0.04	2.05 ± 0.02	2.59 ± 0.11	2.55 ± 0.06	2.55± 0.09
Serum P (nmol\L)	1.37± 0.18	1.54 ± 0.09	2.01 ± 0.32	1.65 ± 0.15	1.82± 0.13
Serum Mg (nmol\L)	1.02 ± 0.05	0.90 ± 0.02	1.05 ± 0.09	1.02 ± 0.05	1.02± 0.08
Serum Vit D (nmol\L)	106.17± 11.97	77.47±3.47	91.26 ± 21.31	85.95±12.50	82.17 ± 33.78
Serum ALP (U\L)	53.63 ± 16.39	128.70 ± 11.06	128.28 ± 39.40	145.25 ± 41.22	135.86± 54.90

Values are presented as (mean ± SD); Ca: calcium; P: phosphorus; Mg: magnesium; Vit D: vitamin D; ALP: alkaline phosphatase

Figure 1: Effect of ginseng, *Lepidium sativum* seed and their combination on biochemical parameters.



Values are mean ± SD. #Significant P<0.05 versus control. *Significant P<0.05 versus OVX.

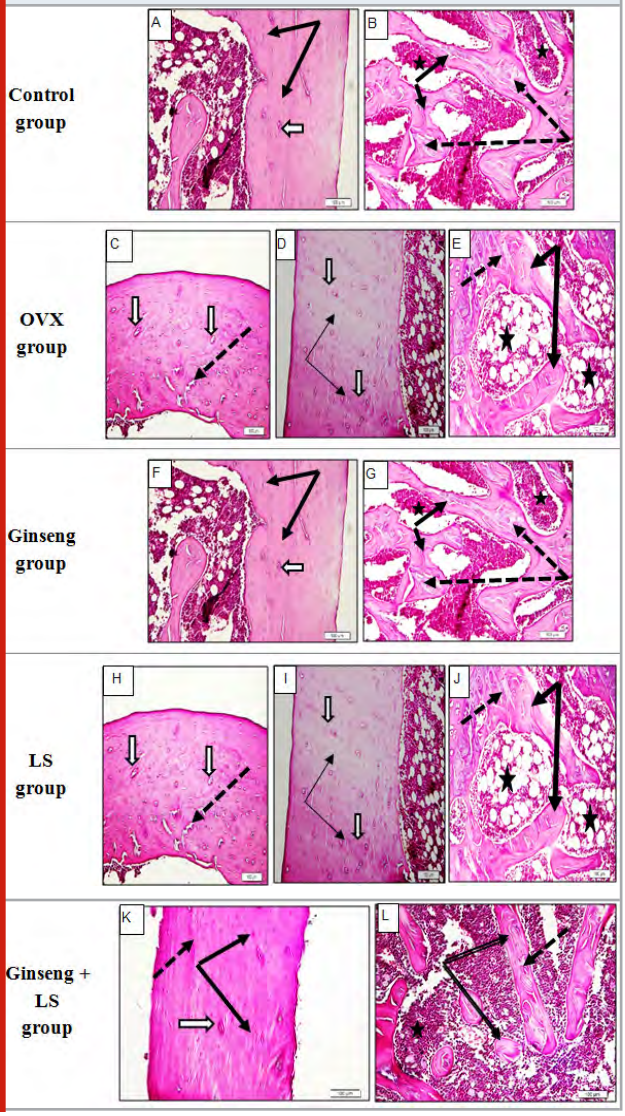
##Significant P<0.01 versus control. **Significant P<0.01 versus OVX. ###Significant P<0.001 versus control. ***Significant P<0.001 versus OVX.

Figure (1 C) showed that serum Mg was significantly decreased in the OVX group compared to the control group, at P<0.01. Comparing to the OVX group; Mg levels were significantly increased in Ginseng group (P<0.001), LS group (P<0.01) and Ginseng + LS group (P<0.01). Due to the ovariectomy, vitamin D was showed a significant decreasing in the OVX group compared to control rats (P< 0.05); while the administration of herbal extracts (Ginseng, LS seed or their combination) caused a none-statistical improvement in its level, Fig. (1 D). The serum ALP levels were significantly increased in ovariectomy rats (OVX group, P<0.01) and all treatment groups (Ginseng, LS and Ginseng + LS) compared with the control group (P<0.01, P<0.001 and P<0.001, respectively), Fig. (1 E). In preceding studies, the researchers demonstrated the ameliorative effect of Ginseng and LS seed against mass reduction and bone strength induced by ovariectomy in rats through assessing the serum levels of Ca, P, Mg, Vit D and ALP. These parameters and other bone turnover markers have been widely used as research tools to measure the effect of various drugs on bone remodel in (Bahlou, 2006; Nathawat et al., 2015; Gabr et al., 2017).

The results of Table 1 showed the ameliorative effect of Ginseng, LS seed and their combined extracts on ovariectomized- induced bone loss. The results revealed that serum calcium level was decreased in OVX rats compared to untreated control rats. Serum calcium indirectly reflected the metabolism of bones. The administration of Ginseng and LS seed extract resulted a significantly increasing in the calcium level. These results are consistent with those that showed the serum Ca level was decreased in ovariectomized rats. These findings are supported by the fact that ovarian hormone deficiency following ovariectomy, is marked by reduced intestinal calcium absorption and may contribute

to the bone loss (Ouichou et al., 2008; Hassan et al., 2013). The results are also supported by the fact that, the level of calcitonin recorded a significant decrease in ovariectomized rats and may be attributed to the concentration of calcium level can be considered the principal stimulus for the secretion of calcitonin by C-cell. Low calcium in blood can stimulate for calcitonin secretion is diminished (Zhao et al., 2021).

Figure 2: Histological sections of female rat femur of five groups, (H&E ×200).



The serum phosphate and magnesium were found significantly increased in the serum of the OVX rats who received the plant extracts compared to untreated OVX rats (Yu et al., 2021). The result of them showed gradually increase comparing with OVX, the highest increase shown in treatment with Ginseng. Serum vitamin D was found to be significantly decreased in OVX compared to control rats. The administration of Ginseng and LS seed extracts by the OVX rats caused none-significant increasing in its level compared to the OVX rats. This increasing in the vitamin D level resulted from the stimulatory effect of the extract in vitamin D synthesis in the cell. The results are in line with the fact that herbal extracts can ameliorate the effect of OVX on biochemical parameters (Zhao et al., 2011). The serum of ALP, among the bone formation markers, increased in its level reflected bone turnover whereas its decreasing indicated to bone formation. In ovariectomized rats, ALP level was found to increase dramatically due to an increase in bone deterioration turnover. The results of this work also indicated a significant increasing in ALP level in the OVX rats compared to control rats, but the level of ALP tended to decrease after administration of herbal extracts. These suggest that herbal extracts possess compounds that stimulate bone density and inhibit bone resorption (Nagaki et al., 2021).

In the present study, histology of Group I (Control group): In the present study, the structure of female rat femur was similar to those described in the literatures. The cortical (compact) bone of femur shaft was showed smooth outer surface (Thick black arrows) and characterized by normal Haversian system architecture (white arrows) (Fig. 2A). Trabecular (spongy) bones showed normal branching and anastomosing trabecula (black arrows) separated by the bone marrow spaces (stars) (Fig. 2B). Histology of Group II (OVX group): In this study, the femur bone of OVX rats showed random irregular cracking of femur cortical (compact) bone (black arrows), widening of Haversian canals (white arrow), areas of bone rarefication (star), lines of calcification (dotted arrows), and in severe cases areas of bone fracture and loss (Fig. 2C & D). Trabecular (spongy) bone showed wide bone marrow cavities (stars) and thin cracked trabeculae (white arrows) a thin cracked trabecula (Fig. 2E). Histology of Group III (Ginseng group): Administration of Ginseng to OVX rats provided marked protection against OVX- induced boney changes in the rat femur. Cortical (compact) bone showed a normal appearance with absence of any bone cracking that seen in untreated rats.

Figure (2 F) showed cortical bone with narrow Haversian canals (white arrow) and normal osteocyte lacunae (black arrows). Although trabecular (spongy) bone looked to be thicker, it showed central regions of incomplete ossification. Figure (2 G) showed normal wide trabeculae (black arrows) with bluish areas of calcification (dotted arrows) and bone marrow cavities (stars). Histology of Group IV (LS group): Administration of LS seeds to OVX rats provided more protection against OVX- induced bone changes in rat femur compared to Ginseng. Compact cortical bone showed a well-organized Haversian system with absence of any bone cracking that seen in untreated rats. Figure (2H&I) showed narrow Haversian canals (white arrow) and normal osteocyte lacunae (black arrows).

Fine cracking was still observed (dotted arrow). Spongy trabecular bone looked thicker and more ossified than Ginseng group. Figure (2 J) showed cortical bone with normal wide trabeculae (black arrows) with bluish areas of calcification (dotted arrows) and bone marrow cavities (stars). Histology of Group V (Ginseng + LS group): The administration of a combination of Ginseng and LS seed

to OVX rats provided more protection against OVX-induced boney changes in rat femur compared to Ginseng or LS seeds alone. Compact cortical bone showed a well-organized Haversian system with absence of any bone cracking. Figure (2 K) showed cortical bone with narrow Haversian canals (white arrow) and normal osteocyte lacunae (black arrows). Spongy trabecular bone looked thicker and more ossified compared to previous groups. Spongy bone showed in Fig. (2 L) normal wide trabeculae (black arrows) with areas of complete calcification (dotted arrows) and bone marrow cavities (stars).

The histological study of both cortical and trabecular femur bone revealed that the bone resorption and trabecular bone loss were increased in the OVX group. The administration of Ginseng, LS and their combined extracts showed a marked improvement in trabecular bone and a significant reduction in bone loss. These results were agreed with the biochemical investigations. Microscopically, the control rats revealed no histopathological alteration in the cortical and trabecular femur bone which is in agreement with Vallet et al., (2021) who demonstrated that the left femur of the normal control rats did not show any histopathological changes in the cortical bone with osteoblasts proliferation as well as normal bony trabeculae. The present study suggested an increase in bone loss which was supported by osteoporotic changes found through histological examination in OVX rats. The histopathological findings in Ginseng group are well supported by the findings of Leiu et al. (2015) as Ginseng stimulates the process that increases bone density by inhibiting bone resorption. The positive effect on bone density of the Ginseng extract is probably due to increasing the protein biosynthesis and nucleic acid (Han et al., 2021).

Previous histopathological studies supported a potential protective role of LS against induced osteoporosis in male and female rats. El-Zawahry et al., (2017), and Abdul-Jabbar et al., (2021) reported that the sections of rats treated with LS extract revealed a marked improvement compared with osteoporotic rats, and the cortical bone thickness was very similar to the normal control group. In both studies, the bony trabeculae were partially recovered near the normal structure and appeared to be more persistent with the smaller bone marrow spaces.

CONCLUSION

In conclusion, the present results indicated that Ginseng and LS seed extracts and their combined extracts reduced the bone loss in ovariectomized rats, possibly through inhibiting bone resorption process. Phytochemical constituents of extracts such as flavonoids, saponins, and phenolics could probably be responsible for the anti-osteopathic activities of plants. Therefore, the extract from Ginseng and LS has the potential to be used in the development of clinical anti-osteoporotic agents.

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Biomedical Communication

Parental Knowledge on Using Sports-Related Mouth-guards in School-age Saudi Children

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ABSTRACT

Sports is considered as one of the most common reasons of the orofacial trauma in school-age children. Sports-related mouth guards minimize the risk of injuries in school-age children. The aim of this study is to assess the knowledge of parents about the use of mouth guards by their children during different activities. Cross-sectional survey composed of a questionnaire with 21 multiple choice questions was randomly distributed to 319 parents who have school-age children (7-12 years old). Out of the 319 subjects responded to this study, there were 77.4% mothers. The distribution of characteristics of children shows 56.1% were male. About 65.8% of the children play sports. The maximum number of children (38.3%) had facial bruising followed by other type of injuries. The primary baby teeth were affected in the injury for 38.3% of children. Only 16 (34%) of these injured children had visited the dentist. The knowledge towards mouth guard was assessed among the parents, where only 17.9% of them were familiar with sports mouth guard. A small number 5(1.6%) of them responded that their children use mouth guard during sports. Those who did not use mouth guard, 248(81.3%) had responded as "lack of information about it" as the main reason and 43.9% of them were aware that mouth guard can prevent oral/dental injury. In conclusion there is a lack of parental knowledge regarding the importance of use of mouth guards in reducing dental injuries in school-age children. Professional and parents should be educated regarding the use of mouth guards during activities.

KEY WORDS: KNOWLEDGE, MOUTHGUARD, PARENT, SPORTS-RELATED, SCHOOL-AGE.

INTRODUCTION

Traumatic dental injuries are common in pre-school, school-age and young adults making up 5% of all injuries of which the patients seek treatment for (Petersson et al., 1997; Andreasen et al., 2007). One-third of all dental injuries found to be related to sport activities (Nowjack & Gift, 1996; Collins et al., 2015). The most common type of injuries of the orofacial area is the dentofacial injuries (Tuna & Ozel, 2014). Dental injuries range from concussions to more serious injuries of the oral cavity involving surrounding tooth structures (Al-Obaida, 2010). Avulsion, which is the total loss of the tooth out of its socket, is the most serious and crucial type of all injuries to the oral cavity. It represents 1-16% of dental trauma in which the highest incidence found in children 7-11 years old, with the maxillary central incisor being 80% the most affected tooth (Al-Shamiri et al., 2015; Goswami et al., 2017, Borris et al., 2019, Li et al., 2021).

The primary teeth injuries showed a prevalence that varies from 11% to 30 %, while the permanent teeth range between 2.6 % - 50 % (Tuna & Ozel, 2014). Moreover, children who are encouraged to participate in contact sports are at great risk of dental injuries (Tuna & Ozel, 2014; Collins et al., 2015). The type of dental trauma is affected by multiple factors, including the force direction, the impaction of the force, and the resilience of the impacting object (Collins et al., 2015). Avulsed teeth, TMJ dysfunction, subluxations, lip laceration, crown fracture, extrusion, intrusion, alveolar bone fracture, and root fracture are common consequences

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to trauma (Petersson et al., 1997; Newsome et al., 2001; Collins et al., 2015).

Full-contact sports such as boxing and football are not the only risks for dental injuries, it also can occur in other sports such as in basketball or baseball (Collins et al., 2015). Among various sports, softball, basketball, and baseball have a relatively high risk of dental injury with low prevalence of mouth guard use. Complications of dentofacial injuries can result in dental crowding, abscesses, and failure of eruption of the permanent teeth, spacing, and hypoplasia (Tuna & Ozel, 2014). Thus, it is important to raise the awareness of sports-related dental damage risk and emphasize on the role of dentists to prevent such accidents by recommending the use of mouthguards in all sorts of sports (Tuna & Ozel, 2014; Green, 2017, Ramakrishnan et al., 2019, Al-Habib, 2019, Ayesha et al., 2020, Li et al., 2021).

The literature supports the use of mouth guards in reducing and preventing dental trauma (Onyeaso, 2004). Mouth guards, also called a gum shield, defined as "a resilient device or appliance placed inside the mouth (or inside and outside), to reduce mouth injuries, particularly to teeth and surrounding structures" (Pribble et al., 2004). Custom-made well-fitting mouth guards which are fabricated by the dentist have shown to deliver the best protection (Mekayarajjananonth et al., 1999). More than one visit to the dentist is needed to receive the custom-made well-fitting mouth guard. The process includes taking dental impression, study models, and laboratory construction steps (Westerman et al., 2002). In addition, a stock mouth guard is a preformed thermoplastic tray that loosely fits over the teeth. They can be found in sports stores and are used without modification. Nevertheless, they provide limited protection (ADA, 2006).

Mouth-formed 'boil and bite' mouth guards can also be bought from the stores. However, they are molded after being merged in hot water and softened to fit the individual's mouth by pressure from the fingers, cheeks and tongue having a higher protection than stock mouth guards (Westerman et al., 2002 Ramakrishnan et al., 2019, Al-Habib, 2019, Ayesha et al., 2020, Li et al., 2021). Pribble et al. (2004) studied the factors that influence parental perceptions regarding mandatory mouthguard use in competitive youth soccer and found that few athletes wear mouthguards during the sport activities and recommended to make more efforts by health specialists and sport organizations to educate the parents about orofacial injuries and mouthguard use. O' Malley et al. (2012) made a survey to assess school and sports club policy on mouthguard use in sport, they concluded that the dental profession and individual practitioners should encourage the use of mouthguard for children during sport and be responsible for the development of policies in schools and sporting organizations. Moreover, Quaranta et al. (2014) conducted their study to assess the knowledge of the parents of the primary school children to plan corrective actions and the results showed that parents lack awareness, knowledge and skills to prevent or manage dental trauma.

In addition, Green (2017) did a meta-analyses study of oral injuries and reported that individuals who don't use mouthguards have a higher incidence by 1.6-1.9 times compared with those who use it. Although this form of preventive device is easy to use, available, effective and inexpensive it has been underutilized (Collins et al., 2015). Therefore, the aim of this study is to assess parental knowledge regarding the use of mouth guard during sport activities among school-age children in Riyadh city.

MATERIAL AND METHODS

A cross sectional study was conducted to assess parental knowledge regarding the use of mouth guard during various sport activities among their school-age children (7-12 years old) in Riyadh city. This study was reviewed and approved by Institutional Review Board (IRB) (no. E-19-4353) of King Saud University in Riyadh, KSA. Three hundred and nineteen participants were selected using stratified-cluster random sampling technique. The city of Riyadh was divided into 5 regions and parents were informed that their participation is voluntary in the study. The questionnaire included questions about the children who were classified as Cl I or II status according to the American Society of Anesthesiologists classification. A power analysis was done to specify the appropriate sample size. To achieve a significance level at the 95th percentile confidence level and power of 80 percent, with 0.5 estimated effect size, the sample size was calculated to be 200 subjects.

A validated, self-report questionnaire containing 21 multiple-choice questions was distributed manually (hand-to-hand) to the parents in which each parent signed consent form declaring that their participation in this study is voluntary and their personal information will not be shared and only be used for study purposes. Furthermore, the purpose of the study was explained to the parents in an understandable language, as well, the questionnaire was written in their familiar language (Arabic). The questionnaire was divided into 3 main sections, the demographic data, the age of the child and his/her educational level, and knowledge, management and experience of the child with dental trauma.

The inclusion criteria are parents of Saudi, ASA Cl I and II, school-age (7-12 years old) children who live in Riyadh. Parents who have more than one child falling in the selected age group, the oldest child will be used as the index child for this study. Non-Saudi, medically compromised children, children not living in Riyadh, and less than 7 or more than 12 years old children will be excluded from this study. Moreover, a pilot study was performed and one of the authors (ZH) assessed the parental understanding of the questionnaire. The data collected were analyzed using descriptive statistics and chi-square test for association.

RESULTS AND DISCUSSION

Data were analyzed using SPSS 24.0 version statistical software. Descriptive statistics (frequencies and percentages) were used to describe the categorical variables. Pearson's Chi-square test was used to observe the association between categorical variables. A p-value of ≤ 0.05 was used to report the statistical significance. Out of the 319 subjects

responded to this study, there were 77.4% mothers, 90% of parents were in age group of 31-40 and >40 years, more than 50% of them had bachelor degree, 81.2% of them with middle level socio-economic status and about 50% of them had 4- 6 children. (Table 1)

Table 1. Distribution of Characteristics of Study subjects (n=319)

Study variables	No. (%)
Parent who responded	
Father	72(22.6)
Mother	247(77.4)
Age of parent (in years)	
20-30	33(10.3)
31-40	145(45.5)
>40	141(44.2)
Level of education	
Illiterate	--
Elementary	--
Intermediate	10(3.1)
Secondary	57(17.9)
Bachelor	215(67.4)
Higher Education	37(11.6)
Socio-economic status	
Low	259(81.2)
Middle	134(42)
High	50(15.7)
Number of children	
1-3	10(3.1)
4-6	157(49.2)
>6	28(8.8)

Table 2. Distribution of characteristics of Children

Study variables	No. (%)
Gender	
Male	179(56.1)
Female	140(43.9)
Age (in years)	
7	56(17.6)
8	34(10.7)
9	51(16)
10	51(16)
11	50(15.7)
12	77(24.1)
Grade of Child(n=317)	
Second	66(20.8)
Third	46(14.5)
Fourth	72(22.7)
Fifth	53(16.7)
Sixth	31(9.8)
Seventh	26(8.2)
Other	23(7.2)

The distribution of characteristics of children shows 56.1% were male and their age was between 7 to 12 years and belongs to the grade between 2nd and 7th. (Table 2).

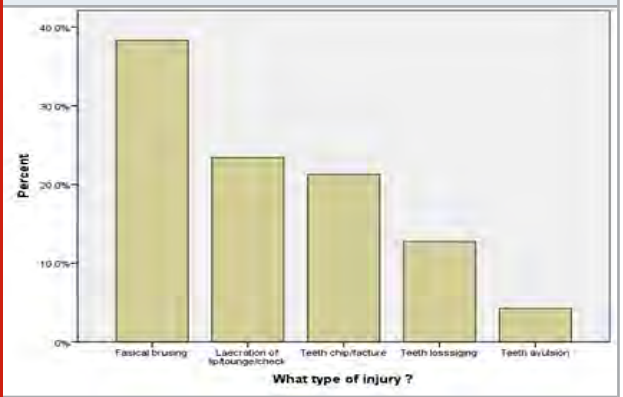
Table 3. Distribution of variables related to Child dental trauma and its management

Study variables	No. (%)
Do your child play sports?	
Yes	210(65.8)
No	109(34.2)
Type of sport Contact/collision (football, martial arts, wrestling, boxing) Limited contact/impact (basketball, cycling, gymnastics, skating, squash, volleyball)	158(49.5)
Strenuous contact (tennis, weightlifting,	61(19.1)
swimming) Moderately strenuous contact (badminton, table tennis)	7(2.2)
Non strenuous contact (archery, golf)	17(5.3)
Other sports	54(16.9)
Did your child ever sustain any facial or dental injury during sports?	
Yes	35(11)
No	272(85.3)
I don't know	12(3.8)
What type of injury? (n=47)	
Facial bruising	18(38.3)
Laceration of lip/tongue/cheek	11(23.4)
Teeth chip/fracture	10(21.3)
Teeth loosening	6(12.8)
Teeth avulsion (tooth/teeth completely fall out of the mouth)	2(4.3)
If you child has dental injury involving his/her teeth, which tooth/teeth was/were affected? (n=47)	
Primary/baby teeth	18(38.3)
Permanent/ adult teeth	5(10.6)
I don't know	24(5.1)
Have you visited a dentist following the accident that involved his/her tooth/teeth? (n=47)	
Yes	16(34)
No	13(27.7)
I don't remember	18(38.3)
If you visited the dentist after the accident, when did the visit take place? (n=46)	
Immediately after the accident	12(26.1)
One day after the accident	6(13)
During the first week of the accident	10(21.7)
After 1 month of the accident	18(39.1)

About 65.8% of the children play sports, in which 49.5% of them play Contact/collision (football, martial arts, wrestling, boxing), 19.1% of them play Limited contact/ impact (basketball, cycling, gymnastics, skating, squash,

volleyball). Only 35 (11%) of the total children had facial or dental injury during sports. The type of injury was expressed as facial bruising, laceration of lip/tongue/cheek, teeth chip/fracture, teeth loosening and teeth avulsion (tooth/teeth completely fall out of the mouth), where the maximum number of children (38.3%) had facial bruising followed by other type of injuries (Fig. 1). The primary baby teeth were affected in the injury for 38.3% of children. Only 16 (34%) of these injured children had visited the dentist, in which 12 (26.1%) had visited immediately after the injury. (Table3).

Figure 1: Types of Injuries



The knowledge towards mouth guard was assessed among the parents, where only 17.9% of them were familiar with sports mouth guard (Fig. 2). A small number 5(1.6%) of them responded that their children use mouth guard during sports. In these 5 responses, 3 of them mentioned about the use of commercial ready-made and 2 were used custom-made at the dentist office. Those who did not use mouth guard, 248(81.3%) had responded as "lack of information about it" as the reason followed by other reasons (it is expensive, it is uncomfortable, and it is not important). About 43.9% of them were aware that mouth guard can prevent oral/dental injury. Towards the use of mouth guard in future for their children, 64.3% of them had responded positively. (Table 4).

The association between characteristics of parents and their familiarity with mouth guard (Yes/No) shows no statistically significant association with the variables (parent, age of parent and socio-economic status). But the level of education of parent is statistically significantly associated with the response towards the familiarity with mouth guard, where higher proportion (32.4%) of the parents with higher education level were familiar with mouth guard when compared with other level of education (p=0.019). (Table 5).

This cross-sectional study was conducted to assess the parental knowledge regarding the use of mouth guard during sport activities among school-age children in Riyadh city. The study design offered an assessment of the parents of Saudi children aged (7-12) years old participating in different sport activities in terms of the knowledge, management and experience of the child with dental trauma. Survey sections were structured to offer a profile to clarify the amount of knowledge the parents possess regarding the use of sports-related mouth guards in school-age children to move dental trauma prevention more into mainstream parental dental education programs. More than half of the parents surveyed had bachelor degree (67.4%) and (81.2%) were of middle level socio-economic status. The latter implies that the practice of physical activities is related to socioeconomic status. Children from lower-income families usually are involved in activities with greater physical contact and violence. However, children with a higher socioeconomic status are more accustomed to using electronic devices (Corrêa Faria et al., 2015, Ayesha et al., 2020, Li et al., 2021).

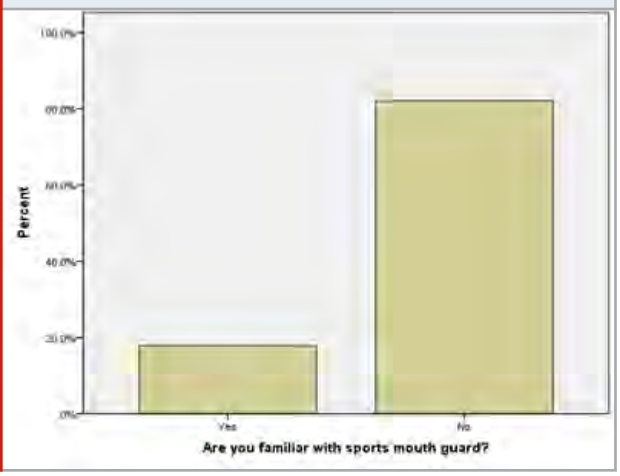
Table 4. Knowledge of about sports mouth guard

Items related to knowledge	No. (%)
Are you familiar with sports mouth guard?	
Yes	57(17.9)
No	262(82.1)
Does your child use mouth guard during sports?	
Yes	5(1.6)
No	314(98.4)
If your answer is yes to question 17, what type of mouth guard your child uses? (n=5)	
Custom-made at the dentist office	2(40)
Commercial ready-made (sports shop brand)	3(60)
If your answer is no to question 17, why are they not using it? (n=305)	
It is expensive	3(1)
It is uncomfortable	12(3.9)
It is not important	14(4.6)
lack of information about it	248(81.3)
Other	28(9.2)
Do you think that mouth guards can prevent oral / dental injury?	
Yes	140(43.9)
No	20(6.3)
I don't know	159(49.8)
Would you consider letting your child use it in the future?	
Yes	205(64.3)
No	8(2.5)
I don't know	106(33.2)

In addition, level of education of the parent showed to be statistically significantly associated with the response towards the familiarity with mouth guard, where higher proportion (32.4%) of the parents with higher education level were familiar with mouth guard when compared with other level of education (p=0.019). In contrast, Fakhruddin et al. (2007) found that mothers' educational level was not significant in association to the use of the mouth guards in their children. This could be explained by the different study design and the sample participated in the study. Participating in sport activities increases the risk of dental injuries among school-age children, as shown in this study,

the males participating in sports accounted more than half of the sample (56.1%).

Figure 2: Familiarity with Mouth guards



This is similar to Tsuchiya et al. (2017) study who reported a significantly higher prevalence (1.5 times) of sports-related dental injuries in male athletes than in female athletes. Sex differences in sports injuries may be caused by a complex mix of intrinsic (e.g., biological differences) and playing environmental factors (e.g., playing management). Out of the 319 participants, 35 (11%) reported that their children had facial or dental injury in which the facial bruising was the most common type among all injuries accounting for (38.3%) followed by the other type of injuries. In contrast, the study done by Goswami et al. (2017) found that chipping and fracture of teeth was the most reported injury with 28.7% more frequent than any other type. This difference could be explained that the participants in our study were the parents of the children not the children themselves, the type of sports the children were involved in at the time of the injury and the attention of the parents to the face when their child sustains a facial injury over other injuries. Moreover, 18 (38.3%) of parents for children who received trauma to their teeth, reported that the affected teeth were the primary teeth, while 5 (10.6%) reported that the permanent teeth were the affected ones.

Table 5. Association between Knowledge of sports mouth guard and characteristics of study subjects

Characteristics	Are you familiar with sports mouth guard		X2- value	p-value
	Yes	No		
Parent who responded				
Father	13(18.1)	59(81.9)	0.002	0.962
Mother	44(17.8)	203(82.2)		
Age of parent (in years)				
20-30	4(12.1)	29(87.9)	1.162	0.559
31-40	25(17.2)	120(82.8)		
>40	28(19.9)	113(80.1)		
Level of education				
Intermediate	2(20)	8(80)	9.963	0.019*
Secondary	4(7)	53(93)		
Bachelor	39(18.1)	176(81.9)		
Higher Education	12(32.4)	25(67.6)		
Socio-economic status	2(20)	8(80)	4.261	0.119
Low	41(15.8)	218(84.2)		
Middle	14(28)	36(72)		
High				

*Statistically significant

On the contrary, Borris et al. (2019) showed that permanent teeth with trauma (64.4%) were more than primary ones (55.6%). This is likely because younger children have immature motor coordination. Almost all the parents who reported their children received different type of injuries also reported they have visited the dentist at different timings post trauma. However, only (26.1%) of parents whom their children received dento-facial trauma visited the dentist immediately. In agreement with our result, Pribble et al. (2004) concluded that small number of parents believed that dento-facial injury is a significant problem. This might be due to the lack of knowledge about dento-facial injury and their consequences. Furthermore, 17.9% of the participants were familiar and had the knowledge about the use of sports mouth guard which coincides with Goswami et al. (2017) who found that level of awareness and knowledge about sports- related orofacial injury is very poor among children in New Delhi. This shows the significance of the current study which focus on the lack of knowledge regarding the use of sports-related mouth guards and how crucial is their use in the prevention and reduction of dental trauma.

In addition, 5 (1.6%) of the parents stated using mouth guard by their children during sports, which agrees with Turagam (2018) who reported that 90% of the children didn't use mouth guards during their sport activities. Additionally, the lack of information about the use of mouth guards in the current study was the main reason for not using it which accounts for (81.3%) while other studies reported that discomfort, peer pressure, difficulty breathing, and the children's coaches did not insist on wearing it were the common barriers for not using mouth guards (Onyeaso, 2004; Pribble et al., 2004). Three participants reported using the mouth guard used commercially ready-made and the other two were using custom-made at the dental clinic. As a protective measure, custom made mouth guard is more preferable than over the counter appliance (Ranalli et al., 1993; Burt & Overpeck, 2001; Newsome et al., 2001; Tuna & Ozel, 2014).

Nevertheless, knowledge about mouth guards does not necessarily mean their utilization during sports. In fact, the study done by Goswami et al. (2017) on 450 children aged 6 to 16 years stated that the recognition alone is not a significant reason to utilize mouth guards or protective appliances in sport activities and recommended that the cooperation between dentist and sports authorities are important to motivate the players and their trainers about the protective appliances in preventing and reducing orofacial injuries (Tuna & Ozel, 2014, Borris et al., 2019, Ayesha et al., 2020, Li et al., 2021).

As well, it is known that trainers have greater influence effect in the attitude of their players (Ramakrishnan et al., 2019). Trainers can educate the players and their parents about the risk of orofacial injuries associated with contact sports and the cost and morbidity they carry (Al-Habib, 2019). Moreover, as parents have the knowledge about the importance of using protective appliances and mouth guards during sport activities, they might be encouraged to seek the use of mouth guards for their children (Pribble et al., 2004). Additionally, almost 64% of the participants reported that they will consider using mouth guards for their children during sport activities indicating that parents and guardians are willing to be educated and gain the information of how to prevent and minimize dento-facial trauma that the children may encounter during different sport activities.

This study has limitations to be considered in future studies which included the questionnaire-based survey, in which some elements of underreporting bias might occurred. Also, the small sample of participants who reported that their children experienced dental trauma during sport activities and having an older age group children who are usually more involved in contact sport activities. Finally, the results of the present study demonstrate the insufficient parental knowledge regarding the use of mouth guards for children during sport activities in Riyadh city. This emphasizes the need to improve the knowledge of the parents and guardians on the importance of the use of mouth guards to prevent and minimize dento-facial trauma to their children when they are involved in different sport activities using a variety of educational methods such as educating the parents or guardians during the children visits to the dental office, distribution of educational flyers in the waiting room, social media posts, and school and sports clubs educational programs.

CONCLUSION

Since dento-facial trauma is common in school-age children while participating in sport activities and using protective appliances such mouth guards can prevent or reduce the effect of trauma, the following measures are recommended: Educational programs to increase the parents, guardians, teachers and coaches awareness by providing them with the information about the usefulness of mouth guards in preventing and reducing the effect of dento-facial trauma. Distribution of educational pamphlets and flyers in dental offices, schools and sports clubs. Educational courses for the dentists and dental students on the important role of protective appliances like the use of mouth-guards to prevent and reduce the effect of dento-facial trauma during sport activities.

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Biotechnological Communication

Efficiency of Using Hydrobiont Meal With Different Preparation Technologies in Feeding Rainbow Trout

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ABSTRACT

The composition of the compound feed for rainbow trout included high-protein feed made from products of processing substandard crayfish in the form of flour. The research was carried out in a closed supply installation. In the course of the experiments, the chemical composition and energy nutritional value of flour from crustaceans of aquatic organisms of various cooking technologies, the chemical composition of muscle tissue, and amino acid score were studied. Studies of the growth and development rates of rainbow trout based on the results of control catches were carried out weekly. At least 10 specimens were weighed on electronic scales. During the experiment, rainbow trout were fed four times a day with pelleted compound feed with a pellet diameter of 6 mm. The composition of the diets differed in several ways. The main component was fish meal and flour made from freshly dried crayfish, and in group 2 from freshly boiled and then dried crayfish. In the experimental groups, the amount of flour studied was 20%. All the changes made in the composition of the feed did not have a significant effect on the energy and protein nutritional value of the feed and the content of fat and phosphorus in them. It was found that the use of crayfish flour in the composition of compound feed for rainbow trout has a positive effect on the increase in live weight of fish, reduces feed costs, does not change the biochemical composition of blood, changes the chemical composition of muscle tissue, and improves the amino acid rate. Due to the relatively low cost compared to fish meal, the use of crayfish meal reduces the cost of compound feed and increases the economic effect of growing rainbow trout.

KEY WORDS: AVERAGE DAILY GAIN, BLOOD, CLOSED WATER SUPPLY INSTALLATION, COMPOUND FEED, CRUSTACEANS.

INTRODUCTION

Currently, fishing in the seas and oceans is the main source of fish products for the population. In some countries, the volume of farmed fish approaches the volume caught from natural reservoirs, and sometimes even exceeds it (Shcherbina & Gamygin 2006; Brug & Ridler 2004). Among the various forms of fish farming, the industrial form has the greatest potential for a rapid increase in production volumes. The success of this form of fish farming largely depends on the balance and quality of the compound feed. Therefore, in recent years in the world, the production of compound feed for fish has been actively developing and there is a constant search for new sources of raw materials (Voronova, 1989, Perednya, 2002, Guseva et al. 2018a, b; Moskalenko et al, 2020; Poddubnaya2020b).

At present, during the processing of crustaceans to obtain gourmet products, up to 80% of non-food waste is formed, which can be divided into three fractions: chitin-containing, protein-containing, and lipid waste (Trukhin 1992, Shiryaev, 1997). The chitin-containing fraction (mainly represented by shells) serves as a raw material for the production of chitin and chitosan, which have adhesive properties in the composition of compound feed for various types of aquatic organisms (Gamygin & Sazonova 1999; Bakhareva et al., 2019, Moskalenko et al, 2020). Besides, other valuable products can be obtained from crustacean waste, such as crayfish meal, crayfish oil, natural pigments, and others (Trukhin 1992, Shiryaev, 1997). Compared to fish meal, crayfish meal contains slightly less protein, but more calcium and phosphorus. Crayfish can be grown under industrial

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conditions in artificial reservoirs (Gamygin & Sazonova 1999; Kiyashko et al. 2016a). The organization of full-fledged feeding of rainbow trout using new traditional feed was also studied by many workers, (Guseva et al., 2018a and Guseva et al. 2018b. Moskalenko et al, 2020).

MATERIAL AND METHODS

In 2017-2021 study was carried out to the effectiveness of using high-protein feeds from crustacean processing products in a closed-loop supply unit when feeding rainbow trout. In this experiment, according to the principle of analogs, 150 rainbow trouts with an average weight of 1240-1276 g were selected and placed by 50 in three polypropylene pools with a volume of 5 m3 each. The duration of the experiment was 120 days. The control group received a complete sinking granulated compound feed (OR). Trout from the 1st and 2nd experimental groups received compound feed with crayfish meal from fresh dried crayfish and crayfish meal from boiled dried crayfish (Table 1).

Table 1. The experiment designs

Group	Number of fish	Feeding type
Control	50	OR
1st experimental	50	OR with crayfish meal from fresh dried crayfish
2nd experimental	50	OR with crayfish meal from boiled dried crayfish

Table 2. Chemical composition and nutritional value of crayfish meal from crustacean aquatic organisms prepared using different technologies

Indicator	Fresh dried crayfish meal	Boiled crayfish meal
Moisture content, %	9.50	9.70
Dry matter, %	90.50	90.30
Raw protein, %	42.28	42.83
Raw fat, %	2.49	2.20
Raw ash, %	28.10	27.67
Carbohydrates, %	5.00	5.82
Chitin, %	12.63	11.78
Energy, kcal	222.00	225.00
Calcium, %	19.47	19.67
Phosphorus, %	0.71	0.75

A weekly study of the growth and development rates of rainbow trout was carried out based on the results of control catches. At least 10 specimens were weighed on an electronic balance. During the experiment, rainbow trout were fed 4 times a day, with an interval of 4 hours at 7.00, 11.00. 15.00 and 19.00. In feeding, we used granulated compound feed with a pellet diameter of 6 mm, which corresponds to the weight of the fish. The feed composition and nutritional value corresponded to the period of fish breeding.

RESULTS AND DISCUSSION

The efficiency of fish rearing is determined by the physicochemical properties of water since the course of all vital functions in them depends on the state of the aquatic environment. During the study, the average value of the water temperature was 13.8 °C, and the water exchange in one pool was 12850 l/h. Indicators of dissolved oxygen values, hydrogen index (pH), also corresponded to the requirements. Only with the use of high-quality feed with sufficient concentration of energy and nutrients can fish grow fast. Table 2 provides data on the chemical composition and energy nutritional value of crayfish meal from crustacean aquatic organisms prepared using different technologies.

Table 3. Composition and nutritional value of compound feed

Indicators	Group 1st (control)	2nd experimental	3rd experimental
Wheat flour, %	7	-	
Sunflower cake, %	22.5	20.0	20.0
Yeast, %	6.4	5.0	5.0
Fish meal, %	51.5	42.0	42.0
Vegetable fat, %	5	6.0	6.0
Fish oil, %	4.6	4.0	4.0
Molasses, %	1.0	1.0	1.0
Premix, %	1.0	1.0	1.0
Peltech, %	1.0	1.0	1.0
Raw crayfish meal, %	-	20	-
Boiled crayfish meal, %	-	-	20
Total, %	100	100	100
Energy, kcal	331.0	329.9	328.5
Protein, g	46.0	45.9	46.0
Fat, g	16.0	15.8	15.8
Fiber, g	3.2	2.8	2.8
Calcium, g	2.4	5.9	5.9
Phosphorus, g	1.7	1.5	1.5
Carbohydrates, g	16.6	11.3	11.3
Ash, g	10.1	13.9	13.6
Price	75	66	67

The crayfish meal was made from substandard crayfish. To included crayfish meal in the compound feed, two compound feed formulations were developed, to which crayfish meal was added. In the 1st experimental group, the crayfish meal was made from freshly dried crayfish, and in the 2nd group from freshly boiled and then dried crayfish. The composition and nutritional value of compound feeds are presented in Table 3.

The composition of the diets differs in several ways. The main component is fish meal and crayfish meal made from freshly dried crayfish, and in group 2 from freshly boiled and then dried crayfish. In the experimental groups, the amount of fish or crayfish meal studied was 20%. All the changes made in the composition of the compound feed did not have a significant effect on the energy and protein nutritional value of the compound feed or the content of fat and phosphorus in them. The largest changes were observed in the level of calcium and carbohydrates. At the same time, the technology itself practically did not affect the chemical composition and nutritional value of the compound feed used. Crayfish meal is much cheaper than fish meal, which is reflected in the cost of compound feed. Evaluation of the effectiveness of the use of crayfish meal in the composition of compound feed was carried out based on the rate of ichthyomass gain, as an indicator of paramount importance for the development of fish (Table 4).

Table 4. Growth rates of hydrobionts

Indicator	Group		
	1st (control)	2nd experimental	3rd experimental
Livestock heads	50	50	50
Total weight of fish at the beginning of the experiment, kg	62.4	62.05	61.3
Average live weight of 1 fish, g	1,248±14.2	12,41±6.3	1,226±11.0
Total weight of fish at the end of the experiment, kg	95.3	98.6	97.55
Average live weight of 1 fish, g	1,906±12.8	1,972±29.0*	1,951±24.4
Weight gain per 1 fish, kg	0.658	0.731	0.725
Average daily gain per 1 fish, g	5.48	6.09	6.04

Figure 1: Muscle tissue of a trout participating in the experiment

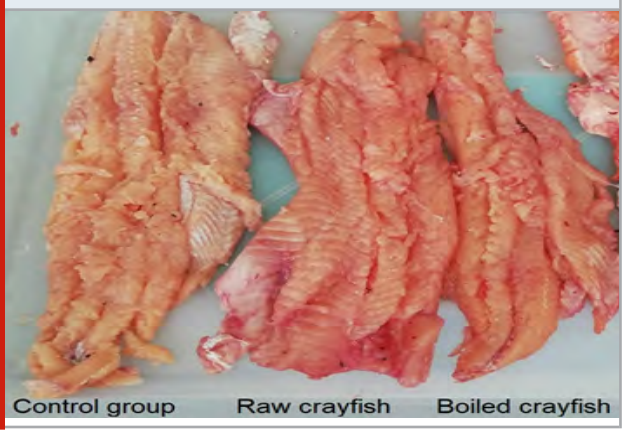

Table 5. Chemical composition of the muscle tissue of rainbow trout, %

\Indicator	Group		
	control	1st experimental	2nd experimental
Initial moisture	72.22 ± 0.45	73.45 ± 0.37	70.53 ± 0.83
Dry matter	26.69 ± 0.43	25.47 ± 0.35	28.22 ± 0.86
Protein	19.87 ± 1.44	20.81 ± 1.62	22.00 ± 0.62
Fat	5.35 ± 0.40	8.05 ± 2.25	5.01 ± 0.56
Calcium	0.06 ± 0.01	0.07 ± 0.02	0.05 ± 0.02
Phosphorus	0.15 ± 0.01	0.14 ± 0.02	0.17 ± 0.01*

*P≥0.95.

The results of the conducted studies show that the total and average live weight of 1 head at the beginning of the experiment in all experimental groups did not differ significantly (P> 0.05). At the end of the study, the fish stock of the second group had a larger ichthyomass and an average live weight of 1 head, 98.6 kg, and 1972 g respectively, versus 95.3 kg and 1906 g in the control group, and 97.55 and 1951 g in the third group. In terms of the average daily gain, the fish of the first group were ahead of their counterparts from the control group by 0.61 g, and from the third group by 0.05 g. The use of raw materials with different technologies for the preparation of crayfish meal did not affect the safety of the fish. The morphological and biochemical composition of blood quickly reacts to the level and quality of nutrients and other substances coming from outside. Therefore, monitoring changes in its indicators can quickly establish irregularities in the organization of fish feeding or the presence of various diseases.

When considering the biochemical parameters of the blood of rainbow trout, during the period of the study, we did not find significant and reliable changes in the studied blood parameters. All of them were within the physiological norms. This confirms the absence of a negative effect of crayfish meal on the work and functions of the internal organs of fish and the absence of pathological processes in the body. To determine the effect of feed additives on the formation of various organs and structures of rainbow trout, a control slaughter was carried out. The fish were dissected, and the head, skin, muscle tissue (Fig. 1), and internal organs were separated.

Edible parts include the muscles, the liver, the caviar, the milt. Inedible parts: the skin, the heart, the scales, the gills, the alimentary tract, the kidneys, the air bladder. Conditionally edible parts are the parts that become edible

after heat treatment. These include the head, the bones, the fins, and the cartilage. They are used for cooking fish soup and aspic. Under the influence of raw crayfish meal in the compound feed, the weight of all edible and conditionally edible parts of trout reached higher values than in the specimens with the diet that included boiled crayfish or the control feed. The weight of the head and fins, the weight of the skin, the weight of bones and muscle tissue in the fish in the experimental group significantly exceeded those parameters in the fish in the control group. It was found that in fish fed with raw crayfish meal, the output of edible parts was almost 2% higher than in the control group, and the output of inedible parts in the experimental group was less than in the control by 4%. The trout from the second experimental group had a 1.62% more output of edible parts than in the control group, and less edible parts by 4.14% than in the control group.

The composition of compound feeds influenced the concentration of amino acids, except for glycine and arginine, the content of which in the muscles of fish of all experimental groups was practically at the same level. In fish with the diet that included raw crayfish meal, the concentration of almost all amino acids had increased in comparison with the control group and the second variant of the experiment. At the same time, the data on the increase in ichthyomass indicate that the addition of boiled crayfish meal to the compound feed gives less effect than raw crayfish meal. Probably, this can be explained by the fact that the process of intermediate protein metabolism was disturbed in the trout in the second experimental group. The transamination reaction, as the main source of the formation of new amino acids, is crucial to the intermediate metabolism of proteins. A disturbance in transamination can result from a deficiency of vitamin B6 in the body, as this vitamin is destroyed during heat treatment.

Determination of the amino acid score allows identifying the limiting amino acid and taking appropriate measures to eliminate its deficiency. It was found that in the muscular tissue of trout with the diet where crayfish meal from raw crayfish had been added, only 2 amino acids, lysine, and isoleucine, were limited, and in the group where crayfish meal from boiled crayfish had been added, the amino acid score corresponded to a complete protein only in terms of serine, histidine, and arginine. Thus, we can conclude that raw crayfish meal provides the compound feed used in rainbow trout feeding with almost all the essential amino acids. At the end of the experiment, we performed a chemical analysis of the muscle tissue of the rainbow trout of the studied groups. The analysis results are presented in Table 5.

Analyzing the data in Table 2, it can be noted that in terms of the protein content in muscle tissue in individuals of the 2nd experimental group, this indicator was higher than in other groups and amounted to 22.0%. The fat content in the 1st experimental group exceeded the values of the control group by 2.7%, and the values in the 2nd experimental group were lower by 0.34% than in the control group. Regarding the content of inorganic substances, such as calcium, no significant differences were found between the groups. The amount of phosphorus was significantly higher in the 2nd experimental group and amounted to 0.17%. Economic efficiency is the main criterion for introducing raw materials into production (Table 6).

Table 6. Economic efficiency

Indicator	Group		
	1st (control)	2nd experimental	3rd experimental
The cost of 1 kg of compound feed, rub.	75	66	67
The amount of compound feed used, kg	39.69	39.478	39.37
Feed costs, rub.	2,976.60	2,605.55	2,637.79
Gross weight gain, kg	32.9	36.55	36.25
Feed costs per 1 kg of weight gain, rub.	90.47	71.29	72.77
Feed costs per 1 kg of weight gain, kg	1.21	1.08	1.09
Feed costs in the cost structure, %	40	40	40
Total cost per 1 kg of weight gain, rub.	226.19	178.22	181.92
The wholesale sale price of 1 kg of fish, rub.	350	350	350
The economic effect, rub.	123.81	171.78	168.08

The cost of compound feed in all groups had certain differences. The difference between the cost of compound feed in the control group and compound feed in the experimental groups is especially noticeable. Due to the higher cost of fish meal, the total cost of compound feed in the first group was 75 rubles/kg, and in the second group, it was 9 rubles less. Due to the cost of cooking crayfish, the price of compound feed in the third experimental group had increased by 1 ruble, compared with the second group, but was still 8 rubles lower than in the control group. During the experiment, all three groups were fed a relatively equal amount of compound feed. Due to the price difference, the total cost of feed during this period was not the same. The difference between the control and experimental groups was 371.05 and 338.81 rubles, respectively. This made it possible to reduce feed costs by 1 kg of live weight gain in the first group by 19.18 rubles, and in the second group by 17.19 rubles.

Considering the existing structure of the cost of weight gain, the largest costs were recorded in group 1. To get 1 kg of weight gain, 226.19 rubles had to be spent. The lowest indicator was obtained in the second experimental group, where it was 178.22 rubles, or 47.97 rubles less than in the control group and 44.27 rubles less than in the 3rd group. The calculation of the economic effect from the use of crayfish meal in compound feed for rainbow trout shows that in the control group it was 123.81 rubles, in the second experimental group it was 171.78 rubles, and in the third experimental group it was 168.08 rubles. The group of economic indicators also includes the cost of feed to obtain 1 kg of live weight gain. Due to the higher rate of weight gain, this indicator in the control group was 1.21 kg, while in the second group it was 1.08 kg, and in the third one 1.09 kg.

Studies have been carried out by several scientists on the use of crab processing products in the diets of sturgeon fish (Bakhareva et al., 2019). It has been proved that the inclusion of 10% crab meal as a substitute for fish meal in the composition of starter and production feed for sturgeon fish allows increasing the weight gain of reared fish by 56% and survival rate up to 81% while reducing feed costs. The presence of shell-forming substances in the flour – chitin and chitosan, as well as the carotenoid – astaxanthin – promotes the synthesis of glycosaminoglycans and helps restore bone and cartilage tissue (Bakhareva et al., 2019; Moskalenko et al, 2020; Poddubnaya et al., 2020a).

The scientist Front (2002) assess the effectiveness of the use of chitosan in fish feed, a natural biopolymer obtained from the chitin of shells by means of a deacetylation reaction. It has been found that chitosan and its preparations, presented in dry form, exhibit high adhesive properties when added to granulated feed for salmon, sturgeon, and cyprinids. The use of freshwater crustacean meal in our studies, as a high-protein component, including chitin, chitosan, and the carotenoid pigment astaxanthin, in the feeding of rainbow trout is another step in finding an alternative to expensive fish meal. It is a cheaper feed component but no less nutritious in fish diets (Trukhin, 1992; Shiryaev, 1997; Shcherbina, & Gamygin, 2006; Moskalenko et al, 2020).

CONCLUSION

Thus, it follows from the results of the studies that the introduction of high-protein feed made from crustacean processing products in the form of crayfish meal into the composition of the rainbow trout compound feed has a positive effect on the increase in the live weight of fish, reduces feed costs, does not change the biochemical composition of blood, changes the chemical composition of muscle tissue, and improves the amino acid score in the 1st experimental group. Due to the relatively low cost compared to fish meal, the use of crayfish meal reduces the cost of compound feed and increases the economic effect of rainbow trout rearing.

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

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

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Dental Communication

A Questionnaire-Based Study to Assess the Knowledge of Parents about Caries Preventive Measures

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ABSTRACT

Preventive interventions, particularly among children, have become extremely important due to increased dental caries and lesions. Because parents have such a significant role in their children's lives, their understanding and attitude toward preventative measures will significantly influence their dental health. Therefore, this study aimed to evaluate parents' knowledge on preventive methods against dental caries. In this cross-sectional study, participating parents completed a comprehensive questionnaire on their demographics, dental history, questions about fissure sealant and topical fluoride therapy. Data were analyzed by Chi-Square tests, analysis of variance and independent t-tests. A total of 206 parents participated in this questionnaire study, with 124 (60.2%) were mothers, and 82 (39.8%) were fathers. The age group of parents included are as follows: 18.9 % in 20 - 29 years age group, 51.9 % in 30 - 39 years age range, and 29.1 % in 40 and older. The current knowledge of parents on the definition of fissure sealants was inadequate, with just 15% of parents having a better description of the term. There was no significant difference between parents' knowledge and gender, education level, and occupational status. The majority of parents in this research had limited knowledge about fissure sealants and fluoride treatment. Dentists play an important role in raising parental awareness. According to the results of this study, parents' knowledge of fissure sealant therapy and fluoride therapy is lacking. Knowledge gained primarily from dentists, followed by the internet, friends, and finally, the media. On the other hand, government services should spend more significant resources on caries prevention programs to provide parents with knowledge on preventive dentistry

KEY WORDS: CHILDREN, DENTAL CARIES, FISSURE SEALANT, FLUORIDE THERAPY, PARENT'S KNOWLEDGE.

INTRODUCTION

Oral health is an essential factor of good general health and plays a significant role in the child's life span (Lawrence and Leake 2001). Dental caries is a major oral health problem affecting 2.43 billion people (35.3% of the population) worldwide in the year 2010 (Vos, et al., 2012). Dental caries is common in Saudi children; approximately 85.77% in the six-year group, 64.98% in the 12 years group, and 71.35% in the 15 years group (Al-Rafee, et al., 2019). The pits and fissures are considered to be at significant risk for dental caries as the control of the accumulation and removal of dental plaque in these deep areas are challenging (Brown, et al., 1996, Liu, et al., 2012).

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The increased prevalence of occlusal caries than smooth surface caries is due to the morphology of the teeth. Lower molars are more prone to caries than anterior teeth and most affected teeth in the entire dentition (Macek, et al., 2003, Hopcraft and Morgan 2006). Since preventing dental caries is a considerable challenge for the public, increasing parental knowledge and utilizing preventive methods, as practiced in developed countries, may lead to decreased dental caries and improved children's health (Downer 1995, Daly, et al., 2013). For example, fluoride therapy and fissure sealants are standard methods to prevent the formation of dental caries (Wright, et al., 2016, Lakshmanan and Gurunathan 2020, Mc Donald et al., 2021).

Pit and fissure sealant application on permanent teeth in the first molar has decreased dental caries from 86% in the first year to 78.6 % in the second and 58.6 % in the 4th year (Wright, et al., 2016). Most scientific evidence indicates that topical fluoride therapy applied by a dentist can effectively

reduce the incidence of dental caries (Merghache, et al., 2011). Topical application of fluoride by a dentist four times a year has been reported to result in an 86 % reduction in dental caries (Mc Donald et al., 2021). However, despite extensive evidence supporting preventive procedures, the percentage of children involved in these services is less (Bhuridej, et al., 2005). One of the barriers to the utilization of preventive dental cares is the lack of public awareness (Centers for Disease Control and Prevention 2020). Several studies have shown that parents know fluoride use as a preventive armory among their children. However, not many parents can convert this knowledge into practice. Surprisingly, some parents reported having refused the fluoride application due to the risk of toxicity or weak beliefs (Chi 2017, Djordjevic 2018, Al-Rafee, et al., 2019, Lakshmanan and Gurunathan 2020, Mc Donald et al., 2021).

Compared to fluorides, the level of knowledge related to dental sealants is lower, as reported in previous studies. Less knowledge has resulted in a lower number of children receiving dental sealants. Differences between mothers and fathers have also been observed in a few studies, where mothers' attitudes were comparatively positive (Lenčová and Duskova 2013). The first step to promote the utilization of preventive cares is to increase the awareness and knowledge of parents about the importance of such cares, as parents play an essential role in developing healthy oral habits in children and have the responsibility of maintaining and improving the child's oral health (Gill, et al., 2001). Therefore, this study was conducted to evaluate the knowledge of parents about preventive measures on their children in Riyadh city, Saudi Arabia.

MATERIAL AND METHODS

Ethical clearance was obtained from the Institutional Review Board and Ethics Committee of the College of Dentistry Research Center (CDRC) and approval IRB. No. E-21-5902. This cross-sectional study was conducted among the parents of children who attended the Pediatric Dental Clinics in Dental University Hospital, Riyadh, Saudi Arabia. The sample size for the study was estimated through power 0.89 and $\alpha = 0.05$ (maximum difference, 0.9). Therefore, the sample size was determined to be a minimum of 200. Participation in the study was voluntary. Before data was collected, the purpose of the study was explained to parents, and the parents of the children obtained formal informed consent. Inclusion criteria were Saudi parents who could answer the questionnaire and whose children were patients at pediatric dentistry clinics. The exclusion criterion was parents not agreeing to participate in the study. A structured and validated questionnaire was modified from questionnaires used previously in studies done by Baradaran Nakhjavani, et al., (2013) and Blumer, et al., (2018).

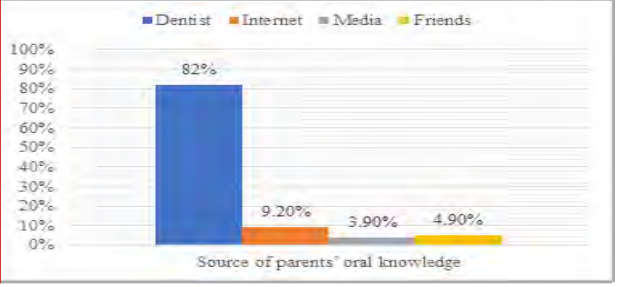
The first section of the questionnaire consisted of demographic information of the parents such as age, gender, occupational status, the highest level of education and number of their children. The second part consists of dental information such as the last dental visit of their children, the reason for visit, personal use of fluoridated mouth rinses and source of information about oral health. Finally, the third part assessed parents' knowledge regarding fissure sealants and fluoride therapy. The questionnaire was constructed in English before being translated into the local language (Arabic) and then back to English to ensure accuracy. Before distributing the questionnaire, a pilot study was conducted on ten parents to check the validity and reliability of the questionnaire; changes were made accordingly. Later, parents were asked to give feedback on the overall questionnaire clarity regarding length and language.

The pilot study responses were not considered in the main study. The questionnaire was an assessed level of knowledge scores varied from 1 to 8. Parents who scored equal to or below four were considered to have less than average knowledge. Others, on the other hand, were found to have more than the average level of knowledge. Descriptive statistics (mean, standard deviation, frequencies, and percentages) were used to describe the quantitative and categorical variables. Chi-Square Tests, analysis, independent t-test and analysis of variance (ANOVA) were used for statistical analysis of the results. A p-value of less than 0.05 was statistically significant. All analyses were performed in SPSS software version 22.0 (IBM Inc., Chicago, Ill, USA).

Table 1. Summary of main demographics.

Variables		n (%)
Parents Gender	Females	124 (60.2)
	Males	82 (39.8)
Parents Age	20-29	39 (18.9)
	30-39	107 (51.9)
	40 and older	60 (29.1)
Education level of parents	Below high school	22(10.7)
	High school	33(16)
	University degree	151(73.3)
Occupation status of parents	Working	131(63.6)
	Not working	75(36.4)
Number of children in family	1	15(7.3)
	2	36(17.5)
	More than 3	155(75.2)

Figure 1: Representing source of parents' oral knowledge.



RESULTS AND DISCUSSION

Two hundred and six questionnaires were filled. Table 1 summarized the main data on demographics. The study sample consisted of 206 parents, of which 82(39.8%) were fathers, and 124 (60.2%) were mothers. Approximately half 52% of the parents were from 30 to 39 age groups. Nearly 73.3% of the parents had university-level education, 36.4 % of the parents were homemakers/unemployed, while 63.6% employees. Mostly 75.2% of families had three or more children. Almost half the children, 50.5% had visited a dentist during the past year. The main reason for the dental visits was routine dental treatment 59.7%, then checkups 24.3%. Only 16% sought emergency treatment at their last dental visit. Figure 1 shows various sources of parental oral health knowledge. Dentists were the primary source for most parents 82%, followed by the internet 9.2%, friends 4.9%, and media 3.9%.

Table 2. Responses of parents to different questions regarding fissure sealants and fluoride therapy.

Questions		n (%)
Which one is the definition of fissure sealants?		
a)	Covering carious fissures of tooth crown by mercury	3(1.5)
b)	Covering deep carious fissures by tooth color material	24(11.7)
c)	Covering deep normal fissures of tooth crown by tooth color material as a foundation*	31(15)
d)	Covering all of the tooth crown by metal sheets to prevent dental caries	20(9.7)
e)	I do not know	128(62.1)
Which teeth are indicated for sealants?		
a)	Deciduous teeth	7 (3.4)
b)	Permanent teeth	47(22.8)
c)	Both*	60(29.1)
d)	I do not know	92(44.7)
What age can you use sealants?		
a)	4-6 years old	35(17)
b)	6-9 years old*	27(13.1)
c)	10 years old and more	29(14.1)
d)	I do not know	115(55.8)
How long do pit and fissure sealants last?		
a)	2 years	19(9.2)
b)	5 years*	15(7.3)
c)	10 years and more	12(5.8)
d)	I do not know	160(77.7)
What age can you use fluoride therapy?		
a)	From tooth emergence up to 6 years of age	42(20.4)
b)	6-12 years old	47(22.8)
c)	After 12 years of age	6(2.9)
d)	All ages*	62(30.1)
e)	I do not know	49(23.8)
How often should you visit a dentist for fluoride therapy?		
a)	Every 4-6 months*	105(51)
b)	Every 12 months	35(17)
c)	Every 2 years	7(3.4)
d)	I do not know	59(28.6)

The result regarding the frequency of responses to questions related to preventive dental procedures in children, for example, fissure sealants and topical fluoride therapy among parents, were summarized in Table 2. Overall knowledge of parents about the definition of fissure sealants was poor. However, only 15 % of parents marked correct answers in define fissure sealants, 62.1 % of parents selected "I do not know" choice, and others gave wrong answers. On the other hand, almost on third, 29.1% of the parents responded correctly to questions on the application of fissure sealant in both permanent and deciduous teeth. Nevertheless, 55.8% of parents responded "I do not know" to questions on what age can use sealants, and 77.7% of parents responded "I do not know" to questions on how long do pit and fissure sealants last in the tooth. Moreover, 13.3% of mothers expected fissure sealants to last for five years, 5.8% thought they remain for ten years or more. About 30.1% of the parents knew the age of fluoride therapy to prevent decay

(all ages to prevent caries). However, 23.8% selected "I do not know". On the other hand, half 51% of the parents had appropriate information about the frequency of fluoride therapy (periods of 4-6 months), but 28.6% of the parents had no idea (Table 2).

There was no significant difference between parents' gender and their knowledge about fissure sealant and fluoride therapy. However, almost all fathers and mothers their knowledge less than average. Furthermore, there was no significant difference in correlation between parents' level of education and their knowledge about fissure sealant and fluoride therapy. While parent with a higher level of education was more knowledgeable, less educated parents' knowledge was less than average but then inadequate. Also, there was no significant difference between parents' occupational status and their information about fissure sealant and fluoride therapy ($p > 0.05$ statistically non-significant, Table 3).

Table 3. Relationships between the parents' knowledge about fissure sealants and fluoride therapy and gender, their level of education and occupational status.

knowledge	Parents Gender		Education level of parents		Occupation status of parents		
	Females	Males	Below high school	High school	University	Working	Not working
Less than average	58.7	38.8	10.7	15.5	71.4	61.7	35.9
More than average	1.5	1	0	0.5	1.9	1.9	0.5
p-value	0.993	0.924	0.229				

*p<0.05 statistically significant; p> 0.05 statistically non-significant NS

Oral health is a critical component of general health and is considered a determinant of the good quality of a child's life (Petersen 2009). Parental knowledge and practices play an essential role in preventing oral diseases and improving dental health in children. In addition, oral health maintenance is initially a parental responsibility, which later involves both parents and children (American Academy of Pediatric Dentistry 2020). Because parents' knowledge is vital in maintaining proper health care for their children at a young age, it's essential to examine their knowledge for preventative measures to avoid dental caries in their children. Therefore, this study aimed to evaluate parental knowledge about preventive measures such as fissure sealants and fluoride therapy. A total of 206 parents participated in this questionnaire study, of which 82 (39.8%) were fathers, and 124 (60.2%) were mothers. In the present study, most parents (82 %) had received information for preventive dental treatments through dentists, which could be attributed to the effectiveness of face-to-face education of dentists and most reliable sources of information, which is similar to the results of other studies (Baradaran Nakhjavani, et al., 2013, Tahani, et al., 2017, Lakshmanan and Gurunathan 2020, Mc Donald et al., 2021).

Given the high percentage of the parent, 50.5% visiting their children visiting the dentist during the last year, encouraging dentists to provide oral health education about preventive treatments is essential. Moreover, according to the proven effectiveness of media in oral health education in other studies, the potential use of this source of information should be considered (Martensson, et al., 2006, Gholami, et al., 2014). In this study, the majority 50.5% of parents reported that the primary reason for their children's visits to the dental clinic in the past year was for routine dental treatment. In a different study conducted in Riyadh, fewer than a third of the participants (28%) went to the dentist for their children even if they were not in pain (Almulhim and Alamro 2016). Increasing parents' awareness of preventative programs may result in the early detection and prevention of dental problems (Kay and Locker 1996). In general, the results showed that most of the parents had a level of knowledge less than average toward fissure sealant and fluoride therapy which was in agreement with the previous studies performed in Saudi Arabia and other countries (Al-Shalan, et al., 2002, Baradaran Nakhjavani, et al., 2013, Almulhim and Alamro 2016, Tahani, et al., 2017, Lakshmanan and Gurunathan 2020, Mc Donald et al., 2021).

In the present study, only 15% of parents knew that fissure sealant was covering deep normal fissures of tooth crown by tooth color material, whereas it was found to be 34% in the study of Baradaran Nakhjavan et al., (2013). This difference may be explained by the fact that information is taken more seriously by school and local media in Tehran. One of the critical preventive measures to be taken against dental caries in children is fissure sealants. As a recommendation by The American Academy of Pediatric Dentistry (AAPD) and the American Dental Association (ADA), it should cover primary and permanent teeth with dental sealants if the patient or the tooth is categorized as high risk for suffering from dental caries in the future. However, it was concluded that fissure sealant was known to only 29.1 % of the parents reported application could be for both dentition and only a smaller percent of 13.1 % of the parents knowing that sealants are used in age between 6-9 years, which indicated the low information of parents toward preventive dental treatment and could partly be explained by lack of parents' awareness, as shown in other studies and in this study (Baradaran Nakhjavani, et al., 2013, Tahani, et al., 2017, Djordjevic 2018, Lakshmanan and Gurunathan 2020, Mc Donald et al., 2021).

Similar to a previous study, the highest parental knowledge mean score was reported in questions associated with fluoride application. In this study, only 30.1 % of parents answered the age correctly, using fluoride therapy. However, 51% of parents were aware that the dental visit should be made within 4-6 months for fluoride therapy (Baradaran Nakhjavani, et al., 2013). This suggests the importance of increasing parental awareness regarding fluoride application. Details on the levels of knowledge score and the associations with the gender, their level of education and occupational status of parents were determined. Knowledge level of parents about preventive dentistry had no significant correlation with gender, education, and occupational status. For instance, a study in Saudi Arabia reported that while knowledge was not affected by age, gender, and parents' education, it was significantly related to socioeconomic status (AL-Shalan 2003). However, other studies have reported opposite finding (Baradaran Nakhjavani, et al., 2013, Blumer, et al., 2018, Lakshmanan and Gurunathan 2020, Mc Donald et al., 2021). Accordingly, Baradaran Nakhjavani et al., said that knowledge of response about preventive measures had statistically significant difference with university degree or occupation (Baradaran Nakhjavani, et al., 2013). Sampling location and the applied tool to assess knowledge could have caused this difference

CONCLUSION

According to the results of this study, parents' knowledge of fissure sealant therapy and fluoride therapy is lacking. Knowledge gained primarily from dentists, followed by the internet, friends, and finally, the media. On the other hand, government services should spend more significant resources on caries prevention programs to provide parents with knowledge on preventive dentistry. The level of knowledge of parents visiting the Pediatric Dental Clinic at the Dental University Hospital was investigated in this study. It might be used as a guide for future government services and caries prevention programs, informing parents about the benefits of sealants and fluoridated products in preventing dental caries in children.

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Dental Communication

Esthetics of Lip Morphology Changes after Filler Injections: A Clinical Assessment

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ABSTRACT

The present study was under taken to evaluate the effects of filler injections on lip morphology changes in three dimensions (vertical, transverse, and sagittal). The subjects included 14 volunteers aged between 23 and 48 years who visited a private dermal clinic for lip enhancement with dermal filler injections. Photographs and video records were captured before (T1) and immediately after filler injection (T2) in frontal rest, frontal smiling, and lateral rest views. After one to two weeks, another set of records was taken before retouch injections (T3). These images were collected and measured using a software program to determine changes in lip morphology over time. In upper and lower lip vermilion heights at rest, lower lip vermilion protrusion, and upper and lower lip indices, there were significant differences between T1 and T2, T2 and T3, and T1 and T3. In upper lip length at rest and while smiling, upper lip vermilion height while smiling, upper incisor displays at rest and while smiling, gingival display while smiling, interlabial gap, smile index, nasolabial angle, upper and lower lip protrusion to the E-line, and upper lip vermilion protrusion, there were significant differences between T1 and T2 and between T1 and T3. There were no significant differences in intercommissural width and buccal corridors between any time points. Most static changes were gained at T3. Filler injection in the lips leads to an increase in lip length at rest and while smiling, lip vermilion height at rest and while smiling, smile and lip indices, lip protrusion to the E-line, and lip vermilion protrusion. Similarly, filler injection in the lips leads to a decrease in upper incisor display at rest and while smiling, gingival display while smiling, interlabial gap while smiling, and nasolabial angle, but it may not alter the intercommissural width (smile width) or buccal corridors.

KEY WORDS: LIP MORPHOLOGY; FILLER INJECTION; COSMETIC; ORTHODONTICS.

INTRODUCTION

The demand for cosmetic procedures has risen over the years. As a result, orthodontic procedures involved in facial esthetics have been increasingly sought after by clients. Amongst them, the most requested procedures include dentition alignment and reconciliation of the patient's profile. Moreover, procedures that attempt to improve smiles are also frequently sought (Polo, 2008).Orthodontists evaluate patient profiles in frontal and vertical planes and in static and dynamic states. Orthodontists are required to critically analyze two main aspects in their patients. The first factor to be considered is soft tissue repose and animation, which is focused around particular details such as the way lips would animate while smiling, the degree of gingival display, crown length, and the quality of the smile. The second factor to be considered is facial amendment over the years throughout a patient's life or the impact of aging on the facial skeletal and soft tissue structures, (Sarver and Ackerman, 2003, Lafaille and Benedetto, de Maio, 2020. Lipko-Godlewska et al., 2021).

Lip shape and fullness strikingly impact facial esthetics. As one of the main determining factors of physical appearance, facial esthetics have been associated with allure, self-love, and overall self-confidence (de Aquino et al., 2013, Litner et al., 2008). Over the past five decades, fuller lips have been considered to be a desirable facial feature in women. A significant number of young females have undergone lip augmentation procedures to achieve the famous sought-after look portrayed by celebrities and fashion magazines (Segall and Ellis, 2007). Moreover, according to a study done by Bisson and Grobbelaar (Bisson and Grobbelaar, 2004), it has been reported that models seem to be more inclined

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towards following the fuller lip trend than women who are not involved in the fashion industry. Lip augmentation with fillers accentuates the overall look by making the lips look fuller and more defined by enhancing lip contours, and by making them look smooth as the procedure also reduces the appearance of fine lines (Lanigan, 2011, Fitzgerald et al 2019). Furthermore, the use of dermal fillers for facial rejuvenation is trending. Such cosmetic procedures promise fascinating esthetic outcomes such as youthful, younger, and wrinkle-free skin without the need for any invasive surgical procedure, (Vlahova et al., 2014b, Dalati et al 2020 De Maio, 2020, Lipko-Godlewska et al 2021).

This study was aimed to evaluate the consequences of filler injections on lip morphology changes in three dimensions (vertical, transverse, and sagittal). Our null hypothesis stated that there would be no distinction in lip measurements before and after filler injection at all time points within the frontal rest view, frontal smile view, or the lateral view.

MATERIAL AND METHODS

Sample selection: All subjects signed an institutional review board-approved informed consent form which was explained to them by doctors, and all photographs were taken by the same doctors. A total of 14 volunteers aged between 23 and 48 years came to a private dermal clinic in the city of Riyadh, Saudi Arabia, for lip enhancement with dermal filler injections. A total of 114 photographs (38 frontal rest views, 38 frontal smiling views, and 38 lateral views) were collected and measured for this study.

Inclusion and exclusion criteria: The inclusion criteria were as follows: voluntary involvement in the study, individuals of both sexes, between 18-50 years of age, and seeking dermal fillers in the lips for lip enhancement for esthetic reasons. However, patients with a cleft lip and/or palate; inability to determine the natural head position (i.e. uncooperative patients, patients with neural conditions); presence of any craniofacial anomalies or alternative pathology; history of significant facial trauma after a permanent or semi-permanent hyaluronic acid (HA) filler or *Botulinum* toxin (BTX-A) within the previous 12 months; infection, disorder, or scar in the lip or mouth area that would prevent adequate study assessments; orthodontic braces or any other orthodontic appliances; active prosthodontic treatment; history of weight reduction surgery were excluded from this study.

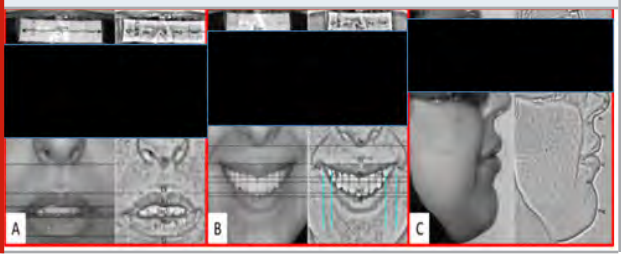
Data collection: Photographs were standardized using a Canon EOS 750D (Canon; Tokyo, Japan) digital camera with a resolution of 1080p and frame rate of 60 frames per second. The camera was mounted on an adjustable LED-ring-lighted tripod and placed in front of the subject. The camera was adjusted in front of the patient's lower face at a distance of 55 cm and continuously registered the face with the lens positioned parallel to the true perpendicular of the face in the natural head position. While the subject was in the natural head position, they wore eyeglasses with a clipped-on reference standard to enable calibration in a digital measurement program (Cooke and Orth, 1990).Three video recordings were made for each subject at each time point using the "P video" setting at 60 frames per second (frontal view of a subject at rest while talking, frontal view of a posed social smile, and lateral right side profile at rest). These videos were recorded pre-injection (T1), post-injection (T2), and at a follow-up one to two weeks later (T3) before retouching.

Filler injection type and technique: In this study, 0.8 mL of HA filler were injected using a 30-gauge needle 12 mm in length. Topical anesthesia was applied before filler injection. The dermatologist used the multiple linear retrograde threading technique. The amount of filler delivered was 0.5 mL to the upper lip and 0.3 mL to the lower lip. The injection points were 0.5 cm away from the corners of each lip.

Image analysis: Using software (OnyxCeph³™ 3.2.100 Build 233; Tigris Chemnitz, Germany), 114 digital photographs were analyzed for changes in all three dimensions. A total of 950 points were traced on the T1, T2, and T3 images.

Measurements for the frontal view at rest (Figure 1A):Included upper lip length (Sn-Sts), upper lip vermilion height (Ls-Sts), upper incisor display (Sts-IncU), and lower lip vermilion height (Sti-Li).

Figure 1: Frontal view measurements (A) frontal view at rest, (B) frontal smiling view and (C) lateral right-side profile view.



Measurements for the frontal smiling view (Figure 1B): Vertical measurements included upper lip length (Sn-Sts), upper lip vermilion height (Ls-Sts), upper incisor display (Sts-IncU), gingival display (UIGM-Sts), and interlabial gap (ILG; Sts-Sti). Transverse measurements included intercommissural width (ICW; smile width; MCR-MCL), premolar width (PMR-PML), buccal corridors ((MCR to MCL) - (PMR to PML)), and smile index (ICW (MCR to MCL) / ILG (Sts to Sti)).

Measurements for the lateral right-side profile view (Figure 1C) included the nasolabial angle (angle between tangent Pn-Sn and tangent LS-Sn), the upper lip protrusion to the E-line of Ricketts (Ls to Pn-Pog' line), lower lip protrusion to the E-line of Ricketts (Li to Pn-Pog' line), upper lip vermilion protrusion (ULP; LS perpendicular to Sls-Ils line), lower lip vermilion protrusion (LLp; Li perpendicular to Sls-Ils line), upper lip index (ULH x ULP), and lower lip index (LLH x LLP).

Examiner reliability: Reliability testing was carried out by having the examiner digitize the landmarks twice in a one-week interval for one randomly selected subject for each of the three different views. No significant differences were found between these examinations. The intra-class correlation coefficient was 1.00, which suggests 100% consistency across results.

Statistical analysis: The Statistical Package for Social Sciences (SPSS version 22.0; IBM, Armonk, NY) was used for statistical analysis. Sample size indicated a power value of 0.85, meaning that the chosen sample size allowed for statistical analysis. Friedman's test was used to detect differences in measurements while the Wilcoxon signed-rank test with Bonferroni correction was applied to test differences between multiple time points for a specific measurement.

RESULTS AND DISCUSSION

Frontal rest measurements: Table 1 shows the lip changes after filler injection for the frontal rest measurements. 1. Upper lip length at rest :The upper lip length at rest was significantly increased between T1 and T2 by a mean value of 0.77 mm; between T2 and T3 also increased by a mean value of 0.39 mm (statistically insignificant). The net change between T1 and T3 was also statistically significant (1.17 mm).1, Upper lip vermilion height at rest.

The upper lip vermilion height at rest was increased between T1 and T2 by a mean value of 1.92 mm; between T2 and T3, decreased by a mean value of -0.12 mm. The net change between T1 and T3 was increased by 1.79 mm mean value.1 Upper incisor display at rest. The upper incisor display at rest was significantly decreased between T1 and T2 by a mean value of -0.84 mm; between T2 and T3 increased by a mean value of 0.04 mm (statistically insignificant). The net change between T1 and T3 was significant and decreased by -0.8 mm mean value. 1 Lower lip vermilion height at rest.The lower lip vermilion height at rest was increased between T1 and T2 by a mean value of 1.2 mm; between T2 and T3 decreased by a mean value of -0.74 mm. The net change between T1 and T3 was increased by 0.45 mm mean value. The details are presented in Table 1.

Table 1. Lip changes after filler injection for the frontal rest measurements.

Frontal at rest	T1 – T2		T2 – T3		T1 – T3	
	Mean	P value	Mean	P value	Mean	P value
upper lip length	0.77857	0.001*	0.39286	0.026	1.17143	0.005*
upper lip vermilion height	1.92143	0.001*	-0.12857	0.011*	1.79286	0.005*
Upper incisor show	-0.84286	0.001*	0.04143	0.023	-0.80143	0.005*
lower lip vermilion height	1.20	0.001*	-0.74286	0.008*	0.45714	0.005*

* Statistical significance at p≤0.017

Frontal smiling measurements: Table 2 shows the lip changes after filler injection for the smiling measurements.

1. Upper lip length: The upper lip length while smiling was significantly increased between T1 and T2 by a mean value of 1.04 mm; between T2 and T3 also increased by a mean value of 0.4 mm (statistically insignificant). The net change between T1 and T3 was significantly increased by 1.45 mm mean value.
2. Upper lip vermilion height while smiling:The upper lip vermilion height while smiling was significantly increased between T1 and T2 by a mean value of 1.23 mm; between T2 and T3 also increased by a mean value of 0.49 mm (statistically insignificant). The net change between T1 and T3 was significant and increased by 1.73 mm mean value.
3. Upper incisor display at a smile:The upper incisor display while smiling was statistically significant decreased between T1 and T2 by a mean value of -1.1 mm; between T2 and T3 increased by a mean value of 0.5 mm (statistically insignificant). The net change between T1 and T3 was also significant and decreased by -0.6 mm mean value.
4. Gingival display while smiling :Gingival display while smiling was significantly decreased between T1 and T2 by a mean value of -0.78 mm; between T2 and T3 also decreased by a mean value of -0.12 mm (statistically insignificant). The net change between T1 and T3 was significant and decreased by -0.9 mm mean value.
5. Interlabial gap while smiling: Interlabial gap while smiling was significantly decreased between T1 and T2 by a mean value of -1.6 mm. However, between T2 and T3 increased by a mean value of 0.49 mm (statistically insignificant). The net change between T1 and T3 was significant decreased by -1.11 mm mean value.
6. Intercommissural width while smiling and Buccal corridors while smiling
7. There was no statistically significant difference for the intercommissural width and buccal corridors at smile between T1 and T2; T1 and T3; and T2 and T3.
8. Smile index:The smile index was significantly increased between T1 and T2 by a mean value of 1.97 mm. However, between T2 and T3 it was decreased by a mean value of -0.49 mm. (statistically insignificant). The net change between T1 and T3 was significant increased by 1.48 mm mean value. The details are presented in Table 2.

Table 2. Lip changes after filler injection for the smiling measurements

Frontal smiling	T1 – T2		T2 – T3		T1 – T3	
	Mean	P value	Mean	P value	Mean	P value
Upper lip length	1.04286	0.004*	0.40714	0.646	1.45000	0.005*
Upper lip vermilion height	1.23571	0.002*	0.49429	0.645	1.73000	0.005*
Upper incisor show	-1.10714	0.005*	0.50143	0.333	-0.60571	0.011*
lower lip vermilion height	-0.78571	0.016*	-0.12143	0.838	-0.90714	0.005*
Interlabial gap	-1.60714	0.001*	0.49571	0.153	-1.11143	0.005*
Intercommissural width	-0.65714	0.035	0.56857	0.314	-0.08857	0.107
Smile index	1.97857	0.001*	-0.49571	0.202	1.48286	0.005*

* Statistical significance at p≤0.017

Table 3. Lip changes after filler injection for the Lateral measurements

Lateral measurements	T1 – T2		T2 – T3		T1 – T3	
	Mean	P value	Mean	P value	Mean	P value
Nasolabial angle	-5.87143	0.001*	3.46857	0.028	-2.40286	0.005*
Upper lip protrusion to E-line	1.32857	0.001*	-0.63857	0.052	0.69000	0.005*
Lower lip protrusion to E-line	0.90714	0.001*	-0.54857	0.065	0.35857	0.005*

* Statistical significance at p≤0.017

Main lateral measurements: Table 3 presents lip changes after filler injection for the Lateral measurements.

1. **Nasolabial Angle:** The nasolabial angle was significantly decreased between T1 and T2 by a mean value of -5.87°; between T2 and T3 the angle increased by a mean value of 3.46° (statistically insignificant). The net change between T1 and T3 was statistically different by -2.4°.
2. **Upper lip protrusion to E-line:** The upper lip protrusion to the E-line was significantly increased between T1 and T2 by a mean value of 1.32 mm; decreased between T2 and T3 by a mean value of -0.63 mm. The net change between T1 and T3 was statistically significant by 0.69 mm.
3. **Lower lip protrusion to E-line:** The lower lip protrusion to the E-line was significantly increased between T1 and T2 by a mean value of 0.9 mm; decreased between T2 and T3 by a mean value of -0.54 mm. The net change between T1 and T3 was statistically significant 0.35 mm.

The present study was aimed to analyze the changes in lip morphology following lip filling injections. The changes were observed in the upper and lower lips of each individual in three dimensions including vertical, transverse, and sagittal planes. A total of 114 photographs were taken and evaluated for this study. The images were taken before and after the procedure.

The measurements show striking differences between images taken immediately after injection and those taken one to two weeks after the procedure. This suggests that the effect of lip fillers fades significantly over time; as a result of which, we considered a third measurement (T3) to determine the actual effect of HA fillers. One of the main reasons behind the changes noted over time following lip filling is the injection site reaction that follows immediately after HA filler injections. According to Chiu et al, injection site reactions are fairly common after HA injections to the lip, despite being short-lived (Chiu et al., 2016). According to Lafaille and Benedetto, the most common side effects related to HA lip fillers are at the local injection site, including pain, redness, edema, ecchymosis, and itching (Lafaille and Benedetto, de Maio, 2020). However, these side effects are usually mild and short-lived , Abduljabbar and Basendwh, 2016, Lipko-Godlewska et al., 2021).

Upper lip length has a significant impact on the way lips equilibrate with one another. A shorter upper lip may decrease the lip seal and increase the interlabial gap at rest. Furthermore, the shorter the upper lip length, the greater the gingival exposure while smiling (Miron et al., 2012, Seixas et al., 2011). Our study suggests that the upper lip can increase by roughly 1.2 mm, which is the length measured from the base of the nose (Sn) to the lowermost border of the upper lip (Sts) after lip filler injections. Subsequently, as a result, the visibility of the upper incisors decreased by only 0.8 mm. Hence, it can be estimated that the reduction in incisor display is roughly around two-thirds of the amount of upper lip length gained. Furthermore, the remaining one-third could be due to a change in the Sn point position as a result of vermilion lip projection. As per our observations, upper lip vermilion height grew by around 1.8 mm whereas

the lower lip vermilion height increased by almost 0.45 mm. The difference between vermilion heights of the upper and lower lip can be attributed to different quantities of HA fillers injected.

The upper lip was injected with 0.5 mL of HA filler whereas the lower lip was injected with 0.3 mL. Concerning vertical smile measurements post-injection, the upper lip length and vermilion height increased by 1.45 mm and 1.73 mm, respectively. As a result, the upper incisor display and gingival display decreased by 0.6 mm and 0.9 mm, respectively. In contrast, BTX-A injections in subjects with large gingival displays (a "gummy smile") resulted in gingival display reductions of 5.2 mm (Polo, 2008). To evaluate the effect of dermal fillers on gingival display reduction, we required subjects with gummy smiles before filler injections. However, only 1 out of the total 14 subjects presented with a gummy smile before filler injection at T1, and they dropped out of the study after T2. Therefore, our findings of gingival display reduction relied on changes between T1 and T3 associated with the amount of upper lip length changes instead of actual gingival display changes.

The observations regarding the amount of reduction in gingival display achieved after lip filler injections are comparable to the report by Goldstein et al. (Goldstein et al., 2009). The use of dermal fillers for lip augmentation in cases with gummy smiles can be very helpful to improve the aesthetics of fixed prosthetic constructions for women with high or medium smile lines (Fitzgerald et al., 2019). Vlahova et al. (2014a) showed pre- and post-procedure pictures that illustrated a decrease in smile line in two patients injected with HA fillers and they concluded that if a gummy smile is solely because of a thin upper lip, then lip-filling HA injections can give satisfactory results by improving the esthetics of a smile. Our suggested results are also in line with findings by Dalati, who stated that dermal fillers are used for lip augmentation and are used by dentists for cases of high lip lines, uneven lips, and to make the perioral area more esthetically pleasing (Dalati and Koussayer, 2020).

Even though all of the studies mentioned have results that can be compared to ours, they did not provide exact measurements. In contrast, our study showed that the mean gingival display reduction was almost 0.9 mm.The transverse dimension of the smile is a major characteristic of smile analysis. The transverse dimension affects smile broadness and buccal corridors (more commonly referred by orthodontists as negative space) (Sarver, 2001).The effect of HA filler on intercommissural width and buccal corridors in our current study did not show a significant difference before and after injection. It can be suggested that the injection technique of terminating the filler injection 0.5 cm away from the corner of the mouth may contribute to these findings.

To visualize and quantify the frontal smile, Ackerman and Ackerman developed a ratio known as the smile index, which describes the area framed by the vermilion borders of the lips during a social smile (Ackerman and Ackerman, 2002). The smile index is determined by dividing the intercommissural width by the interlabial gap while smiling. This ratio helps compare smiles among different patients or over time in a single patient. It was suggested that the lower the smile index, the less youthful the smile appears. Our results for the smile index increased after HA filler injection by almost 1.48 mm. As the intercommissural width did not change significantly in our study, it can be said that it did not affect the smile index, which was mainly affected by changes in the interlabial gap. As per our observations, the interlabial gap decreased by roughly 1.11 mm. Hence, it can be said that HA lip fillers can contribute negatively to the beauty of a smile due to a decrease in smile length, even if the smile index increases.

The nasolabial angle is influenced by the inclination of the columella of the nose and by the position of the upper lip. According to Sarver and Jacobson, one of the factors affecting the nasolabial angle is the soft tissue thickness of the maxillary lip, as a thin upper lip favors a flatter angle and a thicker lip favors an acute angle (Sarver and Jacobson, 2007). Our results showed that the nasolabial angle decreased after filler injection by almost 2.4°. Therefore, the HA filler caused a narrowing of the nasolabial angle.The E-line, or the esthetic line of Ricketts, is drawn from the tip of the nose to the most anterior point on the soft tissue of the chin (Ricketts, 1957). This line is considered to be a very valuable diagnostic tool for an orthodontist to detect the amount of anterior-posterior soft tissue projection.

Our results showed that upper and lower lip protrusion to the E-line increased after filler injection by 0.69 mm and 0.35 mm, respectively. Upper and lower lip vermilion protrusion were measured by perpendicular lines from the most anterior points of the vermilion of the lips to a line connecting the point of greatest concavity between the nose and upper lip and a point of greatest concavity between the chin and lower lip.22 Our results showed that the upper and lower lip vermilion protrusion increased after filler injection by almost 0.91 mm and 0.56 mm, respectively.A simple lip index was proposed to evaluate the clinical effects and duration of dermal fillers (Lemperle et al., 2010). This index is calculated by multiplying the length of the vermilion lip by its protrusion. The upper and lower lip indices for our sample increased after filler injection by almost 15.94 mm2 and 8.31 mm2, respectively.

At the time of writing this manuscript, we could not find any study that measured the impact of dermal fillers on lips with such linear and angular measurements. Therefore, we could not objectively compare the results of this study with others. In this study, we noticed differences in lip measurements before and after filler injection at all time points in the frontal rest view and the frontal smile view, and some differences in the lateral view, and so our null hypothesis was partially rejected.

Ethical Statement: The Board observed that you have complied with the Ethics Codes if the Scientific Research specified by the RCsDP has approved your proposal.The IRB approval number is RC/IRB/2016/586 which you may use as needed in future for conferences,poster presentation and publications.

You are allowed to start your investigation starting 11-19-2017,please comply with the recommendations specified by the IRB.We wish you a successfull project.

CONCLUSION

Within the limitations of the present study, most static changes were gained at T3 (one to two weeks after filler injection), which we consider to be the actual effect of the HA filler. Injecting HA filler to the lips leads to an increase in the following parameters: lip length at rest and while smiling, lip vermilion height at rest and while smiling, smile index, lip protrusion to the E-line, lip vermilion protrusion, and lip index. Injecting HA filler to the lips also leads to a decrease in the following parameters: upper incisor display at rest and while smiling, gingival display while smiling, interlabial gap while smiling, and nasolabial angle. Injecting HA filler to the lips may not alter intercommissural width or buccal corridors.

Conflict of Interest: The author reports no conflicts of interest in this work.

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Ethical Clearance Statement: The Board observed that you have complied with the Ethics Codes if the Scientific Research specified by the RCsDP has approved your proposal.The IRB approval number is RC/IRB/2016/586 which you may use as needed in future for conferences,poster presentation and publications. You are allowed to start your investigation starting 11-19-2017,please comply with the recommendations specified by the IRB.We wish you a successfull project.

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Technological Communication

Experimental Investigation on Performance and Exhaust Emission Characteristics of Variable Compression Ratio Diesel Engine Fueled with Microalgae Biodiesel Blends

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ABSTRACT

In the current competitive situation, microalgae are the finest prominent alternative of biodiesel among all the other available sources. Microalgae are non-eatable and doesn't require crop land for its cultivation which makes the microalgae most attractive source to study. This paper is aimed to investigate experimentally the competency of novel, less considered biodiesel from microalgae species, which will be developed as an alternative fuel in place of traditional diesel. In this study, the most common chemical process, transesterification was used to extract biodiesel from microalgae. As per the requisite of experimentation five microalgae blends (MAB) were prepared on volumetric basis. The experimentation was performed on single cylinder variable compression ratio diesel engine to investigate the impact of microalgae biodiesel and its blends on performance as well as exhaust emission characteristics. All the experiments were carried out as per standard operating procedure and with due precautions. Brake power, brake specific fuel consumption and brake thermal efficiency were the performance characteristics focused during the experimentation. Microalgae blends indicated reduction in torque and hence brake power which resulted in average reduction of 7.14 % in the brake thermal efficiency. Brake specific fuel consumption increased by 11.54 % for microalgae as equated to traditional diesel. As a need of time emission characteristics must be evaluated along with performance. So, the main emission characteristics were also investigated in this work. As blending ratio increased, considerable reduction in exhaust emission characteristics of carbon monoxide (CO) plus hydrocarbon (HC) were recorded. Conversely, for all microalgae biodiesel blends, nitrogen oxides (NOx) and carbon dioxide (CO_2) increased little which was compatible and in acceptable range. The performance as well as emission characteristics using microalgae biodiesel were found satisfactory.

KEY WORDS: ALGAL BIODIESEL, EMISSION CHARACTERISTICS, FUEL PROPERTIES, PERFORMANCE CHARACTERISTICS, VCR DIESEL ENGINE.

INTRODUCTION

The current status of traditional energy sources is quite fickle and the world economy depends upon it. Rapidly depleting petroleum derivatives, on the other hand percentage increase in their cost likewise with disturbing increase in pollution levels are significant points of concerns for the general public in this scenario. To counter this situation, lots of biodiesel alternative are identified but they denied complete replacement for traditional diesel. This may be due to their unattractive physiochemical properties (Tayari et al. 2020).

In addition to this an enormous number of vehicles is being offered on the roads regularly which increase stress on petroleum industries and environment. So as per BP Statistical Review of World Energy, it is a need of time for offering new varieties of alternative fuel to rout the exhaustion of petroleum products and expansion in environment pollution. World Energy 2020 Statistical Review by BP pointed towards a gap of 4287 thousand barrels per day between oil utilization and its supply for our country. Considering the food sock for mankind, it is always desirable to go with non-edible sources because of its several advantages over edible sources. As per the report of petroleum ministry, the yearly measured petroleum products consumption in India is nearly 120 million tonnes, and it is increasing (Kale et al. 2021).

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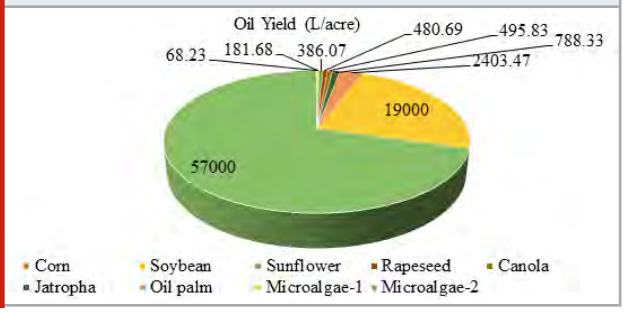
MATERIAL AND METHODS

Raw Algae sample was collected from river outskirts at Salai (Mendha) Nagpur district, Maharashtra (Figure 2a). This raw alga was washed very clearly (Figure 2b). After washing these algae were grounded in a motor mixer to form homogeneous paste (Figure 2c). The grounded homogeneous algae paste was dried out at 800C for emancipating water (Figure 2d). 20ml Hexane plus 20 ml ether solution were mixed with this dried grounded homogeneous alga. This mixture was set aside for 24 hours to get settle down. After total settlement, the mixture was filtered to separate biomass and after separation it was measured. The filtered biomass contained hexane and ether so it was evaporated in vacuum to make it free from hexane and ether. Afterwards 0.25 grams of NaOH (as a catalyst) was mixed with this extracted methanol. Proper stirring using magnetic stirrer was carried out to achieve uniform mixing. Then this mixture of catalyst plus methanol was dispensed into the algae oil. The standard operating procedure given by American society for testing and materials standards (ASTM) was adopted during this biodiesel production. The most popular transesterification chemical reaction was implemented to get decent quality of microalgae biodiesel (Kale et al. 2021).

The conical flask containing solution of methanol, catalyst and algae oil was shaken at 300rpm and then set aside to settle down. The biodiesel was separated from sedimentation and remaining amount of residue, containing glycerine, was assessed through colours. Biodiesel was washed by 5% water and then dried using dryer to make it totally water free. Biodiesel production was measured and stored safely. For experimentation purpose, microalgae biodiesel was blended with diesel on volume basis to get microalgae biodiesel blends as represented in figure 4 (Kale et al. 2020). Implementing above methods with precision, the microalgae biodiesel blends MAB10, MAB20, MAB30, MAB40, and MAB50 were prepared as shown in Figure 5.

Figure 1: Comparison of oil yield (L/acre) from various sources of biodiesel



RESULTS AND DISCUSSION

The oil yield from some edible and non-edible biodiesel sources are presented in figure 1. From the figure, one thing is very clear that, for a yield like soybean or palm, a colossal level of crop land is needed to substitute petroleum diesel entirely. Hence, the use of this assessable land for biodiesel purpose was not feasible. On the other hand, while considering microalgae oil yield per acre, it has been prophesied that nearby 02 to 03 % of entire crop land is sufficient for providing sufficient microalgae biodiesel to supersede all petroleum diesel concern of our country. Undoubtedly, microalgae feedstock is major option for vast biodiesel production (Subhaschandra et al. 2019).

Figure 2: Steps in microalgae biodiesel extraction (Kale et al. 2021).

The steps followed in biodiesel preparation are given in figure 2. The chemical reaction used for biodiesel preparation is provided in figure 3. The techniques used for blends formation is illustrated in figure 4. Figure 5 shows the actual photographs of microalgae blends prepared on volumetric basis.

Figure 3: Transesterification reaction for biodiesel extraction (Kale et al. 2021).

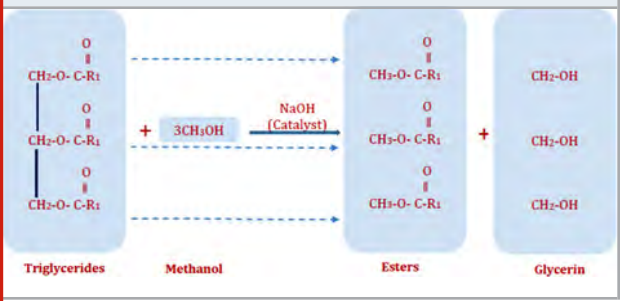


Figure 4: Microalgae biodiesel blends preparation on Volume basis

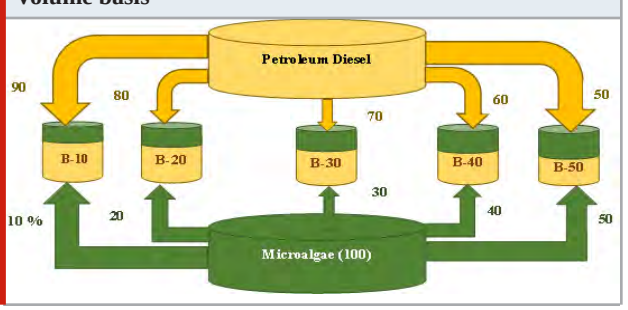


Figure 5: microalgae biodiesel blends with petroleum diesel (Actual photographs)

Properties of Test Fuel: It is very important to know the physical plus chemical properties of biodiesel and its blend fuel so as to avoid any harm to engine and proper reading (Tayari et al. 2020). Following the ASTM Standard, the key properties found out in institute laboratory that were viscosity, cetane number, density, flash point, fire point and heating value. These properties were put forth in table 1.

Table 1. Microalgae blends chemical and physical properties

Properties	Petroleum diesel	Algae Biodiesel	MAB10	MAB20	MAB30	MAB40	MAB50
Kinematic Viscosity at 104 oF (mm2/s)	2.7	3.160	2.746	2.792	2.838	2.884	2.930
Cetane Number	49	48	48.8	48.72	48.66	48.59	48.52
Density @ 150C (kg/m3)	830	881	835.1	840.2	845.3	850.4	855.5
Flash point (0C)	64	150	72.6	81.2	89.8	98.4	107
Fire point (0C)	71	83	72.2	73.4	74.6	75.8	77
Heating Value (MJ/kg)	42	40.5	41.85	41.83	41.78	41.73	41.69

Experimental Test Rig: Variable compression ratio CI Engine test rig situated in thermal engineering laboratory of Institute is shown in figure 6. This test rig was used for experimentation. A single cylinder engine was coupled with eddy current dynamometer for loading. The setup was with facility of changing compression ratio from 12.5 to 18:5. The detailed specification of experimental setup were brief in table 2. ARAI Approved MARS Multi Gas analyser (Model: MN-05,) as shown in figure 7 was used for emission parameters measurement. Measurement range along with its accuracy was also provided in table 3.

Figure 6: Actual photograph of experimental test rig

Performance characteristics: Brake Thermal Efficiency (BTE) Figure 8 represents the variation of brake thermal efficiency with respect to load applied. As the load on engine increased the brake thermal efficiency also increased but it declined with fuel blends when compared with petroleum diesel. Specific fuel consumption (SFC) increased for microalgae biodiesel blends because of its lower calorific value. This obviously decreased the brake thermal efficiency. With an increase in blending proportion, there was a reduction in the rate of heat release which finally declined the brake thermal efficiency of the engine (Kalsi et al. 2017; Tayari et al. 2020).

Table 3. Technical specification of Experimental Test Rig

Parameter	Specification
Model	TV1
Manufacturer	Kirloskar Oil Engines
Form	4-Stroke
Total No. of cylinder	01
Bore Diameter (m)	0.0875
Stroke Length (m)	0.11
Cubic capacity (cm3)	661
Standard CR	17.5:1 (Variable)

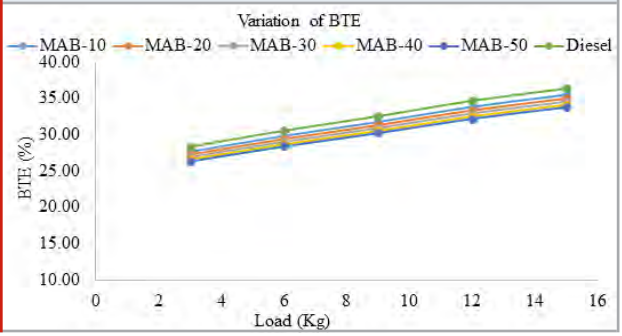
Figure 7: Actual photograph of multi gas analyser (MARS, Model: MN-05)

Table 3. Measurement Range Resolution of Multi Gas analyser

Parameter	Range	Accuracy
CO	0 to 9.99% Volume	0.001% Volume
CO_2	0 to 20% Volume	0.01% Volume
HC (propane)	0 to 15000 ppm	01 ppm
O_2	0 to 25% Volume.	0.1% Volume
NOx	0 to 5000 ppm	1 ppm Volume
Engine RPM	500 to 6000 rpm	1 rpm
Lambda	0.200 to 2.000%	0.001

According to the experimental results brake thermal efficiency obtained was 32.09% for diesel fuel conversely 31.32%, 30.87%, 30.49%, 30.10% and 29.80% for MAB10, MAB20, MAB30, MAB40 and MAB50 microalgae biodiesel blends respectively. On an average, the reduction was observed in case of brake thermal efficiency for MAB10 by 2.41% and MAB50 by 7.14 % in comparison with diesel fuel. As the loading on engine increased, it was observed that brake thermal efficiency also increased. Analogous results trends were attained by earlier researchers in their work (Elsanusi et al. 2017; Datta et al. 2017; Srihari et al. 2017: Can et al. 2017; Tayari et al. 2020).

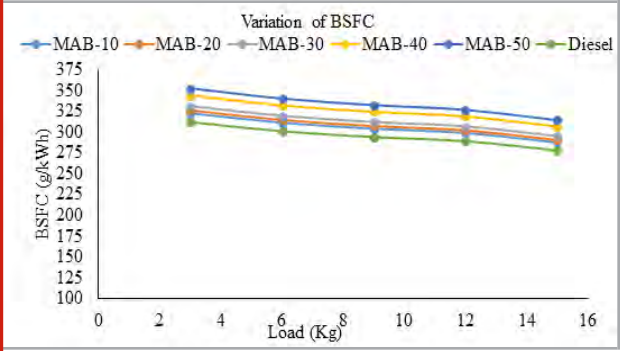
Figure 8: Brake Thermal Efficiency (BTE) variation with respect to load



Brake specific fuel consumption (BSFC): Brake specific fuel Consumption was subjected directly on energy value (Calorific /Heating Value) of the fuel. The heating value significantly affected the complete combustion process of the fuel. Because of inferior heating value, biodiesel and its blends directly influenced the increase in brake specific fuel consumption values (Elsanusi et al. 2017; Datta et al. 2017). Moreover, the variation in brake specific fuel consumption in accordance to engine ran on different loading was represented in figure 9. BSFC was minimum for diesel fuel, trailed by all microalgae biodiesel blends at applied loads. In overall, there was an increase in BSFC for MAB10 by 3.43 % and MAB50 by 11.54 % compared with diesel fuel. For Individual microalgae biodiesel blends, BSFC was decreasing with higher load since combustion efficiency increased and this was also proved from the figure 9. The analogous propensity of result was also observed in a referred study (Srihari et al. 2017: Can et al. 2017; Tayari et al. 2020).

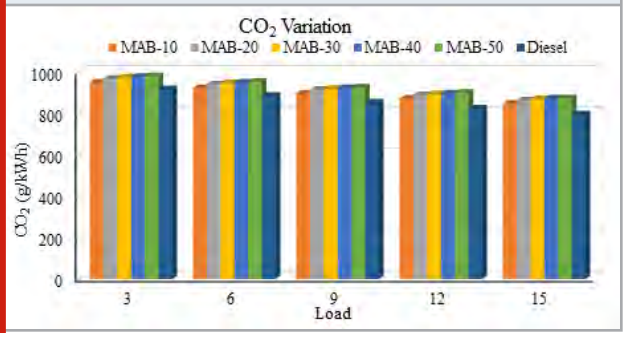
Emission Characteristics: Engine exhaust emission characteristics such as CO, CO_2, HC, NOX, emissions in accordance with various loads provided on test engine were found experimentally for microalgae biodiesel blends and equated it with petroleum diesel fuel. Carbon dioxide (CO_2) exhaust emissions, viscosity, process of atomization, compression ratio, oxygen, rpm of engine etc. were the factors which influence CO_2 emissions from engine exhaust (Muralidharan et al. 2011; Rahman et al. 2013; Celik et al. 2017; Gharehghani et al.2017 Shrivastava et al. 2019). Figure 10 depicts CO_2 emissions variation in accordance to engine loading. CO_2 was obtained to be higher for microalgae biodiesel blends in comparison with diesel. From the figure 10, it can be stated that, CO_2 emission was increasing with an increase in blending proportion i.e. From B10 to B50 and decreased with engine load increase.

Figure 9: Brake Specific Fuel Consumption (BSFC) variation with respect to load



The CO_2 exhaust emissions was 853.96 g/ kWh for petroleum diesel whereas it was 896.47g/kWh for MAB10 microalgae biodiesel blend. There was an increment of 4.74 % for B10 microalgae biodiesel blend in CO_2 exhaust emissions, 6.52 % for MAB20, 7.07 % for MAB30, 7.43 for MAB40 and 7.70 % for MAB50 microalgae biodiesel blend. Though the CO_2 Emissions were higher but quite compatible with diesel fuel.

Figure 10: CO_2 exhaust Emissions Variation with respect to load



Nitrogen oxides (NOX) Exhaust Emissions: Combustion temperature, oxygen contents of the test fuel and the actual space of combustion zone were the aspects which directly

dominated NOX exhaust emissions (Zehra et al. 2014). Stoichiometry, flame temperatures, delay in ignition, composition of fatty acids for fuel, rate of heat removal (HRR), premixing, combustion space, fuel cetane number, injection timing and thermo-physical properties of the fuel were different aspects which influence NOX exhaust emissions (Rajak et al. 2018; Shrivastava et al. 2019; Subhaschandra et al. 2019). Fig. 11 illustrates the NOX exhaust emission variations for microalgae biodiesel blends and petroleum diesel fuel in accordance with engine loads. It was witnessed that, as the engine load increased, NOX exhaust emission also gradually increased. The average measured values for NOX emissions were 648 ppm for petroleum diesel fuel, 669.77 ppm for MAB10,694.20 ppm for MAB20, 714.09 ppm for MAB30, 741.37 ppm for MAB40, and 722.19 ppm for MAB50. This may correlate to the greater content of oxygen level in case of microalgae biodiesel as well as its various blends which increased the combustion gas temperature and resulted in increasing NOx development by giving surplus oxygen (Song et al. 2002; Shrivastava et al. 2019).

Figure 11: NOX exhaust emission Variation with respect to load

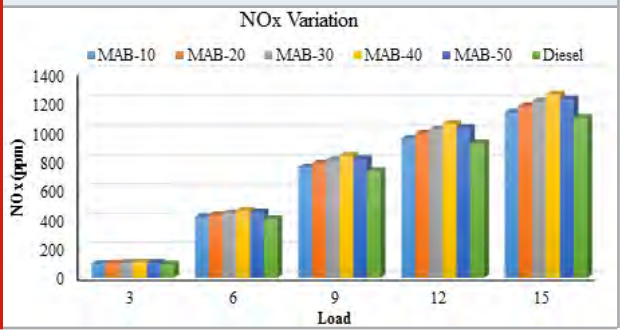
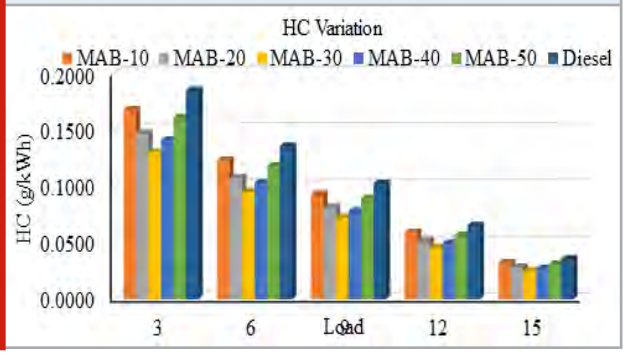
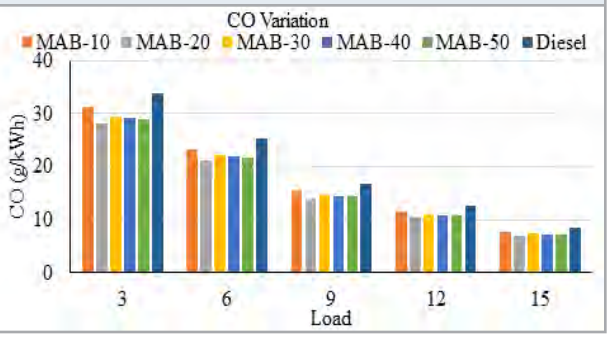


Figure 12: HC exhaust emission Variation with respect to Load



Hydrocarbon (HC) Exhaust Emissions: Figure 12 depicts hydrocarbons (HC) exhaust emissions variations for all the microalgae biodiesel blends fuels as well as petroleum diesel in accordance with engine load. The average HC emissions were found as 0.1045 g/kWh in case of petroleum diesel fuel, whereas for MAB10 it is 0.0949 g/kWh, 0.0830 g/kWh for MAB20, 0.0736 g/kWh for MAB30, 0.0796 g/kWh for MAB40, and 0.0909 g/kWh for MAB50 microalgae biodiesel blends. The average reduction in HC emissions with respect to diesel fuel was 9.2% for MAB10, 20.58% for MAB20, 29.62% for MAB30, 23.84% for MAB40, and 13% for MAB50 Microalgae biodiesel blends. This might be correlated with the greater kinetic viscosity of biodiesel blends, impeding in fuel atomization and therefore benefits the HC emissions (Celikten et al. 2012; Shrivastava et al. 2019).

Figure 13: CO exhaust emission Variation with respect to load



Carbon Monoxides (CO) Exhaust Emissions: Figure 13 illustrates CO exhaust emissions variations for all tested microalgae biodiesel blends and petroleum diesel fuel with respect to the different engine loading conditions. A massive reduction in CO emissions at higher engine load was witnessed for microalgae biodiesel blends. The average CO emissions were found as 19.41 g/kWh for petroleum diesel fuel, 17.92 g/kWh for MAB10, 16.15 g/kWh for MAB20, 16.96 g/kWh for MAB30,16.83 g/kWh for MAB40 and 16.59 g/kWh for MAB50. This reduction in CO emission might be explained in linkage with fact that microalgae Biodiesel and its blends were an oxygenated fuel and they had additional oxygen atoms which comforts the complete combustion reaction, thus transforming CO to CO_2 molecules, consequently a noteworthy amount of drop in CO emission was observed in case of Biodiesel Blends with diesel (Celikten et al. 2012; Shrivastava et al. 2019).

CONCLUSION

The findings of the present study illustrated that the brake thermal efficiency and brake specific fuel consumption were increased for all microalgae biodiesel blends. Increment of in CO_2 emissions in case of all microalgae biodiesel blends was recorded. Moreover, it was witnessed that, with an increase in engine load, NOX exhaust emission also gradually increased. The average reduction in HC and specific CO emissions with respect to diesel fuel was observed. Microalgae biodiesel blend MAB30 gave the finest results in terms of performance plus exhaust emission characteristics are concerned.

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

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Biotechnological Communication

Production of Tannin Acyl Hydrolase and its Purification from *Klebsiella pneumoniae*

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ABSTRACT

Tannases or Tannin acyl hydrolases (EC 3.1.1.20) belonging to the superfamily of hydrolase finding their applications in food, brewing, chemical, and pharmaceutical industries etc., A number of fungal tannase were very well characterised. However, little is known about the extra cellular bacterial tannases and their properties. The present investigation was undertaken with an objective of tannic acid degradation from bacterial tannase. The bacterium was cultivated in MSM medium containing 0.3% tannic acid as sole carbon source and produced extracellular tannases from early lag phase (4 h) and increased exponentially till 10 h (*Klebsiella pneumoniae* BH49). The strain was confirmed by different biochemical tests and16S rDNA phylogenetic analysis. The crude enzyme showed maximum activity at pH 6.0 at 30 ºC and the activity was retained 99.33% at 40 ºC. The Km of the crude enzyme showed 0.0518 mM and 0.0389 mM for methyl gallate and propyl gallate as substrate respectively. The specific activity of the crude enzyme was found to be 0.943U/mg for methyl gallate. In addition, the activity was significantly increased in presence of K^{+}, Mg^{2+}, and Ca^{2+} similarly, the activity was inhibited by Fe^{2+} Mn^{2+} and Cu^{2+}. The enzyme was purified by ammonium sulphate fractionation followed by DEAE-Cellulose (Ion exchange chromatography). In gel staining confirmed major tannase enzyme and the SDS-PAGE analysis of the purified enzyme showed, the molecular weight of 55 kDa. Our investigations would give potential source for efficient production of extracellular tannase and can used for tannery effluent degradation, pharmaceutical and industrial applications.

KEY WORDS: GALLIC ACID, METHYL GALLATE, PROPYL GALLATE, RHODANINE.

INTRODUCTION

Tannins are generally considered recalcitrant to biodegradation and major effluents of tanning industries and they pose potential threats to human health as well as the environment (Bari et al. 2015; Adamczyk et al. 2017). However, despite their toxic effects, some microorganisms have evolved to use gallotannins as carbon sources for growth by the action tannin acyl hydrolases, commonly known as tannases (Tannin acyl hydrolase E. C. 3.1.1.20). Tannase, that hydrolysis of ester and depside bonds in varied substrates like gallotannins, gallic acid esters (Govindarajan et al. 2016a, Govindarajan et al. 2016b; Tripathi et al. 2018; Xu et al. 2019). Microorganisms are the main source of industrial enzymes due to their biochemical diversity and their amenability to genetic modifications. A significant number of tannase-producing microorganisms especially fungi and bacteria were identified (Thiyonila et al. 2020).

Fungal tannases have been well documented for their potential bioconversion and specifically for the biotransformation of tannic acid to gallic acid. To date, commercially available tannases are mainly produced by fungal species (Tripathi et al. 2016; Dhiman et al. 2018). However, the usage in industrial purpose is limited due to its lower catalytic efficiency in front of bacterial tannase. Previous studies demonstrated that tannases from yeast have been only verified in Sporidiobolus ruineniae, *Candida* sp., *Aureobasidium, Blastobotrys adeninivorans*, and *Kluyveromyces marxianus* (Beniwal et al. 2010; Dhiman et al. 2018). Bacterial strains that belong to genera such as *Staphylococcus, Lonepinella, Lactobacillus, Pseudomonas, Serratia, Bacillus, Azobacter, Klebsiella, Citrobacter, Pantonea*, and *Enterobacter were predominant* (Tripathi et al. 2016; Thiyonila et al. 2020).

Meanwhile, the degradation of natural tannins by bacterial tannase were found to be very effective due to its industrial

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applications and its rapid catalytic degradation potential (Wang et al. 2019). It is crucial to isolate and identify the potential source for tannase-producing bacteria. In the present study, we have purified and biochemical properties were investigated for extracellular tannase from *Klebsiella pneumoniae* BH49 potential source for pharmaceutical and industrial applications.

MATERIAL AND METHODS

Tannic acid, Gallic acid, Methyl gallate, Propyl gallate, and Rhodanine were obtained from Sigma Chemical Co., St Louis, MO, USA. The culture media components used (Hi-Media, Mumbai), other chemicals used were of analytical grade. For the isolation of the microorganism, soil samples were collected from near Slaughter house habitat (Tannery Rd, Richards Town, Bengaluru, Karnataka, India). About 1 gram of tannic acid was enriched to soil was serially diluted by suspending in 10 ml of sterile distilled water and the suspension was swirled about for a period of one to two hours. The serially diluted tubes (dilutions of 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}) were plated on a LB Agar containing tannic acid. In total, four plates were made each plate containing a different concentration of tannic acid namely, 0.1%, 0.2%, 0.3% and 0.4% respectively.

For the identification of the microbial strain, morphological characteristics of the bacteria were determined by performing by microscopic observations, and the isolated pure strain was subjected to various biochemical tests according to Bergey's manual of determinative bacteriology (Baird-Parker 1974). Further, the pure strain was confirmed by 16S rDNA and phylogenetic analysis (Sambrook and Russell 2001). For the analysis of substrates and products by thin layer chromatography and to determine the fate of the accumulated metabolites, the cells were pelleted by the configuration at 10,000 rpm for 10 min at 4 °C. The supernatant containing gallic acid was extracted with ethyl ether (1:5 v/v), further the extracts were dried and resuspended in methanol. Thin layer chromatography was carried out by spotting the methanol extract with authentic substrate (tannic acid) and metabolite (gallic acid). The plates were developed using pentane saturated acetonitrile and toluene (2:1 v/v) containing 1.25% formic acid. The metabolites were visualized by exposing the plates to iodine vapors (Ferry et al. 1991).

For the production of extracellular Tannase by *Klebsiella pneumoniae* BH49 was analyzed in MSM supplemented with 0.3% tannic acid (filter sterilized). Extracellular tannase (Spent medium) was pooled in batch cultures in 100 ml Erlenmeyer flasks in three independent replicates. The crude enzyme at different time points were chilled and centrifuged at 10,000 rpm for 10 min at 4°C for further analysis. For enzyme assay, after implicating a few modifications, the activity of tannase was assayed by using methyl gallate as substrate. The activity was measured in terms of micromoles of gallic acid formation (Sharma et al. 2000). For the biochemical characteristics of extracellular Tannase, temperature optima, the crude tannase activity was carried out by incubating enzyme and substrate at various temperatures ranging from 4°C to 100°C, the percentage activity and optimum temperature were determined spectroscopically.

For temperature stability, enzyme stability was determined by incubating the enzyme along with the buffer for 20 min at different temperatures ranging from 4°C to 100°C. Further, the substrate was added and incubated at room temperature for an enzyme reaction. For pH optima, the effect of pH on the enzyme activity was studied by conducting the reaction at various pH ranging from 3-10 and assayed for tannase to determine the pH optimum. For pH stability, enzyme stability was studied by pre-incubating the enzyme along with the buffer pH ranging from 3-10 for 20 min. Further, the substrate was added to the above solutions and incubated for 20 min. To determine the effect of metal ions on tannase activity, different metal like Fe^{2+}, Zn^{2+}, Ca^{2+}, Mn^{2+}, Mg^{2+}, Co^{2+}, K^{+} and Cu^{2+} were dissolved in citrate buffer (50 mM pH 6.0). The enzyme was preincubated in a buffer containing different metal ions for 15 min, and the reaction was initiated by adding substrate.

For the determination of Km and Vmax, different concentrations of methyl and propyl gallate were used for enzyme activity to determine Km and Vmax of the tannase was determined by Lineweaver-Burk plot. For the purification of extracellular Tannase, initially, the spent medium was subjected to ammonium sulfate fractionation (80%), followed by centrifugation for 12000 rpm for 15 min at 4 °C. The precipitated enzyme was resuspended and dialyzed in 10 mM citrate buffer pH 6.0 at 4 °C. The enzyme was further purified by DEAE-Cellulose column was equilibrated with 10 mM citrate buffer pH 6.0. The bound enzyme fractions were eluted (step-wise) by 200, 300, 500, and 700 mM NaCl in citrate buffer pH 6.0. Further, the enzyme fractions were pooled, dialyzed, and assayed for tannase.

Protein content was estimated by the Lowry method using bovine serum albumin as standard (Lowry et al. 1951). For In-gel staining of tannase activity, the electrophoresed gel (Native-PAGE) was incubated in methyl gallate (10 mM in 10 mM citrate buffer pH 6.0) for 30 min. The gel was washed with the same buffer and incubated in 0.667% methanolic rhodanine for 30 minutes at RT, further, 0.5 N KOH was added for color development. For the molecular weight determination, the purified tannase was analyzed by SDS-PAGE (Laemmli 1970) and protein bands were stained by Coomassie Brilliant Blue R-250. The molecular weight of the enzyme protein was determined.

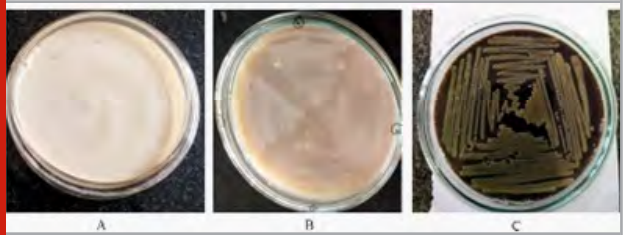
RESULTS AND DISCUSSION

Selection of bacterial strains: Eight strains of bacterial origin isolated from tannic acid-induced soil samples were evaluated for tannase. A total of 4 strains showed a colour change (dark colour) on LB agar plate with tannic acid after 48 h (Figure 1), were found to be positive tannase production and total activity was measured (Supplementary Figure 1). Based on the above observations Isolate C1 was selected for further studies. Similar studies were made for different bacterial strains (Sharma et al. 2000; Osawa et al.

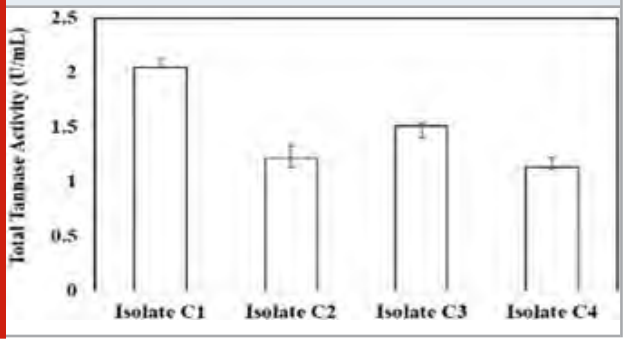
2006, Murugan et al. 2007; Sivashanugam and Jayaraman 2011; Chartchai et al. 2020; Govindarajan et al. 2021).

Characteristics of the Isolate C1 (potential) strain: Microscopic studies of Isolate C1 were found to be gram-negative cocci, non-motile and capsulated (Supplementary Figure 2 and Supplementary Table 1). Fermentation results revealed that dextrose, lactose, and sucrose were sole sources of carbon. The biochemical studies showed positive results for methyl red, Voges Proskauer, citrate, and production of catalase/oxidase (Table 1 and Supplementary Figure 3). It has been observed that the optimal growth in MSM media with 0.3% tannic acid (pH 7.0) at 37°C. Based on the above observations and 16S rRNA studies revealed that the strain was found to be *Klebsiella pneumoniae* BH49 (Figure 2). Similar observations were also made for *Klebsiella pneumoniae* MTCC 7162 and *Bacillus subtilis* KMS2-2 (Sivashanmugam and Jayaraman 2011; Chartchai et al. 2020; Govindarajan et al. 2021).

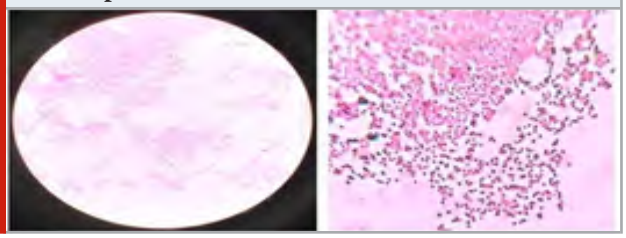
Figure 1: Utilization of the Tannic Acid on LB Agar: Colour change (Dark) in LB agar plates containing 0.3% tannic acid.



Supplementary Figure 1: Analysis of total tannase activity from different isolates



Supplementary Figure 2: Gram staining of Isolate C1- *K. pneumoniae* BH49



In Supplementary Table 1.Colony Characteristics (Morphological Tests) of *K. pneumoniae* BH49 (Isolate C-1).

Colony	Morphological Observations
Size	0.5 cm
Margin	Entire
Shape/form	Circular
Elevation	Unborate
Surface texture	Smooth
Consistency	Butter-ferous
Opacity	Trans-lucent
Chromo- genesis	Creamy white, off white

Table 1: Results of various biochemical tests carried out for tannic acid degrading bacteria *K. pneumoniae* BH49 B(Isolate C-1)

Biochemical test	Result
Lactose Fermentation	Positive
Dextrose Fermentation	Positive
Sucrose Fermentation	Positive
H2S Production	Negative
Citrate Utilization	Positive
Indole Test	Negative
MR Test (Methyl Red)	Positive
VP Test (Voges Proskauer)	Positive
Urease Activity	Negative
Catalase Activity	Positive
Oxidase Activity	Positive
Gelain Liquification	Negative
Starch Hydrolysis	Negative

Supplementary Figure 3: Various Biochemical Tests of Tannic Acid Degrading Bacteria *K. pneumoniae* B (Isolate C-1).

Production Tannase from *K. pneumoniae* BH49: It was observed that the bacteria can able to grow in tannic acid (0.3%) as a sole source of carbon. The results indicated that there was a significant increase in production of tannase at

early lag phase (4 h) and the maximum degradation of tannic acid was observed in the exponential phase. The production rate was continuing till 10 h and was steadily decreased and the total enzyme activity was found to be the 2.14 U/mL of the crude extract (Figure 3). Similar observations were made by group of bacterial strains of taxa *Lactobacillus* (0.3 U/ml), *Bacillus* sp. (1.03 U/ml), *K. pneumoniae* (0.75 U /ml), *Enterobacter cloacae* strain 41, *Pseudomonas aeruginosa* IIIB 8914 (13.65 and 12.90 U/ml) using amla and keekar leaves (2% w/v) respectively under SmF, *K. pneumoniae* MTCC 7162 (3.45 U/ml) and *B. tequilensis* K34.2 (0.60 U/mL) (Deschamps et al. 1983; Hadi et al. 1994; Kar et al. 2003; Selwal et al. 2010; Sivashanmugam and Jayaraman 2011; Govindarajan et al. 2019; Chartchai et al. 2020).

Figure 2: Neighbor-joining tree showing the position of isolate *Klebsiella pneumoniae* BH49.

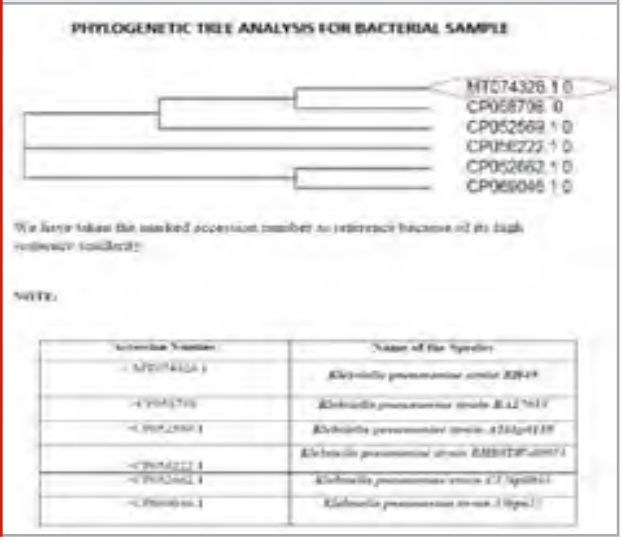
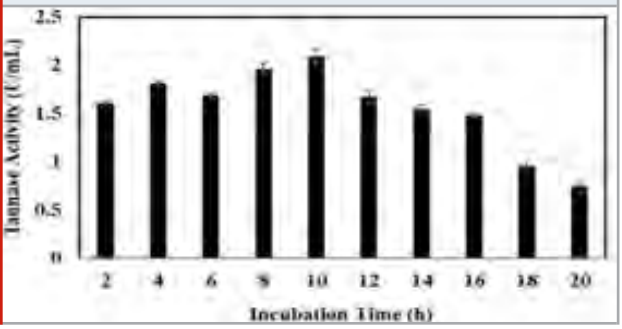


Figure 3: Analysis of Tannase activity at different time points.



Analysis of metabolites by Thin Layer Chromatography: The chromatogram revealed that the production of gallic acid, indicating that the hydrolysis was progressed with significant production of tannase. The Rf values gallic acid and pyrogallol were comparable with the Rf values of purified samples (Figure 4). Similar observations were made for the degradation of tannic acid in ruminal bacterium, *Lactobacilli, Rhodococcus* NCIM 2891, *Enterococcus faecalis* and *Enterobacter cloacae* (Nelson et al. 1995; Lee et al. 2008; Goel et al. 2011; Nadaf and Ghosh 2011; Govindarajan et al. 2019).

Figure 4: TLC profile of degradation of tannic acid: Lane 1-Standard tannic acid, Lane 2-Standard gallic acid, Lane 3-gallic acid and glucose.

Figure 5: Effect of temperature on tannase activity, results were obtained mean values of ± SD (n = 3).

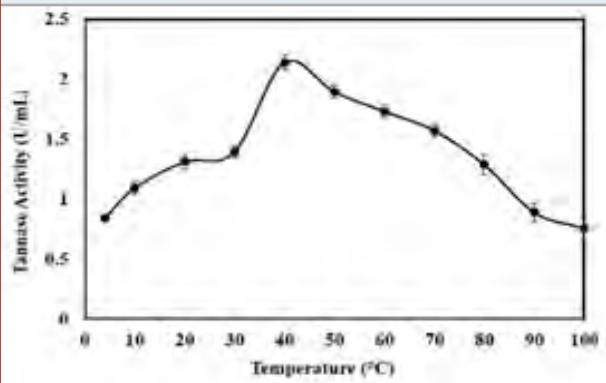


Figure 6: Effect of temperature stability of tannase activity, results were obtained mean values of ± SD (n = 3).

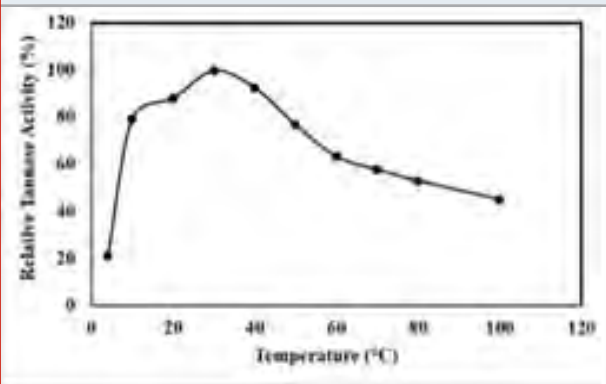
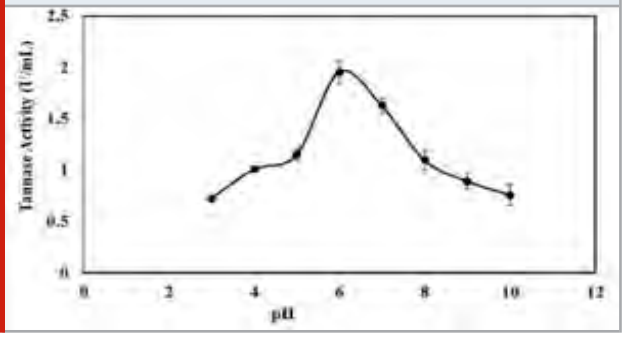


Figure 7: Effect of pH on tannase activity, results were obtained mean values of ± SD (n = 3).



Properties of Extracellular Crude Tannase

Determination of optimum temperature: The results revealed that the tannase was active in the temperature range of 4°C-100°C with an optimal activity (2.14 U/mL) at 40 °C (Figure 5), which was similar for *K. pneumoniae* MTCC 7162 at 40 °C, A. niger ATCC 16620, *Bacillus licheniformis* KBR 6 and for *Bacillus subtilis* KMS2-2 at 50 °C (Mondal and Pati 2000; Sabu et al. 2005; Sivashanmugam and Jayaraman 2011; Govindarajan et al. 2021).

Thermal stability of tannase: From the graph, it was revealed that the enzyme retained its activity above 99.5% at 40 °C (Figure 6). A similar result was observed for tannase produced from *K. pneumoniae* MTCC 7162 and *Bacillus subtilis* KMS2-2, which was thermally stable and retained its activity more than 50% at 60 °C and 80% at 50 °C (Sivashanmugam and Jayaraman 2011; Govindarajan et al. 2021). The obtained result suggests that the extracellular tannase from *K. pneumoniae* BH 49 persists its activity for a broad temperature range.

pH optima for tannase activity: The optimum pH for crude extract was found to be pH 6.0. It was observed that the enzyme showed a pH range of 3-10 with an optimal activity at pH 6.0 (1.96 U/mL) (Figure 7). Further, tannase activity was gradually decreased as pH reached the alkaline range. Optimum pH of 5.0-7.0 was reported for tannase from *Paecilomyces variotii*, pH 5.0 for *P. chrysogenum*, pH of 5.5 for *K. pneumoniae* MTCC 7162 and pH 6.0 for *Bacillus subtilis* KMS2-2 (Mahendran et al. 2006; Hamdy 2008; Sivashanmugam and Jayaraman 2011; Govindarajan et al. 2021). Some extracellular tannase from bacterial sources has been previously reported that the maximum activity at pH levels close to neutral (Mondal et al. 2001b; Batra and Saxena 2005; Sabu et al. 2006; Enemuor and Odibo 2009; Mahapatra et al. 2009; Chhokar et al. 2010).

pH Stability of Tannase: The pH stability results of extracellular tannase revealed that the enzyme was stable from a pH range of 4-7 (Figure 8). Tannases from yeast and *A. oryzae* was stable in a wide range of pH (3.5-8.0), whereas tannases from *P. chrysogenum* and *A. oryzae* were stable in the narrow ranges of 4.5-6.0 and 5.0-5.5, respectively (Abdel-Naby et al. 1999; Pan et al. 2020). Stability of above 99.33% was retained by the crude enzyme at pH 6.0. Even at pH 4.0 and 8.0, the enzyme exhibited more than 70% of activity (Figure 8). Stability of above 81.3% and 80% was retained in tannase produced from *K. pneumoniae* MTCC 7162 at pH 6.0 and tannase from Acid Stable Yeast (Sivashanmugam and Jayaraman 2011; Kanpiengjai et al. 2021).

Figure 8: Effect of pH stability of tannase activity, results were obtained mean values of ± SD (n = 3).

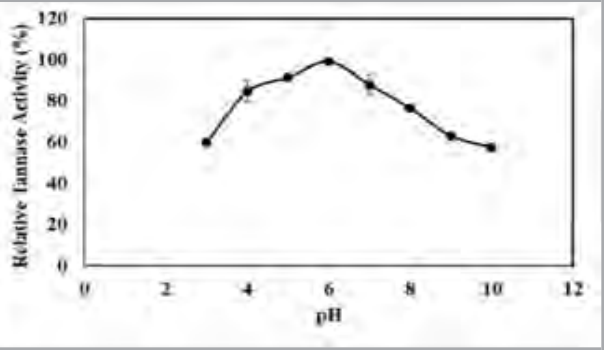
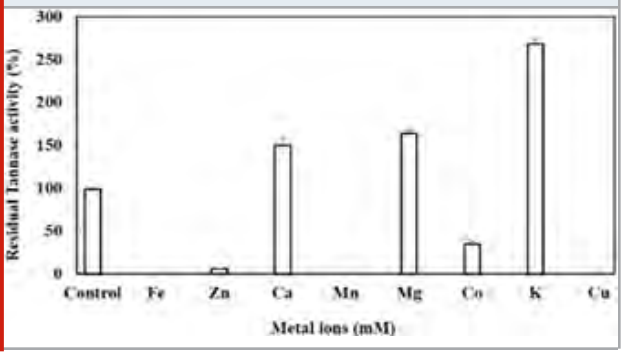


Figure 9: Effect of metals on Tannase activity, results were obtained mean values of ± SD (n=3).



Effect of heavy metals: The tannase showed complete inhibition by Fe^{2+}, Mn^{2+} and Cu^{2+}, 95% by Zn^{2+} and 70% by Co^{2+} in 10 mM concentration. The obtained results and LB plots emphasis that the metal ions act as competitive inhibitors. However, the activity was almost doubled in presence of 10 mM K^{+}, by 64% in presence of Mg^{2+} and up to 50% in presence of Ca^{2} (Figure 9). Previously it has been investigated that tannase from *Aspergillus oryzae* and *P. chrysogenum* was showed maximum inhibition in the presence of Fe^{2+}, Zn^{2+}, and Cu^{2+} (Ibuchi et al. 1968; Rajakumar and Nandy 1983). However, Tannase from *A. niger* was significantly inhibited by Cu^{2+} and to a lesser extent by Fe^{2+}, Zn^{2+} (20 mM) (Barthomeuf et al. 1994). Tannase from *Bacillus subtilis* KMS2-2 was inhibited by Cu^{2+} (40%), Fe^{2+} (56%), Fe^{2+} (31%), Ba^{2+} (39%), Hg^{2+} (48%) and K+ (32%), whereas, Zn^{2+} (08%), Ag^{2+} (23%) and Na^{+} (6%) showed moderate inhibitions. Among the metal ions studied, the Na^{+} significantly increased activity by 10% at 1 mM concentration; however, the activity was generally inhibited in the presence of large concentrations of ions, except Ca^{2+} ions. In the presence of Mg^{2+}, the activity of tannase was enhanced (Kar et al. 2003; Chaitnyakumar and Anbalagan 2016; Govindarajan et al. 2021).

Kinetic Parameters: Based on the the LB-Plot we obtained higher Km value for methyl gallate (0.0518 mM) and

lower Km value for propyl gallate (0.0389 mM). However, the values of Km suggest *Klebsiella pneumonia* BH49 tannase showed more specific towards propyl gallate. The Km value is very low as compared to tannases from *Selenomonas ruminantium* and *Cryphonectria parasatica* using methyl gallate (1.6 mM and 7.49 mM respectively) (Farias et al. 1994). The Km values for tannase produced by *Rhodococcus* NCIM 2891, *Cryphonectria parasitica* and *Enterobacter cloacae* (0.34 mM, 0.94 mM and 3.0 mM). Similarly, tannases from many fungi showed Km values of 0.20 to 1.03 mM for tannic acid (Farias et al. 1994; Ramirez-Coronel et al. 2003; Sabu et al. 2005; Mukerjee and Banerjee 2006; Nadaf and Ghosh 2011; Govindarajan et al. 2019).

Table 2. Purification table of tannase isolated from *K. pneumoniae* BH49

Purification steps	Total volume (mL)	Total activity (U)	Total Protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude enzyme	20	41.8	112	0.373	100	1
Ammonium sulfate precipitation (0-80%)	10	27.5	85	0.323	65.78	0.865
DEAE – Cellulose	10	9.84	10.45	0.941	23.54	2.522

Figure 10: The elution profile of DEAE-Cellulose Ion exchange chromatography of Tannase: Protein fractions (-) and Enzyme fractions (---).

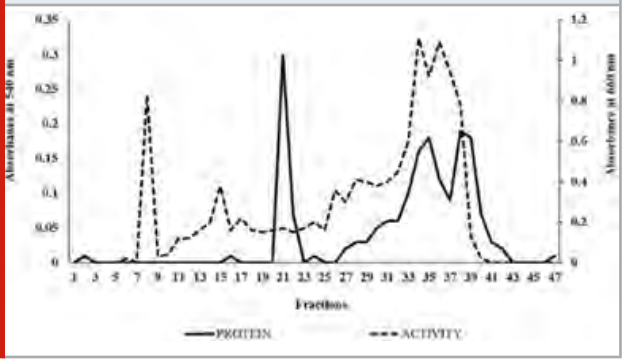
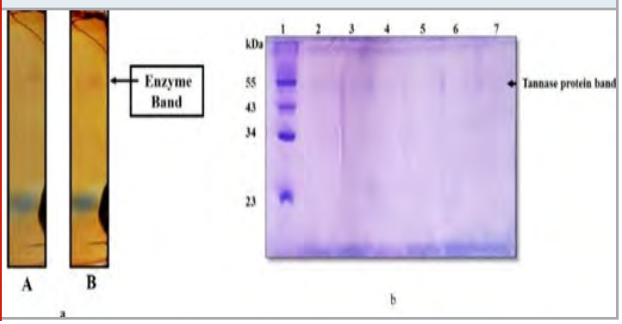
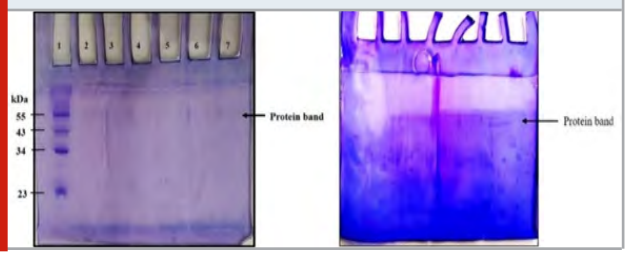


Figure 11: a, Native-PAGE pattern of Tannase activity; A-Crude enzyme, B-Purified tannase. b, SDS-PAGE profile of purified tannase; Lane1-Molecular weight marker, Lane-2 to 7 purified tannase.



Supplementary Figure-4: SDS-PAGE profile of purified tannase; Lane1-Molecular weight marker, Lane-2 to 7 purified tannase.



Purification of extracellular tannase: Tannase has been purified from *Klebsiella pneumoniae* BH49 to homogeneity by using the different steps (Table 2). The elution profile of extracellular tannase by DEAE-Cellulose showed the best yield of the enzyme (23%) (Figure 10). The specific activity of purified tannase was found to 0.943 U/mg for methyl gallate. Our results were compared to the purified tannase from *Klebsiella pneumoniae* MTCC 7162 and *Bacillus subtilis* KMS2-2 (Sivashanmugam and Jayaraman 2011; Govindarajan et al. 2021). The yield of 7% was lower than the value reported for tannases from *Penicillium chrysogenum* and *Cryphonectria parasitica* (Farias et al. 1994; Rajakumar and Nandy 1983). The fold purification, on the other hand was identical to that of purified tannase from a variety of fungi and bacteria (Rajakumar and Nandy 1983; Sharma et al. 1999; Govindarajan et al. 2019).

In-gel staining of tannase activity: Tannase activity of both crude and purified enzyme (In-gel) was visualized by formation of chromogen by methanolic rhodanine in KOH, the chromogenic bands that were obtained (Figure 11a).

Molecular weight determination of Extracellular purified Tannase: The SDS-PAGE profile of purified tannase showed one major band with the molecular mass of about 55 kDa (Figure 11b and Supplementary Figure 4). It was shown that most of the purified fungal tannases range from168 to 310 kDa and multimeric (Aguilar 2001; Ramirez 2003). Similarly, it has been reported the isoforms of tannase from *Paecilomyces variotii* of 87.3 kDa and 71.5 kDa (Battstin and Macedo 2007). Tannase from *Verticillium* sp. P9 had two subunits with molecular masses of 39.9 and 45.6 kDa (Kasieczka 2007). In addition, the molecular mass of tannase from *K. pneumoniae* MTCC 7162 and *Bacillus*

subtilis KMS2-2 by SDS-PAGE revealed that 46.5 kDa and 43 kDa respectively (Sivashnmugam and Jayaraman 2011; Govindarajan et al. 2021).

CONCLUSION

The findings of the present study explored the production of tannase from *Klebsiella pneumonia* BH49 has shown a higher affinity for tannic acid compared to other bacterial tannases. The bacteria produced a high amount of extracellular tannase in the exponential phase to be a conventional strain compared to fungal strains. The biochemical properties like temperature stability, pH stability and other kinetic parameters further insights to potential source for efficient production of extracellular tannase and can be used for tannery effluent degradation, pharmaceutical and industrial applications. In addition, the production of gallic acid (antioxidant) was another significant end product and could the novel discovery used to remove tannins from food fodder and thus improve the health of the animals.

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Conflicts Of Interests: Authors declare no conflicts of interests to disclose.

Author Contributions: Sharadamma N designed the study, analysed the results, and prepared the manuscript. All authors performed the experiments and analysed the results, reviewed the results and approved the final version of the manuscript.

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Ecological Communication

Body Parameters of Wolves (*Canis lupus campestris*) in the Steppes of Kazakhstan

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ABSTRACT

The study of the morphometric characteristics of the wolf was aimed at identifying the potential properties of the species and intraspecific individual geographic and age variability depending on the habitat. The carcasses of 36 male and 26 female wolves were examined. This paper presents the results of morphometric studies of wolves of the Kazakh steppes, *Canis lupus campestris*. The animals of three identified areas (western, central, and eastern sections) were compared within their habitat. It was discovered that wolves throughout the habitat had practically no significant differences and were almost identical in appearance. At the same time, there was an increase in body length from west to east and in body weight from east to west. The morphological features of wolves from different areas were as follows: the wolves of the eastern area were larger, the animals of the central area were higher on their legs and had a squarer shape, and the animals of the western area were heavier. Pronounced sexual dimorphism was characteristic of the entire studied habitat. Comparison of the results obtained with the literature data showed that the steppe Kazakh wolves were morphologically similar to the wolves of the southeast of Ukraine and significantly different from the wolves of the Altai Territory. Besides, it turned out that representatives of subspecies *C. l. campestris* and *C. l. desertorum* were similar in body weight and length. It was also found that wolves in the steppes of Kazakhstan were subjected to intense hunting pressure, which is why the population core is actively renewed. The main result of the study is the clarification of the intraspecific structure depending on the habitat.

KEY WORDS: KAZAKHSTAN, MEASUREMENTS, MORPHOMETRY, STEPPE TERRITORY, WOLF CANIS LUPUS CAMPESTRIS.

INTRODUCTION

Separate works have been devoted to the intraspecific polymorphism of wolves and their subspecies systematic status (Bondarev, 2012, 2013; Yudin, 2013, Wagner and Ruf, 2019, Alvares et al., 2019). For Kazakhstan, the taxonomy of wolves was considered poorly developed as early as in the middle of the last century. The situation has not changed to this day. Some authors distinguish only two ecological forms of the wolf (forest wolf and desert-steppe wolf, others believe that the borders of Kazakhstan include the habitat of four subspecies, or races (Geptner, 1967) (Siberian forest wolf *C. l. lupus* and *C. l. altaicus*, desert wolf *C. l. desertorum*, steppe wolf *C. l. campestris*, mountain, or Tibetan, wolf *C. l. chanco*). The geographic variability of Kazakhstani wolves is high, but it has not been studied enough (Boitani et al., 2018; Shmalenko, 2020; Bergström et al, 2020).

The object of our research is the steppe subspecies of the wolf *Canis lupus campestris*, which occupies the steppe territory of Kazakhstan. Its habitat runs from the Altai and Tarbagatai mountains in the east to the Caspian Sea in the west; and in the north of the Caspian Sea, it continues further west into the southern Russian steppes (Geptner, 1967). The southern border of its distribution is in the area of the desert zone, the Betpak-Dala plateau, and the Ustyurt plateau. In the north, the area is limited to the forest-steppe zone. Outside the republic, the steppe wolf is found in the east of Lebanon (Yudin, 2013), in the

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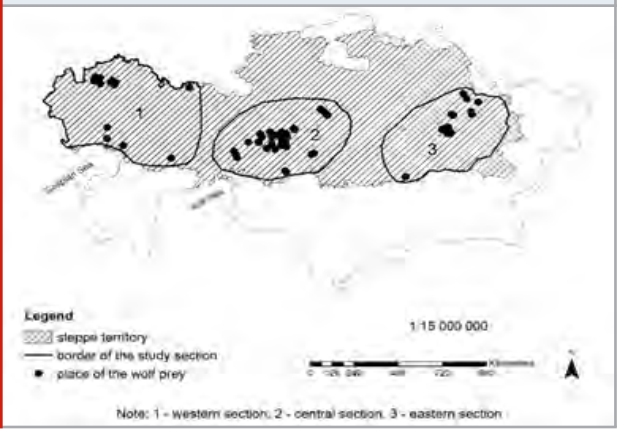
southern and eastern parts of Ukraine (Geptner, 1967), in the south of the European part of Russia, in the southern regions of Western Siberia (Bondarev, 2017, Bondarev & Malikov, 2018; Boitani et al., 2018; Shmalenko, 2020), in Transbaikalia and Mongolia, as well as in the north of China and the south of the Amur region (Yudin, 2013; Shmalenko, 2020).

Large-scale studies of steppe wolves in Kazakhstan have not been carried out yet. There are only a few regional data on the Aktobe region. This work is being performed for the first time. Its result demonstrates the exterior features of the physique and their variations in *Canis lupus campestris* in the steppe biogeocenoses of Kazakhstan, along their entire length. The purpose of this work is to study the parameters of the physique of wolves and their geographic variability in the steppe territory of Kazakhstan.

MATERIAL AND METHODS

In the ecological aspect, the area of collection of the material was a steppe. In Kazakhstan, the steppe occupies the northern and central regions, covering about 160 million ha, which is 59% of the republic's area, of which about 123 million ha are natural habitats. The immediate research area is located between 46° and 51° north latitude, 47° and 81° east longitude and has a length of 2.5 thousand km and a width of about 0.5 thousand km. Such a vast area of the steppe territory, as well as differences in the living conditions of animals, suggest the existence of morphometric differences in different ecological groups of wolves of the steppe subspecies. These circumstances prompted us to distinguish three areas within the studied region: the western, central, and eastern sections (Figure 1).

Figure 1: The area of the wolf study in Kazakhstan. Source: the figure was created by authors.



The western section is about 40 million ha and occupies the territory of the Caspian lowland and the Ural-Emba plateau, including the southern outskirts of Obshchy Syrt. The central section is about 30 million ha and occupies the plain of the Northern Aral Sea region and the Turgai plain. The eastern section is also about 30 million ha and is located on the territory of the Central Kazakhstan Uplands. A common feature of the climate in Kazakhstan is pronounced aridity and a high degree of continentality, with large daily and annual air temperature amplitudes and a significant moisture deficit. The object of the study was the wolf (*Canis lupus campestris* Dwigubski, 1804). The material was carcasses of wolves hunted by hunters from November to March in the period from 2017 to 2020. All animals were legally shot based on special permits for the hunting of animal species, the number of which is subject to regulation (KZ94VQQ00002234 dated 29.12.2017, KZ18VQQ00002244 dated 29.12.2017, KZ60VQQ00001100 dated 12.04.2017, KZ43VQQ00000533 dated 14.02.2017, KZ54VQQ00001305 dated 27.04.2017, KZ23VQQ00004191 dated 02.03.2018, KZ64VQQ00007333 dated 18.02.2019, KZ39VQQ00007298 dated 18.02.2018, KZ55VQQ00007107 dated 18.02.2019, KZ28VQQ00007302 dated 18.02.2019, KZ67VQQ00006988 dated 18.02.2019, KZ24VQQ00007083 dated 18.02.2019, KZ49VQQ00007118 dated 18.02.2019, KZ68VQQ00006970 dated 15.02.2019, KZ75VQQ00009948 dated 22.05.2019, KZ92VQQ00009448 dated 14.03.2019, KZ22VQQ00009447 dated 14.03.2019, KZ49VQQ00009446 dated 14.03.2019, KZ38VQQ00011584 dated 15.01.2020, KZ64VQQ00013832 dated 17.01.2020, KZ72VQQ00016695 dated 23.01.2020).

Special permits for the withdrawal of animal species, the number of which is subject to regulation, were issued by the territorial divisions of the Committee for Forestry and Wildlife at the request of individuals (hunters) through the electronic government web portal www.elicense.kz in electronic form. A total of 61 killed wolves were measured, of which 36 were males and 25 females (Table 1).

According to the degree of tooth wear, all studied individuals were divided into three age groups: young or less than a year old (from 7 months to 1 year), semi-mature or yearlings (from 1.5 years to 2 years), mature or adults (over 2.5 years). Among the mature wolves in the studied sample, only two males from the eastern section and one female from the western section were older than 6 years. Determination of morphological parameters of the physique was carried out by measuring the body and its parts using a centimeter elastic tape with a scale of 1 mm, a 150 mm mechanical caliper (accuracy up to 0.02 mm), and a 500 mm mechanical caliper (accuracy up to 0.1 mm). Determination of total body weight was carried out using an electronic balance (accuracy up to 5 g). Measurements were taken according to generally accepted methods.

The data obtained were transferred to an electronic database, where they were grouped according to the gender and age of the animal, taking into account if they belonged to the western, central, or eastern section. Further statistical processing of the parameters was carried out in the Excel program using biometric techniques, and body build indices were calculated. After that, the results were compared with each other, as well as with the data of morphological parameters of other populations from published literature sources. The criterion for the selection of such populations was the territorial proximity of the region to Kazakhstan, as

well as the historical distribution area of the steppe wolf. Thus, to compare the data, wolves were selected from the adjacent territory of the southeast of Kazakhstan plains, southeast of Ukraine, as living within the habitat of *C. l. campestris* (Geptner, 1967), and the wolves of the Altai Territory, where the penetration of migrant steppe wolves from the territory of Kazakhstan to the territory of Russia is observed (Bondarev, 2013; Rakin, & Bondarev, 2020).

Table 1. Distribution of the studied wolves by regions of their killing

Gender and age groups	Number of measured animals by region, individuals				
	Western section	Central section	Eastern section	Total:	%
males (Mature: yearlings: less than a year old)	8 (4:3:1)	17 (8:5:4)	11 (6:4:1)	36 (18:12:6)	59
females (Mature: yearlings: less than a year old)	6 (1:0:5)	16 (2:6:8)	3 (1:2:0)	25 (4:8:13)	41
Total: (Mature: yearlings: less than a year old)	14 (5:3:6)	33 (10:11:12)	14 (7:6:1)	61 (22:20:19)	100

Table 2. Results of using the Chi-square (χ2) test to assess the distribution of the studied sample by gender and age groups

Indicator	Mature wolves	Yearlings wolves	Wolves less than a year old	Note
all wolves				
Observed (O)	22	20	19	N=61
Theoretically expected at 1:1:1(E)	20.33	20.33	20.33	N=61
(O-E)2/E	0.14	0.14	0.14	χ2=0.41
males				
Observed (O)	18	12	6	N=36
Theoretically expected at 1:1:1(E)	12	12	12	N=36
(O-E)2/E	3	0	3	χ2=6.0
females				
Observed (O)	4	8	13	N=25
Theoretically expected at 1:1:1(E)	8.33	8.33	8.33	N=25
(O-E)2/E	2.25	0.01	2.61	χ2=4.87

Table 3. Body weight of wolves of different gender and age groups in the context of regions, kg

Age group	Western section		Central section		Eastern section	
	n	M±m	n	M±m	n	M±m
Males mature	4	33.4±1.51	6	31.9±2.13	4	34.0±3.07
yearlings	2	27.7±0.35	4	32.5±2.07	3	31.1±0.81
wolves less than a year old	1	25.5	3	26.3±2.45	1	27.9
Females						
mature	1	23.5	2	24.7±1.85	1	27.6
yearlings	-	-	6	23.9±0.81	2	28.0±0.5
wolves less than a year old	5	26.1±0.76	7	25.2±0.49	-	-

RESULTS AND DISCUSSION

The poor knowledge of the steppe wolf was noted by Geptner (1967). Our studies of the subspecies showed the following results. The average age of wolves in the adult group is 3.5+ years, which indicates the intensive hunting of the population. This is confirmed by the low share of fertile animals in the sample (22%). Consequently, the population core is being actively updated. In our study, the ratio of the number of males to the number of females, in general, equaled 1.44:1. This ratio varies by age group: 1:2.2 among wolves less than a year old, 1.5:1 among yearlings, and 4.5:1 among the mature wolves. A similar ratio of males to females 1.4:1 was recorded by Tsyndyzhapova (2003) for the Baikal National Park. Smirnov and Korytin (1985), based on an analysis of numerous literature data on hunted wolves and an examination of museum collections, also found the predominance of males (1.22: 1 in the young wolves, 1.4:1 in the yearlings, and 1.28:1 in the mature ones). The ratio of mature wolves, yearlings, and young wolves in our material is 36%:33%:32%, which corresponds to a 1:1:1 ratio. Separately, by gender, the ratios are directly opposite: 50%:33%:17% for males, which is close to 3:2:1, and 16%: 32%: 52% for females, which coincides with 1:2:3. In a healthy population, underyearlings always predominate, with a decrease in the number of individuals of subsequent age categories (Yudin, 2013; Gankhuyag et al., 2021).

However, in our case, the number of young animals is practically equal to the number of semi-mature animals. One of the reasons may be the practice of wolf cub removal from the dens by the locals in the eastern part of the territory, as well as the extermination of the wolf in the early autumn period (due to damage to cattle breeding), which is not so developed in the central and western areas. Since young animals, due to their inexperience, are easier prey than adults, by the winter season, most of them are already dead in the eastern part of the habitat, the result of which we observe in the sample from this site. An increase in the proportion of young females over adults, as well as a twofold excess of females over males in the migrant group, might be a reaction of the genetic mechanism for regulating the gender ratio with a decrease in the density of animals. A similar picture was observed by Yudin (2013) in intensively developed populations. At the same time, the influence of the characteristics in the behavior of animals cannot be ruled out. Adult females, as more cautious individuals, are more likely to avoid being shot from a snowmobile than adult males, who, according to some hunters, often take on the role of distracting the pursuer. For this reason, there are probably fewer adult females in the sample than could be (Dhargupta, et al., 2020; Gankhuyag et al., 2021).

However, taking into account the ratio of five young wolves per fertile female, we assume that our sample is representative in terms of the gender and age structure of the entire population in the autumn-winter season. Taking into account the above, we carried out a statistical analysis of the studied sample of steppe wolves using the generally accepted Chi-square ($\chi 2$) test by calculating it and comparing the obtained value with the table value taking into account the number of degrees of freedom (Peker and Kubat, 2021). The observed distribution by gender does not differ from the theoretically expected one, since the actual value of the Chi-square test is less than the standard value with the number of degrees of freedom = 1. The observed distribution by age groups of the entire sample, and females in particular, also does not differ from the theoretically expected value, since the observed value of the Chi-square test is less than the standard value with the number of degrees of freedom = 2. At the same time, the analysis of age groups of males showed a significant difference between the observed distribution and the theoretically expected one in a ratio of 1:1:1, since the observed value of the Chi-square test corresponds to the tabulated value at P = 0.05 and the number of degrees of freedom = 2 (Table 2).

Body weight: The wolf that lives in the central regions of Kazakhstan has an average body size and weighs up to 55 kg (Kuznetsov, 1948). In our studies, the maximum weight of animals did not exceed 42 kg. The heaviest male at the age of 2.5+ years was obtained in the eastern section. The lightest male (25.3 kg) was obtained in the central section at the age of 2.5+ years. The average weight of adult males throughout the steppe territory equaled 32.9 ± 1.26 kg, with the heaviest animals living in the eastern section and the lightest in the central. The difference in weight between these populations was 6%. A female with a maximal weight of 29 kg was obtained in the central section at the age of about 9 months. A female with a minimum weight of 21 kg was obtained in the same area at the age of 1.5+ years.

The average weight of adult females was 25.1 ± 1.16 kg, which is 1/5 less than that of males. The males of the young wolves group had an average weight of 22.0 ± 1.28 kg, that is, 28.8% lower than that of the next age group of yearlings. In yearling males, the arithmetic mean weight corresponded to 30.9 ± 1.1 kg, which is 6% lower than the average weight of adult males. However, in the central area, the mass of yearling males was 1.8% higher than the mass of mature males. In females, the average weight of the young wolves was practically equal to the weight of the mature ones (25.6 ± 0.43 kg), and the mass of the yearlings was slightly lower (24.9 ± 0.91 kg). Unfortunately, single specimens of adult females by region cannot provide sufficient representativeness of the sample. We can unambiguously state that the animals of the eastern section had the largest body weight and that there was a decrease in this indicator in the central and western parts of the habitat (Table 3).

The coefficient of variation in males is almost two times higher than that of females and amounts to 14.7%, while in females it deviates only by 7.8%. This fact fits into the framework of the concept of gender differentiation by Geodakyan (1981), whose central position is the conclusion about the greater phenotypic variance (diversity) of males as compared to females. This

statement applies to most of the linear measurements we have made. Taking into account the presence of the acceleration effect in the wolf, noted in the 20th century, we compared the data on the mass of animals in the Aktobe region with our results (Table 4).

The Aktobe region is located within the western part of the study area, which is why the wolves weighed in the 1980s and wolves in the western part of our sample are animals of the same population, separated by a 30-35-year time interval. It makes no sense to analyze the change in mass in adult females and young males because of the single individuals in our sample. However, the change in the age dynamics of the mass of animals is evident. Thus, modern young wolves demonstrate a significant increase in weight in comparison with animals of the same age (for females up to 19.18%), while for mature males and yearling males, this indicator practically has not changed (0.6-0.7%).

Table 4. Changes in body weight of wolves in the western population over the past 30-35 years, kg

Observation period	Average indicator		Adults		Yearlings		Wolves less than a year old	
	males	females	males	females	males	females	males	females
1985	29.0±0.89	24.1±0.67	33.6±0.95	29.7±0.85	27.5±1.16	24.8±0.5	23.6±1.3	21.9±0.95
2017-2020	30.6±1.56	25.7±0.76	33.4±1.51	23.5	27.7±0.35	-	25.5	26.1±0.76
Acceleration effect,%	+5.52	+6.64	-0.60	-20.88	+0.73		+8.05	+19.18
t-test	0.87	1.53	-0.11		0.16			3.38

Table 5. The main indicators of linear measurements and body build indices of adult (mature) wolves

Parameter	Male indicators			Female indicators		
	Western section (n=3-4)	Central section (n=7-8)	Eastern section (n=5-6)	Western section (n=1)	Central section (n=2)	Eastern section (n=1)
Body length, cm	115.7±5.04	122.4±4.56	125.4±2.54	108.0	117.0±5.00	113.0
Oblique body length, cm	73.0±2.94	72.6±1.21	78.0±2.30	64.0	69.5±1.50	72.0
Height at the withers, cm	66.3±1.89	67.1±1.72	66.0±0.84	58.0	63.5±3.5	60.0
Chest circumference, cm	74.5±1.71	72.3±2.39	71.0±2.68	64.0	68.8±1.25	68.5
Foot length, cm	23.4±1.07	23.4±0.48	24.3±0.35	23.0	23.5±0.50	23.0
Stretch (Format) index, %	110.4±4.64	108.8±2.59	118.2±3.06	110.3	109.9±8.42	120.0
Massiveness index, %	112.9±5.18	108.1±2.21	107.5±3.34	110.3	108.5±4.01	114.2
Long-muzzle index, %	38.4±2.11	40.0±1.07	39.4±0.51	39.7	42.9±0.25	39.6
Broadhead index, %	49.7±3.87	48.7±2.49	51.8±2.17	51.4	47.6±0.50	50.6

Thus, the average total weight of males in the population showed an increase of 5.5%, and the weight of females by 6.6%, mainly due to the greater weight of young wolves. The increase in the bodyweight of young animals in our studies can be explained by the better provision of the forage base in 2017-2020 during the period of rearing the offspring, compared with the 1980s. Thus, in 1983-84 and 1984-85 massive deaths of saigas (Saiga tatarica) were observed (Nurushev, & Bajtanaev, 2018). Besides, from 1983 to 1988, according to the data of the Mangistau anti-plague station (Kaijrbaev, et al., 2019; Stasenko, & Zhupkali, 2019; Gankhuyag et al., 2021), a deep depression in the abundance of the great gerbil (Rhombomys opimus) was observed. Saiga and gerbils make up more than 70% of the diet of wolves in this area (Leontyev, 2017); therefore, a significant reduction in these food items is most likely reflected in the weight gain of young wolves (Stasenko, & Zhupkali, 2019; Gankhuyag et al., 2021).

Body length: The body length of adult males increases from west to east, with an intermediate value in the central section. As in mature males, the elongation of the body in the eastern direction is observed in males and females of the groups of yearlings and young wolves.

In adult females, the dynamics of an increase in body length from the western section to the central one is also traced, but this indicator decreases in the eastern section (Table 5). Probably, this is due to the small size of the sample which contained single individuals of extreme populations. Thus, in general, for steppe wolves, there is a clear tendency to increase in body length from west to east.

Oblique body length: In adult males, the oblique body length is maximum in the east and minimum in the central part of the habitat, with an average value in the western part. In adult females, this value decreases from east to west. The dynamics of this measurement is similar to that of mature males in terms of yearlings and young wolves in both genders. Accordingly, the smallest indicator of the oblique body length of the steppe wolves is observed in the central section and the largest one in the eastern section.

Height at the withers: The highest height at the withers among adults for both genders is possessed by animals of the central section: 67.1 ± 1.72 cm for males, 63.5 ± 3.5 cm for females (Table 5). However, in the groups of semi-mature (yearling) and young (less than a year old) wolves in males and females, the maximum height at the withers is noted for the eastern section, with a decrease in this indicator in the western direction. Thus, the geographic-latitudinal dynamics of the average population height at the withers do not correspond to the dynamics of that in mature individuals and demonstrates an increase from west to east. It is possible that this feature is not significant in the geographical difference between the populations under consideration, and due to small samples, it cannot be unambiguously distinguished.

Chest circumference: In adult males, an increase in chest circumference from east to west by 3.5 cm is observed (Table 5). In adult female wolves, as well as in males and females of the yearling group, the largest chest circumference is presented for the central region, the middle one for the eastern region, and the smallest for the western region. In the group of young wolves, both genders tend to increase this indicator from west to east, that is, inversely proportional to the dynamics of the chest circumference of mature males. Thus, according to this indicator, a clear pattern common for all age and gender groups is not expressed, either.

Table 6. Comparison of the main parameters of adult wolves of lowland Kazakhstan, south-east of Ukraine and Altai Territory

Parameter	Indicator	Value							
		the steppe of Kazakhstan		South-East of the lowland Kazakhstan		South-East of Ukraine** (old – more than 6 years****)		Altai territory***	
		males	females	males	females	males	females	males	females
Body weight, kg	M±m	32.9±1.26	25.1±1.16	29.7	26.1	31.4±1.385 (35.3±2.793)	29.7±1.538 (29.9±1.757)	39.87±0.81	33.98±1.03
	t					0.80 (-0.78)	-2.39 (-2.28)	-4.65	-5.72
	The superiority of the steppe wolves of Kazakhstan, %			+9.72	-3.98	+4.56 (-7.29)	-18.33 (-9.12)	-21.19	-35.38
	SDD, %	+23.7		+12.1		+5.4 (+15.3)		+14.8	
Body length, cm	M±m	122.1±2.52	113.8±2.95	115.4	109.4	114.4±2.155 (123.8±3.043)	113.9±2.615 (109.6±1.327)	126.41±2.04	119.6±3.16
	t					2.32 (-0.43)	-0.03 (1.30)	-1.33	-1.34
	The superiority of the steppe wolves of Kazakhstan, %			+5.49	+3.87	+6.31 (-1.39)	-0.09 (3.69)	-3.53	-5.10
	SDD, %	+6.8		+5.2		+0.4 (+11.4)		+5.4	
Height at the withers, cm	M±m	66.6±0.88	61.3±1.97			76.5±0.5	(73±4.163)	78.91±1.23	72.22±0.81
	t					-9.78	(-2.54)	-8.14	-5.13
	The superiority of the steppe wolves of					-14.86	(-1.09)	-18.48	-17.81

SDD, % (the sexual dimorphism degree) is the ratio of the value of the parameter of males to the value of the parameter of females (borrowed from V.G. Yudin, 2013).

** based on materials by Smirnova and Domnich (2012).

*** based on materials by Bondarev (2013).

**** only for the southeast of Ukraine (in addition to the group of adult animals, the authors distinguished old individuals over 6 years into a separate group).

Foot length: The length of the foot in mature males increases from west to east by 0.9 cm (Table 5). However, in females of this group, with the minimum value of this indicator for the western and eastern parts of 23.0 cm, the maximum length was determined for the central part of 23.5 ± 0.5 cm, which is only 0.5 cm more (Table 5). In males and females of yearlings, as well as young females, the change in foot length corresponds to that of adult males. In the mature males, the dynamics are the opposite: the lengthening of the foot is observed from east to west. Despite the insufficient amount of the studied material, there is a tendency to increase the length of the foot from west to east. Many authors quite reasonably consider absolute indicators to be insufficient for assessing conformational characteristics, and use, as more reliable, body type indices, reflecting the proportions of the body (Yudin, 2013).

Stretch (Format) index: The stretch (format) index (the ratio of wolves body length to its height) for males and females of the adult group is characterized by the maximum index for the eastern section and the minimum for the central one. The stretch of mature animals in the eastern part of the habitat is 6-8% more than in animals in the western section, at the same time, this index in the central area is less than in the western section only by 0.3-1.3% (Table 5). The similar nature of the dynamics is expressed in semi-mature and young females. However, according to the yearlings and young males, an opposite picture is observed. The maximum stretch indicator was found in the central section, and the minimum one in the eastern section. Young animals do not yet have a fully formed physique, so the data on them can only supplement the result obtained for adults. Thus, the stretch index has a maximum average value in the eastern section and a minimum in the central section.

Massiveness index: In males of the adult group, there is a regularity in a decrease in the index of massiveness from west to east, while in adult females the minimum indicator is in the central section, and the maximum is in the eastern one (Table 5). On the contrary, in both genders of the yearling group, as well as young males, the maximum value was noted for the central section and the minimum for the eastern one. In young females, an increase in this index is observed in the western direction. In general, for the population, there is an increase in the massiveness of animals with movement to the west.

Long-muzzle index: The ratio of muzzle length to head length in adult wolves is characterized by a maximum value in the central part of the habitat and minimum in the western and eastern parts (Table 5). At the same time, in females of yearlings, this indicator is maximal in the east, and in males of the young group and the group of yearlings, it increases to the west. Young females have a longer muzzle in the central part of the habitat, like wolves of the adult group. Thus, the ratio of muzzle length to head length is maximal for animals of the central part of the habitat.

Broadhead index: The ratio of the head width to its length in mature males and females is minimal in the central part of the habitat (Table 5). Similar dynamics are manifested in males of yearlings and young females. In females of yearlings and young males, there is a tendency towards an increase in this index to the west. Thus, the minimum indicator of the ratio of the width of the head to its length is characteristic of the central region. Comparison of the results of linear measurements, body weight, and body parts in wolves in the context of the populations under consideration undoubtedly gives an idea of the change in one parameter or another in different geographic groups of animals. Based on the considered exterior characteristics of wolves, it can be noted that the wolves of the eastern section are larger and have a longer body; wolves of the central section are higher on the legs, have a more square body and an elongated muzzle; and the wolves of the western section are more massive. When assessing the level of reliability of differences according to Student's test (t-test) of mean population values, the only reliably confirmed difference is in the weight of females in the eastern and central regions, but the insufficient sample size for females in the eastern region allows us to neglect this fact. For the rest of the indicators, no significant differences in body parameters were found between the geographical groups of wolves, based on which it can be concluded that all three populations are very close to each other.

At the same time, wolves in the eastern part of the habitat have a greater morphological difference from the animals in the western and central sections than the animals of these two sections between them. Morphological similarities and differences are determined by the habitat conditions and the species composition of the main food objects (Yudin, 2013). Thus, in our study, the main objects of food for wolves in the western and central sections are saiga and rodents, while in the stomachs of animals from the eastern section, we often found the remains of wild and domestic ungulates. Besides, the eastern section of the steppes is a hilly area with mountain ranges and interspersed forests, while the territory of the western and central sections is mainly low treeless plains (Shmalenko 2020; Gankhuyaga et al 2021).

The closeness of the Kazakh steppe wolves with wolves from other regions can be revealed by comparing their morphological data (Table 6). Comparison of the main parameters of mature wolves of lowland Kazakhstan, south-east of Ukraine, and Altai Territory is shown in Table 6. It shows that the wolves of the Kazakh steppes *C. l. campestris* are similar in body weight and length to the desert wolves *C. l. desertorum* in the southeast of flat Kazakhstan. The differences in measurements fall within the difference in the average values of these parameters between the considered populations of wolves of steppe Kazakhstan. This fact confirms the morphological closeness (if not identity) of the steppe and desert wolves of the Republic of Kazakhstan. Steppe wolves are similar in body weight and length to wolves in southeastern Ukraine, but have significant differences, mainly in males, in height at the withers and chest girth. Thus, if

Ukrainian wolves are on average 13% taller on their legs, then Kazakhstani wolves have a longer body by 6% and a larger chest circumference by 11% (Table 6).

The wolves of the Altai Territory are distinguished, being much heavier (up to a third of the mass of Kazakh wolves), and 18-19% higher on their feet; they are also slightly longer and with a large chest circumference. That means that they surpass the Kazakh wolves in all respects. Thus, in terms of external parameters, the wolves of the Altai Territory are more distant from the Kazakh steppe wolves than the wolves of the southeast of Ukraine. According to the above criteria, adult wolves of the Altai Territory reliably exceed the wolves of steppe Kazakhstan in body weight and height at the withers, while the differences are not significant in body length and chest circumference. Adult male wolves of southeastern Ukraine under the age of 6+ years, in comparison with mature males of steppe Kazakhstan, have significantly lower indicators of height at the withers and chest girth. Based on morphometric data, it can be assumed that the genetic relationship of wolves in the steppe regions of Kazakhstan and Ukraine is preserved, as well as the unity of *C. l. campestris* and *C. l. desertorum* on the territory of Kazakhstan.

Sexual dimorphism is most pronounced in Kazakh steppe wolves, reaching maximum values in terms of the difference in weight and body length. In Altai wolves, the difference in chest circumference and height at the withers is best expressed. Ukrainian wolves have the smallest difference between linear measurements and bodyweight of males and females. According to Yudin (2013), a more pronounced sexual dimorphism characterizes the state of a population at the stage of intensive processes of evolutionary development. As a confirmation, the number of wolves in southeastern Ukraine over 40 years (from 1970 to 2009) increased from 7 individuals (!) to 1,276 individuals (Smirnova, & Domnich, 2012), which suggests the influence of gene drift (the consequences of a "bottleneck"), probably a high proportion of inbreeding and a low degree of genetic diversity in the population. On the contrary, the wolves of steppe Kazakhstan allow us to conclude that they have a higher genetic diversity (Stasenko, & Zhupkali, 2019; Gankhuyag et al., 2021).

SDD, % (the sexual dimorphism degree) is the ratio of the value of the parameter of males to the value of the parameter of females, borrowed from Yudin (2013).

On the territory of the steppes of Kazakhstan, males predominate in the sexual structure of wolves, and their ratio to females is characterized as 1.44:1. The average age of adult animals is 3.5+ years. The ratio of mature wolves to young wolves under the age of two is expressed as 1:3.5. The ratio of mature wolves, yearlings, and young wolves for the entire sample corresponds to a ratio of 1:1:1, but separately for males it equals 3:2:1 and for females 1:2:3. This indicates intense pressure from the selective hunting press, and an active renewal of the population core. All three populations of steppe wolves in Kazakhstan are almost identical in terms of the studied parameters of body build. At the same time, there are slight differences between mature animals:

- the mature wolves of the eastern section have bigger weight, in comparison with the wolves of the central and western sections, by 6.2% and 1.8% for males and by 10.5% and 14.8% for females, respectively;
- the mature wolves of the eastern section have a higher stretch index relative to the wolves of the central and western sections (by 7.9% and 6.6% for males and by 8.4% and 8.0% for females, respectively);
- the mature wolves in the central section have a greater height at the withers compared to the wolves in the western and eastern sections (by 1.2% and 1.6% for males and by 8.7% and 5.5% for females, respectively);
- the mature wolves of the central section have a more elongated muzzle relative to the length of the head, compared with wolves of the western and eastern sections (by 4.0% and 1.5% for males and by 7.5% and 7.7% for females, respectively);
- the mature males in the western section weigh more than the males in the central section (by 4.2%) and the males in the eastern section (by 4.8%).

CONCLUSION

An increase in the average weight of wolves of the western population over the past 30-35 years has been noted in males by 5.5% and in females by 6.6%, mainly due to the greater weight of modern young wolves. In the steppe wolves of Kazakhstan, the degree of sexual dimorphism is more pronounced than in the wolves of the south-east of Ukraine and the Altai Territory. In wolves of subspecies *C. l. campestris* and *C. l. desertorum* on the territory of Kazakhstan, no significant differences in body weight and length were found. The wolves of steppe Kazakhstan are most similar in morphometric parameters to the wolves of the southeast of Ukraine.

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Biotechnological Communication

In silico Molecular Docking of α-Glucosidase with Prangenidin and Columbin As An Anti-Hyperglycemic Strategy

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ABSTRACT

Diabetes is considered to be a fatal disease as it brings many malfunctioning to the body. Currently used therapy is found to possess lots of side effects. Hence, we need to turn to more potent and safer options. An alternative choice can be phytochemicals of common use that will be cost-effective and safe. Therefor the aim of this research was to identify and analyze plant based metabolites as glusocidase inhibitors. This enzyme is known to enhance carbohydrate digestion and responsible to increase the level of glucose in blood circulation. Hence inhibitors of glucosidase are in limelight all over the world to regulate type-2 diabetes by reducing carbohydrate digestion and absorption. In the present study two phytochemicals prangenidin and columbin were selected as ligands. α-glucosidase was chosen as ligand's receptor from Protein Data Bank. Molecular docking of ligand & receptor was carried out by using PyRx molecular docking software. The docking results are found to be quite promising with the binding affinity of -6.6 kcal to -7.7 kcal and -6.4 to -8.1 kcal for prangenidin and columbin respectively. It can be concluded from the results of the present study that these phytochemicals have a high affinity to bind with α-glucosidase and may be used in the near future to deal with diabetes type II. In addition, the present study may be helpful to initiate in vitro/ in vivo research by using these phytochemicals. Such evidence based researches will provide a platform to start clinical trials for the treatment of fatal diseases like diabetes.

KEY WORDS: BINDING AFFINITY, DIABETES TYPE II, MOLECULAR DOCKING, PHYTOCHEMICALS, A-GLUCOSIDASE.

INTRODUCTION

α-Glucosidase (EC 3.2.1.20) is considered as a key enzyme that regulates the conversion of carbohydrates into glucose. Therefore, it is known to regulate the blood sugar level and control type–II diabetes (Venable and Aschenbrenner 2002; Park et al. 2008; Gamblin et al. 2009; Rawling et al. 2009). Previous studies suggested that inhibition of α-glucosidase activity may be utilized to design promising therapy for type-II diabetes. Scientists have synthesized several molecules as an inhibitor of α-glucosidase. However, synthetic molecules are found to possess many side effects such as abdominal discomfort, diarrhea, flatulence, and hepatotoxicity (Campbell et al. 2000; Krentz and Bailey, 2005; Hsiao et al. 2006; Nathan et al. 2006; Rehman et al. 2019; Kato-Schwartz et al. 2020).

As a result, molecular docking tools may provide a promising route to design and identify novel inhibitor for the α-glucosidase enzyme (Raghu et al. 2019; O'Keefe et

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al. 2019). There have been several α-glucosidase inhibitors including acarbose, voglibose, and miglitol obtained from natural sources with clinical implications (Lefebvre and Scheen 1994; Scott and Spencer 2000; Playford et al. 2013). However, very few α-glucosidase inhibitors are commercially available. Therefore the search of novel natural inhibitors of the α-glucosidase enzyme is still going on. In recent years, projects undertaken to discover potent non-sugar based α-glucosidase inhibitors from natural sources have received tremendous attention (Chang et al. 2013; Mata et al. 2013). A majority of the compounds reported contain flavonoid and terpene ring structures (Humphries et al. 1986; Hwangseo et al. 2008; Rudd et al. 2008; Yin et al. 2014; Yousuf et al. 2020; Kato-Schwartz et al. 2020).

The ligand of the present study i.e., Prangenidin is also known as Alloimperatorin and belongs to the coumarin compound. It is an active chemical content of the plant Aegl emarmelos (Indian name- Bael) and known to be extracted from *Angelica dahurica*. It is found to have anti-inflammatory, anti-oxidant, and anti-neoplastic activity (Chen et al. 2008; Li et al. 2016). Another ligand of the present study is Columbin, an organic heterocyclic compound. It is an active chemical content of the plant *Tinospora cordifolila* (Indian name- guduchi) and known to have anti-inflammatory and anti-cancer properties (Abdelwahab et al. 2012). Furthermore, a search on the pub-med timeline has shown an increasing trend of research on diabetes (Chung et al. 2019; Zhang et al. 2020; Chen et al. 2021). Therefore, in the present study, Prangenidin and Columbin ligands were docked with the α-glucosidase enzyme to find out their binding potential with enzyme and to explore future outcomes to control diabetes type –II.

MATERIAL AND METHODS

We performed molecular docking between ligands (Prangenidin and Columbin) & enzyme α-glucosidase. The enzyme molecule was obtained from PDB in its PDB format and its PDB ID is 5DKY. Protein structure was viewed in BIOVIA Discovery Studio Visualizer, and hetero-atoms, water molecules, ligand groups and nucleic acid groups were removed. Polar hydrogens were added and nonpolar hydrogens were merged. Missing atoms were checked and repaired before applying Kollman charges. The macromolecule was saved in PDBQT file format for the further application. The ligand structures were obtained from chemsketch in its SAG format and were checked for drug likeness and its physiochemical properties by using Lipinski rule of 5. The software used for molecular docking was PyRx, a useful virtual screening software for computational drug discovery to screen libraries of compounds against potential drug targets. PyRx includes docking wizards with an easy-to-use set up which makes it a valuable tool for Computer-Aided Drug Design (Tuli et al. 2021).

RESULTS AND DISCUSSION

Molecular Docking or QSAR is a technique to explore the binding affinity of the ligand to its receptor. In this method, a scoring function used to find the highest affinity position for the tested ligand in the active site of the receptor. In molecular docking, it has been known that the lesser the energy relates to high affinities between ligand and receptor. QSAR analysis of molecules has gained importance to find a convenient ligand before performing expensive and time-consuming wet laboratory experiments. The physio-chemical properties of the selected ligands (Prangenidin & Columbin) have described in table 1. Prangenidin on docking with α-glucosidase gave the best binding energy affinity of -7.7. It interacted with two amino acids of α-glucosidase, to be exact at GLY-228 and GLU-271. Table 2 represented α-glucosidase amino acids interactions with ligand Prangenidin and the distance between the interacted ligand poles with an amino acid. Whereas Table 3 represented the complete molecular docking results of ligand and receptor α-glucosidase. The docked pose of ligand and α-glucosidase has been shown in Figure 1.

Table 1: Physio-chemical Properties of ligands with Lipinski rule of 5* (Molecular mass less than 500 Dalton, High lipophilicity (expressed as LogP less than 5), Less than 5 hydrogen bond donors, less than 10 hydrogen bond acceptors, Molar refractivity should be between 40-130).

Ligands Properties	Prangenidin	Columbin
Chemical Formula	$C_{16}H_{14}O_4$	$C_{20}H_{22}O_6$
Source	*Angelica dahurica*	*Tinospora cordifolila*
pH	3.1	1.3
Molecular Weight	270.28 g/mol 358.4 g/mol	
Hydrogen Bond Donor	1	1
HydrogenBond Acceptors	4	6
LogP	3.7	2.2

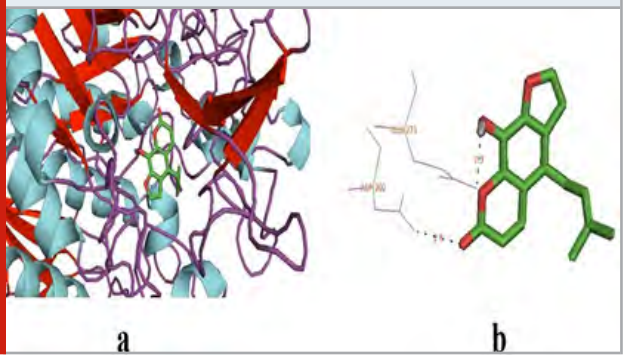
Table 2. The table shows interaction between the active poles of the ligand and the amino acid of the receptor (α-glucosidase).

Receptor	Ligand	Amino acid interacted	Distance between the amino acid and the ligand pole (Å)
α-glucosidase	Prangenidin	ASP-202	2.8
α-glucosidase	Prangenidin	GLU-271	2.3

Columbin on docking with α-glucosidase gave the best binding energy affinity of -8.1. It interacted with two amino acids of α-glucosidase, to be exact at ASN-301. The amino acids of α-glucosidase interacted with columbin, and complete docking results were shown in table 4. Furthermore, the interaction between the active poles of the

ligand and the amino acid of the receptor (α-glucosidase) i.e ASN-301 was observed with a distance of 2.9Å. It represents strong H-bonds formation between ligand and receptor. Figure 2 represented the docked pose of Columbin and α-glucosidase receptors in the software. The results of the present study are found to be consistent with previously published docking results that showed binding energies in the range of -7.7 to -8.1 kcal may be considered as promising results (Wang et al. 2017; Rehman et al. 2019).

Figure 1: (a) The docked pose of ligand (prangenidin) in the binding pocket of the enzyme which is used as a receptor; α-glucosidase. The ligand is shown in the stick structure and the enzyme is shown in cartoon model structure. (b) This figure depicts the H-bond interaction between the ligand, prangenidin with the residues of α-glucosidase which are GLU-271 and ASN-202. The blue dots show the H-bonding between the ligand and the residue. The numbers with the H-bonds represents the distance between the residue and the ligand interacting pole (Source: PDB, PyRx & BIOVIA).



In discussion, there have been a variety of pharmacologically active metabolites reported from plant sources. Evidences have suggested that these bioactive metabolites are majorly a part of our diet and demonstrated their role in the treatment of several dreadful diseases including cardiovascular, cancer and neruro-degeneration (Kumar et al. 2015; Kashyap et al. 2016; Kashyap et al. 2018a; Kashyap et al. 2018b; Aggarwal et al. 2019; Kashyap et al. 2019; Tuli et al. 2019; Sharma et al. 2019). Therefore, diet modification may contribute to preventing a significant number of such dreadful diseases. Furthermore, existing conventional approaches of drug discovery are very much time consuming and labor-oriented. Moreover, diagnosis and progression rate of such diseases are very fast. Therefore, in such a drastic scenario computational biology may help the pharmaceutical industry to fast the drug discovery processes (Wooller et al. 2017). Computational tools of docking can not only tell the interactions of the ligand with receptor but also define the possibility of drug synthesis from a large number of chemical database (Hasselgren and Myatt 2018; Thomford et al. 2018; Mochizuki et al. 2019; Patel et al. 2020; Gupta et al. 2021). Therefore in order to understand the interactions and binding affinities of ligands with glucosidase receptor, we performed molecular docking studies by using PyRx software. Out of tested ligand columbin was found to interact more firmly with glucosidase receptor with more docking score as well as H-bond formation capability.

Table 3. This table shows the receptor and the ligand docked, force field with their binding affinities at different position and the best energy minimization being -7.7.

Ligand	Binding Affinity	rmsd/ub	rmsd/lb
a-glucosidase_69502	-7.7	0	0
a-glucosidase_69502	-7.2	10.031	6.86
a-glucosidase_69502	-7.2	11.653	9.153
a-glucosidase_69502	-7	11.202	9.483
a-glucosidase_69502	-7	16.273	14.211
a-glucosidase_69502	-6.7	12.724	10.256
a-glucosidase_69502	-6.7	16.999	13.74
a-glucosidase_69502	-6.7	12.748	9.634
a-glucosidase_69502	-6.6	12.966	9.359

Table 4. This table shows the receptor and the ligand docked, force field with their binding affinities at different position and the best energy minimization being -8.1.

Ligand	Binding Affinity	rmsd/ub	rmsd/lb
a-glucosidase_442015	-8.1	0	0
a-glucosidase_442015	-7.3	3.166	2.054
a-glucosidase_442015	-7.3	6.81	2.048
a-glucosidase_442015	-7.3	34.481	31.079
a-glucosidase_442015	-7.3	34.359	32.715
a-glucosidase_442015	-6.8	21.467	19.651
a-glucosidase_442015	-6.8	6.676	3.846
a-glucosidase_442015	-6.6	34.255	31.272
a-glucosidase_442015	-6.4	33.31	31.553

Furthermore results of present study explore the binding affinity of both the ligands for glucosidase receptor with active interacting amino acid residues GLU 271, ASP 202, and ASN 301. Previously also *in silico* studies of phytochemicals or their synthetic derivatives such as oriciacridone F and O-methylmahanineand 2-(benzo[d] [1,3]dioxol-5-yl)-4H-chromen-4-one, respectively have been carried to find α-Glucosidase Inhibitors (Zafar et al. 2016; Meena et al. 2019). More recently, three compounds named as 50-hydroxymethyl-10-(1, 2, 3, 9-tetrahydro-pyrrolo(2,1-b)quinazolin-1-yl)-heptan-10-one(1),-terpinyl-glucoside (2), and machaeridiol-A were extracted from *Psychotria malayana* and docked with glucosidase (Nipun et al. 2020). Results revealed that four hydrogen bonds formed at ASP352, ARG213, ARG442, GLU277, GLN279, HIE280, and GLU411 and energy minimizarion were in the range of -7.6, and -10.0 kcal/mole. Therefore results of present study are in good agreement with previously published work so as to find out active interacting residue as well binding affinities.

Figure 2: (a) The docked pose of ligand (Columbin) in the binding pocket of the enzyme which is used as the receptor; α-glucosidase. The ligand is shown in the stick structure and the enzyme is shown in cartoon model structure. (b). This figure depicts the H-bond interaction between the ligand, columbin with the residues of α-glucosidase which is ASN-301. The blue dots show the H-bonding between the ligand and the residue. The number with the H-bonds represents the distance between the residue and the ligand interacting pole (Source: PDB, PyRx & BIOVIA).

CONCLUSION

The findings of the present study revealed molecular docking between ligands (Prangenidin and Columbin) and α-glucosidase. We found that the binding energies of these two ligands ranging from -6.4 to -8.1 and also the hydrogen bond interaction are quite strong. Diabetes mellitus is one of the biggest threats to human health which is increasing at an alarming rate. This work is an effort to put forward these two ligands as future anti-hyperglycemic agents.

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

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Biotechnological Communication

Exogenous Application of *Chenopodium album* Aqueous Extracts on Salt-Stressed Wheat Seedling

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ABSTRACT

Biostimulants are substances when applied to plants seeds, or soil stimulates the natural process to improve water and nutrient use effectively and increase tolerance to biotic and abiotic stress by enhancing primary and secondary metabolism. Application of plant biostimulants in a state of environmental stress can reduce the effects of stress and improve soil water holding capacity, root growth, and yield. Particularly, they reduce the application of mineral fertilizers by increasing the number of micro-and macronutrients taken up by plants, positively affecting root morphology and plant growth. In this study, aqueous extract of different parts like shoot and bud of *C. Album* is used for observing role in salt stress tolerance of wheat seedling grown in vitro. The wheat seedling was germinated in moistened filter paper in petriplates, with 3 petriplates for each treatment and 10 seeds in each petriplate (the study was done in triplicates). Then observing seed germination in control set in which no plant extract was used and two concentration of salt was taken i.e., 50mM and 100mM, along with no salt stress (0mM). In another set, the same salt stress was used but seeds were also treated with shoot and bud extract of *C. album*. After their growth for 7 days, seedlings were measured for root length, shoot length, wet weight, and dry weight. Again the same sets of experiments were repeated for measuring biochemical parameters like protein, sugar, and proline. It was observed that aqueous extract of *Chenopodium* can induce salt stress by increasing protein, proline, and sugar content along with better growth characteristics of seedlings. In conclusion, an aqueous extract of *Chenopodium* can be used as a biostimulant for fighting stress tolerance in the wheat seedling. Further, this study can be extended to other plant species other than wheat, and a gene expression study can be done for further validation.

KEY WORDS: BIOSTIMULANTS, CHENOPODIUM ALBUM, PHYSICAL AND BIOCHEMICAL PARAMETERS, SALT STRESS, WHEAT.

INTRODUCTION

Land plants are living in an intrinsically harsh atmosphere ever since their appearance as variety of physical or chemical factors are antagonistic to them, including low or high temperature, undersupplied or excessive water, high salinity, heavy metals, and ultraviolet (UV) radiation, among others. These stresses jointly referred to as abiotic stresses, are posing a brutal threat to agriculture and the ecosystem, accounting for great crop yield loss (Wang et al. 2003). Abiotic stresses lead to a sequence of morphological and physiological, biochemical, and molecular changes that significantly influence plant productivity (Wang et al. 2004; Wania et al. 2016; Sarker and Oba 2020).

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Salinity is the chief stress restraining the rise in the requirement for food crops. More than 20% of cultured land worldwide (~ about 45 hectares) is exaggerated by salt stress and the quantity is escalating day by day. Therefore, salinity generally is one of the fierce environmental stresses that slow down crop productivity universally (Flowers 2004; Tester and Munns 2008). A plant biostimulant is any substance or microorganism useful for plants to improve nutrition effectiveness, abiotic stress tolerance, and/or crop quality traits, despite its nutrients content (Patrick 2015; Gupta et al. 2021).

In current years, plant biostimulants are being widely used in farming and cultivation. Positive effects of its application have yielded unexpected results as confirmed by many studies (Poincelot 1993; Jelacic et al. 2007; Smolen and Sady 2010; Smolen et al. 2010; Matzsiak et al. 2011; Beata et al. 2013). Plant biostimulants are organic materials that come into view to impact some metabolic procedures such as respiration, photosynthesis, nucleic acid synthesis, and ion

uptake and when applied in small quantities, improve the plant growth and development or specifically, a blend of two or more PGRs or a mixture of these with other substances (amino acids, nutrients, vitamins) is acknowledged as a plant growth promoter or plant biostimulant. Plant biostimulants are effective when applied in small doses, consequently leads to plant growth, and production enhancement (Li and Ni 1996; Castro and Vieira 2001; Saa-Silva et al. 2013; Gupta et al. 2021).

Moreover, the application of plant biostimulants in a state of environmental stress can reduce the effects of stress and improve soil water holding capacity, root growth, and yield. The performance of endophytic fungi applied to crops as a supplement to plant genetics or soil management to alleviate salt stress in crops. They reduce the application of mineral fertilizers by increasing the number of micro-and macronutrients taken up by plants, positively affecting root morphology and plant growth (Fisher and Wilson 1975; Kunicki et al. 2010; Ziosi et al. 2013; Nardi et al. 2009; Ertani et al. 2013). The salt-sensitive and tolerant A. tricolor variety behaved differently under salt stress regarding growth, anatomical, physiological, ROS accumulation, enzymatic and non-enzymatic antioxidative defence mechanisms, and attributes associated with tolerance to oxidative stress (Sarker and Oba 2020). As there are many reports of biostimulants playing role in promoting growth and development of plants, in this study a weed plant *C. album* is chosen as it grows in bulk amount in field and they coexist in the field with the wheat (Gupta et al. 2021). Therefore, this study has explored its effect in *in vitro* grown salt-stressed wheat seedlings.

MATERIAL AND METHODS

Grains of *Triticum aestivum* (Wheat) was collected from RARI, Durgapura, Jaipur, Rajasthan. *Chenopodium album* weed was selected as the accessibility of this weed was easy and grown with Wheat seeds. Plants of *Chenopodium album* was collected from the garden of Dr B. Lal Institute, Jaipur, and Rajasthan. Different parts of *C. album* was separated and washed three times with distilled water and then transferred in a beaker containing $HgCl_2$ and was left for 1 min, again washed 3-4 times with distilled water. Plant material was being spread on blotting paper and was left 10-15 days for drying (Abid et al. 2017). After drying different parts of the plant were crushed separately in mortar and pestle and were stored in a well labelled clean and dry falcon. To prepare the extract 1gm powder of plant material was soaked overnight in 100ml of autoclaved distilled water. During this soaking, the plant metabolites are released in distilled water after that the filtrate is filtered with the help of filter paper and stored at 4°C.

From the latter extract, different concentrations of the shoot (50 mg and 200mg) and bud (50 mg and 100mg) was prepared using distilled water as these concentrations of plant parts showed the best results in both morphological parameters and biochemical parameters when compared to the control of all the concentrations used of *C. album* in the previous study done by us. A homogenous set of grains of the wheat plant was selected for uniformity of size, shape, and viability. Before germinating, sterilization of seeds was done under laminar airflow, where seeds were washed 3-4 times with autoclaved distilled water then with $HgCl_2$ for 1 min, and then again washed with distilled water 3-4 times. The grains were transferred to sterile petriplates containing two sheets of filter paper, in between a thin layer of cotton. Each petriplate contained 10 grains and each treatment was replicated 3 times.

The petriplates were moistened with different concentrations of NaCl solutions (50mM and 100 mM) and different concentrations of plant extract of the shoot (50 mg and 200mg) and bud (50 mg and 100 mg). These petriplates were wrapped in aluminium foil and incubated in dark for two days at an average room temperature of 25-27° C. After two days these petriplates were placed in plant tissue culture racks under 16 hours of photoperiod. The results of root length, shoot length, wet weight, and dry weight of both roots, shoots and different biochemical parameters of salt-stressed wheat seedlings was calculated after 7 days of transfer to plant tissue culture racks.

Protein evaluation was done according to Bradford's method (Bradford 1976). Firstly preparation of individual components was done: Bradford's Reagent (100mL): 0.01g G-250 in 5 ml, ethanol+8.5 mL ortho – Phosphoric acid + 87.5 ml Distilled water. Standard solution of BSA: 50 mg (0.05 g) BSA in 2.5 ml Bradford Reagent and 5 ml Buffer was added in each tube. Blank: 0.1 ml Distilled water + 0.9 ml buffer + 5 ml Bradford reagent. Test sample: 0.1 ml sample in 0.9 ml buffer and add 5 ml Bradford reagent. After that protein estimation was carried out using the following steps: 0.1 ml of sample solution was taken and 0.9 ml 0.1M Na-P buffer was added. Suitable aliquots of BSA solution were pipetted out. Blank was prepared for calibration. 5 ml Bradford reagent was added to each tube. Absorbance was recorded at 595 nm. Protein concentration was determined by the standard curve of Bovine Serum Albumin (BSA) by using the following equation: $y = 1.073x – 0.068$; where y is absorbance recorded of the sample and x is protein concentration in mg/ml. Sugar estimation was done according to Dubois method (Dubois et al. 1956). 1 ml of plant extract was taken in a test tube and 3 ml of 96% H_2SO_4 was added to it after that 1 ml of 5% Phenol was added to it. The solution was mixed and was kept in a water bath for 20 min at 25-30 ^{0}C. Absorbance was taken at 490nm. Blank was prepared using 1 ml D.W+ 3ml 96% $H_2SO_4^{+1}$ ml phenol.

The total concentration of sugar was determined by using the equation from the standard curve: $y = 0.033x – 0.003$; where y is absorbance recorded of the sample and x is sugar concentration in mg/ml. Proline estimation was done according to Bates (Bates et al. 1973). 0.5g of plant material was extracted by homogenizing in 10 ml of 3% aqueous Sulphosylic acid. The homogenous was filtered through filter paper. 2 ml of filtrate was taken in a test tube and 2ml of glacial acetic acid and 2 ml of ninhydrin was added into it. The solution was heated in the boiling water bath for 1 hr. The reaction was ended by placing the tube in an ice bath. 4 ml of toluene was added to the reaction mixture and was stirred well for 20-30 sec. The toluene layer was

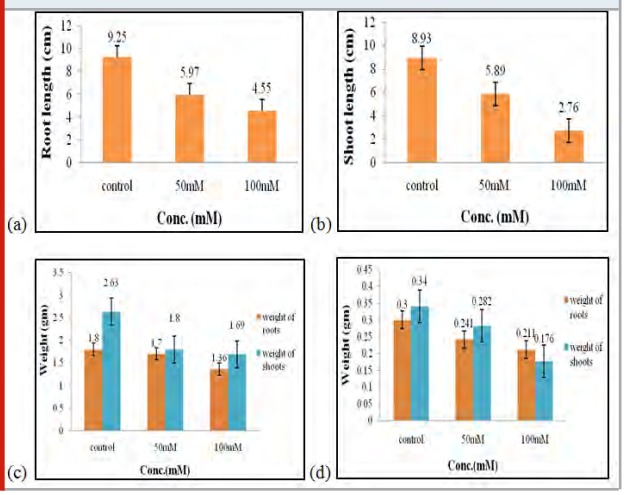
separated and was warmed at room temperature. The red colour intensity was measured at 520nm. Amount of proline in the test sample was calculated from the standard curve by using the following equation: Proline = [(µg proline/mL × mL toluene)]/ [115.5 µg/umole]/ [(g sample/5].

RESULTS AND DISCUSSION

Morphological Analysis: Different concentrations of extracts of shoot and bud of *C. album* showed varied results in the following physical parameters like root length, shoot length, fresh and dry weight of roots and shoots when treated with different concentrations of salt.

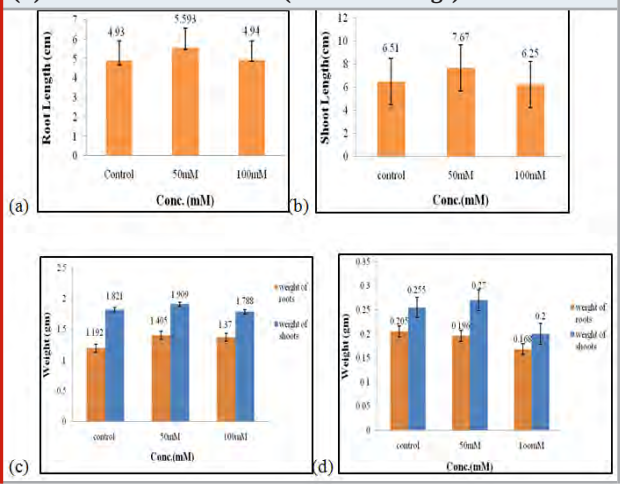
Effect of different salt concentrations on wheat seedlings (Control set): In control set, two concentration of salt was taken i.e., 50mM and 100mM and no plant extract was used. Stimulatory effects were seen in control in all the parameters that are root length, shoot length, fresh and dry weight of roots and shoots while at higher concentration of salt (100mM) it showed inhibitory effects (Fig.1). Out of two concentrations of salt, 50mM was found to be as optimum concentration for the growth of the wheat seedlings in the control set (Figure 1). CHS1 ameliorated the adverse effect of high NaCl stress and rescued soybean plant growth by regulating the endogenous plant hormones and antioxidative system. CHS1 isolate could be exploited to increase salt resistant and yield in crop plants (Asaf et al. 2018; Zelm et al. 2020).

Figure 1: Effects of salt stress (control set) on root length (a) shoot length (b), fresh weight of roots and shoots (c), and dry weight of roots and shoots of wheat seedlings (d). Values are mean ± SD (n = 30 seedlings).



Effect of salt stress on physical parameters of wheat seedlings containing 50mg/ml of shoot extract: The maximal root length (5.59 cm, Fig. 2a), shoot length (7.67 cm, Fig. 2b), fresh weight of shoots and roots (1.9 gm and 1.4 gm, Fig. 2c), shoot dry weight (0.27 gm, Fig. 2d), were obtained in 50 mM of salt concentration, which increased by 0.66 cm,1.16 cm, 0.08 gm, and 0.5 gm correspondingly when compared to control. The optimal dry weight of roots (0.2 gm, Fig. 2d) was found to be in control one where no salt stress was given. Therefore, 50mM of salt concentration was found to be the suitable concentration when 50mg/ml of shoot extract was used for the growth of wheat seedlings (Figure 2). Salt induces multiphase changes in growth rate, as well as changes in root system architecture and a salt avoidance response of the main root. These responses are mediated by several hormones, including auxin and ABA (Zelm et al. 2020).

Figure 2: Effects of salt stress on physical parameters of wheat seedlings containing 50mg/ml of shoot extract on root length (a) shoot length (b), fresh weight of roots and shoots (c), and dry weight of roots and shoots of wheat seedlings (d). Values are mean ± SD (n = 30 seedlings).



Effect of salt stress on physical parameters of wheat seedlings containing 200mg/ml of shoot extract: The maximal fresh weight of shoots and roots (2.56 gm and 1.85 gm, Fig. 3c), dry weight of shoots (0.34 gm, Fig. 3d) were obtained in 50mM of salt concentration, which increased by 0.55 gm, 0.69 gm, 0.06 gm correspondingly when compared to control. The optimal root length and shoot length (11.4 cm, 10.7 cm, Fig. 3a, 3b), dry weight of roots (0.26 gm, Fig. 3d) were found to be in 100mM of salt concentration. Hence, both 50mM and 100mM of salt concentration were found to be the suitable concentration when 200mg/ml of shoot extract was used for the growth and development of wheat seedlings (Figure 3). *C. crassa* is a highly tolerant taxon to salinity that can also survive the presence of a low to moderate amount of Cd and Pb at the seedling stage (Samiei et al. 2020).

Effect of salt stress on physical parameters of wheat seedlings containing 50mg/ml of bud extract: The maximum root and shoot length (4.52 cm and 5.99 cm, Fig. 4a, 4b), fresh weight of shoots and roots (1.44 gm and 1.04 gm, Fig. 4c), dry weight of shoots (0.21 gm, Fig. 4d) were obtained in 100mM of salt concentration, which increased by 1.65 cm, 2.02 cm, 0.36 gm, 0.34 gm, 0.03 gm respectively when compared to control. The optimal dry weight of roots (0.16 gm, Fig. 4d) was found to be in 50mM of salt concentration. Therefore, 100mM of salt concentration was found to be the suitable concentration when 50mg/ml of bud extract could effectively improve the growth performance of wheat seedlings (Figure 4). The salinity stress does not only hinder the growth and

development of plants but also affects some physiological and metabolic activities as it affects osmolyte and ionic concentrations in plants. To prevent the crops from these losses various mitigation strategies are adopted to combat the harmful effects of saline stress (Bhardwaj and Kumar 2020).

Figure 3: Effects of salt stress on physical parameters of wheat seedlings containing 200mg/ml of shoot extract on root length (a) shoot length (b), fresh weight of roots and shoots (c), and dry weight of roots and shoots of wheat seedlings (d). Values are mean ± SD (n = 30 seedlings).

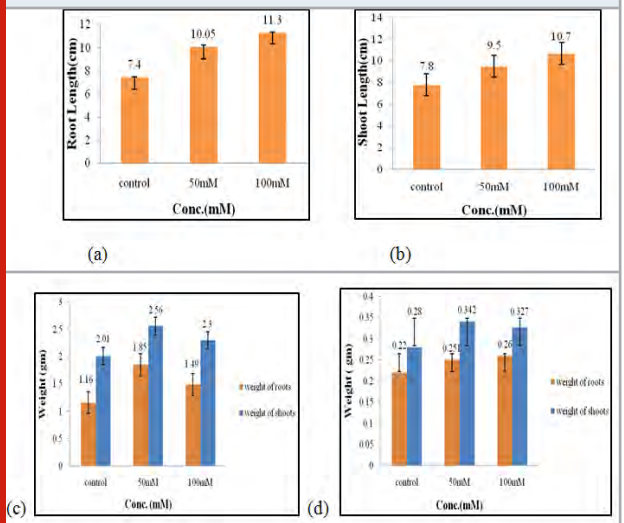
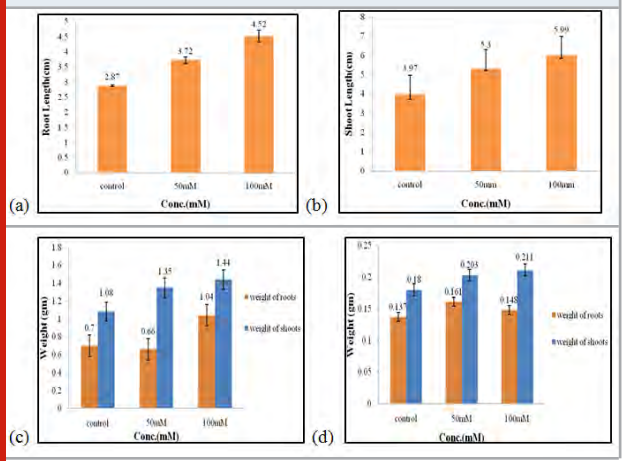
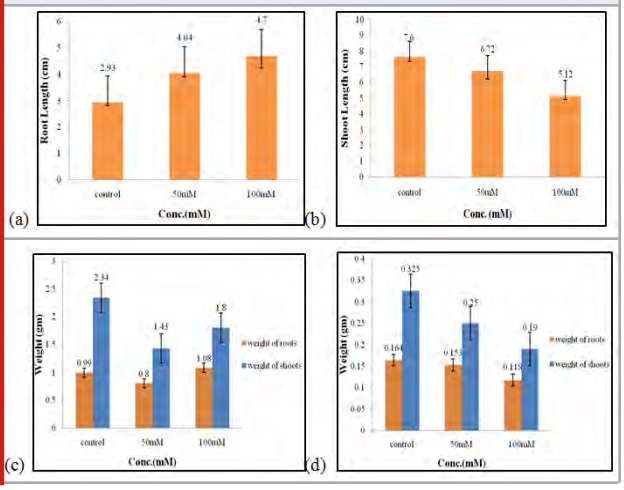


Figure 4: Effects of salt stress on physical parameters of wheat seedlings containing 50mg/ml of bud extract on root length (a) shoot length (b), fresh weight of roots and shoots (c), and dry weight of roots and shoots of wheat seedlings (d). Values are mean ± SD (n = 30 seedlings).



Effect of salt stress on physical parameters of wheat seedlings containing 100mg/ml of bud extract: The maximum root length (4.7 cm, Fig. 5a), fresh weight of roots (1.08 gm, Fig. 5c) were obtained in 100mM of salt concentration, which increased by 1.77 cm, 0.9 gm respectively when compared to control. The optimal shoot length (7.6 cm, Fig. 5b), fresh weight of shoots (2.34 gm, Fig. 5c), and dry weight of shoots and roots (0.32 gm and 0.16gm, Fig. 5d) were found to be in control. Therefore, the case of 100mg/ml of bud extract control set shown the best results in the growth and development of wheat seedlings whereas 100mM of salt concentration was inhibiting its effect (Figure 5). The application of ascorbic acid and humic acid as a foliar spray and inoculation with PGPR treatment gave the highest significant yield, and chemical constituents as it may provide a useful way to reduce the adverse effects of salinity stress on wheat plants grown in saline soil (El-Sayed and Hagab 2020).

Figure 5: Effects of salt stress on physical parameters of wheat seedlings containing 100mg/ml of bud extract on root length (a) shoot length (b), fresh weight of roots and shoots (c), and dry weight of roots and shoots of wheat seedlings (d). Values are mean ± SD (n = 30 seedlings).

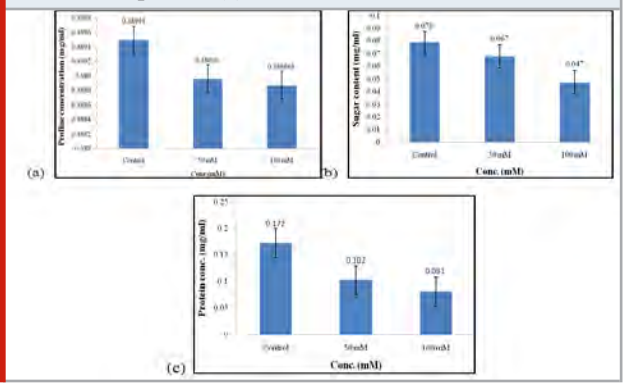


Biochemical Analysis: The biochemical analysis of the extract of *C. album* (shoot and bud) total soluble sugar estimation, protein estimation, and proline estimation was carried out using as mentioned in materials and method. Biochemical analysis of plants was necessary to check the quality of seeds when they are exposed to a different extract of weed of varying concentrations. In this study, along with the control two concentrations were used as selected for performing the above physical analysis.

Effect of salt stress on biochemical parameters of wheat seedlings (Control set): In the control set, different biochemical parameters were measured when wheat seedlings were treated with varying concentrations of salt that are 50mM and 100mM, and no plant extract was used in them. Proline content in wheat seedlings was found to be highest in control as compared to the 50mM and 100mM of salt concentration. Content of proline in control was 0.389 mg/ml, (Fig. 6a), respectively. Increased Proline concentration generally protects the plant from a different kind of stress. Sugar content was also found to be highest in control when compared to the other concentration of salt used which is 0.078 mg/ml (Fig. 6b). Sugar was mainly the essential component of plant nutrition found during photosynthesis and its amount gets decreased as it was highly sensitive to environmental stress (Ami et al. 2020).

Protein concentration also gets increased in control i.e., 0.172 mg/mL (Fig. 6c) as compared to other concentrations of salt used. Protein concentration in plants gets decreased due to the inhibition of incorporation of amino acids caused due to stress. Protein content also increases due to the activity of genes involved in various enzymatic activities get increased. Of all, proline, sugar, and protein content was found to be highest in control as compared to the other concentration of salt used i.e., 50mM and 100mM, and also at higher concentrations of salt i.e. at 100mM the quality of seed was getting inhibited. Salt stress can be partially alleviated by proline in the drought-resistant cultivar MBB, even though it is relatively salt-sensitive (Ami et al. 2020).

Figure 6: Effect of salt stress on biochemical parameters of wheat seedlings (Control set) Proline (a), soluble sugar (b), and soluble protein (c).



Effect of salt stress on biochemical parameters of wheat seedlings containing 50mg/ml of shoot extract: Wheat seedlings when treated with 50mg/ml of shoot extract gave the following results, Proline content in wheat seedlings was found to be almost similar in all control, 50mM and, 100mMof salt concentration. Content of proline did not show any significant effect when treated with 50mg/ml of shoot extract (Fig. 7a). Sugar content was also found to be highest in 100mM when compared to the other concentration of salt used which was 0.015 mg/ml (Fig. 7b). Protein concentration gets increased in 50mM of salt concentration i.e., 0.123 mg/mL (Fig. 7c) as compared to other concentrations of salt used. Of all, proline, sugar content was found to be highest in 100mMand protein content was found to be highest in 50mM as compared to the other concentration of salt used i.e., control and 50mM. The production of phytohormones, particularly auxins, have been demonstrated by PGPR, even the pathogenic bacteria and fungi which also modulate the endogenous level of auxins in plants, subsequently enhancing plant resistance to various stresses (Ullah et al. 2021).

Effect of salt stress on biochemical parameters of wheat seedlings containing 200mg/ml of shoot extract: Wheat seedlings when treated with 200mg/ml of shoot extract gave the following results, Proline content in wheat seedlings was found to be highest in 100mM i.e., 0.389mg/ml (Fig. 8a) and almost equal in control, and 50mM of salt concentration. Here also, the content of proline did not show any considerable effect when treated with 200mg/ml of shoot extract. Sugar content was also found to be highest in 100mM when compared to the other concentration of salt used which was 0.013 mg/ml (Fig. 8b). Protein concentration was found to be increased in control i.e., 0.531 mg/mL (Fig. 8c) as compared to other concentrations of salt used. Of all, proline and sugar content was found to be highest in 100mM as compared to the other concentration of salt used i.e., control and 50mM, and protein content did not show any significant effect as it was highest in control. Salt stress increased total soluble sugars (TSS) in all parts of the kiwifruit genotypes. The High accumulation of TSS in roots explains the overproduction of TSS by leaves transported and stored in roots via the phloem. (Abid et al. 2020).

Figure 7: Effect of salt stress on biochemical parameters of wheat seedlings containing 50mg/ml of shoot extract, Proline (a), soluble sugar (b), and soluble protein (c).

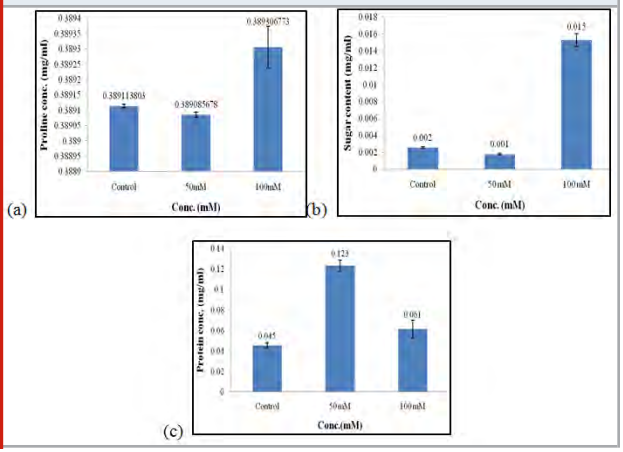
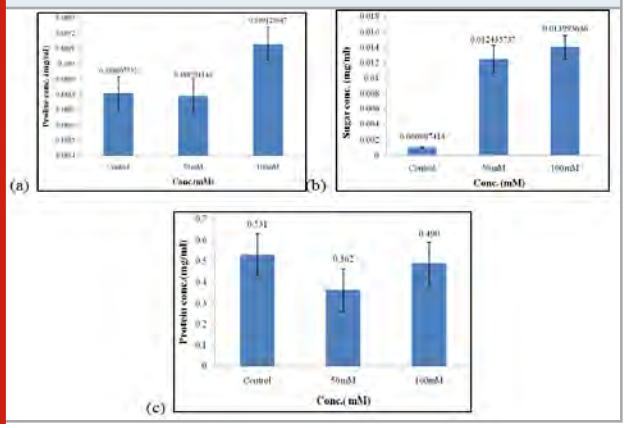


Figure 8: Effect of salt stress on biochemical parameters of wheat seedlings containing 200mg/ml of shoot extract, Proline (a), soluble sugar (b), and soluble protein (c).



Effect of salt stress on biochemical parameters of wheat seedlings containing 50mg/ml of bud extract: Wheat seedlings when treated with 50mg/ml of bud extract gave the following results, Proline content in wheat seedlings was found to be highest in 50mM and 100mM i.e., 0.391mg/ml (Fig. 9a) of salt concentration. Sugar content was found to be highest in 50mM when compared to the other concentration of salt used which was 0.056 mg/ml (Fig.

9b). Protein concentrations were found to be increased in control i.e., 0.422 mg/mL (Fig. 9c) as compared to other concentrations of salt used but it gets decreased at higher salt concentrations.

Figure 9: Effect of salt stress on biochemical parameters of wheat seedlings containing 50mg/ml of bud extract, Proline (a), soluble sugar (b), and soluble protein (c).

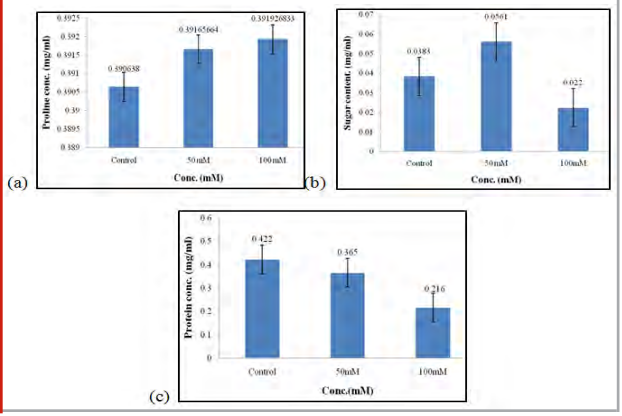
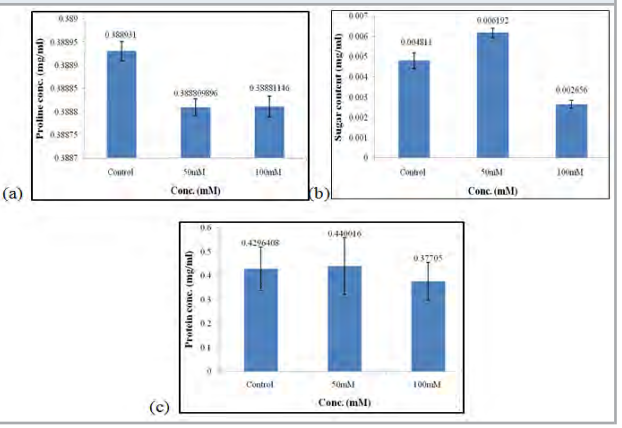


Figure 10: Effect of salt stress on biochemical parameters of wheat seedlings containing 100mg/ml of bud extract, Proline (a), soluble sugar (b), and soluble protein (c).



Effect of salt stress on biochemical parameters of wheat seedlings containing 100mg/ml of bud extract: Wheat seedlings when treated with 100mg/ml of bud extract gave the following results, Proline content in wheat seedlings didn't show any considerable effect as it was similar in all the concentrations of salt used and also in control i.e., 0.388mg/ml (Fig. 10a) of salt concentration. Sugar content was found to be highest in 50mM when compared to the other concentration of salt used which was 0.006 mg/ml (Fig. 10b). Protein concentration was found to be increased in 50mM of salt concentration i.e., 0.440 mg/ml (Fig. 10c) as compared to other concentrations of salt used but it gets decreased at higher salt concentrations. Of all, proline, sugar, and protein content were found to be highest in 50mM as compared to the other concentration of salt used i.e., control and 100mM. Cysteine treatments had a beneficial role in alleviating the adverse effect of salinity stress on the soybean plant (Sadak et al. 2020). Vanillic acid significantly improves salinity tolerance and plant growth performance by involving the actions of plant antioxidant defence and glyoxalase systems (Parvin et al. 2020).

CONCLUSION

The findings of the present study shows that bud and stem extract mitigates salt stress in wheat seedlings. It was shown by physical, and various biochemical characteristics like sugar, protein, and proline. We saw at the maximum places the increase in these biochemical parameters in the presence of *C. album* extracts. Plants growing in the desert have the potential to tolerate adverse climatic change. *Chenopodium album* grows naturally as a weed in fields of wheat, barley, etc. Therefore, this study can find the potential of weed plants that can be used for abiotic stress tolerance, and these plant extracts combined with reduced doses of herbicides could be the promising strategy not only for abiotic stress tolerance but for sustainable agriculture in the future.

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

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Food Science Communication

On the Sensory, Colour, Texture and Physiochemical Characteristics of Foxtail Millet and Green Pea Pasta

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ABSTRACT

Millets have great nutritional properties and health benefits, and can thus be utilized as a pasta supplement. Thus, for the health-conscious genre of the present world, minor millet especially foxtail millet is perhaps one more addition to the proliferating list of healthy foods, owing to its nutritional superiority. They are convenient, easy to make, quick to cook, relatively inexpensive, and have a long shelf life. The extrusion process was chosen for making pasta which comprises foxtail millet and green pea flour to improve nutritional value, particularly in terms of protein level. The objective of the study is to formulate foxtail millet and green pea incorporated pasta and to evaluate for its physicochemical, textural profile analysis, colour analysis, and sensory analysis. Four variants of pasta were developed by mixing the following flours like foxtail millet (30-60%), green pea (10%), and wheat flour (30-60%) at different ratios. The developed pasta physiochemical, textural, colour, and sensory parameters were assessed using standard procedure. The results of the study was to revealed that chemical analysis, colour, textural, and sensory analysis of pasta showed a 1% level of substantial difference ($p<0.01$) as compared to the standard as the level of flour addition was increased. The pasta made with 40% foxtail millet and 10% green pea flour achieved the highest sensory scores as compared to regular pasta. The sensory assessment of the pasta samples showed that the variations differed significantly. This study was concluded that pasta has a universal demand and is a strong carrier of nutrients for a segment of the population that is health-conscious. Foxtail millet and green pea flour were a good replacement for wheat flour in terms of improving the nutritional properties of pasta manufacture and use.

KEY WORDS: COLOUR, FOXTAIL MILLET, GREEN PEA, PHYSIOCHEMICAL AND SENSORY.

INTRODUCTION

Millets stand out among cereals due to their high calcium, dietary fibre, polyphenol, and protein content. Millets are gluten-free, making them a good choice for celiac disease sufferers who are bothered by wheat and other gluten-containing cereal grains. The sixth-highest yielding crop, foxtail millet (*Setaria italica* (L.)), has been established as a major millet in terms of global output (Gélinas, 2008; Saleh et al. 2013; Devi 2014). Like most millets, foxtail millet is high in crude fibre, which aids in digestion and induces bowel movement, resulting in a laxative effect that is useful to a healthy digestive system. In China, foxtail millet is used to make noodles, nourishing gruel or soup, brewing alcoholic beverages, cereal porridges, and pancakes due to its nutritional characteristics (Krishna 2013; Yang et al. 2013). Aside from its nutritional value, foxtail millet has been found to provide several health benefits, including cancer prevention, hypoglycemic, and hypolipidemic effects (Zhang et al. 2015).

Green pea (*Pisum sativum*) is the second most important highly nutritious crop in terms of production. Peas are known for being a low-fat (3%), high-protein (24%), high-carbohydrate (58%), and high-dietary fibre (12%) carrying food (Iqbal et al. 2006). Pea containing a significant amount of vitamin A, vitamin C, vitamin B complex, iron, calcium, copper, zinc and manganese. No significant value of anti-metabolites or toxicity has been reported in pea (Garg et al. 2015; Narayanan et al. 2015; Ettoumi and Chibane 2015; Laureati et al. 2020).

Solubility, emulsifying and foaming characteristics, gelling ability, and water holding capacity are some of the functional qualities of pea flour and pea protein. These functional qualities are desirable in a variety of foods to improve shelf life and stability. Pasta products are well-known in many countries, and they are consumed and enjoyed worldwide.

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Researchers and food producers are becoming more interested in developing pasta products that are high in minerals, vitamins, fibre, and have a low glycemic index as a result of increased demand from health-conscious customers. Pasta is considered an effective medium for the incorporation of nutrition supplements by the World Health Organization (WHO) and the Food and Drug Administration (FDA) (Chillo et al. 2008; Ettoumi and Chibane 2015). According to Bustos et al. (2015), pasta is an ideal vehicle for the advancement of health among functional foods due to its low cost, long shelf life, and widespread consumption. These studies show that consumers are becoming more interested in using pasta as a functional meal. The addition of healthy ingredients to the pasta will lower the glycemic index and provide customers with additional health benefits (Bustos et al. 2015; Laureati et al. 2020).

Foxtail millet and green peas play an important role in the preparation of pasta due to their nutritional value. The study's goals were to identify the best combinations as well as to investigate the physical, chemical, textural, colour properties, and sensory analysis of pasta made with foxtail millet and green pea flour. The goal of this research is to develop pasta and assess it for physicochemical, textural profile, colour, and sensory properties.

MATERIAL AND METHODS

The raw materials such as foxtail millet (*Setaria italica*), wheat, and green pea (*Pisum sativum*) were received from the local market in Salem, Tamil Nadu. To exclude foreign materials, the collected materials were thoroughly washed. The cleaned grains such as foxtail millet, wheat, and green peas were grounded well into fine flour for future usage. For extrusion of pasta, the following flours like foxtail millet flour were mixed at different levels of 30 (V1), 40 (V2), 50 (V3), and 60% (V4) were used to replace with wheat flour at the level of 60 (V1), 50 (V2), 40 (V3) and 30% (V4). About 10% of green pea flour was in every four variations of the mixture. A 150ml of water was added and 0.2g of salt was added according to its taste.

Totally 5 variations (4 variants with 1 standard) of pasta were formulated. All the grounded flours (100g) were mixed with the optimum amount of water containing 2% salt and were mixed in the pasta extruder chamber for 10minutes to distribute water uniformly throughout the flour particles. The moist flour aggregate was placed in a metal extruder attachment of the pasta machine fitted with an adjustable die-size. After the preparation of pasta, the formulated pasta was dried in a food-grade dryer at 60°C for about 3hours. The developed pasta was analyzed for Energy by Parr Oxygen Bomb Calorimeter Method, Protein by micro Kjeldahl procedure, crude fat using Soxhlet extraction, Carbohydrate by anthrone method, iron by colourimetric method, calcium by colourimetric method, and vitamin C by 2,6 di-chlorophenolendophenol.

All the analyses were carried out in triplicate using standard procedures of (AOAC, 2000). The hardness, strength, stringiness, and adhesiveness of dry pasta and cooked pasta were evaluated by a texture profile analysis (TPA). A 35 mm diameter cylindrical probe (dry pasta) and 75 mm cylindrical probe (cooked pasta) was used to compress single pasta at a constant deformation rate of 1 mm/s to 80% of the thickness. The measurement mode settings for double-cycle compression (pre-test, test, and post-test) were set to a speed of 1.0 mm/sec; trigger type at auto-10 g; and data rate: 200 PPS. The colour profile was measured for dry and cooked pasta using the Lovibond tintometer. The measurements determined chromatic coordinates of L*, a*, and b*. In the range of 0 to 100, a coordinate L* described the brightness (black to white). The yellowness-blueness balance was specified by a coordinate b*, which was in-minus for blue and in-plus for yellow.

The yellowness-blueness balance was specified by a coordinate b*, which was in-minus for blue and in-plus for yellow. Measurements were taken in five replicates for each sample. All the developed pasta were evaluated for sensory quality based on appearance, colour, flavor, taste, texture, and overall acceptability using a 9-point Hedonic scale by a panel of 10 judges scorecard with scores ranging from 9 to 1, where 1 = dislike extremely, 5= neither like nor dislike and 9= like extremely was used. Samples were coded and presented in a random sequence to the panelists. All experiments in the present analysis were conducted in triplicate and mean values were reported. Data were subjected to analysis of variance (ANOVA), and the means were compared using Duncan's Multiple Range Test at 0.05% significance to find the best variations. Paired t-test was used to compare the standard with that of the variants of pasta.

RESULTS AND DISCUSSION

Chemical analysis of foxtail millet and green pea incorporated pasta: The chemical composition of pasta indicates that the energy content was ranged from 346.7 to 357.7kcal, in this high energy content was noticeable in V4 (357.7kcal) and the lowest energy content was observed in V1 (346.7kcal) pasta sample compared to standard pasta ($p<0.05$) (Table-1). The protein content was showing an increasing level where the incorporation level increases and it ranged from 10.07 to 13.89g. V2 pasta had a higher protein level than regular pasta, with a protein value of 13.89g. The high protein content of semolina led to the formation of a strong protein-starch matrix in pasta, which determined the cooking and quality criteria (Rizkalla et al. 2004). Few investigations have concentrated on expanding the dietary benefits of pasta in terms of protein content (Fuda et al. 2010; Adegunwa et al. 2012). Cooking loss is a critical metric for determining the quality of pasta products. The high quality of pasta is associated with less cooking loss. Cooking loss is caused by the gluten-starch network's ability to maintain the physical uprightness of pasta throughout cooking (Lu et al. 2016).

As reported by Santos et al. (2015), the lipid content was 2.44 kg 100kg-1, in pasta prepared with refined wheat flour, eggs, and salt, which is similar to this study. The protein network of pasta can be strengthened by thermal protein denaturation to improve the firmness of cooked pasta. Pasta is a healthy food that contains protein, vitamins and is an

important source of carbohydrates with virtually no fat (Malcolmson 2003; Krishnan et al. 2012 and Foschia et al. 2015; Santos et al. 2015). The high amount of zinc content, calcium, phosphorus, vitamin A and iron content was detected in V4 1.94mg/100g, 44.95mg, 247mg, 4.45mcg, and 2.86mg respectively. The proximate analysis and the formulated pasta samples showed a significant difference at the 0.05% level, according to Duncan's Multiple Range test results.

Table 1. Chemical analysis of pasta

	Standard	V1	V2	V3	V4
Energy (k.cal)	345.9±1.79^{a}	346.7±1.89^{b}	348.7±2.14^{a}	356.7±1.82^{de}	357.7±2.04^{a}
T value	-	4.85	7.62	5.35	3.67
Significance	-	0.00**	0.02**	0.41^{NS}	0.06^{NS}
Protein (g)	10.07±0.74^{b}	13.27±0.64^{c}	13.89±1.81^{b}	12.39±0.55^{f}	13.87±0.71^{d}
T value	-	8.85	12.35	9.58	9.54
Significance	-	0.00**	0.02**	0.00**	0.34^{NS}
Fat (g)	2.84±0.64^{a}	2.44±0.64^{ac}	2.58±0.55^{c}	2.84±0.31^{bc}	3.13±0.32^{e}
T value	-	11.62	6.58	9.72	8.87
Significance	-	0.00**	0.02**	0.39NS	0.56^{NS}
Zinc	1.7±0.43^{a}	1.7±0.53^{bc}	1.82±0.85^{bc}	1.90±0.52^{a}	1.94±0.87ac
T value	-	11.39	15.38	16.38	9.45
Significance	-	0.00**	0.35^{NS}	0.41^{NS}	0.089^{NS}
Calcium (mg)	43.6±0.54^{bc}	44.17±0.74^{b}	44.19±0.64^{ac}	44.82±0.63^{b}	44.95±0.41f
T value	-	3.67	6.47	5.66	4.14
Significance	-	0.36NS	0.02**	0.91NS	0.76^{NS}
Phosphorus (mg)	243.5±2.41^{a}	153.5±2.41^{d}	158±1.77^{ac}	142.5±2.45^{a}	247±1.14bc
T value	-	5.31	2.35	3.35	4.65
Significance	-	0.00**	0.00**	0.00**	0.46^{NS}
Iron (mg)	3.63±0.75^{a}	3.33±0.35^{ab}	2.84±0.21^{b}	2.35±0.55^{ac}	2.86±0.8a
T value	-	6.22	3.65	4.26	5.38
Significance	-	0.00**	0.01**	0.68^{NS}	0.97^{NS}

Values are the means ± standard errors of means (SEM) of 3 determinants. Means with same superscript are not significantly different using Duncan's Multiple Range Test ($p < 0.05$)

Table 2a: Textural properties of Dry pasta

Textural properties	Variations	Mean SD	T value	Significance
Hardness (N)	Standard	12.74±2.34^{a}	-	-
	V1	11.47±1.503^{bc}	8.75	0.00**
	V2	15.40±1.50^{ac}	11.35	0.01**
	V3	13.19±0.43^{de}	13.68	0.00**
	V4	17.70±0.78^{e}	19.54	0.00**
Strength (N/mm)	Standard	6.37±1.21^{a}	-	-
	V1	5.73±0.75^{c}	7.80	0.00**
	V2	7.70±0.75^{bc}	12.35	0.01**
	V3	6.59±0.22^{f}	11.57	0.00**
	V4	8.85±0.39^{de}	13.25	0.87^{NS}

Values are the means ± standard errors of means (SEM) of 3 determinants. Means with the same superscript are not significantly different using Duncan's Multiple Range Test ($p < 0.05$)

Textural properties of foxtail millet and green pea incorporated pasta: Texture profile analysis of the pasta is presented in Table-2a and 2b. Compare with standard pasta, V4 pasta shows high hardness and strength where the pasta samples showed a significant difference at ($p<0.01$) level. As time moves, the dryness of the pasta was changed due to the protein matrix and the surface moisture was also evaporated too quickly. According to Jayasena and Nasar (2012) and Bustos et al. (2015), the use of chemicals in the production of pasta, particularly high-protein pasta, can alter not only the culinary characteristics but also the texture. The firmness and minimal stickiness of pasta are significant attributes for customers (Susanna and Prabhasankar 2013). According to the literature, protein additions used in the making of pasta, such as egg and broad bean protein, increase stiffness, which is in line with the results found (Laleg et al. 2007; Jayasena and Nasar 2012; Bustos et al. 2015; Camelo et al. 2016).

Table 2b. Textural properties of cooked pasta

Textural properties	Variations	Mean SD	T value	Significance
Hardness (N)	Standard	13.65 ± 0.46^{a}	-	-
	V1	9.70 ± 0.13^{bc}	3.67	0.00**
	V2	9.48 ± 0.20^{d}	15.62	0.01**
	V3	9.69 ± 0.11^{f}	11.47	0.00**
	V4	9.66 ± 0.08^{b}	12.32	0.89^{NS}
Stringiness (mm)	Standard	6.69 ± 0.23^{a}	-	-
	V1	6.94 ± 0.12^{ac}	22.41	0.00**
	V2	6.15 ± 0.75^{gh}	2.85	1.35^{NS}
	V3	6.98 ± 0.10^{ab}	3.64	0.00**
	V4	6.90 ± 0.10^{d}	5.68	0.97^{NS}
Adhesiveness (J)	Standard	2.03 ± 0.23^{a}	-	-
	V1	1.60 ± 0.60^{b}	7.98	0.00**
	V2	0.08 ± 0.04^{ac}	8.65	0.01**
	V3	1.05 ± 0.373^{de}	5.69	0.00**
	V4	1.01 ± 0.37^{g}	7.12	0.36^{NS}

Values are the means ± standard errors of means (SEM) of 3 determinants. Means with the same superscript are not significantly different using Duncan's Multiple Range Test ($p < 0.05$)

The hardness and strength of green pea incorporated pasta showed a significant difference at a 5% level while comparing to a standard one. During pasta cooking, the gelatinization of starch and denaturation of proteins causes the main structural changes in pasta's texture (Aravind et al. 2012; Camelo et al. 2016). A decrease in the cohesiveness or an increase in the adhesiveness of the pasta indicates changes in the texture quality of the pasta made with unripe apple flour or oat bran, and it can be used to determine the product's consumer acceptability. Various texture studies have been reported in spaghetti-type pasta added with different types of flour (Hernández et al. 2009; Osorio et al. 2014). Hatcher et.al. (2005) stated in their study that amylopectin content and retrogradation rate and although it has been reported that addition of gluten content may decrease significantly adhesiveness values of noodle. The stringiness of cooked pasta samples ranged from 6.15mm to 6.94mm. The high stringiness was found in V1 (6.94mm). There was a significant difference ($p<0.01$) between the stringiness of the samples in variations than standard. The adhesiveness of the pasta samples ranged from 0.08J to 2.03J and showed a statistically significant. Lee et al. (2005) found no substantial variations in the adhesiveness of Chinese fresh noodles made from wheat flours as the percentage of garbanzo bean substitutions increased from 0 to 30%. Chen et al. (2005) found significant associations between amylose content and the adhesiveness of Chinese fresh noodle made from wheat flours. Results on Duncan's Multiple Range test showed that there was a significant difference between all the pasta samples developed using foxtail millet and green pea flour.

Cohesiveness and springiness parameters indicated how the sample holds together upon cooking, which interpreted the higher values recorded for the chickpea fortified pasta than the control sample (Lee et al. 2005; Chen et al. 2005; Kosovićet al. 2016).

Colour analysis of foxtail millet and green pea incorporated pasta: Lei et al. (2004) have reported that elasticity was significantly related to TPA's springiness. Tang et al. (1999) also reported that adding gluten into soft wheat flour could improve the elasticity of noodles. The elasticity decrease in wheat-SPF noodles might result from the dilution of gluten in noodle dough. Laleg et al. (2016) reported in his literature indicate that protein additives

used in the production of pasta, such as egg and broad bean protein, increase the firmness, which is consistent with the obtained results (Tang et al. 1999; Lei et al. 2004; Laleg et al. 2016).

Table 3a. Colour analysis of dry pasta

Colour	Variations	Mean SD	T value	Significance
L*	Standard	70.3±0.24^{b}	-	-
	V1	67.1±1.20^{ac}	6.74	0.00**
	V2	74.9±2.12^{ab}	7.52	0.01**
	V3	71.7±0.97^{a}	6.38	0.00**
	V4	71.9±0.75^{d}	9.65	0.99^{NS}
a*	Standard	4.4±1.40^{a}	-	-
	V1	4.0±0.97^{b}	8.56	0.00**
	V2	4.7±0.82^{d}	5.64	0.01**
	V3	4.1±0.75^{ac}	5.67	0.68^{NS}
	V4	4.0±1.04^{b}	6.58	0.021*
b*	Standard	15.6±0.87^{a}	-	-
	V1	16.6±0.41^{b}	1.38	0.00**
	V2	15.0±0.85^{ac}	5.69	0.01**
	V3	15.9±0.94^{a}	7.65	0.031*
	V4	15.7±0.72^{ab}	9.45	3.37^{NS}

Values are the means ± standard errors of means (SEM) of 3 determinants. Means with the same superscript are not significantly different using Duncan's Multiple Range Test ($p < 0.05$)

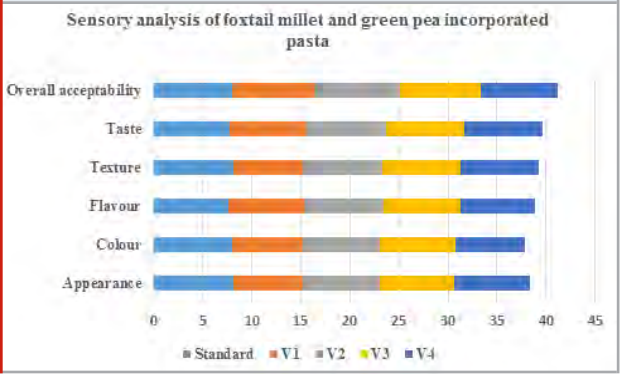
Table 3b. Colour analysis of cooked pasta

Colour	Variations	Mean SD	T value	Significance
L*	Standard	56.5±0.41^{a}	-	-
	V1	55.5±0.88^{ac}	8.64	0.00**
	V2	56.5±0.67^{ab}	5.64	0.01**
	V3	57.1±0.73^{d}	7.61	0.002**
	V4	55.4±0.75^{c}	6.39	0.004**
a*	Standard	2.4±0.27^{b}	-	-
	V1	1.8±0.14^{c}	8.94	0.00**
	V2	1.7±0.34^{a}	7.41	0.01**
	V3	1.8±0.26^{b}	6.58	0.41^{NS}
	V4	1.7±0.22^{ac}	8.94	0.01**
b*	Standard	11.3±0.84^{a}	-	-
	V1	12.7±0.67^{bc}	9.47	0.00**
	V2	12.6±0.51^{d}	7.65	0.01**
	V3	13.2±0.81^{b}	8.56	0.00**
	V4	12.8±0.34^{a}	7.14	0.01**

Values are the means ± standard errors of means (SEM) of 3 determinants. Means with the same superscript are not significantly different using Duncan's Multiple Range Test ($p < 0.05$)

The colour of pasta is one of the foremost basics discriminates of its quality. It depends mainly on the raw materials used for the development of pasta. In considering the results of the dry and cooked pasta colour coordinates (Table -3a and 3b). Colour changes in food ingredients (including noodles) can be known based on the values L* a* and b. The L* value donates the brightness level of the noodle, the a* value donates the greenish colour while the b* value represents the yellow colour (Ginting et al. 2015; Sirichokworrakit et al. 2015). As suggested by Güler et al. (2002), changes in starch during high-temperature drying have been shown to influence the consistency of cooked pasta. As pierced out by Chanu and Jena (2015) in pasta incorporating wholemeal rye flour, this kind of monotony occurred due to the higher concentration of fibre, pigments, and other structural components that are naturally present on external corn layers (Güler et al. 2002; Chanu and Jena 2015).

Figure 1



As the legume components in the pasta increased, the lightness (L* value) of all raw and cooked samples declined. This result is in agreement with studies conducted in the past. (Zhao et al. 2005; Wood 2009; Petitot et al. 2010). The darker colour of the legume-supplemented pasta may be attributed to the higher content of ash and the specific colour of the legume flour. After cooking, the redness and yellowness of legume-fortified pasta decreased, whereas the brightness (L*) increased, according to Petitot et al. (2010). Cooked pasta samples using 30-60% foxtail flour and wheat flour with 10% green pea flour had a significant increase in colour parameter a*.

Sensory analysis of foxtail millet and green pea incorporated pasta: The sensory evaluation was carried out based on colour, flavour, taste, appearance, and overall acceptability of the developed product. The sensory evaluation of the pasta samples revealed that there were significant differences among the variations for the organoleptic qualities compare with the standard. Overall acceptability of pasta ranged from 7.90 to 8.80. The V2 (8.80) incorporated pasta has got the highest overall acceptability than standard (figure-1). Regarding appearance, colour, flavor, texture, taste, and overall acceptability of the pasta incorporated levels of 40% foxtail millet and 10% green pea flour has appeared to have the highest overall acceptability score than standard pasta.

Yilmaz and Buket (2012) stated that sensory evaluation (SE) has been used to evaluate the products sensory characteristics and consumer acceptance. Hence, determination of the consumer expectations and the expression of consumer demands are classified into scientifically sound sensory descriptors. It allows for the description and comparison of food samples, as well as the matching of consumer requests. It is a complex sensory dimension that includes tactile, visual, and auditory perceptions and plays a key role in defining consumer responses (Yilmaz and Buket 2012; Laureati et al. 2020).

CONCLUSION

The findings of the present study suggests that pasta mixed with foxtail millet and green pea flour forms a pasta variant that is satisfactory in terms of physiochemical, colour, texture, and sensory aspects. Increased foxtail millet incorporation resulted in a significant change ($p<0.05$) in proximate and overall acceptability of the pasta samples, although textural properties reduced when compared to the standard, with a 5% level of significant difference. Increasing the amount of nutrient-dense foods significantly improves the health of the great majority of health-conscious people. The findings revealed that cereals have the ability and functionality to enrich wheat-based pasta for the manufacture of dietetic pasta with high protein content and all nutritional benefits. As a result, foxtail millet and green pea flour are being used in innovative pasta products aimed at health-conscious customers who always promote cereal-based foods as the greatest nutritional supplement.

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

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Ecological Communication

Avian Diversity Assessment of a Tropical Forest in Central India

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ABSTRACT

Pandharkawada forest division is located in biodiversity rich landscape vidharbha region of Maharashtra, India. 296 birds distributed in different habitats are recorded from this forest division belongs to 19 orders and 69 families. From the recorded 296 species, 10 species are near threatened, 2 vulnerable and 1 is endangered (IUCN red data book). 74% species are occurred as common, 17% as uncommon, 5% rare and 14% as very rare. 56% spices are distributed as resident, 21.28%, are winter visitors 7.50% vagrant, 6.41% are distributed from winter to summer, 6.41% are recorded from monsoon to winter, 4 are monsoon to summer, 2 are monsoon visitor and 1 is summer visitor. Main food habit of recorded species are insectivores and carnivorous followed by frugivores, granivores, terrestrial grazer, nectarivores, folivores, aquatic grazer, and scavengers. Study of their habitat reveals that, 56% are arboreal, 22% as shore birds 10% water birds 8% ground birds and 3% as arial birds. Their nesting type is recorded as, 48% as tree nesters, 17% in ground scrap, 12% built nests floating on water 8% in ground, 8% in tree hole and 7% in rock cleft. In a way they directly impacts human health, economy, food production as well as millions of other species. Therefore, it is important to know about local bird diversity and its ecology. This study establishes that Pandharkawada forest division is rich in avifaunal diversity due to varied habitats like forest, grassy patches, shrubs, perineal waterbodies and seasonal wetlands. Due to this, it attracts varieties of local resident and migratory species and found distributed in these various habitats different seasons of the year.

KEY WORDS: BIO-INDICATORS, CONSERVATION, CONSUMER, ECOLOGY, POLLINATOR.

INTRODUCTION

Avian population has a central role in ecosystem functioning and ecosystem services. Birds are bio-indicators and appraise the health of environment and ecosystems, they are capable of determining environmental integrity using their functions and populations. Birds play pivotal ecological roles both in forest and farmland ecosystems, notably pollination, seed dispersal, and pest control (Whelan et al. 2008; Mulwa et al. 2012). In addition to above, there is positive role of birds in nutrient cycling and soil formation. They also richly contribute to the recolonization and restoration of disturbed ecosystem (Sekercioglu et al. 2004; Sekercioglu 2006). A study inferred that, birds are also act as mobile links that transfer energy both within and among ecosystems that are crucial for maintaining ecosystem function and resilience (Lundberg and Moberg 2003).

In a way it directly impacts human health, economy, food production as well as millions of other species. Therefore, it is imporant to know about local bird diversity and its ecology (Ndang'ang'a et al. 2013). In Vidarbha region of Maharashtra 413 species are recorded by various authors. The annotated checklist of Nagpur area represented 284 species of birds. 171species are studied from Pohara-Malkhed reserve Forest, district Amravati (Wadatkar and Kasambe 2002; Kasambe 2009). 135 species of birds are observed in and around Ambazari Lake Nagpur (Kedar 2012). Total 312 species of birds recorded from the nearby area of Navegaon National Park Gondia 76 species of birds are reported from Chaprala wild life sanctuary, Gadchiroli (Paliwal 2013; Chauhan and Dhamani 2014; Wagh and Tiwari 2020) 92 species of birds are studied from Tamkarada forest near Malegaon tehsil of Washim district (Ingle et al. 2015). 99 species of birds are listed from Junona lake Chandrapur.

Increasing anthropogenic pressure, change in land use patterns, commercial exploitation of waterbodies and seasonal wetlands, forest fire, rampant grazing, overuse of pesticides and herbicides rapidly eats up natural habitats of birds. Most deforestation has happened in biodiversity-rich tropical forests (Asner et al. 2009: Hansen et al. 2013; Harney 2015). These areas are expected to face even more

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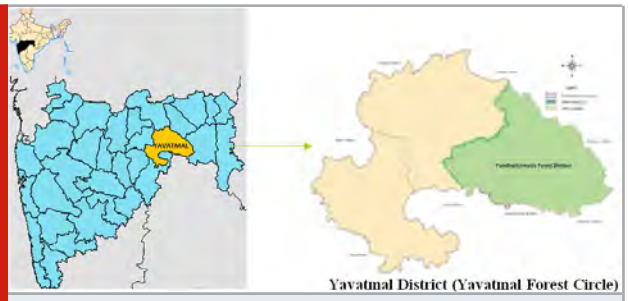
pressures in the future, largely due to agricultural expansion and increased industrialization (Tilman and Fargione 2001: Dobrovolski et al. 2011: Wagh and Tiwari 2020).

In this context, the conservation of this ecologically important fauna is paramount and, this research exercise of systematic study on bird diversity assessment in different habitat is undertaken from June 2015 to December 2020 to understand and document local diversity, distribution, habit, habitat, nesting type and conservation status of birds of Pandharkawada forest division. This will help to device policies and practice of conservation of birds and their habitats in this forest division.

MATERIAL AND METHODS

Pandharkawada forest division, located at south-eastern part of Maharashtra and situated in the geographic coordinates of 78^0 14′ & 79^0 13′ East and 19^0 45′ & 20^0 20′ North, spread over 655.336 square kilometer. It constitutes a compact patches of dense forest cover, open forest, shrubs, grassy belts and associated waterbodies and seasonal wetlands having great value from the point of view of wildlife and bio-diversity conservation. The main portion of forests constitutes the dry Teak bearing forests bearing 60 percent of the total crop composition, so classified as Dry Teak Bearing forests. The bird survey was conducted from June 2015 to December 2020 using standard point count method. Binocular (Nikon 10x40 8.2 0) and camera (Nikon D700, 150-500 Sigma lens) was used for bird watching and to photograph them.



Source: https://www.veethi.com/places/maharashtra-yavatmal-district-405.htm

They were identified and classified on the basis of the "The Book of Indian Birds" by Ali (eds.1996) and "Pocket Guide of Birds of the Indian Subcontinent" by Grimmet and Inskipp (eds.2000). Diversity of bird was taxonomically classified and categorized on threaten scale by using latest IUCN Red list (Ali 1996; Grimmet and Inskipp 2000). The data collected from the surveys were used to assess habit, habitat, nesting type, occurrence and distribution. Surveys were conducted four days in a week, from sunrise to 4 hours after sunrise and from 4 hours before sunset until sunset. Morning and evening counts were altered between sites. Each of the sites was surveyed daily depending on weather conditions. Birds seen were identified and recorded along the habitat type and status and checklists were prepared.

RESULTS AND DISCUSSION

296 species of birds were studied from Pandharkawada forest division from June 2015 to December 2020. Habit, habitat, nesting type, occurrence, distribution and IUCN status of these recorded species were studied.

296 species of birds belonged to 19 orders and 69 family were recorded and photographed from Goplpur Nursery, Shibla Forest Range, Mandar Forest Range, Shindola scrubby forest, Saykheda dam, Wai dam, Mucchi dam, Karanwadi dam, Chilai dam, Bhimnala dam, Navargaon dam, Warud dam, Jam dam Nilgiri ban, Krishna Tekadi of Pandharkawada forest division dominated by order Passeriformes (Table 1 (ABCDEFGH parts), Fig.1). 219 (74%) were common, 49(17%) uncommon, 15(5%) rare and 13(4%) were very rare (fig.4). Locally rare recorded species were White Bellied Minivet, Spotted Creeper, Ultra-marine Flycatcher, Greater Flamingo, Pallied Harrier, Pied Harrier, Indian Spotted eagle, Common Black Bird, Stork Billed Kingfisher, Grey Headed Canary Flycatcher, Graylag Goose and Forest Wagtail, Eurasian Curlew, Whimbrel, Broad Billed Sandpiper, Lesser Adjutant, Orange headed Green Pigeon, Gull Billed Tern, Egyptian Vulture, Peregrine Falcon, Amur falcon, Red-necked Falcon, Grey-necked Bunting, Black eagle as very rare.

Main food habit of recorded species were insectivores and carnivorous followed by frugivores, granivores, terrestrial grazer, nectarivores, folivores, aquatic grazer, and scavengers (fig.2). Study of their habitat reveals, 166 (56%) were arboreal, 66 (22%) as shore birds 31(10%) water birds 24 (8%) ground birds and 9 (3%) as arial birds (fig.3). Their nesting type is recorded as, 143 (48%) birds were recorded building their nests on tree, 51(17%) in ground scrap, 37 (12%) built nests floating on water 22 (8%) in ground, 22(8%) in tree hole and 21(7%) in rock cleft.166 (56%) were resident and were found distributed in various habitat (21.28%), 63 were winter visitors and most amongst them were waterbirds, they spent winter at different waterbodies and seasonal wetlands in this division. 22 (7.50%) were Vagrant, 19 (6.41%) were distributed from winter to summer,19 (6.41%) was recorded from monsoon to winter, 4 were monsoon to summer, 2 were monsoon visitor, Greater flamingo was a summer visitor. (fig.5). IUCN red data book conservation status of birds studied from Pandharkawada forest division shown that, Alexandrine Parakeet, Curlew Sandpiper, Great Thick-knee, Painted Stork, Grey Headed Fish Eagle, Black Tailed Godwit, Red-neck Falcon, Pallid Harrier these 10 species were near threatened. 2 species, Lesser adjutant, River tern were vulnerable and Egyptian Vulture is endangered.

This study established that Pandharkawada forest division is rich in avifaunal diversity due to varied habitats like forest, grassy patches, shrubs, perineal waterbodies and seasonal wetlands. This attracts varieties of local resident and migratory species and different species were found distributed in different seasons of the year.

The results showed that in comparison to the avian diversity in the adjoining areas, the diversity in Pandharkawada area

is significantly better. Major support systems for all kind of flora and fauna in general and birds in particular were forests and waterbodies. Waterbodies and seasonal wetlands were among the most productive ecosystems on the earth and attracts varieties of species of bird. These were under tremendous anthropogenic pressure due to commercial overexploitation and increasing lean period agriculture. Increasing anthropogenic pressure influenced the number of wintering waterbirds, their distribution, foraging behavior and interspecies competition (Chen, et al. 2011; Zhao et al. 2013; Zhou et al. 2010; Alice et al. 2020).

Table 1 (Part-A). Bird Diversity of Pandharkawada Forest Division

Sr. No.	Order	Family	Scientific Name	Common Name	Habitat	Nest	Food	Occurr-ence	Distri bution	IUCN Status
1.	Galliformes	Phasianidae	Francolinus pictus	Painted francolin	GrB	GrNt	Grv, Ins	C	RD	LC
2.			Francolinus pondicerianus	Grey francolin	GrB	GrNt	Grv, Ins	C	RD	LC
3.			Perdicula asiatica	Jungle Bush Quail	GrB	GrNt	Grv, Ins	C	RD	LC
4.			Perdicula argoondah	Rock Bush Quail	GrB	GrNt	Grv, Ins	C	WV	LC
5.			Coturnix coturnix	Common Quail	GrB	GrNt	Grv, Ins	C	WV	LC
6.			Coturnix coromandelica	Rain Quail	GrB	GrNt	Grv, Ins	C	RD	LC
7.			*Pavo cristatus*	Indian peafowl	GrB	GrNt	Grv, Ins	C	RD	LC
8.	Charadriiformes	Turnicidae	*Turnix tanki*	Yellow-legged buttonquail	GrB	GrNt	Grv, Ins	UC	M-W	LC
9.			*Turnix suscitator*	Barred buttonquail	GrB	GrSc	Grv, Ins	C	RD	LC
10.			*Turnix sylvaticus*	Small buttonquail	GrB	GrSc	Grv, Ins	UC	VG	LC
11.	Anseriformes	Anatidae	*Anser anser*	Grey-lag goose	WtB	WtFl	Frv, Grz (Gr)	R	WV	LC
12.			*Anser indicus*	Bar-heaed goose	WtB	WtFl	Frv, Grz (Gr)	UC	WV	LC
13.			*Dendrocygna javanica*	Lesser Whistling Duck	WtB	TrHl	Car (Mn), Grz (Gr)	C	RD	LC
14.			*Tadorma ferruginea*	Ruddy Shelduck	WtB	RoCl	Car (Mn), Grz (Gr)	C	W-S	LC
15.			*Sarkidiornis melanotos*	Comb duck	WtB	TrHl	Car (Mn), Grz (Gr)	UC	WV	LC
16.			*Nettapus coromandelianus*	Cotton pygmy-goose	WtB	TrHl	Car (Mn), Grz (Gr)	C	RD	LC
17.			*Anas strepera*	Gadwall	WtB	WtFl	Car (Mn), Grz (Gr)	C	WV	LC
18.			*Anas Penelope*	Eurasian wigeon	WtB	WtFl	Grz (Gr)	C	WV	LC
19.			*Anas poecilorhyncha*	Spot-billed duck	WtB	WtFl	Car (Mn), Grz (Gr)	C	RD	LC
20.			*Anas clypeata*	Northern shoveler	WtB	WtFl	Car (Mn)	C	WV	LC
21.			*Anas acuta*	Northern pintail	WtB	WtFl	Car (Mn)	C	WV	LC
22.			*Anas querquedula*	Garganey	WtB	WtFl	Car (Mn)	C	WV	LC
23.			*Anas crecca*	Common teal	WtB	WtFl	Car(Mn), Grz (Gr)	C	WV	LC
24.			*Rhodonessa rufina*	Red-crested pochard	WtB	WtFl	Car (Mn)	C	WV	LC
25.			*Aythya ferina*	Common pochard	WtB	WtFl	Car (Mn) & (Mj)	C	WV	LC
26.			*Aythya fuligula*	Tufted duck	WtB	WtFl	Car (Mn) & (Mj)	R	WV	LC
27.			*Aythya nyroca*	Ferruginous Pochard	WtB	WtFl	Car (Mn) & (Mj)	R	WV	LC
28.	Piciformes	Picidae	*Jynx torquilla*	Eurasian wryneck	ArB	TrHl	Ins	C	W-S	LC
29.			*Dendrocopos nanus*	Brown-capped pygmy woodpecker	ArB	TrNt	Ins	C	RD	LC
30.			*Dendrocopos mahrattensis*	Yellow-crowned woodpecker	ArB	TrNt	Ins, Nct	C	RD	LC
31.			*Dinopium benghalense*	Lesser goldenback	ArB	TrNt	Ins, Nct	C	RD	LC
32.			*Chrysocolaptes festivus*	White-naped woodpecker	ArB	TrHl	Ins, Nct	C	RD	LC
33.		Ramphastidae	*Megalaima haemacephala*	Coppersmith barbet	ArB	TrHl	Ins, Frv	C	RD	LC
34.			*Megalaima virens*	Brown headed barbet	ArB	TrHl	Ins, Frv	UC	RD	LC
35.	Bucerotiformes	Bucerotidae	*Ocyceros birostris*	Indian grey hornbill	ArB	TrHl	Ins, Frv	UC	RD	LC
36.		Upupidae	*Upupa epops*	Common hoopoe	ArB	RoCl	Ins	UC	RD	LC

Table 1 (Part-B) Bird Diversity of Pandharkawada Forest Division

Sr. No.	Order	Family	Scientific Name	Common Name	Habitat	Nest	Food	Occurr-ence	Distribu-tion	IUCN Status
37.	Coraciiformes	Coraciidae	*Coracias benghalensis*	Indian roller	ArB	TrNt	Ins, Car (Mj)	C	RD	LC
38.			*Coracias garrulus*	Europian roller	ArB	TrHl	Ins, Car (Mj)	UC	WV	LC
39.		Alcedinidae	*Alcedo atthis*	Common kingfisher	ArB	TrNt	Car (Mn), & (Mj)	C	RD	LC
40.			*Halcyon smyrnensis*	White throated kingfisher	ArB	TrNt	Car (Mn), & (Mj)	C	RD	LC
41.			*Ceryle rudis*	Pied kingfisher	ArB	RoCl	Car (Mn), & (Mj)	C	RD	LC
42.			*Pelargopsis capensis*	Stork-billed Kingfisher	ArB	TrNt	Car (Mn), & (Mj)	R	VG	LC
43.		Meropidae	*Merops orientalis*	Green bee-eater	ArB	RoCl	Ins	C	RD	LC
44.			*Merops philippinus*	Blue-tailed bee-eater	ArB	GrSc	Ins	C	WV	LC
45.			*Meropspersicus*	Blue-cheeked Bee-eater	ArB	RoCl	Ins	UC	WV	LC
46.	Cuculiformes	Cuculidae	*Clamator jacobinus*	Jacobin cuckoo	ArB	TrNt	Ins, Frv	C	MV	LC
47.			*Hierococcyx varius*	Common hawk cuckoo	ArB	TrNt	Ins, Frv	C	RD	LC
48.			*Cuculus canorus*	Eurasian cuckoo	ArB	TrNt	Ins, Frv	C	M-W	LC
49.			*Cacomantis passerines*	Grey –bellied cuckoo	ArB	TrNt	Ins, Frv	C	RD	LC
50.			*Eudynamys scolopaceus*	Asian koel	ArB	TrNt	Ins, Frv	C	RD	LC
51.			*Taccocua leschenaultia*	Sirkeer malkoha	ArB	TrNt	Ins, Frv	C	RD	LC
52.			*Centropus (sinensis) parroti*	Southern coucal	ArB	TrNt	Ins, Car (Mj)	C	RD	LC
53.	Psittaciformes	Psittaculidae	*Psittacula eupatria*	Alexandrine parakeet	ArB	TrHl	Frv, Grv	C	RD	NT
54.			*Psittacula krameria*	Rose ringed parakeet	ArB	TrHl	Frv, Grv	C	RD	LC
55.			*Psittacula cyanocephala*	Plum-headed parakeet	ArB	TrHl	Frv, Grv	C	RD	LC
56.	Strigiformes	Tytonidae	*Tyto alba*	Barn owl	ArB	RoCl	Ins, Car (Mj)	C	RD	LC
57.		Strigidae	*Otus bakkamoena*	Indian scops owl	ArB	RoCl	Ins, Car (Mj)	C	RD	LC
58.			*Bubo bengalensis*	Indian eagle owl	ArB	RoCl	Ins, Car (Mj)	C	RD	LC
59.			*Ketupa zeylonensis*	Brown fish owl	ArB	RoCl	Car (Mn), & (Mj)	C	RD	LC
60.			*Strix ocellata*	Mottled wood owl	ArB	TrHl	Car (Mn), & (Mj)	C	RD	LC
61.			*Glaucidium radiatum*	Jungle owlet	ArB	TrHl	Ins, Car (Mj)	UC	RD	LC
62.			*Athene brama*	Spotted owlet	ArB	TrHl	Ins, Car (Mj)	C	RD	LC
63.	Caprimulgiformes	Apodidae	*Cypsiurus balasiensis*	Asian palm swift	AiB	RoCl	Ins,	C	RD	LC
64.			*Apus affinis*	House swift	AiB	RoCl	Ins,	C	RD	LC
65.			*Hemiprocne coronate*	Crested treeswift	AiB	TrNt	Ins,	C	RD	LC
66.		Caprimulgidae	*Caprimulgus asiaticus*	Indian nightjar	ArB	GrSc	Ins	C	RD	LC
67.			*Caprimulgus affinis*	Savannah nightjar	GrB	GrSc	Ins,	UC	WV	LC
68.			*Caprimulgus indicus*	Jungle Nightjar	GrB	GrSc	Ins	UC	WV	LC
69.	Columbiformes	Columbidae	*Columba livia*	Rock pigeon	GrB	RoCl	Grv, Ins	C	RD	LC
70.			*Streptopelia orientalis*	Oriental turtle dove	ArB	TrNt	Grv, Frv	UC	WV	LC
71.			*Stigmatopelia senegalensis*	Laughing dove	ArB	TrNt	Grv, Frv	C	RD	LC
72.			*Stigmatopelia chinensis*	Spotted dove	ArB	TrNt	Grv, Frv	C	RD	LC
73.			*Streptopelia tranquebarica*	Red collard dove	ArB	TrNt	Grv, Frv	C	M-W	LC
74.			*Streptopelia decaocto*	Eurasian collared dove	ArB	TrNt	Grv, Frv	C	RD	LC
75.			*Treron phoenicopterus*	Yellow-footed green pigeon	ArB	TrNt	Grv, Frv	C	RD	LC
76.			*Treron bicinctus*	Orange-breasted Green Pigeon	ArB	TrNt	Grv, Frv	VR	VG	LC

Table 1 (Part-C) Bird Diversity of Pandharkawada Forest Division

Sr. No.	Order	Family	Scientific Name	Common Name	Habitat	Nest	Food	Occurr-ence	Distribu-tion	IUCN Status
77.	Gruiformes	Rallidae	*Amaurornis akool*	Brown crake	ShB	TrNt	Grv, Car (Mn)	C	RD	LC
78.			*Zapornia pusilla*	Baillon's Crake	ShB	WtFl	Ins, Car (Mn)	UC	WV	LC
79.			*Porzana porzana*	Spotted crake	ShB	WtFl	Ins, Car (Mn)	UC	WV	LC
80.			*Amaurornis phoenicurus*	White breasted waterhen	ShB	WtFl	Ins, Car (Mj)	C	RD	LC
81.			*Porphyrio porphyrio*	Purple swamphen	ShB	WtFl	Ins, Frv	C	RD	LC
82.			*Gallinule chloropus*	Common moorhen	ShB	WtFl	Car(Mn), Frv	C	RD	LC
83.			*Fulica atra*	Common coot	ShB	WtFl	Omn, Grv	C	RD	LC
84.	Pterocliformes	Pteroclidae	*Pterocles indicus*	Painted sandgrouse	GrB	GrSc	Ins, Grv	C	RD	LC
85.			*Pterocles exustus*	Chestnut-bellied sandgrouse	GrB	GrSc	Grv, Grz (Gr)	UC	WV	LC
86.	Charadriiformes	Scolopacidae	*Gallinago gallinago*	Common snipe	ShB	GrSc	Car (Mn), Ins	C	RD	LC
87.			*Gallinago stenura*	Pintail Snipe	ShB	GrSc	Car (Mn), Ins	C	WV	LC
88.			*Limosa limosa*	Black tailed godwit	ShB	GrSc	Car (Mn), Ins	C	W-S	NT
89.			*Tringa erythropus*	Spotted redshank	ShB	GrSc	Car (Mn), Ins	C	W-S	LC
90.			*Tringa totanus*	Common redshank	ShB	WtFl	Car (Mn), Ins	C	W-S	LC
91.			*Tringa stagnatilis*	Marsh sandpiper	ShB	WtFl	Car (Mn), Ins	C	WV	LC
92.			*Tringa nebularia*	Common greenshank	ShB	GrSc	Car (Mn), Ins	C	W-S	LC
93.			*Tringa ochropus*	Green sandpiper	ShB	GrSc	Car (Mn), Ins	UC	WV	LC
94.			*Tringa glareola*	Wood sandpiper	ShB	GrSc	Car (Mn), Ins	C	M-W	LC
95.			*Actitis hypoleucos*	Common sandpiper	ShB	GrSc	Car (Mn), Ins	C	M-W	LC
96.			*Limicola falcinellus*	Broad billed sandpiper	ShB	GrNt	Car (Mn), Ins	VR	VG	LC
97.			*Calidris minuta*	Little stint	ShB	GrSc	Car (Mn), Ins	C	W-S	LC
98.			*Calidris temminckii*	Temminck's stint	ShB	GrSc	Car (Mn), Ins	C	WV	LC
99.			*Calidris alpina*	Dunlin	ShB	GrSc	Car (Mn), Ins	UC	WV	LC
100.			*Calidris ferruginea*	Curlew sandpiper	ShB	GrSc	Car (Mn), Ins	C	W-S	NT
101.			*Philomachus pugnax*	Ruff	ShB	GrSc	Car (Mn), Ins	UC	W-S	LC
102.			*Numenius arquata*	Eurasian curlew	ShB	GrSc	Ins, Car (Mn)	VR	VG	NT
103.			*Numenius phaeopus*	Whimbrel	ShB	GrSc	Car (Mn), Ins	VR	VG	LC
104.		Rostratulidae	*Rostratula benghalensis*	Greater painted snipe	ShB	GrSc	Ins, Car (Mn)	C	RD	LC
105.		Jacanidae	*Hydrophasianus chirurgus*	Pheasant tailed jacana	ShB	WtFl	Grz (Wt), Car (Mn)	C	RD	LC
106.			*Metopidius indicus*	Bronze winged jacana	ShB	WtFl	Grz (Wt), Car (Mn)	C	RD	LC
107.		Burhinidae	*Burhinus indicus*	Indian thick-knee	GrB	GrSc	Ins, Car (Mn)	C	RD	LC
108.			*Esacus recurvirostris*	Great thick-knee	ShB	GrSc	Car (Mn), Ins	C	RD	NT
109.		Recurvirostridae	*Himantopus Himantopus*	Black-winged stilt	ShB	GrSc	Ins, Car (Mn)	C	M-S	LC
110.		Charadriidae	*Charadrius dubius*	Little ringed plover	ShB	GrSc	Ins, Car (Mn)	C	RD	LC
111.			*Charadriushiaticula*	Common Ringed Plover	ShB	GrSc	Ins, Car (Mn)	UC	VG	LC
112.			*Pluvalis fulva*	Pacific golden plover	ShB	GrSc	Ins, Car (Mn)	UC	WV	LC
113.			*Pluvalis squatarola*	Grey plover	ShB	GrSc	Ins, Car (Mn)	UC	WV	LC

Table 1 (Part-D) Bird Diversity of Pandharkawada Forest Division

Sr. No.	Order	Family	*Scientific Name*	Common Name	Habitat	Nest	Food	Occurr-ence	Distribu-tion	IUCN Status
114.		Charadriidae	*Charadriusmongolus*	Lesser sand plover	ShB	GrSc	Ins, Car (Mn)	UC	WV	LC
115.			*Chadrius alexandrinus*	Kentish plover	ShB	GrSc	Ins, Car (Mn)	C	M-W	LC
116.			*Vanellus malarbaricus*	Yellow wattled lapwing	ShB	GrSc	Ins, Car (Mn)	C	RD	LC
117.			*Vanellus cinereus*	Grey headed lapwing	ShB	GrSc	Ins, Car (Mn)	R	WV	LC
118.			*Vanellus indicus*	Red wattled lapwing	GrB	GrSc	Ins, Car (Mn)	C	RD	LC
119.		Glareolidae	*Glareola pratincole*	Collared pratincole	ShB	GrSc	Car (Mn), Ins	C	WV, MV	LC
120.			*Glareola maldivarum*	Oriental pratincole	ShB	GrSc	Car (Mn), Ins	C	VG	LC
121.			*Glareola lactea*	Small pratincole	ShB	GrSc	Ins, Car (Mn),	C	RD	LC
122.			*Cursorius coromendalicus*	Indian Coursor	GrB	GrSc	Ins, Car (Mn)	C	RD	LC
123.		Laridae	*Larus brunnicephalus*	Brown-headed gull	ShB	GrSc	Car (Mn), Ins	UC	WV	LC
124.			*Larus ridibundus*	Black headed gull	ShB	GrSc	Car (Mn), Ins	C	WV	LC
125.			*Sterna aurantia*	River tern	ShB	GrSc	Car (Mn), Ins	C	RD	VU
126.			*Sterna albifrons*	Little tern	ShB	GrSc	Car (Mn), Ins	C	RD	LC
127.			*Childonias hybridia*	Whiskered tern	ShB	GrSc	Car (Mn), Ins	C	W-S	LC
128.			*Gelochelidon nilotica*	Gull- billed Tern	ShB	GrSc	Car (Mn), Ins	VR	VG	LC
129.	Accipitriformes	Accipitridae	*Neophron percnopterus*	Egyptian Vulture	ArB	TrNt	Scv	VR	VG	EN
130.			*Pernis ptilorhyncus*	Oriental honey buzzard	ArB	TrNt	Car (Mj)	C	RD	LC
131.			*Butastur teesa*	White-eyed buzzard	ArB	TrNt	Car (Mj)	C	RD	LC
132.			*Accipiter nisus*	Eurasian sparrowhawk	ArB	TrNt	Car (Mj)	UC	VG	LC
133.			*Accipiter badius*	Shikra	ArB	TrNt	Car (Mn), & (Mj)	C	RD	LC
134.			*Elanus caeruleus*	Black-winged kite	ArB	TrNt	Car (Mj)	C	RD	LC
135.			*Milvus migrans*	Black kite	ArB	TrNt	Car (Mj)	C	RD	LC
136.			*Pandion haliaetus*	Osprey	ArB	TrNt	Car (Mj)	C	W-S	LC
137.			*Aquila rapax*	Towny eagle	ArB	TrNt	Car (Mj)	C	VG	LC
138.			*Aquila fasciata*	Bonelies eagle	ArB	TrNt	Car (Mj)	C	RD	LC
139.			*Ichthyophaga ichthyaetus*	Grey headed fish eagle	ArB	TrNt	Car (Mj)	UC	RD	NT
140.			*Circaetus gallicus*	Short toed snake eagle	ArB	TrNt	Car (Mj)	C	RD	LC
141.			*Spilornis cheela*	Crested serpent eagle	ArB	TrNt	Car (Mj)	C	RD	LC
142.			*Circus aeruginosus*	Western marsh harrier	ArB	TrNt	Car (Mn), & (Mj)	UC	W-S	LC
143.			*Circus macrourus*	Pallid harrier	ArB	GrNt	Car (Mj)	R	WV	NT
144.			*Circus melanoleucos*	Pied Harrier	ArB	GrNt	Car (Mj)	R	WV	LC
145.			*Circus pygargus*	Montagu's harrier	ArB	GrNt	Car (Mj)	UC	WV	LC
146.			*Ictinaetus malayensis*	Black eagle	ArB	TrNt	Car (Mj)	VR	VG	LC
147.			*Aquila hastata*	Indian-spotted Eagle	ArB	TrNt	Car (Mj)	R	VG	LC
148.			*Hieraaetus pennatus*	Booted eagle	ArB	TrNt	Car (Mj)	UC	WV	LC
149.			*Spizaetus cirrhatus*	Changeable hawk eagle	ArB	TrNt	Car (Mj)	C	RD	LC

Table 1 (Part-E) Bird Diversity of Pandharkawada Forest Division

Sr. No.	Order	Family	Scientific Name	Common Name	Habitat	Nest	Food	Occurr-ence	Distribu-tion	IUCN Status
150.	Falconiformes	Falconidae	*Falco tinnunculus*	Common kestrel	ArB	TrNt	Car (Mj), Ins	C	WV	LC
151.			*Falco amurensis*	Amur Falcon	ArB	TrNt	Car (Mj), Ins	VR	VG	LC
152.			*Falco perigrinus*	Peregrine Falcon	ArB	TrNt	Car (Mj), Ins	VR	WV	LC
153.			*Falco chicquera*	Red-necked Falcon	ArB	TrNt	Car (Mj), Ins	VR	VG	NT
154.	Phoenicopteriformes	Podicipedidae	*Tachybaptus ruficollis*	Little grebe	WtB	WtFl	Car (Mn), & (Mj)	C	RD	LC
155.	Pelecaniformes	Anhingidae	*Anhinga melanogaster*	Darter	WtB	WtFl	Car (Mn), & (Mj)	C	RD	LC
156.		Phalacrocoracidae	*Phalacrocorax niger*	Little cormorant	WtB	WtFl	Car (Mn), & (Mj)	C	RD	LC
157.			*Phalacrocorax fuscicollis*	Indian cormorant	WtB	WtFl	Car (Mn), & (Mj)	C	RD	LC
158.			*Phalacrocorax carbo*	Greater cormorant	WtB	WtFl	Car (Mn), & (Mj)	C	RD	LC
159.		Ardeidae	*Egretta garzetta*	Little egret	ShB	WtFl	Ins, Car (Mn)	C	RD	LC
160.			*Ardea cinerea*	Grey heron	WtB	WtFl	Car (Mn), & (Mj)	C	RD	LC
161.			*Ardea purpurea*	Purple heron	WtB	WtFl	Car (Mn), & (Mj)	C	RD	LC
162.			*Casmerodius albus*	Great egret	ShB	TrNt	Ins, Car (Mn)	C	RD	LC
163.			*Mesophoyx intermedia*	Intermediate egret	ShB	TrNt	Ins, Car (Mn)	C	RD	LC
164.			*Bubulcus ibis*	Cattle egret	ShB	TrNt	Ins, Car (Mn)	C	RD	LC
165.			*Ardeola grayii*	Indian pond heron	WtB	TrNt	Ins, Car (Mn)	C	RD	LC
166.			*Butorides striata*	Striated heron	WtB	TrNt	Car (Mn), & (Mj)	C	RD	LC
167.			*Nycticorax nycticorax*	Black crowned night heron	WtB	TrNt	Car (Mn), & (Mj)	C	RD	LC
168.			*Ixobrychus sinensis*	Yellow bittern	WtB	TrNt	Car (Mn), & (Mj)	C	RD	LC
169.			*Ixobrychus cinnamomeus*	Cinnamon bittern	WtB	WtFl	Car (Mn), Ins	C	RD	LC
170.			*Dupetor flavicollis*	Black bittern	WtB	WtFl	Ins, Car (Mn)	C	RD	LC
171.		Threskiornithidae	*Plegadis falcinellus*	Glossy ibis	ShB	TrNt	Car (Mj), (Min)	C	RD	LC
172.			*Threskiornis melanocephalus*	Black-headed ibis	ShB	TrNt	Car (Mj), (Min)	C	RD	LC
173.			*Pseudibis papillosa*	Black ibis	ShB	WtFl	Car (Mj), (Min)	C	RD	LC
174.			*Platalea leucorodia*	Eurasian spoonbill	ShB	WtFl	Car (Mj), (Min)	C	RD	LC
175.		Ciconiidae	*Mycteria leucocephala*	Painted stork	ShB	TrNt	Car (Mj), (Min)	C	RD	NT
176.			*Anastomus oscitans*	Asian openbill	ShB	WtFl	Car (Mj), (Min)	C	RD	LC
177.			*Ciconia episcopus*	Woolly necked stork	ShB	TrNt	Car (Mj), (Min)	C	RD	NT
178.			*Ciconia nigra*	Black Stork	ShB	TrNt	Car(Mj), (Min)	C	WV	LC
179.			*Leptoptilos javanicus*	Lesser adjutant	ShB	TrNt	Car (Mj), (Min)	VR	VG	VU
180.		Phoenicopteridae	*Phoenicopterus ruber*	Greater flamingo	WtB	WtFl	Car (Mn) & (Mj)	R	SV	LC
181.	Passeriformes	Pittidae	*Pitta brachyura*	Inian pitta	GrB	TrNt	Ins	UC	S-M	LC
182.		Laniidae	*Lanius isabellinus*	Isabelline shrike	ArB	TrNt	Ins, Car (Mj)	UC	WV	LC
183.			*Lanius cristatus*	Brown shrike	ArB	TrNt	Ins, Car (Mj)	C	WV	LC
184.			*Lanius vittatus*	Bay backed shrike	ArB	TrNt	Ins, Car (Mj)	C	RD	LC
185.			*Lanius schach*	Long tailed shrike	ArB	TrNt	Ins, Car (Mj)	C	RD	LC
186.			*Lanius tephronotus*	Grey-backed Shrike	ArB	TrNt	Ins, Car (Mj)	C	VG	LC
187.			*Lanius meridionalis*	Southern grey shrike	ArB	TrNt	Ins, Car (Mj)	UC	RD	LC

Table 1 (Part-F) Bird Diversity of Pandharkawada Forest Division

Sr. No.	Order	Family	*Scientific Name*	Common Name	Habitat	Nest	Food	Occurr-ence	Distribu-tion	IUCN Status
188.		Corvidae	*Dendrocitta vagabunda*	Rufous treepie	ArB	TrNt	Ins, Car (Mj)	C	RD	LC
189.			*Corvus splendens*	House crow	ArB	TrNt	Scv,Car (Mn)	C	RD	LC
190.			*Corvus culminatus*	Indian Jungle crow	ArB	TrNt	Scv, Car (Mn)	C	RD	LC
191.		Oriolidae	*Oriolus (oriolus)kundoo*	Indian golden oriole	ArB	TrNt	Ins, Frv	C	RD	LC
192.			*Oriolus xanthornus*	Black hooded oriole	ArB	TrNt	Ins, Frv	UC	RD	LC
193.		Campephagidae	*Coracina macei*	Large cuckooshrike	ArB	TrNt	Ins, Car (Mn)	UC	RD	LC
194.			*Coracina melanoptera*	Black headed cuckooshrike	ArB	TrNt	Ins, Car (Mn)	C	RD	LC
195.			*Pericrocotus cinnamomeus*	Small minivet	ArB	TrNt	Ins, Car (Mn)	C	RD	LC
196.			*Pericrocotus erythropygius*	White bellied minivet	ArB	TrNt	Ins, Car (Mn)	R	VG	LC
197.		Rhipiduridae	*Rhipidura aureola*	White-browed fantail	ArB	TrNt	Ins, Car (Mn)	C	RD	LC
198.			*Rhipidura albicollis*	White-throated fantail	ArB	TrNt	Ins, Frv	C	RD	LC
199.		Dicruridae	*Dicrurus macrocercus*	Black drongo	ArB	TrNt	Ins	C	RD	LC
200.			*Dicrurus caerulescens*	White bellied drongo	ArB	TrNt	Ins, Frv	C	RD	LC
201.			*Dicrurus leucophaeus*	Ashy drongo	ArB	TrNt	Ins	C	WV	LC
202.		Monarchidae	*Hypothymis azurea*	Black naped monarch	ArB	TrNt	Ins, Car (Mn)	C	RD	LC
203.			*Terpsiphone paradisi*	Asian paradise flycatcher	ArB	TrNt	Ins, Car (Mn)	C	RD	LC
204.		Cloropseidae	*Cloropsis jerdoni*	Jerdon's leafbird	ArB	TrNt	Ins, Frv	UC	W-S	LC
205.		Aegithinidae	*Aegithina tiphia*	Common iora	ArB	TrNt	Ins, Frv	C	RD	LC
206.		Vangidae	*Tephrodornis pondicerianus*	Common woodshrike	ArB	TrNt	Ins, Car (Mn)	C	RD	LC
207.		Turdidae	*Turdus merula*	Common blackbird	ArB	TrNt	Ins, Car (Mj)	R	VG	LC
208.			*Zoothera citrina*	Orange headed thrush	ArB	TrNt	Ins, Car (Mj)	C	RD	LC
209.			*Monticola solitarius*	Blue rock thrush	ArB	RoCl	Ins, Car (Mj)	C	M-S	LC
210.		Muscicapidae	*Muscicapa dauurica*	Asian brown flycatcher	ArB	TrNt	Ins, Car (Mj)	C	RD	LC
211.			*Ficedula parva*	Red-breasted flycatcher	ArB	TrHl	Ins, Car (Mn)	C	WV	LC
212.			*Ficedula albicilla*	Red-throated Flycatcher	ArB	TrHl	Ins, Car (Mn)	UC	WV	LC
213.			*Ficedula superciliaris*	Ultramarine flycatcher	ArB	TrNt	Ins, Car (Mn)	R	WV	LC
214.			*Eumyias thalassinus*	Verditer flycatcher	ArB	TrNt	Ins, Car (Mn)	UC	RD	LC
215.			*Cyornis tickelliae*	Tickell's blue flycatcher	ArB	TrHl	Ins, Car (Mn)	C	RD	LC
216.			*Luscinia svecica*	Bluethroat	ShB	GrNt	Ins, Car (Mn)	C	WV	LC
217.			*Copsychus saularis*	Oriental magpie robin	ArB	TrNt	Ins, Car (Mn)	C	RD	LC
218.			*Saxicoloides fulicatus*	Indian robin	ArB	TrNt	Ins, Car (Mn)	C	RD	LC
219.			*Phoenicurus ochruros*	Black redstart	ArB	TrNt	Ins, Car (Mn)	C	M-W	LC
220.			*Saxicola torquatus*	Common stonechat	ArB	TrNt	Ins, Car (Mn)	C	RD	LC
221.			*Saxicola caprata*	Pied bushchat	ArB	TrNt	Ins, Car (Mn)	C	RD	LC
222.			*Cercomela fusca*	Brown rock chat	ArB	TrNt	Ins, Car (Mn)	C	RD	LC
223.			*Oenanthe isabelilina*	Isabelline Wheatear	ArB	RoCl	Ins, Car (Mn)	UC	WV	LC
224.		Stenostiridae	*Culicicapa ceylonensis*	Grey headed canary flycatcher	ArB	TrNt	Ins	R	WV	LC

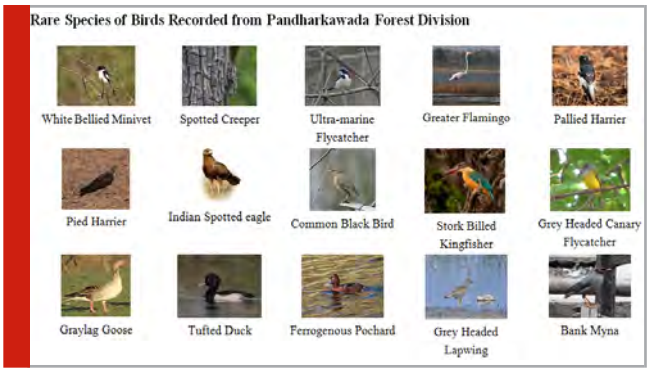
Table 1 (Part-G) Bird Diversity of Pandharkawada Forest Division

Sr. No.	Order	Family	*Scientific Name*	Common Name	Habitat	Nest	Food	Occurr-ence	Distribu-tion	IUCN Status
225.		Sturnidae	*Pastor roseus*	Rosy starling	ArB	TrNt	Ins, Frv	C	W-S	LC
226.			*Sturnia pagodarum*	Brahminy starling	ArB	TrNt	Ins, Frv	C	RD	LC
227.			*Sturnia malabarica*	Chestnut-tailed starling	ArB	TrHl	Ins, Frv	C	W-S	LC
228.			*Gracupica contra*	Asian pied starling	ArB	TrNt	Ins, Frv	C	RD	LC
229.			*Acridotheres tristis*	Common myna	ArB	TrNt	Ins, Frv	C	RD	LC
230.			*Acridotheres albocinctus*	Bank Myna	ArB	RoCl	Ins, Frv	R	VG	LC
231.		Paridae	*Parus major*	Great tit	ArB	TrHl	Ins, Frv	C	RD	LC
232.			*Parus xanthogenys*	Black-lored tit	ArB	TrNt	Ins, Frv	C	RD	LC
233.		Sittidae	*Sitta cinnamoventris*	Chestnut-bellied nuthatch	ArB	TrHl	Ins, Frv	UC	RD	LC
234.		Certhiidae	*Salpornis spilonotus*	Spotted Creeper	ArB	TrNt	Ins	R	W-S	LC
235.		Hirundinidae	*Riparia paludicola*	Plain martin	AiB	RoCl	Ins	C	RD	LC
236.			Ptyonoprogne concolor	Dusky Crag Martin	AiB	RoCl	Ins	C	RD	LC
237.			*Hirundo rustica*	Barn swallow	AiB	RoCl	Ins	C	WV	LC
238.			*Hirundo smithii*	Wire tailed swallow	AiB	RoCl	Ins	C	RD	LC
239.			*Cecropis daurica*	Red rumped swallow	AiB	RoCl	Ins	C	RD	LC
240.			*Petrochelidon fluvicola*	Streak throated swallow	AiB	RoCl	Ins	UC	M-W	LC
241.		Pycnonotidae	*Pycnonotus cafer*	Red vented bulbul	ArB	TrNt	Frv, Ins	C	RD	LC
242.			*Pycnonotus luteolus*	White browed bulbul	ArB	TrNt	Frv, Ins	C	RD	LC
243.		Cisticolidae	*Cisticola juncidis*	Zitting cisticola	ArB	TrNt	Ins	C	RD	LC
244.			*Prinia hodgsonii*	Grey breasted prinia	ArB	TrNt	Ins	C	W-S	LC
245.			*Prinia socialis*	Ashy prinia	ArB	TrNt	Ins	C	RD	LC
246.			*Prinia inornata*	Plain prinia	ArB	TrNt	Ins	C	RD	LC
247.			*Prinia sylvatica*	Jungle prinia	ArB	TrNt	Ins	C	RD	LC
248.			*Orthotomus sutorius*	Common tailorbird	ArB	TrNt	Ins	C	RD	LC
249.		Zosteropidae	*Zosterops palpebrosus*	Oriental white eye	ArB	TrNt	Ins, Frv	C	RD	LC
250.		Acrocephalidae	*Acrocephalus dumetorum*	Blyth's reed warbler	ArB	TrNt	Ins	C	WV	LC
251.			*Acrocephalus Agricola*	Paddy field Warbler	ArB	TrNt	Ins	C	RD	LC
252.			*Iduna caligata*	Booted Warbler	ArB	TrNt	Ins	UC	WV	LC
253.			*Acrocephalus stentoreus*	Clamorous reed warbler	ArB	TrNt	Ins	C	WV	LC
254.		Phylloscopidae	*Phylloscopus collybita*	Common chiffchaff	ArB	TrNt	Ins	C	W-S	LC
255.			*Phylloscopus griseolus*	Sulphur bellied warbler	ArB	TrNt	Ins	UC	WV	LC
256.			*Phylloscopus trochiloides*	Greenish warbler	ArB	TrNt	Ins	UC	WV	LC
257.		Sylviidae	Sylvia hortensis	Orphean warbler	ArB	TrNt	Ins, Car (Mj)	UC	WV	LC
258.			*Chrysomma sinense*	Yellow eyed babbler	ArB	TrNt	Ins, Car (Mj)	C	RD	LC
259.			*Turdoides caudata*	Common babbler	ArB	TrNt	Ins, Car (Mj)	C	RD	LC
260.			*Turdoides malcolmi*	Large grey babbler	ArB	TrNt	Ins, Car (Mj)	C	RD	LC
261.			*Turdoides striatus*	Jungle babbler	ArB	TrNt	Ins, Car (Mj)	C	RD	LC
262.			*Dumetia hyperythraa*	Tawny-bellied Babbler	ArB	TrNt	Ins	C	RD	LC
263.			*Sylvia curruca*	Lesser Whitethroat	ArB	TrNt	Ins	C	W-S	LC
264.			*Mirafra cantillans*	Singing bushlark	ArB	TrNt	Ins	C	RD	LC

Table 1 (Part-H) Bird Diversity of Pandharkawada Forest Division

Sr. No.	Order	Family	*Scientific Name*	Common Name	Habitat	Nest	Food	Occurr-ence	Distribu-tion	IUCN Status
265.		Alaudidae	*Mirafra erythroptera*	Indian bushkark	ArB	TrNt	Ins, Grv	C	RD	LC
266.			*Eremopterix griseus*	Ashy crowned sparrow lark	ArB	TrNt	Ins, Grv	C	RD	LC
267.			*Ammomanes phoenicurus*	Rufous tailed lark	GrB	TrNt	Ins, Grv	C	RD	LC
268.			*Calandrella brachydactyla*	Greater short toed lark	GrB	TrNt	Ins, Grv	C	M-W	LC
269.			*Galerida cristata*	Crested lark	GrB	GrNt	Ins, Grv	UC	M-W	LC
270.			*Galerida deva*	Sykes's lark	GrB	GrNt	Ins, Grv	C	M-W	LC
271.			*Alauda gulgula*	Oriental skylark	GrB	GrNt	Grv	UC	M-W	LC
272.		Nectariniidae	*Leptocoma zeylonica*	Purple rumped sunbird	ArB	TrNt	Nct, Ins	C	RD	LC
273.			*Cinnyris asiaticus*	Purple sunbird	ArB	TrNt	Nct, Ins	C	RD	LC
274.		Passeridae	*Passer domesticus*	House sparrow	ArB	TrNt	Grv , Ins	C	RD	LC
275.			*Gymnoris xanthocollis*	Chestnut shouldered petronia	ArB	TrHl	Ins, Grv	C	RD	LC
276.		Motacillidae	*Motacilla alba*	White wagtail	ShB	GrNt	Ins, Car (Mn)	C	M-W	LC
277.			*Motacilla maderaspatensis*	White browed wagtail	ShB	GrNt	Ins, Car (Mn)	C	M-S	LC
278.			*Motacilla citreola*	Citrine wagtail	ShB	GrNt	Ins, Car (Mn)	C	M-W	LC
279.			*Motacilla flava*	Yellow wagtail	ShB	GrNt	Ins, Car (Mn)	C	M-S	LC
280.			*Motacilla cinerea*	Grey wagtail	ShB	GrNt	Ins, Car (Mn)	C	M-W	LC
281.			*Dendronanthus indicus*	Forest wagtail	ArB	TrNt	Ins	VR	VG	LC
282.			*Anthus rufulus*	Paddyfield pipit	ArB	GrSc	Ins, Fol	C	RD	LC
283.			*Anthus godlewskii*	Blyth's pipit	ArB	GrSc	Ins, Fol	C	WV	LC
284.			*Anthus hodgsoni*	Olive-backed Pipit	ArB	TrNt	Ins	UC	WV	LC
285.			*Anthus richardi*	Rechard's Pipit	ArB	GrSc	Ins, Fol	UC	M-W	LC
286.			*Anthus compestris*	Tawny Pipit	ArB	GrSc	Ins	C	M-W	LC
287.		Ploceidae	*Ploceus philippinus*	Baya weaver	ArB	TrNt	Ins, Frv	C	RD	LC
288.		Estrildidae	*Amandava amandava*	Red avadavat	ArB	TrNt	Frv, Grz (Gr)	C	RD	LC
289.			*Euodice malabarica*	Indian silverbill	ArB	TrNt	Grv, Frv	C	RD	LC
290.			*Lonchura punctulata*	Scaly breasted munia	ArB	TrNt	Grv, Frv	C	RD	LC
291.			*Lonchura malacca*	Black headed munia	ArB	TrNt	Grv, Ins	C	RD	LC
292.		Fringillidae	*Carpodacus erythrinus*	Common rosefinch	ArB	TrNt	Grv, Nct	C	M-W	LC
293.		Emberizidae	*Melophus lathami*	Crested bunting	ArB	GrNt	Grv, Ins	UC	RD	LC
294.			*Emberiza buchanani*	Grey necked bunting	ArB	TrNt	Ins	VR	WV	LC
295.			*Emberiza melanocephala*	Black headed bunting	ArB	TrNt	Grv, Ins	C	M-W	LC
296.			*Emberiza bruniceps*	Red headed bunting	ArB	TrNt	Grv, Ins	C	WV	LC

- Habitat: Water Birds - WtB; Shore Birds - ShB; Ground Birds – RrB; Arboreal bird – ArB; Aerial bird – AiB
- Nesting: Groud Scrap - GrSc; Ground Nest - GrNt; Tree Nest – TrNt; Tree Hole – TrHl; Rock Cleft – RoCl; Water Floating – WtF
- Food:Granivores - Grv; Frugivores – Frv; Nctariores – Nct; Foliores - Fol; Grazer (Terrestrial)- Grz (Gr); Grazer (Aquatic) - Grz (Wt); Insectiores- Ins; Omnivores – Omn; Carniores (Minor) - Car (Mn); Carniores (Major) - Car (Mj); Scavanger - Scv
- Occurrence: Common - C; Uncommon -UC; Rare - R; Very rare - VR
- Distribution: Resident –RD; Winter Visitor – WV; Monsoon Visitor – MV; Summer Visitor – SV; Vagrant – VG; Winter to summer W-S, Winter to monsoon W-M; Summer to monsoon - S–M; Summer to winter - S-W; Monsoon to winter - M- W, Monsoon to summer –M-S.
- IUCN Status: List concerned - LC; Near threatened – NT; Vulnerable – VU; Endangered – EN; Critically endangered – CR



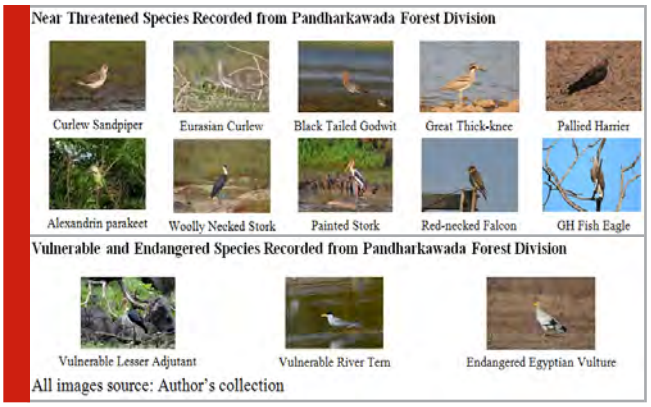


Figure 1: Family wise species Diversity of Pandharkawada Forest Division

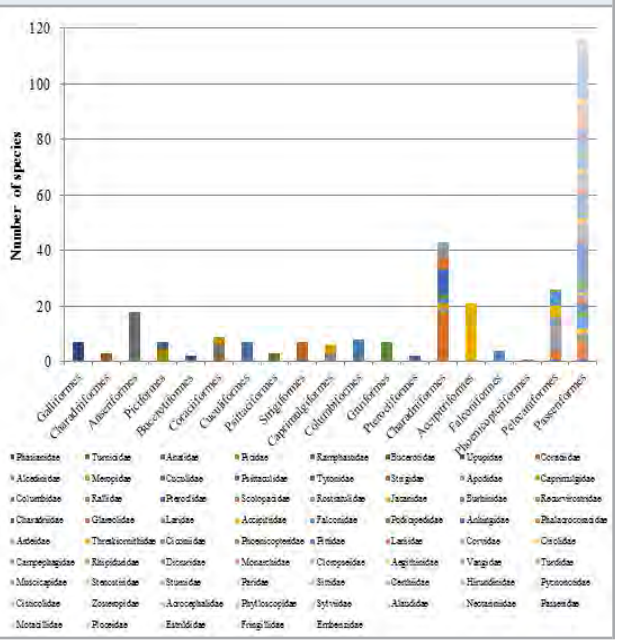


Figure 2: Food Habit

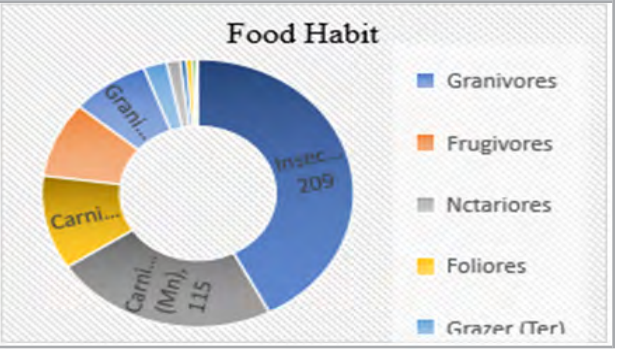
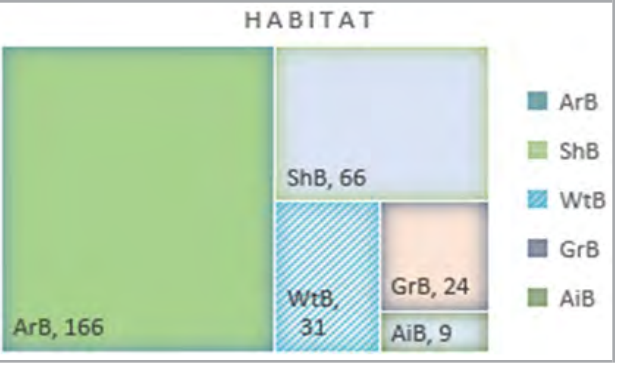


Figure 3: Habitat



Changes in tropical wetlands leads to the change in structure and function of the wetland ecosystems, which affect the delivery of important ecosystem services, this loss of ecosystem services impacts human welfare (Alice et al. 2020). After enactment of forest right act 2006, there is tremendous increase in encroachments for individual and community forest rights. More recently, conversion of tropical forests to farmland is a main threat to extinction of bird species from a region, especially intact old-growth forest to agricultural and pastoral lands is among the greatest threats to biodiversity including birds (Gibbs et al. 2010; Tilman et al. 2001; Laurance et al. 2014; Hughes et al.2020; Rurangwa et al. 2021).

Increasing anthropogenic pressure, change in land use patterns, commercial exploitation of waterbodies and seasonal wetlands, forest fire, rampant grazing, overuse of pesticides and herbicides rapidly eating up natural habitats of birds in this forest division. To conserve the diversity in general and highly sensitive avifauna in particular, habitat conservation on landscape level is an essential instrument (Socolar and Wilcove 2019; Sayer et al. 2017; Hughes et al. 2020).

Figure 4: Occurrence

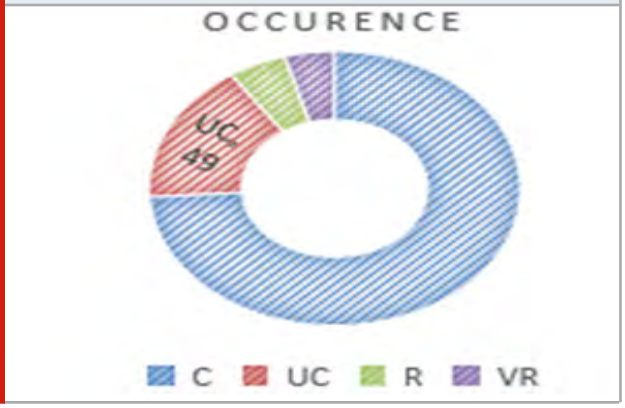


Figure 5: Distribution

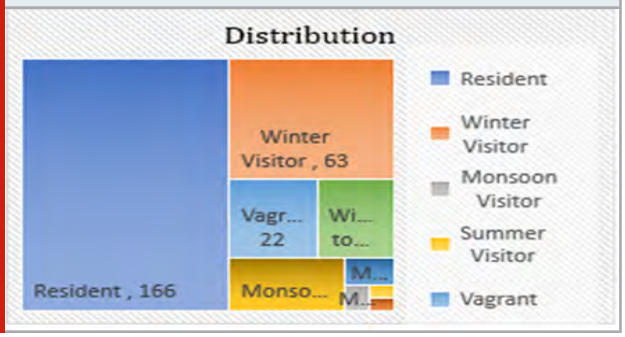


Figure 6: Nesting type

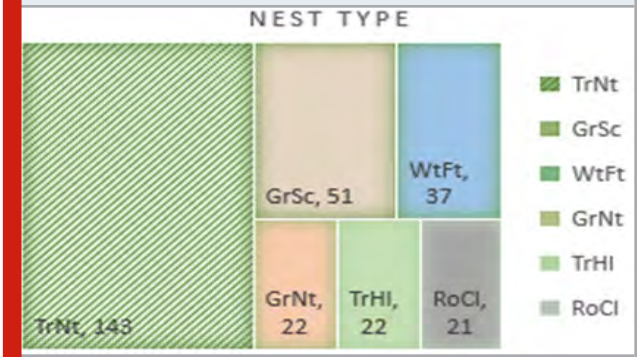
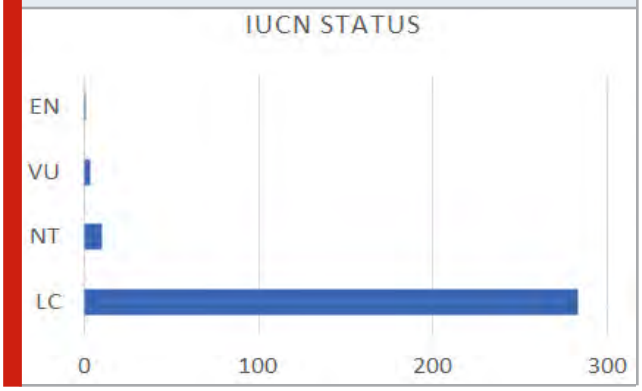


Figure 7: IUCN Status



CONCLUSION

The findings of the present study will help in understanding local diversity, distribution, and conservation status of birds in this landscape which, will help in drawing sustainable conservation strategies by balancing human demand and ecological services provided by these flying denizens.

ACKNOWLEDGMENTS

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

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Technological Communication

Development of the Technology for Obtaining a Probiotic Fermented Milk Product Enriched with Magnesium and Whey Proteins

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ABSTRACT

The problem of an increase in the level of alimentary-dependent diseases has a global scale, and the fulfilment of tasks to solve it is included in the state programs of most developed countries, including the Russian Federation. Enrichment of food products of mass consumption with essential micronutrients is a modern, most economically profitable, effective and physiological way to improve the health of the population. The research is aimed at development of the technology of probiotic fermented milk product enriched with magnesium-containing whey protein concentrate for the prevention of alimentary-dependent diseases. The experiments were aimed at studying the effect of various doses of WPC-Mg on the main organoleptic, physicochemical and microbiological indicators of the enriched fermented milk product. As a result of the conducted research, it has been determined that introduction of WPC-Mg to the milk base in the amount of 10% has a stimulating effect on the biochemical processes in the production of fermented milk drinks. It has been found that the structural and mechanical characteristics of WPC-Mg promote the formation of stronger intermolecular bonds in the fermented milk clot, which significantly improves the rheological characteristics of the product and makes the consistency of the drink similar to that of the products with a high mass fraction of fat. Based on the experimental data obtained, the technology of obtaining a probiotic fermented milk product enriched with magnesium and whey proteins has been developed. The obtained results open up broad perspectives for creating probiotic enriched products for functional and therapeutic nutrition.

KEY WORDS: FERMENTED MILK PRODUCT, MAGNESIUM, PROPIONIC ACID BACTERIA, WHEY PROTEINS.

INTRODUCTION

(Ince-Coskun & Ozdestan-Ocak, 2020) Modern physiologists increasingly attribute magnesium to the priority micronutrients for the human organism (;Trisvetova, 2012; Al Alawi et al., 2018; Glasdam et al., 2016; Guerrero-Romero et al., 2016; Sarrafzadegan et al., 2016; Farsinejad-Marj et al., 2016; Li et al., 2016; Kirkland et al., 2018; Joy et al., 2019). Being a necessary macroelement for the cells and tissues, magnesium is involved in many physiological processes that ensure normal vital activity of the organism: in the synthesis of enzymes (ATP substrate, ADP, creatine kinase, hexokinase, etc.), direct activation of enzymes, regulation of the cell membrane function (stabilization of cell membranes, cell adhesion, the transmembrane flow of electrolytes), antagonism with calcium (muscle contraction/relaxation, the release of neurotransmitters, the excitability of the specialized cardiac conduction system), and plastic processes (synthesis of protein and catabolism, metabolism of nucleic acids and lipids, and mitochondria) (Al Alawi et al., 2018; Glasdam et al., 2016; Trisvetova, 2012). According to the WHO, magnesium deficiency takes one of the leading places in human pathologies caused by disorders of mineral metabolism (Glasdam et al., 2016; Al Alawi et al 2018; Severino et al., 2019; Hernández-Becerra et al., 2020).

Low magnesium level in the organism is associated with such diseases as osteoporosis, high blood pressure, blockage of arteries, hereditary heart diseases, diabetes, and apoplectic attack (Al Alawi et al., 2018; Glasdam et al., 2016; Guerrero-Romero et al., 2016; Sarrafzadegan et al., 2016; Farsinejad-Marj et al., 2016; Li et al., 2016; Kirkland et al., 2018; Hernández-Becerra et al., 2020; Joy et al., 2019; Trisvetova, 2012, Ince-Coskun & Ozdestan-Ocak, 2020). Like other macroelements, magnesium is received with food and water. The need for magnesium cannot always be satisfied through nutrition. In this case, mineral supplements and magnesium-containing preparations are prescribed. The effectiveness of

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drugs containing magnesium depends mainly on two factors: the amount of "elemental" magnesium in the compound and its bioavailability (the ability to be assimilated in the organism). High bioavailability is characteristic of chelated forms of magnesium —compounds of magnesium with amino acids (Glasdam et al., 2016; Al Alawi et al., 2018; Severino et al., 2019; Hernández-Becerra et al., 2020).

Whey proteins are advised for consumption by the authors as a source of amino acids for obtaining chelate complexes. By their amino acid composition, whey proteins are among the most valuable proteins of animal origin (they are sources of essential amino acids, exhibit immunomodulatory, antagonistic, and anticarcinogenic activity; they are responsible for transporting fat-soluble vitamins and microelements in the organism) (Gordienko et al., 2015; Nechaev et al., 2007; Khramtsov, 2011). Whey proteins contain significant amounts of branched-chain amino acids and are physiologically beneficial: for example, consuming whey proteins in combination with power training accelerates fat loss in humans (Wang et al., 2020; Lockwood et al., 2017). Besides, whey proteins are widely used for technological purposes, such as forming gels (Egan et al., 2014; Oztop, 2014), changing viscosity (Patocka et al., 2006), and fat substitution (Akalın et al., 2008). Chelated complexes of magnesium with amino acids from whey proteins are obtained through thermal denaturation with the use of magnesium salt as a coagulant, followed by fermentation of the protein mass by probiotic cultures (Shchekotova & Khamagaeva, 2017; Ince-Coskun & Ozdestan-Ocak, 2020).

The studies performed in recent years have clearly shown that probiotics have a beneficial effect on gut microbiota and mineral metabolism (Skrypnik & Suliburska, 2018). Microflora is involved in the metabolism of many micro- and macroelements, including magnesium (Skrypnik & Suliburska, 2018). Biotechnological processing of whey proteins with probiotic cultures will improve their functional properties after thermomagnesium precipitation. Normalization of the intestinal microflora will cause acidification of the medium in the large intestine and ensure better magnesium absorption (Glasdam et al., 2016; Skrypnik & Suliburska, 2018; Al Alawi et al., 2018; Hernández-Becerra et al., 2020). The presented literature data show that joint enrichment of dairy products with magnesium, WPC, and probiotic cultures will allow obtaining functional food for various purposes, including the prevention of nutrition-related diseases. The work is aimed at developing a technology for obtaining a probiotic fermented milk product enriched with the magnesium-containing WPC.

MATERIAL AND METHODS

Experimental studies were performed at the Technology of Dairy Products. Merchandising and Examination of Goods Department of the HE FSBEI East Siberia State University of Technology and Management (ESSUTM) in Ulan-Ude, Russia, during the period from May to December 2019. The objects of research at different stages were whole milk, fermented WPC-Mg, probiotic fermented milk product. Pure cultures of *Propionibacterium freundenreichii subsp. freundenreichii* AC-2585 obtained from the All-Russian Collection of Industrial Microorganisms of Federal Institution "State Research Institute of Genetics and Selection of Industrial Microorganisms of the National Research Center" Kurchatov Institute" (Russia) were used to obtain probiotic yeast.

The fermented WPC-Mg was used for enriching the fermented milk drink. Unclarified curd whey was used as the raw material for the production of the fermented WPC that was obtained by thermal coagulation with the addition of magnesium salt as a coagulant, followed by the fermentation of proteins with propionic acid bacteria of the *P. freundenreichii* subsp. freundenreichii species and drying. Before the introduction, WPC-Mg was preliminarily dissolved in a small amount of pasteurized milk cooled to (60 – 65) ^{0}C. The content of magnesium in the fermented WPC was 236 ± 0.7 mg/100g, the mass fraction of moisture was 70 – 80%, and the mass fraction of protein was 13 ± 0.6%. Cow's raw milk was used for the production of fermented milk product. The technological process for the production of the enriched fermented milk product included milk acceptance, purification, heating, normalization to mass fraction of fat of 2.5%, homogenization, pasteurization at (93 ± 2) ^{0}C for (15-20) sec, cooling of normalized mixture up to (30 ± 2) ^{0}C, introduction of WPC-Mg, fermentation of the mixture with 5% starter culture until the acidity reached (70-90) ^{0} T, cooling and bottling. In order to exclude undesirable relationships among microorganisms, fermentation of the normalized mixture was carried out with an active ferment based on the same probiotic cultures that were used for fermentation of WPC-Mg – *P. freundenreichii subsp. freundenreichii* AC-2585.

The research scheme included study of the effect of various doses of fermented WPC-Mg in a milk base on the fermentation process of fermented milk product (assessment of titratable acidity and the number of viable cells of propionic acid bacteria); assessment of the organoleptic properties of fermented milk product with different content of WPC-Mg (taste, smell, color, consistency); study of the effect of WPC-Mg on the structural, mechanical and rheological characteristics of fermented milk clots (assessment of dynamic viscosity, clot density, degree of seneresis); establishment of shelf life and quality indicators of the enriched fermented milk product; development of technology for a probiotic fermented milk product enriched with whey proteins and magnesium.

When performing the experimental part of the work, standard and generally accepted methods of physicochemical, organoleptic, microbiological analysis were used. Organoleptic indicators were determined visually, as well as by smelling and tasting the product. The titratable acidity was determined by titration: the method was based on the neutralization of the acids contained in the product with sodium hydroxide solution in the presence of phenolphthalein indicator. The rheological characteristics of acid clots were determined on a Brookfield RVDV-II + Pro rotational viscometer (United States, Brookfield Engineering Labs. Inc., 2009). The clot density was determined by measuring the immersion depth of a plate with certain

weight and area, exerting pressure on the clot for (30-60) sec (Krekker et al., 2016). The plate with the weight of 12.4 g and the base area of 1.6 cm^3 was used in the experiment. The clot density was calculated by the equation: D = (0.5 • q • hc) / (d • hn), where D was the clot density, g/cm^3, q was the load created by the plate (weight of the plate, g), hc was the clot height in the glass (mm), d was the plate base area (cm^2), hn was the plate immersion depth (mm).

The syneresis was determined by the filtration method through measuring the amount of whey released during filtering 100 cm^3 of the decomposed clot through a paper filter for eight hours at room temperature.The mass fraction of magnesium was determined by the method of capillary electrophoresis on a Kapel-105M device (Russia, St. Petersburg, Lumex-Marketing LLC, 2012). The method was based on sample dilution, further separation, identification and quantitative determination of the mass concentration of magnesium (mg/L) by capillary electrophoresis (Lumex, 2013). The mass fraction of fat in the enriched product was determined by the acid-butyrometric method based on the separation of fat from the fermented milk product under the action of concentrated sulfuric acid and isoamyl alcohol, followed by centrifugation and measurement of the volume of released fat in the graduated part of the butyrometer (Gosstandart of the USSR, 1990).

The mass fraction of protein was determined by the Kjeldahl method. The method was based on the mineralization of the analyzed product sample with concentrated sulfuric acid in the presence of a catalyst with the formation of ammonium sulfate, its conversion into ammonia, distillation of the latter into a boric acid solution, quantitative accounting of ammonia by the titrimetric method and calculation of the mass fraction of protein in the analyzed sample (Rosstandart, 2018). The number of cells of propionic acid microorganisms was determined by the method of limiting dilutions (Rosstandart, 2014b). The method was based on sowing propionic acid bacteria in certain dilutions in (on) selective nutrient media for submerged cultivation, their cultivation at a temperature of (30 - 1) ^{0}C for 48 hours with limited oxygen access and subsequent quantitative calculation of the content of propionic acid bacteria in the product.Bacteria of the *E. coli* group were determined by the signs of growth in liquid Kessler medium (Rosstandart, 2014a). Yeast and molds were determined by sowing the product on a solid nutrient medium (Sabouraud's agar) (Rosstandart, 2015).

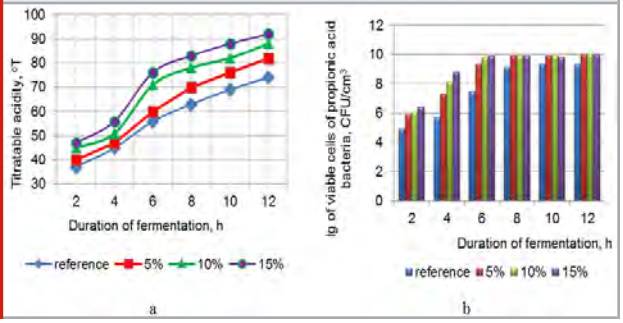
All experiments were carried out 3-5 times. The data obtained were processed using personal computer in Microsoft Excel 14 with the calculation of arithmetic mean values and corresponding errors (M ± m). The significance of differences between the compared indicators in the groups was assessed by Student's t-test. Differences were considered statistically significant at P<0,05. Graphical dependencies in the figures were presented after the experimental data processing. Calculations, plotting and specification of diagrams were performed using Microsoft Office 14 and Excel 14 applications on Windows 10.

RESULTS AND DISCUSSION

At the first stage of the studies, the authors studied the effect of fermented WPC-Mg in a milk base on the fermentation process of the fermented milk product. WPC-Mg was introduced into normalized milk after heat treatment in the amounts of 5, 10, and 15%. The ripening process was monitored by the increase in acidity Fig. (1a) and the growth of propionic acid bacteria Fig. (1b) in the product. During ripening, the authors tracked the time required for the acidity of the tested samples of the product to reach (70 – 90) 0T. The reference was normalized milk with the fat mass fraction of 2.5%. Fig. (1) shows that adding WPC-Mg accelerated the fermentation process in the normalized mixture of the fermented milk product. The required acidity (70 – 90 °T) of the fermented milk product clot was reached after six hours of cultivation with the addition of 10 and 15% of WPC-Mg Fig. (1a). An increase in acid formation with increasing the dose of WPC-Mg in the tested samples was explained by the change of the buffer capacity of the mixture and its enrichment with additional sources of nitrogenous nutrition and the macroelement for propionic acid bacteria (Skrypnik & Suliburska, 2018; Cousin et al., 2011; Begunova et al., 2019; Vorobjeva et al., 2008). An intensive increase in the titratable acidity with the introduction of WPC-Mg at a dosage of 10 and 15% allowed shortening the process by (2 – 4) hours, compared to the reference Fig. (1a).

Quantitative accounting of the probiotic cultures showed more intensive growth of microorganisms in the milk with the addition of WPC-Mg Fig. (1b). The number of viable cells of propionic acid bacteria in the experimental samples with WPC-Mg after six hours of cultivation was (2•109 – 9•109) CFU/cm3, which was two orders of magnitude greater than in the reference Fig. (1b). The high biological value of whey proteins probably created favorable conditions for the development of propionic bacteria. The presence of lactose, peptides, and free amino acids in WPC-Mg provided the possibility of the propionic bacteria's faster growth during cultivation.

Figure 1: The effect of the mass fraction of fermented WPC-Mg on the dynamics of acid formation in the normalized mixture and the growth of propionic acid bacteria in the fermented milk product



Thus, summarizing the results obtained, a conclusion can be drawn that adding WPC-Mg to normalized milk intensifies the fermentation process and increases the

number of probiotic microorganisms in the fermented milk product. This fact indicates the prebiotic properties of the fermented WPCs enriched with magnesium. It was noted that a significant increase in the titratable acidity and the number of viable cells of propionic acid bacteria had been observed after adding 10 – 15% of WPC-Mg to the normalized mixture. The joint presence of whey proteins and the macro element in WPC-Mg contributes to a synergistic effect, enhancing their positive effect on the activity of propionic acid bacteria in the fermented milk product. This fact opens up wide opportunities for using WPC-Mg in the fermented milk product technology, providing symbiotic properties and functional orientation to it.

Table 1. Organoleptic assessment of the milk base after the introduction of various amounts of WPS-Mg

The studied property	The dose of WPC introduced			
	reference	5%	10%	15%
taste and odor	the taste and odor are clean with sour milk aftertaste	the taste and smell are clean, with a sour milk aftertaste and a subtly bitter flavor		the taste and smell are those of fermented milk with a pronounced bitter aftertaste
color	cream			
consistency	Liquid, homogeneous, glossy, and delicate	homogeneous, glossy, and thickish	homogeneous, glossy, and thick	

Table 2. The effect of WPC-Mg on the structural, mechanical, and rheological properties of fermented milk clots

The studied property	The dose of WPC introduced			
	reference	5%	10%	15%
Dynamic viscosity, Pa•s	33 ± 0.3	38 ± 0.5	42 ± 0.2	47 ± 0.3
Clot density, 102 g/cm3	1.20 ± 0.01	1.36 ± 0.03	1.78 ± 0.02	1.96 ± 0.02
Syneresis degree, %	67 ± 0.5	59 ± 0.2	52 ± 0.4	40 ± 0.3

According to the literature (Al Alawi et al., 2018; Glasdam et al., 2016; Guerrero-Romero et al., 2016; Sarrafzadegan et al., 2016), the process of product enrichment with various magnesium-containing salts and additives may affect the organoleptic properties of the product. In this regard, in further experiments, the organoleptic properties of the test samples with various contents of WPC-Mg were assessed Table (1). The analysis of the data in Table (1) showed that the introduction of fermented WPC-Mg to the fermented milk drink affected the taste of the product: a bitter aftertaste appeared. After the introduction of (5 – 10) % of WPC-Mg, this change in the taste was barely noticeable and was not a defect; with increasing the dose, the bitter taste increased, which significantly reduced the consumer properties of the fermented milk drink. This defect may be explained by the bitter taste of magnesium salts. It should be noted that in all studied samples, the introduction of fermented WPC-Mg improved the consistency of the products. This fact is especially important for producing fermented milk drinks with a low mass fraction of fat. The introduction of WPC-Mg ensured the consistency of the drink similar to that of the products with a high mass fraction of fat, even without the use of stabilizing systems.

An objective assessment of the consistency of fermented milk products was provided by the rheological properties that were determined by the type of structure and mechanical properties of the product. These properties were sensitive to the changes in the chemical composition of the product, physical parameters, and processing conditions (Ababkova et al., 2016). In this regard, the next series of experiments was devoted to studying the effect of WPC-Mg on the structural, mechanical, and rheological properties of fermented milk clots Table (2). The analysis of the data in Table (2) showed that the presence of WPC-Mg in the product contributed to forming stronger bonds between the structural elements of the fermented milk clot. This was confirmed by the 0.8 – 1.4 times increased viscosity and 1.1 – 1.6 times increased strength of the resulting lumps. With increasing the dose of introduced WPC-Mg, a decrease in the acid clots syneresis ability was observed, compared to the reference (Table (2). In the studied samples, the degree of syneresis decreased from 67% to 40%. The high water-binding capacity of WPC-Mg was explained by the presence of amino acids that adsorbed water from hydrophilic elements. Usually, hydration of the native whey proteins is weak, however, the thermal denaturation during WPC-Mg production might have significantly increased this ability, which had a positive effect on the water-binding ability of the fermented milk clots of the product.

It should be noted that samples of fermented milk products enriched with WPC-Mg retained uniformity of consistency and a high number of viable cells (108 – 109 CFU/cm3) during storage (for 10 – 12 days), in contrast to the reference sample. The homogeneity of the consistency of the samples

with the WPC, compared to the reference, was explained by the stabilizing properties of whey proteins, which had a water-holding ability and improved the quality of the products and their storage life. The obtained results allowed concluding that fermented WPC-Mg not only enriched the fermented milk drink with protein, easily digestible chelated magnesium, and probiotic cultures, but also intensified the production process, prolonged the shelf life of the product, and improved its structural and mechanical properties, which fact was especially important in the production of fermented milk drinks with low fat content. A comprehensive study of the organoleptic, physicochemical, and rheological parameters of the fermented milk drink made it possible to conclude that a sample with the 10% content of WPC-Mg had the best consumer properties.

Within the study, a technology for the production of a probiotic fermented milk product enriched with magnesium and whey proteins was developed. The process envisaged the milk acceptance, purification, normalization, homogenization, heat treatment and introduction of WPC-Mg into normalized milk in the amount of 10%. This method of introduction was explained by the fact that in the case of using the fermented WPC, subsequent pasteurization of the mixture was not advisable (due to the death of probiotic cultures), as well as homogenization, which could affect the structure of the whey concentrates after mixing the components. This was followed by fermentation of a mixture of 5% starter culture, fermentation, cooling and bottling. The ripening time according to the developed technology was only 6-7 hours. The enriched fermented milk product was characterized by good organoleptic properties and contained a high number of viable cells of propionic acid bacteria (109 CFU/g). The qualitative characteristics of the developed fermented milk product enriched with WPC-Mg are shown in Table (3).

Table 3. The qualitative characteristics of the enriched fermented milk drink

Indicators		Characteristic
Appearance and consistency		Thick, stretchy, glossy, and homogeneous consistency
Taste and odor		The taste and odor are clean, of fermented milk, with a light, barely noticeable, bitter aftertaste
Color		Milky-white, homogeneous, with a creamy shade
Mass fraction of fat, % not less than		2.5 ± 0.01
Mass fraction of protein, % not less than		3.4 ± 0.03
Mass fraction of nonfat milk solids, % not less than		7.8 ± 0.04
Acidity, °T, not more than		89 ± 0.2
Mass fraction of magnesium, mg/l, not less than		256 ± 0.5
The number of propionic acid bacteria cells, CFU/cm³		$(7-9)\cdot10^9$
The volume (cm³) in which are not allowed:	Coliform bacteria	0.1
	pathogenic (including salmonella)	25
	S. aureus staphylococci	1
	L. monocytogenes listeria	-
Yeast, mold, CFU/cm³ (g), not more than		D-50, P-50

The data in Table 3 show that the obtained fermented milk product had good organoleptic properties, contained a prophylactic dose of magnesium in an easily digestible form, and had high protein content and a great number of viable cells of propionic acid bacteria. The consumption of 0.25 liters of the developed product will satisfy the daily need of an adult in macro elements by (16 – 18) %, and consumption of 0.5 liters – by (32 – 36) %, respectively. These values are within the safe levels of product enrichment for magnesium (10 – 40%), as recommended by the leading nutritionists and physicians. At present, a number of effective dairy products enriched with WPC are available in our country and abroad (Khramtsov & Nesterenko, 2004; Lawrence, 1993; Lelievre, 1990; Cozzolino, 2003; Patocka, 2006; Smirnova et al., 2014; Lagrange et al., 2015; Henriques et al., 2012, 2017; Nastaj et al., 2020). Protein concentrates, isolated by various expensive membrane methods, prevail among the used WPCs. These concentrates, with all their advantages, have one significant drawback - high allergenic activity (Kattan et al., 2011; Botteman & Detzel, 2016; Vonk, 2017; Abbring, 2020). In this work, to enrich a fermented milk product, the authors propose to use WPC obtained by thermal coagulation with the addition of magnesium salt as a coagulant, followed by fermentation of protein clots with probiotic cultures.

Biotechnological processing of WPC-Mg using propionic acid bacteria allows increasing the functional properties of the protein concentrate obtained and reducing the allergenic effect of whey proteins. It should be noted that in the literature, the authors did not find data on the production of fermented WPC simultaneously enriched with probiotic cultures and any essential elements. Therefore, the use of fermented concentrates for the enrichment of dairy products, obtained by the method of thermal coagulation with the addition of magnesium salt as a coagulant, is a relevant and cost-effective solution (Minj & Anand, 2020). The proposed biotechnological methods for obtaining a fermented milk product can shorten the production process and significantly improve the quality indicators of the product. The authors have proved the stimulating effect of fermented WPC-Mg on biochemical processes in the production of a fermented milk product: acid formation during fermentation, improvement of structural, mechanical, rheological characteristics and shelf life. The enriched product, developed according to the proposed technology, is of greater importance in

dietary nutrition. The introduction of probiotic fermented milk drinks enriched with magnesium and whey proteins into production and their promotion on the market will significantly expand the range of products for the prevention and correction of alimentary-dependent diseases, as well as allow implementing the principle of waste-free production at dairy enterprises and reducing environmental pollution as a result of utilization of whey protein.

CONCLUSION

As a result of the studies, a new technology for producing the probiotic fermented milk drink has been developed, which has made it possible to obtain an enriched dairy product with functional properties. It has been found that the use of WPC-Mg in the production of the fermented milk drink not only enriches it with an easily digestible macro element and whey proteins but also intensifies the fermentation process and increases the number of probiotic microorganisms in the fermented milk product. This fact is the evidence of prebiotic properties of the fermented WPCs enriched with magnesium. This fact opens up wide opportunities for using WPC-Mg in the fermented milk product technology, providing symbiotic properties and functional orientation to it. The introduction of WPC-Mg into the milk base improves the structural and mechanical properties of the finished product: the density and viscosity of the fermented milk clots increase, and the syneresis slows down. This circumstance is of particular importance in the production of fermented milk drinks with low fat content since it allows excluding or significantly reducing the number of stabilizers and/or thickeners used in such cases. The use of the fermented WPC in the production of the fermented milk drink has allowed increasing the shelf life by up to 10 – 12 days without significant changes in the organoleptic, microbiological, and structural and mechanical properties, which increases the economic efficiency of the developed product.

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Dental Communication

Impacts of Malocclusion on Oral Health-Related Quality Among Saudi Orthodontic Patients

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ABSTRACT

The literature contains contradictory evidence on the association between malocclusion and oral health-related quality of life (OHRQoL) in different populations. OHRQoL is a multi-dimensional construct that comprises a subjective assessment of how an individual 's oral health impacts their comfort, functional, psychological, social well-being and overall quality of life. This study aimed to evaluate the relationship between gender, age, malocclusion severity and OHRQoL in Saudi patients seeking orthodontic treatment at the King Saud University Dental Hospital in Riyadh, Saudi Arabia. A cross-sectional study was done on a random sample of 108 orthodontic patients aged 14–25 years. The orthodontic treatment needs of each participant were assessed using the Dental Health Component of the Index of Orthodontic Treatment Needs (IOTN-DC). While, the oral heath quality of life was evaluated by asking the participant to complete the Oral Health Impact Profile (OHIP-14) questionnaire. The participants generally had good OHRQoL. No association was found between their OHIP-14 scores and IOTN-DC grades. The oral health quality of life of participants with "borderline treatment needs" was strongly affected by psychological disability and psychological discomfort. In particular, Females with 'borderline need of treatment' showed positive impact on oral health than males. Overall, malocclusion did not have a major impact on OHRQoL. This study found that malocclusion had no discernible detrimental effects on OHRQoL and its domains.

KEY WORDS: OHRQOL, OHIP-14, IOTN-DC, MALOCCLUSION, ORTHODONTICS

INTRODUCTION

In recent years, oral health-related quality of life (OHRQoL) has gained traction amongst healthcare professionals in general and orthodontists in particular. According to a US surgeon general, OHRQoL is a multi-dimensional construct that comprises a subjective assessment of how an individual 's oral health impacts their comfort, functional, psychological, social well-being and overall quality of life (DeGuzman et al., 1995). In the World Oral Health Report (2003), the World Health Organization (WHO) recognised the influence of oral health on quality of life and presented it as an essential component of its Global Oral Health Program. Multiple-item questionnaires are the most widely used instruments to assess the impact of personality traits as well as functional and psychosocial aspects on OHRQoL (Feu et al., 2010).

Several instruments have been carefully verified to evaluate the psychometric factors, such as validity and reliability. The shortened version of the Oral Health Impact Profile (OHIP-14) is the most efficient and commonly used method for assessing OHRQoL (Slade et al., 1997; Olkun and Sayar, 2019; Baidas et al., 2020; Kolawole and Ayodele-Oja, 2021). Since malocclusion can be perceived differently by affected individuals (de-Oliveira and Sheiham, 2004;

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Hassan et al., 2014) and a person's self-awareness of the malocclusion reported in a manner that does not reflect its severity (Borzabadi-Farahani and Borzabadi-Farahani, 2011), the OHIP-14 can be useful for quantifying how malocclusion impacts an individual's well-being. Thus, oral health practitioners are encouraged to apply the OHIP-14 in the clinical practice, dental research and dental education. Several indices have been established to address malocclusion, including the Index of Treatment Need (IOTN) and the Index of Complexity Outcome and Need (ICON; Borzabadi-Farahani and Borzabadi-Farahani, 2011).

The IOTN has been widely utilised to assess actual treatment needs in people with varied ethnic backgrounds, owing to its ease of use and its greater diagnostic popularity in the Middle East than the ICON (Hasan and Amin, 2010). Despite the fact that malocclusion is neither a disease nor a harmful condition, it has well-documented physical, social and psychological effects on natural life. According to systematic and meta-analyses, malocclusion has a detrimental influence on an individual's overall quality of life (Andiappan et al., 2015; Dimberg et al., 2015; Kragt et al., 2016).

The association between malocclusion and OHRQoL has been studied in different populations and age groups (Elmahgoub and Abuaffan 2015; Singh et al., 2019; Paes-da-Silva et al., 2020; Elyashkhil et al., 2021; Kolawole and Ayodele-Oja, 2021). For example, one study reported that the impact of malocclusion on OHRQoL in children likely differs from that of adults because of variations in their self-perception and awareness of various oral conditions (de Oliveira and Sheiham, 2004). Moreover, the literature has demonstrated that gender greatly influences the impact of malocclusion on OHRQoL, although perceptions of malocclusion remain a controversial issue (Elmahgoub and Abuaffan, 2015; Elyaskhil et al., 2021; (Kolawole and Ayodele-Oja, 2021).

Crucially, there are conflicting data on the relationship between malocclusion and OHRQoL in adolescents and children. Certain studies found that some adolescents with normative orthodontic treatment needs (as measured by the IOTN-DC) do not have their OHRQoL negatively impacted by malocclusion (de-Oliveira and Sheiham, 2004; Elmahgoub and Abuaffan, 2015). Overall, the relationships between clinical indicators of malocclusion (IOTN-DC) and subjective indicators of malocclusion impact (OHIP-14) require further investigation (Onyeaso, 2009; Olkun and Sayar, 2019). Hence, the present study evaluated the relationships between gender, age, malocclusion severity and OHRQoL in Saudi patients seeking orthodontic treatment at the King Saud University Dental Hospital.

MATERIAL AND METHODS

This cross-sectional study enrolled 108 adolescents and young adults aged 14–25 years who were either self-referred or referred by their general dental practitioners to the orthodontic clinics at the Faculty of Dentistry, King Saud University which is one of the kingdom's largest dental treatment and referral hospitals. These clinics provide high-quality care to insured university students, employees and their families. Before they began any orthodontic treatments, the participants were recruited based on their orthodontic screenings. The sample size calculation was carried out using G* Power Software (3.1.19.4 ed.) and based on prior studies (Hassan and Amin, 2010; Hassan et al., 2014).

A sample size of 100 participants was required to show a significant change in their OHRQoL (α=0.05), with an effect size of 0.4 and a power of 90%. This study included participants in good dental and general health who had not undergone any previous orthodontic treatments. Moreover, it excluded individuals who were undergoing active orthodontic treatment and/or required a surgical intervention, had medical conditions, had previously received orthodontic treatment, possessed severe dentofacial anomalies such as cleft lip and palate, had untreated dental caries and/or had poor periodontal health. These criteria were selected to ensure an unbiased assessment of the participants' quality of life and achieve a homogeneous population.

Ethical Statement: This study was approved by the Clinical Dental Research Centre (FR0276) and the ethics board Date Dec 2015. All eligible participants or their caregivers gave their written consent after they were fully informed of the nature of this study and had agreed to participate.

OHIP-14: The OHIP-14 is a self-administered questionnaire that measures quality of life using 14 items in seven domains: functional limitation, physical pain, psychological discomfort, physical disability, psychological disability, social disability and handicap. Each dimension is measured with two questions. The participants were asked how frequently they had experienced negative effects in these dimensions. The questionnaire uses a five-point Likert scale: 0=never; 1=hardly-ever; 2=occasionally; 3=fairly often and 4=very often. The participants were asked to rate how frequently they experienced oral health issues. The sum of the domain scores can range from 0 to 8, while the OHIP-14 scores can range from 0 to 56. High OHIP-14 scores represent a strong negative impact of oral health issues on OHRQoL (Demirovic et al., 2019; Baidas et al., 2020).

The English version of the OHIP-14 was translated into Arabic and then linguistically and culturally adapted using the forward-back translation technique (Demirovic et al., 2019; Baidas et al., 2020). In this procedure, two bilingual dentists independently translated the English version to Arabic. Next, they conferred and produced an Arabic version, which was then translated back to English by two professional translators who had never seen the original version. The conceptual equivalence between the English version of the OHIP-14 and the back-translated version was confirmed by an expert committee of five dental consultants with different specialties. The final Arabic version was pilot tested on a convenience sample of 10 participants who were not included in the study sample.

The comprehensiveness of the instrument was tested by interviewing each participant after they had filled out

the questionnaire. The goal was to identify whether they had understood the meaning of each questionnaire item and their chosen response. Based on the participants' responses, changes were made to some questions to improve intelligibility. Cronbach's alpha indicated reasonable internal consistency (α=0.896) for Arabic OHIP-14 and acceptable reliability.

IOTN-DC: The IOTN-DC instrument assesses the need for orthodontic treatment in patients, and it determines a grade based on the most severe malocclusion feature: grade 1=no treatment needed, grade 2=minimal treatment needed, grade 3=borderline treatment needed and grades 4 and 5=definite treatment needed. In this study, each participant was clinically examined, and their casts were measured on the missing, overjet, crossbite, displacement and overbite (MOCDO) hierarchical scale to identify their most severe features (Guillemin et al., 1993; Shaw et al., 2007). The IOTN-DC calibration exercises were conducted at the orthodontic clinic by an expert orthodontist. Ten orthodontic study models were evaluated by two examiners using two-week intervals to support inter-examiner and intra-examiner reliability (k=0.86).

Statistical analysis: The data were analysed using the Statistical Package for Social Studies (SPSS 22; IBM Corp., New York, NY, USA). Continuous variables were expressed as means, standard deviations and confidence intervals, whereas categorical variables were expressed as percentages. Cronbach's alpha was used to assess the reliability and internal consistency of the items in the questionnaire. The t-test compared the differences in the total mean scores of the OHIP-14 between the gender and age groups. One-way analysis of variance (ANOVA) and post-hoc tests evaluated the differences between the domains of the OHIP-14 according to the orthodontic treatment need grades. Multiple linear regression and simple linear regression determined the association between the ordinal factors (i.e., age, gender and treatment need) and the OHIP-14 scores.

RESULTS AND DISCUSSION

As shown in Figure 1, most of the 108 participants were female (75.93%). Moreover, 52.78% of the participants were young adults (20–25 years) and 47.22% were adolescents (14–19 years). Regarding malocclusion severity, 41% of participants had grade 4 indicating a definitive need for treatment. In addition, 24% of the participants had grade 3, 18% had grade 2 malocclusion, 12% had grade 5 malocclusion, and 5% had grade 1 (Figure 2).

Table 1: displays the means and standard deviations of the OHIP-14 scores and their seven domains. Psychological disability had the highest mean score (2.15), whereas functional limitation had the lowest score (0.81). Table 2: compares the OHIP-14 scores of the different gender and age groups. There was a statistically significant difference between the mean OHIP-14 scores of the male and female participants (p=0.002), while the difference between the age groups was not statistically significant (p>0.05). Table 3: displays the results of the one-way ANOVA test. Statistically significant differences were found between the OHIP-14 (IOTN-DC) scores of participants with different severities of malocclusion. Clearly, psychological discomfort and psychological disability were significantly affected domains in grade 3 participants. In contrast, only psychological discomfort was significantly affected in grade 4 participants.

Figure 1: Distribution of the participants' genders and ages

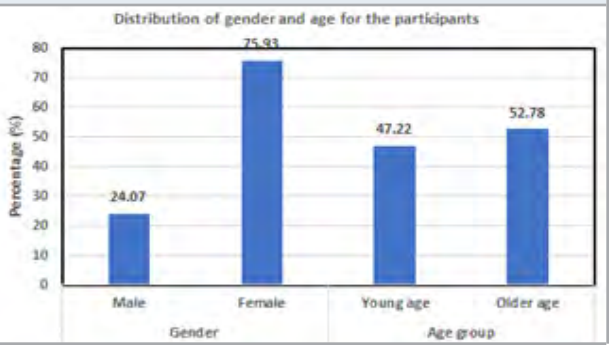

Figure 2: Distribution of the participants' treatment need grades

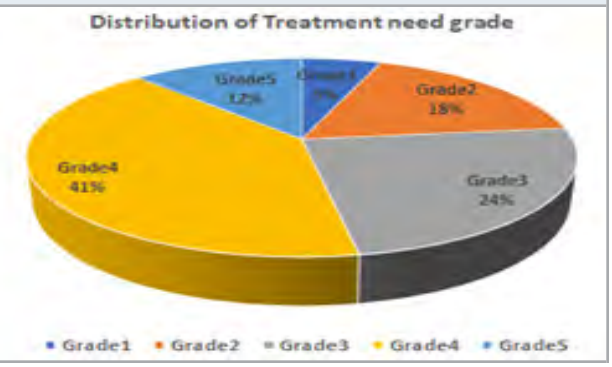

Table 1. Means and standard deviations of the OHIP-14 scores

Domain	Mean*	SD
Functional limitation	0.81	1.32
Physical pain	1.89	1.94
Psychological discomfort	1.91	2.52
Physical disability	1.13	1.61
Psychological disability	2.15	2.17
Social disability	1.40	1.90
Handicap	1.36	1.78
OHIP-total	10.67	9.91

*Mean score for every domain out of 8 and for the total OHIP score out of 56; SD=standard deviation

Table 4: shows a pairwise comparison of the IOTN-DC grades with the domains of the OHIP-14. When the OHIP-14 domains were compared with the IOTN-DC grade pairs

(grade 2 vs grade 4, grade 3 vs grade 4 and grade 5 vs grade 3), significant differences were found (p=0.49, p=0.003 and p =0.026, respectively). When grade 3 was compared to grade 4 with respect to psychological disability, the results showed a significant difference (p=0.011). Likewise, another significant difference was found regarding the total OHIP-14 scores (p=0.025). The correlation analysis did not find an association between the OHIP-14 scores and the IOTN-DC grades (r=0.147; p=0.128). Table 5 shows the results of the multivariate regression analysis on the factors (i.e., gender, age group and treatment need) that affected the OHIP-14 scores. The results showed that gender was significantly and positively associated with OHIP-14 score. Female participants had higher impact scores than male participants (B=9.650; p=0.012). This association was also strong in the simple linear regression model (B=5.690; p=0.010; 95% CI=1.383–9.998). There was approximately 6.1% variability amongst genders (r2= 0.061; Table 6).

Table 2. Comparison of the OHIP-14 scores between gender and age groups

Group	Mean	SD	Mean Difference	95% CI		t test	df	p value
				Lower	Upper			
Gender								
Male	6.35	6.78	-5.69	-10.00	-1.38	-2.62	106.00	0.002*
Female	12.04	10.38	-5.69	-9.20	-2.18	-3.24		
Age group								
Young age	9.33	8.74	-2.53	-6.30	1.25	-1.33	106.00	0.182
Older age	11.86	10.79	-2.53	-6.26	1.21	-1.34		

* Significant p-value<0.05; CI: Confidence interval of the difference

Table 3. Comparison of the IOTN-DC grades and the OHIP-14 domains

OHIP-14 domains	IOTN-DC										
	Grade 1		Grade 2		Grade 3		Grade 4		Grade 5		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	p value
Functional limitation	0.00	0.00	0.63	1.16	0.81	1.23	0.91	1.48	1.15	1.41	0.448
Physical pain	1.33	1.21	2.11	2.05	2.38	2.02	1.61	1.97	1.77	1.79	0.506
Psychological discomfort	1.83	2.23	2.89	2.73	3.23*	2.96	1.05*	1.93	0.77	1.54	0.001*
Physical disability	0.50	0.84	0.84	1.68	1.46	1.70	1.18	1.70	1.00	1.22	0.604
Psychological disability	1.67	1.63	2.58	2.52	3.19*	2.51	1.45	1.55	2.00	2.24	0.018*
Social disability	1.33	1.75	1.95	2.32	2.08	2.24	0.82	1.33	1.23	1.79	0.055
Handicap	1.17	1.33	1.74	2.16	2.04	2.18	0.91	1.31	1.08	1.55	0.094
Total OHIP score	7.83	7.60	12.74	11.67	15.26*	11.40	7.93	7.88	9.00	8.24	0.027*

*ANOVA; significant p value<0.05

Table 4. Pairwise comparison of the IOTN-DC grades and the OHIP-14 domains

Dependent Variable			Mean Difference	Std. Error	p value	95% CI	
						Lower	Upper
Psychological discomfort	Grade2	Grade 4	1.85*	0.64	0.049	0.00	3.70
	Grade 3	Grade 4	2.19*	0.58	0.003	0.52	3.85
	Grade 5	Grade 3	-2.46*	0.80	0.026	-4.74	-0.18
Psychological disability	Grade 3	Grade 4	1.74*	0-.52	0.011	0.26	3.22
Social disability	Grade3	Grade 4	1.26	0.46	0.071	-0.06	2.57
Total OHIP-14 scores	Grade 3	Grade 4	7.34*	2.37	0.025	0.53	14.14

* CI=Confidence interval; post-hoc test; significant p value<0.05

How patients evaluate their health-related quality of life is becoming increasingly important to clinicians, and it may prove useful in cosmetic and elective procedures alike (Tsakos et al., 2006; Mandall et al., 2006). Although malocclusion has well-known physical and psychological implications, research on these effects is still contradictory, which could be due to a lack of standardised assessment methods. The OHIP-14 have been used in both general populations and individuals with specific oral diseases (Mandall et al., 2006). Different studies applied it to evaluate the effects of malocclusion on quality of life (Hassan and Amin, 2010; Borzabadi-Farahani and Borzabadi-Farahani, 2011; Hassan et al., 2014; Andiappan et al., 2015; Dimberg et al., 2015; Elmahgoub and Abuaffan, 2015; Kragt et al., 2016; Singh et al., 2019; Paes-da-Silva et al., 2020; Elyaskhil et al., 2021; Kolawole and Ayodele-Oja, 2021).

Table 5. Multivariate linear regression model showing the association between OHIP-14 score and gender, age group and treatment need grade

Independent Variable	B	t	95.0% CI for B		p value
			Lower	Upper	
Gender	9.650	2.634	2.262	17.038	0.012*
Age group	-1.391	-.377	-8.823	6.041	0.708
Treatment need grade	-1.150	-.635	-4.802	2.501	0.529

* Significant p value<0.05; dependent variable: OHIP-14; reference category; young age, male

Table 6. Simple linear regression model showing the association between OHIP-14 score and gender, age group and treatment need grade

Independent Variable	R^2	B	t	95.0% CI B		p value
				Lower	Upper	
Gender	0.061	5.690	2.619	1.383	9.998	0.010*
Age group	0.016	2.526	1.327	-1.249	6.302	0.187
Treatment need grade	0.022	-1.353	-1.535	-3.101	.395	0.128

* Significant p value<0.05; dependent variable: OHIP-14; reference category; young age, male

Both cross-sectional and longitudinal investigations tested the OHIP's sensitivity and specificity. Notably, previous studies used the validated Arabic version of the OHIP-14 and the IOTN-DC in Saudi orthodontic patients (Al-Jundi et al., 2007; Hassan and Amin, 2010; Hassan et al., 2014; Baidas et al., 2020). The participants in this study had lower OHIP14 scores, than other studied populations, signifying that their perception of how malocclusion affected their OHRQoL was limited (Elmahgoub and Abuaffan, 2015; Olkun and Sayar, 2019; Singh et al., 2019; Paes-da-Silva et al., 2020; Elyaskhil et al., 2021; Kolawole and Ayodele-Oja, 2021). The participants with grade 3 orthodontic treatment needs reported higher scores on the OHIP-14 scale than those with other grades. The OHIP-14 domains of psychological disability and psychological discomfort were found to significantly affect OHRQoL amongst the grade 3 participants. This observation is consistent with the findings of other studies (Elmahgoub and Abuaffan, 2015; Olkun and Sayar, 2019; Baidas et al., 2020; Elyaskhil et al., 2021).

Moreover, the grade 3 participants had significantly higher psychological discomfort scores than the grade 4 participants. Furthermore, their reported psychological disability scores were significantly higher than those of the grade 4 participants, which seems unreasonable from a clinical perspective. This could be due to the higher percentage of women in the grade 3 group. According to previous research, women are more self-conscious about their dental appearance than men (Elmahgoub and Abuaffan, 2015; Olkun and Sayar, 2019). Surprisingly, the social disability scores of the grade 3 group were higher than those of the grade 4 group, with a weak but significant difference. This result contradicts previous studies (Hassan and Amin, 2010; Hassan et al., 2014). Overall, this study found that the patients' perceptions of malocclusion strongly impacted their OHRQoL regardless of their malocclusion severity, which varied from person to person.

The current study found no correlation between orthodontic treatment need and OHRQoL, implying that increased malocclusion severity had no impact on OHRQoL. These findings are in line with previous studies (Elmahgoub and Abuaffan, 2015; Kolawole and Ayodele-Oja, 2021). However, these findings contrast with those of other

studies that associated malocclusion severity with a great impact on OHRQoL (Onyeaso, 2009; Hassan and Amin, 2010; Hassan et al., 2014; Demirovic et al., 2019; Olkun and Sayar, 2019; Paes-da-Silva et al., 2020; Elyaskhil et al., 2021). Hence, this study's findings could be attributed to an IOTN-related shortcoming. The displacement rank may have exaggerated the DHC scale, resulting in high scores for normal occlusion. Furthermore, the sample, which included different IOTN-DC grades, was randomly collected from patients seeking orthodontic treatment at specific intervals, and this may have influenced the results. Although, the result of this study showed no association between orthodontic treatment need and oral heath related quality of life. It was observed that the standard deviations of all question domains were large when the OHIP-14 scores were assessed with IOTN-DC, gender, and age, suggesting that malocclusion alone did not determine OHRQoL and that other factors may have contributed (Elmahgoub and Abuaffan, 2015; Demirovic et al., 2019; Paes-da-Silva et al., 2020; Kolawole and Ayodele-Oja, 2021).

Most participants in this study were female, which could be because most patients seeking orthodontic treatment are female. In the present study, female participants reported that malocclusion more significantly impacted their quality of life than male participants, which is in agreement with other studies (Elmahgoub and Abuaffan, 2015; Olkun and Sayar, 2019). The regression analysis indicated that gender impacted OHRQoL and female participants reported negative OHRQoL scores. Moreover, aging seemed to decrease quality of life in all aspects. The age groups included in the present study (14–19 and 20–25 years old) who are less reliable in their perception of malocclusion than adults. This could explain why this study found no significant difference between the age groups regarding their OHIP-14 scores. This finding contradicts previous studies that claimed age positively impacts OHRQoL (Hassan and Amin, 2010; Olkun and Sayar, 2019; Elyaskhil et al., 2021). Because this study's sample was taken from a single clinical setting, the findings should be interpreted with caution relative to the total Saudi population. To confirm our findings, additional multi-centred studies with larger samples should be conducted.

CONCLUSION

This study found that malocclusion had no discernible detrimental effects on OHRQoL and its domains. Only the borderline treatment group reported that malocclusion had a significant negative impact on psychological discomfort and psychological disability. No variations in the influence of malocclusion on oral health quality were identified between the age groups. However, the female participants reported a significant detrimental impact of malocclusion on their oral health quality.

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Biotechnological Communication

Physical and Antioxidant Properties of Oyster Mushroom *Pleurotus florida* in Response to Different Drying Methods

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ABSTRACT

Drying is a traditional unit operation employed for shelf-life extension of perishable food item. Oyster mushroom being a potent source of bioactive components is highly perishable owing to its high moisture content. The aim of this work was to extend the shelf life of oyster mushroom and to preserve its bioactive components. In this study the impact of different drying methods on physical and bioactive components was analysed. The prepared mushroom slices were subjected to different drying methods viz., sun, solar, oven (40°C), microwave (300 W), freeze (-60°C) and osmotic drying (14% salt solution). The highest and lowest L* value of 81.02 and 58.19 were recorded for freeze and microwave dried oyster mushroom, respectively. All the drying methods caused a significant reduction in the water activity (aw) which is the principle underlying the preservation by drying. The least water activity (0.45) was recorded for freeze-dried oyster mushroom, while as sun dried sample depicted highest value (0.62). The reduction in water activity in response to drying methods followed the order as Freeze drying<Osmotic drying<Microwave drying<Oven drying<Solar drying<Sun drying. The antioxidant potential in terms of total phenols, total flavonoids, DPPH scavenging activity (IC50) and reducing power (EC_{50}) was found to be highest for freeze-dried oyster mushroom corresponding to values of 408.562 mg GAE/100g, 146.231 mg QE/100g, 0.068 mg/ml and 0.142 mg/ml, respectively. All the drying methods affected physical and bioactive components significantly. The freeze dying resulted in better retention of antioxidant potential and colour attributes of oyster mushroom in contrast to other drying methods.

KEY WORDS: DPPH SCAVENGING ACTIVITY, FREEZE DRYING, MICROWAVE DRYING, REDUCING POWER.

INTRODUCTION

Oyster mushroom, a popular food product, is a rich source of proteins containing all the essential amino acids. It belongs to class Basidomycetes and family Agaricaceae with *P. ostreatus, P. florida, P. eryngii, P. tuberegium* and *P. sajor-caju* being common species (Kues and Liu 2000; Deepalakshmi and Mirunalini 2014). These mushrooms are low in fat (0.8-7%) and serve as an excellent source of non-starchy carbohydrates, dietary fiber, minerals and vitamins. Pleurotus mushrooms apart from providing traditional nutrients offer health promoting benefits due to the presence of biologically active substances like alkaloids, phenols, terpenes, antioxidants and, consequently, are regarded as functional foods. They also exert anti-diabetic, anti-carcinogenic and hepatoprotective benefits when consumed (Randive 2012; Thatoi and Singhdevsachan 2014; Maheshwari et al. 2020).

However, the presence of large amounts of water (80-85%) makes them highly perishable with shelf life of only 2-3 days which is further reduced at temperatures of 18°C and above (Randive 2012; Thatoi and Singhdevsachan 2014; Maheshwari et al. 2020). Of all the preservation methods, drying is an important preservation technique which can be employed for long-term preservation of oyster mushroom as it offers a number of advantages like low operating cost, mass and volume reduction of food product thereby minimizing packaging, handling, storage and transport costs (Arumuganathan et al. 2010; Piskov et al. 2020).

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Drying is a unit operation involving simultaneous mass and heat transfer thereby reducing water activity and consequential shelf-life extension. However, drying induces a no. of changes in physical and chemical composition of foods like colour deterioration, enzymatic changes, degradation of antioxidant components. A number of drying methods can be employed for the preservation of oyster mushroom like oven drying, sun, solar, microwave osmotic and freeze drying (Piskov et al. 2020). Each of these drying methods has got their own advantages and limitations specific to them. The uncontrolled operating condition like temperature and relative humidity during sun and solar drying accelerate the physical and chemical changes occurring in foods. The high temperatures employed during oven and osmotic drying, reduce the drying times but at the same time degrade heat sensitive components like vitamin C and antioxidants (Gąsecka et al. 2020). The microwave drying, despite being faster, induces physical and textural changes in food materials (Bashir et al. 2020).

The freeze drying method employs vacuum and low temperatures thereby retaining physical and chemical properties of food, but the associated high cost and longer drying times limit its application (Izli 2017). A study carried out by Ucar and Karadag (2019) concluded freeze drying as most the favourable methods for retention of physical components in contrast to vacuum drying. However,5 the studies comparing impact of the six drying methods (sun, solar, oven (40°C), microwave, freeze and osmotic drying on the bioactive and physical components of oyster mushroom (*P. florida*) are limited (Izli 2017; Ucar and Karadag 2019). The purpose of the study was to compare the effect of six different drying methods (sun, solar, oven (40°C), microwave, freeze and osmotic drying) on quality parameters of oyster mushrooms with focus on bioactive components.

MATERIAL AND METHODS

Freshly harvested oyster mushrooms (*Pleurotus florida*) were obtained in a single lot from the Division of Plant Pathology, SKUAST- Jammu. The procured oyster mushrooms were trimmed and washed thoroughly under running water to remove adhering soil and dirt. The washed mushrooms were drained and sliced into small pieces (Bashir et al. 2020). The oyster mushroom slices (500g) were then divided into six lots and subjected to different drying methods as given in Table 1. The moisture loss of samples was recorded periodically with an electronic moisture analyzer (Citizon MB 50C). The colour attributes (L*, a*, b*) of samples were measured in accordance with the method described in the past (Roueita et al. 2020). The water activity of samples was measured as per the method of AOAC, 2005 using an Aqualab water activity meter (Model series 3TE) with the readings corrected at 20°C. For determination of anti-oxidant components, a methanolic extract of samples was prepared according to the method described in previous studies (Jeena et al. 2014; Roueita et al. 2020).

Table 1. Details of drying experiment

Treatment	Drying method	Temperature
T1	Sun drying	Ambient
T2	Solar drying	Ambient
T3	Oven drying	40˚C
T4	Microwave drying using Samsung CE137NEL microwave convective oven with the technical specifications of 230 V, 50 Hz and 3100 W.	40˚C
T5	Freeze drying using a freeze drier (Martin Christ Type 101041) with chamber temperature of -60°C, under vacuum (<13 Pa of total pressure) and a condenser temperature of -50°C	-60˚C
T6	Osmotic dehydration (brining) 14 per cent salt solution followed by oven drying	40˚C

The anti-oxidant activity in terms of free radical scavenging activity (IC_{50}; mg/ml) and Reducing power (EC_{50}; mg/ml) was estimated as per methods described by Jeena et al. (2014) and Mujic et al. (2009), respectively. The Folin-Ciocalteau (FC) method based on electron transfer as given by Ahmed and Abozed (2015) was employed for estimation of total phenolic content of samples. A suitable calibration curve prepared from different concentrations of standard Gallic acid solution was prepared, and the total phenolic content of samples was expressed as mg Gallic acid equivalents (GAE) per gram of sample. A method described by Dewanto et al. (2002) was used for quantification of the total flavonoid content of sample extract using standard calibration curve of Quercetin and was expressed as mg Quercetin (QE) per gram of sample. Statistical analysis was carried out using Opstat software. A completely randomized design with three replications with three sub-samples of all experiments was carried out. Significant differences between different drying methods were determined at the significance level of $p<0.05$ (Dewanto et al. 2002; Mujic et al. 2009; Jeena et al. 2014; Ahmed and Abozed 2015; Bashir et al. 2020).

RESULTS AND DISCUSSION

Physical parameters: Colour: In dried foods colour is a quality indicator as it gives an idea related to the comparative change in colour of fresh and dried material

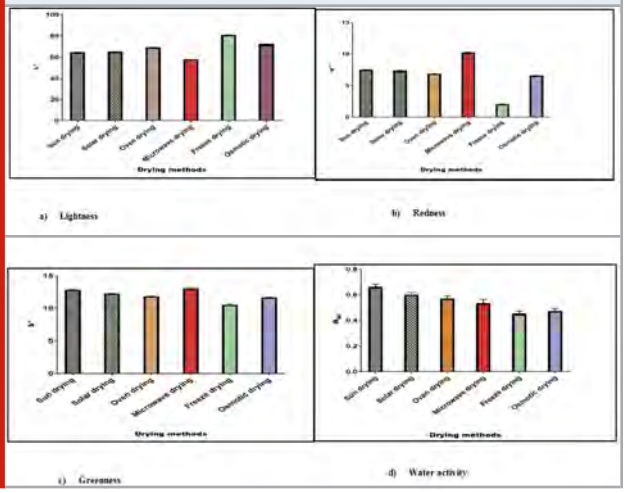
(Bansal et al. 2013; Xu et al. 2021). The different drying methods resulted in decrease in L* (lightness), an increase of a * (greenness) and b* (redness) values [(Figure 1 (a), (b) and (c)]. This is in accordance with the findings of previous studies (Duan and Xu 2015). During the drying process, the colour changes in oyster mushroom could be correlated with enzymatic and non-enzymatic (maillard) reactions. The freeze-dried samples exhibited the highest (81.02) L* value followed by osmotic, oven, sun, solar and microwave dried mushrooms. The least a* value (2.09) was reported in freeze-dried oyster mushroom followed by osmotic, oven, solar, sun and microwave dried mushroom powders.

The highest b* (12.89) value was reflected by sun dried oyster mushroom and the least b* (10.41) was recorded for freeze-dried sample. The b* value of osmotic, oven, solar, sun and microwave dried mushroom was recorded in increasing order corresponding to values of 11.68, 11.85, 12.29, 12.89 and 13.09, respectively. The least colour change was reflected by freeze-dried oyster mushroom while microwave dried mushroom exhibited the highest colour change. Ali et al. (2016) while working on guava drying reported similar results of minimum colour change in freeze-dried samples followed by oven, sun and microwave dried guava slices (Duan and Xu 2015; Ali et al. 2016; Xu et al. 2021). Coklar et al. (2018) also reported greater colour change in microwave and oven-dried hawthorn fruit than freeze-dried. The minimum colour changes during freeze-drying might be because of the sublimation of frozen ice directly to vapour, reduced availability of oxygen and inactivation of polyphenoloxidase enzyme due to low temperature preventing enzymatic browning reactions thereby stabilizing colour (Henriquez et al. 2013). The osmotic dried oyster mushrooms in comparison to oven-dried mushrooms were less dark in colour exhibiting higher values of L* and lower a* and b* values (Henriquez et al. 2013; Coklar et al. 2018; Nowak and Jakubczyk 2020).

The solute uptake during osmotic treatment reducing oxygen transfer to surface results in lesser oxidation of colour pigments and leaching of soluble substances during steeping step of osmotic drying that otherwise act as a substrate for browning reactions might be responsible for better colour retention in osmotic dried oyster mushroom (Velickova et al. 2014). Kaur et al. (2014) while working on osmotic convective drying of oyster mushrooms also reported that osmotic treatment resulted in better retention of colour. The sun and solar dried oyster mushrooms reflected greater colour change than the oven-dried sample, which might be because of the accelerated browning reactions, mainly oxidation of phenolic compounds by polyphenolase enzyme catalysed UV- radiations of sun light (Ginat and Alghamdi 2013; Kaur et al. 2014; Nowak and Jakubczyk 2020).

Furthermore, in comparison to sun and solar drying, higher temperatures employed during oven drying might have resulted in inactivation of enzyme catalysing browning reactions (Karabulut et al. 2007). The sun-dried samples exhibited lesser colour change than the microwave dried samples similar to findings already available, while studying colour changes in apricot pestil in response to different drying treatments (Suna et al. 2014; Nowak and Jakubczyk 2020).

Figure 1: Effect of different drying methods on physical parameters of oyster mushroom



Water activity (aw): The different drying methods decreased the water activity of oyster mushrooms to different levels represented in Figure 1(d). The least water activity (0.45) was reflected by freeze-dried oyster mushroom followed by osmotic and microwave dried oyster mushrooms corresponding to values of 0.47 and 0.52, respectively. Similar results were reported in past studies, while studying the effect of different drying methods on water activity of apple slices, respectively (Baysal et al. 2015; Nowak and Jakubczyk 2020).

The least water activity value of freeze-dried mushroom might be because of greater removal of moisture content as a result of sublimation of moisture. The highest value of water activity was recorded for the sun-dried sample depicting a value of 0.62 (Ali et al. 2016). This might be because of fluctuating temperature and humidity resulting in less effective heat transfer, thereby leading to lesser removal of water (Muyanja et al. 2012). The osmotic drying decreased water activity to a greater extent than microwave and oven drying, which might be because of solute uptake during steeping in brine solution resulting in a concentration gradient thereby facilitating greater water removal (Salim et al. 2016). Similar results were reported in the past studies during the drying of oyster mushrooms (Aishah and Rosli 2013; Sharma and Bhat 2018; Nowak and Jakubczyk 2020).

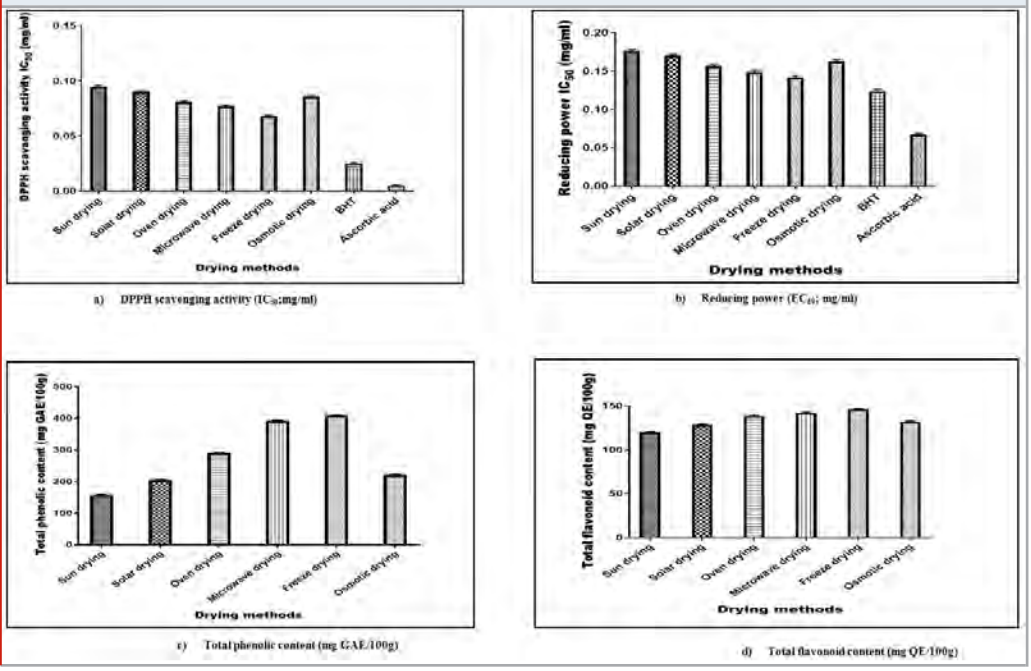
Bioactive components: Antioxidant activity: The data pertaining to the effect of drying methods on antioxidant activity of oyster mushroom is presented in Figure 2 (a). The drying methods in general resulted in decrease of antioxidant activity of oyster mushroom. The decrease in antioxidant activity during drying might be attributed to thermal degradation of ascorbic acid and polyphenols mainly responsible for antioxidant activity. Lim and Murtijaya (2007) also reported a decrease in antioxidant activity of *Phyllanthus amarus* extract in response to various drying

methods (Lim and Murtijaya 2007; Ruvini et al. 2017). The DPPH scavenging activity was expressed in terms of IC50, the concentration required for 50 per cent inhibition. The lower the value of IC50, the greater is the antioxidant activity. The IC50, value of DPPH scavenging activity followed the order of sun drying>solar drying>osmotic drying>oven drying>microwave drying>freeze drying.

The highest antioxidant activity was reflected by freeze-dried oyster mushroom corresponding to IC_{50} value of 0.068 mg per ml. Zhang et al. (2009) while studying the effect of drying methods on antioxidant activities of shitake mushroom also reported better retention of antioxidant activity in order of freeze-dried shitake mushroom followed by microwave, oven and sun-dried mushroom, thus confirming our results. Ji et al. (2012) also reported higher radical scavenging activity in freeze-dried button mushroom followed by hot air- and sun-dried button mushroom similar to our results. The oven-dried mushroom exhibited higher antioxidant activity and lower IC50, value of 0.081 mg per ml in comparison to sun and solar dried samples. Mechlouch et al. (2012) while studying the effect of sun and solar drying on antioxidant activity of tomato also reported higher radical scavenging activity in solar dried sample than sun dried one similar to our findings. The microwave dried mushroom reflected higher radical scavenging activity compared to oven-dried oyster mushrooms (Nowak and Jakubczyk 2020).

The osmotic dried mushroom reflected higher IC50, value of 0.086 mg per ml indicating lesser antioxidant activity than oven-dried mushroom. Similar results were reported in past studies in guava (Patel et al. 2016). The reductones/ reducers in a substance act as an antioxidant by donating the hydrogen atom, thereby terminating the free radical chain or by reacting with precursors of peroxide and preventing their formation (Ma et al. 2013). The reducing power of dried mushroom extracts was expressed in terms of EC_{50}, values, i.e., concentration at 50 per cent of absorbance. The lower the value of EC50, the greater is the reducing power or antioxidant potential.

Figure 2: Effect of different drying method on bioactive components of oyster mushroom



The reducing power of dried oyster followed the order of freeze-dried oyster mushroom>microwave dried oyster mushroom>oven-dried oyster mushroom>osmotic dried oyster mushroom>solar dried oyster mushroom>sun dried oyster mushroom as reflected in Fig 2(b). Similar trend was reported by Ji et al. (2012) in *Robinia pseudoacacia* flowers. Chan et al. (2009) while studying the effect of different drying methods on antioxidant activity of *Alpinia zerumbet* leaves observed greater loss of reducing power in oven-dried material than microwave dried samples. Que et al. (2008) also reported higher reducing power in freeze-dried pumpkin powder than the oven-dried one. Siriamornpun et al. (2014) while working on papaya also reported greater loss of reducing power during osmotic drying than oven drying (Siriamornpun et al. 2014; Nowak and Jakubczyk 2020).

The better retention of antioxidant activity in freeze drying might be attributed to low temperature and vacuum associated with freeze drying that does not cause thermal degradation and oxidation of polyphenolic substances (Zhang et al. 2009). The lesser antioxidant activities reflected by sun and solar dried samples might be because of inefficient inactivation of polyphenolase enzyme and accelerated oxidation catalyzed by UV-radiations of sun leading to excessive loss of phenolic substances and ascorbic acid contributing to antioxidant activity. The disruption of cellular structure and release of bound phenolics might

be responsible for higher antioxidant activity during microwave drying (Incheun et al. 2010; Kankara et al. 2014; Liu et al. 2020). The leaching of soluble antioxidant components like vitamin C and some phenolic acids from food matrix during osmotic drying might be responsible for low antioxidant activity in osmotic dried mushroom in comparison to oven (Phisut et al. 2013; Liu et al. 2020).

Total phenolic content: The effect of different drying methods on total phenolic content of oyster mushrooms is represented in Figure 2 (c). The drying process caused a decrease in the total phenolic content of oyster mushroom. The exposure of food products to high temperatures for longer times and the enzymatic processes catalysed by light and air might be responsible for reduction in total phenolic content during drying (Youssef and Mokhtar 2014). This is in accordance with the findings of Henriquez et al. (2013) reporting a decrease in total phenolic content of apple peel subjected to different drying processes. The reduction in total phenolic content of oyster mushrooms was highest in sun drying followed by solar drying, osmotic drying, oven drying, microwave drying and freeze drying. The highest total phenolic content was reflected by freeze-dried oyster mushroom corresponding to a value of 408.562 mg GAE per 100 g dry matter followed by microwave and oven-dried oyster mushroom depicting a value of 392.001 and 290.172 mg GAE per 100g, respectively (Liu et al. 2020).

This is similar to the findings of Assefa and Keum (2017) revealing the highest total phenolic content in freeze-dried yuzu fruit than the microwave and oven-dried sample. The total phenolic content of osmotic dried oyster mushroom was found to be lesser than the oven-dried mushroom, which is similar to the findings of Djendoubi et al. (2013). Among the dried oyster mushrooms, the sun-dried sample exhibited the least total phenolic content of 157.210 mg GAE per 100 g. Vu et al. (2017) reported least phenolic content in the sun-dried banana peel than the oven, microwave and freeze-dried samples. The freeze-drying method resulted in better retention of total phenolic content which might be because of the low temperatures and vacuum employed during freeze drying that inhibit thermal and oxidative degradation of phenolic compounds (Zhang et al. 2013; Ozay-Arancioglu et al. 2021).

Furthermore, during freeze drying the formation of ice crystal in the cellular structure of sample may disrupt the same allowing easy access of solvents and better extraction of phenolic components leading to higher values of total phenolic content (Orphanides et al. 2013). The intense heat generated during microwave drying resulted in high vapour pressure and high temperature in food matrix leading to cellular disruption and consequential release of bound phenolics might be responsible for greater total phenolic content of microwave dried samples. The migration of phenolic compounds in osmotic solution may be responsible for higher reduction of total phenolic in osmotic drying (Stojanovic and Silva 2007; Izli et al. 2018). In sun and solar drying the prolonged drying periods facilitating oxidation and enzymatic degradation might be responsible for greater loss of phenolic components (Orphanides et al. 2013; Ozay-Arancioglu et al. 2021).

Total flavonoid content: Figure 2 (d) reveals the effect of different drying methods on the total flavonoid content of oyster mushrooms. The decrease in total flavonoid content during drying might be because of thermal degradation and oxidation during drying (Chauhan et al. 2015). The flavonoid loss during drying might also be correlated with polymerization and oxidation catalysed by various factors like temperature, pH and enzymes (Si et al. 2016). The loss of total flavonoid content was found to be the least in freeze drying followed by microwave, oven, osmotic, solar and sun drying. Vu et al. (2017) while working on banana peels reported total flavonoid content in order of freeze-dried peel>microwave dried banana peel>oven-dried banana peel>sun dried banana peel quite consistent with the findings of the present study. The greater total flavonoid content of the freeze-dried oyster mushroom might be because of lower temperatures and vacuum associated with freeze drying resulting in better retention (Ibrahim et al. 2013; Vu et al. 2017; Ozay-Arancioglu et al. 2021).

The greater total flavonoid content of microwave in comparison to oven, sun and solar drying might be because of shorter drying times associated with microwave drying. The least total flavonoid content of 120.092 mg QE per 100 g was recorded for sun dried mushroom consistent with the past findings (Shahat et al. 2016). The longer drying times and exposure to oxygen associated with sun and solar drying might be responsible for greater loss in flavonoid content as flavonoid degradation is dependent on exposure time, enzymes, light and oxygen catalysing their breakdown (Ibrahim, et al. 2013). The osmotic dried mushroom in comparison to oven-dried mushroom exhibited lower total flavonoid content which might be because of weakening of cellular structure during osmotic treatment resulting in greater leaching of flavonoid components (Zainol et al. 2009; Ozay-Arancioglu et al. 2021).

CONCLUSION

The findings of the present study depict that the different drying methods had a significant impact on physical parameters and bioactive components of oyster mushrooms. The oyster mushrooms subjected to freeze drying gave best results in respect of antioxidant activity (DPPH scavenging activity; IC_{50}: 0.068 mg per ml and reducing power; IC_{50}: 0.142 mg/ml), total phenols (408.562 mg GAE/100g), total flavonoids (146.231 mg QE/100g). Furthermore freeze-dried oyster mushroom recorded least colour change corresponding to highest L* value of 81.02 and least a* and b* values of 2.09 and 10.41, respectively. The water activity value of dried oyster mushroom ranged from 0.45 to 0.62. The freeze-dried mushroom powder loaded with bioactive components can be used to formulate functional foods and in conventional foods like cakes, breads and noodles as a functional ingredient.

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Biotechnological Communication

Impacts on Foliar Application of Copper Nanoparticles for the Growth in *Zea mays*

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ABSTRACT

The aim of the present study is to analyse the growth of *Zea mays* L. supplemented with different concentrations (20 ppm, 40 ppm and 60 ppm) of copper nanoparticles. Green synthesis of copper nanoparticles was obtained from the leaf extract of Zea mays L. The synthesized nanoparticles were characterized and confirmed using UV-Vis spectrometric analysis, SEM, EDAX and FTIR analysis. Copper nanoparticles were conformed based on the colour change (sea green) and the peak at 600 nm using UV Visible spectrophotometer is due to Surface Plasmon resonance of copper nanoparticles. Scanning electron microscope analysis of copper nanoparticles reveals that the size ranges between 150-200 nm. The present investigation involves a novel method of synthesizing the nanoparticles from the leaf extract of *Zea mays* L. and utilizing the same for the growth analysis of the same plant. Seed germination of *Zea mays* L. supplemented with copper nanoparticles showed maximum growth in root and shoot length at 20 ppm concentration of nanoparticles. In vivo growth analysis of *Zea mays* L. supplemented with copper nanoparticles at one time exhibited maximum growth at 20 ppm concentration. Similarly In vivo growth analysis of *Zea mays* L. supplemented with copper nanopartilces continuously for 15 days also revealed that 20 ppm concentration of copper nanoparticles with optimal growth characteristics in *Zea mays* L. However, with increasing concentrations (40 ppm and 60 ppm) of copper nanoparticles resulted in the decrease of growth and protein content. This reveals the toxicity of copper nanoparticles in *Zea mays* L. Accumulation of silver nanoparticle in the plant was measured using atomic absorption spectroscopy.

KEY WORDS: COPPER NANOPARTICLES, IN VIVO, SEED GERMINATION, *ZEA MAYS* L.

INTRODUCTION

Nanotechnology opens an oversized scope of novel application within the fields of agricultural industries and biotechnology, as a result of their distinctive physiochemical properties, i.e., tunable pore size, high extent, high reactivity, and particle morphology. Although fertilizers are very important for plant growth and development, most of applied fertilizers are rendered unavailable due to many factors such as leaching, degradation by photolysis, hydrolysis and decomposition. Hence, it's necessary to reduce the nutrient losses in fertilization and increase the crop yield through exploitation of latest applications with facilitate of applied science and nanomaterials (Siddiqui et al. 2015; Milewska et al. 2016).

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Nanotechnology has the ability to increase the yield of nutrient values and also plays a vital role in developing improved systems for monitoring ecological conditions and increasing the capacity of crops to absorb nutrients. There are majority of nano-material which is known for its plant growth promoting effects. Recent researches on effects of nanoparticles on various plant crops have reported with increased germination and growth of seeds (Farooqui et al. 2016). In recent years, numerous studies have been conducted to analyze and describe the influence of nanoparticles on plant growth. Many studies have been performed on the impact of nanoparticles on the physiological and metabolic processes that directly influence plant growth and development. An investigation of the response of six crop species: barley, maize, rice soybean, switchgrass, tomato and tobacco showed that nanomaterials accelerate seed germination and enhance the growth (Milewska et al. 2016; Patil et al. 2018; Rajkumar et al. 2019).

The elements including Copper, Magnesium, Nickel and Zinc play vital functions in plant cells. Micronutrients are

essential for biosynthesis and function of nucleic acids, growth substances, chlorophyll and secondary metabolites, as well as for growth and stress resistance, on the other hand they are required in a small trace, whereas turns toxic at higher concentrations. Lower concentrations of copper sulphate and copper sulphate nanoparticles induced increasing in shoot length, root length and fresh weight of *Verbena bipinnatifida* Nutt. Copper enhanced the seedling growth of *Vigna radiate* at low level supplementation. High level retarded the growth of Vigna radiate by interfering the normal cellular metabolic events (Genady et al. 2016). The effect of copper nanoparticles on germination and growth of soybean and chickpea seeds revealed that lower concentration of nanoparticles increased the growth, however high levels become phytotoxic for morphological, physiological and biochemical changes in plants (Mustafa et al. 2017; Patil et al. 2018; Rajkumar et al. 2019).

The Cu deficiency in plants is expressed as curled leaves, petioles bent downwards and light chlorosis along with permanent loss of turgor in the young leaves. Chronic Cu deficiency develops a rosette form of growth. Diagnosis of Cu deficiency in plants is an important as it results in yield losses, with little evidence of the characteristic symptoms. Cu deficiency may become more prevalent in coming future, the applications made 10 to 30 years ago would be running out and increased use of nitrogenous fertilizers will lead to severity of Cu deficiency (Shobha et al. 2014). Copper nanoparticles possess broad range of applications like antimicrobial materials, heat transfer systems, sensors, catalysts and super strong materials. They are very reactive because of their high surface to volume ratio and can easily interact with other particles. Copper nanoparticles possess a strong antibacterial activity and were able to decrease the microorganism concentration by 99.9% (Shobha et al. 2014; Sriram and Pandidurai 2017; Patil et al. 2018).

For the synthesis of copper nanoparticles, both the precursor (plant extract) and the reducing agent (copper sulphate) were mixed in a clean tube in 1:1 proportion. For the reduction of copper ions, 5ml of freshly prepared aqueous plant extract was mixed with 5 ml of freshly prepared 0.001M aqueous copper sulphate solution. It was then kept for incubation for 1hour. After the incubation period color change to sea green from dark brown was noted. This color change indicates synthesis of copper nanoparticles (Sriram and Pandidurai 2017). To investigate the size, shape and composition of copper nanoparticles Scanning electron microscopy and X ray diffraction studies were employed. The outcome of these studies will take a step closer in designing simple, environmentally friendly and low-cost synthesis method of copper nanoparticles (Patil et al. 2018; Rajkumar et al. 2019).

Zea mays L. (Maize or Corn) is a cereal belonging to the Poaceae family. The plant has rich source of carbohydrate, fat, vitamins, minerals and protein. Reports reveal that biomolecules from maize are responsible for the formation nanoparticles. Maize is considered as the most emerging versatile crop possessing wider adaptability under varied agro-climatic condition. Maize is the third most important cereal in the world with wide market potential (Sriram and Pandidurai 2016; Rajkumar et al. 2019). Although soil and climatic conditions of Pakistan favour successful production of maize but inappropriate planting methods significantly reduce the maize production. Planting method is an important agronomic practice for enhancing crop yield (Tanveer et al. 2014; Rajkumar et al. 2019). The present investigation involves a novel method of synthesizing the nanoparticles from the leaf extract of *Zea mays* L. and utilizing the same for the growth analysis of the same plant. Seed germination and in vivo growth of *Zea mays* L. supplemented with various concentrations (20 ppm, 40 ppm and 60 ppm) of copper nanoparticles was analysed based on several parameters like root, shoot leaf length, leaf surface area and whole plant weight. Chlorophyll content of the leaf was also measured.

MATERIAL AND METHODS

Maize leaves were collected from Tamil Nadu Agricultural University, Madurai. The collected leaves were washed with double distilled water and air dried. Then it was cut into small pieces and homogenized with the help of mortar and pestle and dispensed in 100 ml of distilled water and heated for 5 minutes at 70-80oC. The extract was then filtered using Whatman's No.1 filter paper. The filtrate was collected in a clean and dried conical flask by standard sterilized filtration method and was stored at 30º C. The copper nanoparticles were synthesized by mixing the precursor (*Zea mays* L. leaf extract) and the reducing agent (copper sulphate) in 1:1 proportion in a clean conical flask. For this reaction 50 ml of freshly prepared 0.01 M aqueous copper sulphate solution was mixed with 50 ml of filtered plant extract. The resultant solution was observed for its color change after 1 hour at room temperature (Rajput et al. 2018).

For the purification of synthesized copper nanoparticles, the solution was centrifuged for 10 minutes at 10, 000 rpm after the color change. Pellet were collected and concentrated. It was mixed with equal volume of distilled water. Centrifugation process was repeated many times to get better separation of other entities of the copper nanoparticles. For UV-Visible spectra analysis, UV-Visible spectrophotometer was used for monitoring the reduction of copper to nanoparticles. The sample was mixed with distilled water and UV-Visible spectral analysis was done between the range of 340-600 nm. The analysis was done in every 1, 3, 6, 12 and 24 hours) (Sriram and Pandidurai 2017; Rajput et al. 2018). Fourier transform infrared spectroscopy (FTIR) spectrophotometer is used to analyze the functional groups present in the sample. For this analysis the sample and potassium bromide were mixed in the ration of 1:100 respectively. It was incubated for overnight at 110oC. The mixture was cooled and compressed with hydraulic press. The resultant was scanned with FTIR in the range of 500-4000cm-1. The elemental composition of sample was determined by Energy Dispersive X-ray analysis (EDAX). The EDAX analysis system works as an integrated feature of a scanning electron microscope (SEM) and cannot operate on its own without the latter (Ghosh et al. 2020).

The embedded copper nanoparticles in the filtrate were subjected to Scanning Electron Microscope (SEM)

analysis after drying under vacuum. Scanning Electron Microscope was used to examine the powdered sample of copper nanoparticles. The surface and it internal structures of copper nanoparticles are visualized using this study (Wu et al. 2020). For the seed germination analysis, seeds of *Zea mays* L. were obtained from Tamil Nadu agricultural College, Madurai. Seed germination study was carried out in a Petri dish placed with a water porous filter paper. To each 5ml of copper nanoparticles with various concentrations (20ppm, 40ppm and 60ppm) was added. The seeds were incubated in dark and germination was monitored during 6th and 12t^{h} day. A total of 50 seeds were used for studying the seed germination analysis (Almutairi and Alharbi 2015). The parameters used are Root length, Shoot length, Fresh weight (12th day), Dry weight (12th day).

For the *In vivo* growth analysis of *Zea mays* L, the experimental soil for raising the cultivars was sandy loam. The soil was sterilized by solar sterilization method for 5 days. It was then analyzed for its physio chemical properties. The analyzed soil was taken in earthen pots of size 30x33 cm and filled in for about two-third of their height (5 kg of soil per pot). *In vivo* growth of *Zea mays* L. supplemented with nanoparticles by foliar spray was carried out by two different methods

(I) Different concentration of nanoparticles (20 ppm, 40 ppm and 60 ppm) supplemented at one time.
(II) Different concentration of nanoparticles (20 ppm, 40 ppm and 60 ppm) supplemented continuously for 15 days. On 15th day the following parameters were analyzed for the growth of *Zea mays* L. using nanoparticles. The parameters used are Root length, Shoot length, Leaf length, Leaf surface area, Fresh weight & Dry weight (Tanveer et al. 2014). For the analysis of chlorophyll content, 1 gram of finely cut leaf was homogenized using a mortar and pestle. The leaf material was added with 0.5 grams of Magnesium carbonate and 20 ml of 80% acetone. Grinding was continued (Tanveer et al. 2014; Wu et al. 2020).

The leaf material was then refrigerated for 4 hours at 4°C. Centrifugation was carried out at 500 rpm for 5 minutes. After centrifugation in a volumetric flask the supernatant was alone transferred. 80% of acetone was used to made up the final volume to 100 ml. The solution was estimated using a spectrophotometer with absorbance at 663 and 645 nm. Acetone (80%) was used as blank (Plaksenkova et al. 2019).

Chlorophyll content was measured using the formula

Chlorophyll a = 11.75 x A_{662} -2.35xA_{645} Chlorophyll b= 18.61 x A_{645} -3.96xA_{662}
Where A_{662} is Absorbance at 662 nm, A_{645} is Absorbance at 645 nm.

For statistical analysis, each experiment was repeated three times and each treatment had 10 replicates. All data obtained were subjected to Standard deviation and one way analysis of variance (ANOVA). For the analysis of copper nanoparticles in plant material, the nanoparticles accumulated in experimental plants were assayed after 15 days. Copper concentrations in plants were analyzed using the method of Baker et al. (1994). The plant sample as a whole was washed, dried in oven at 1600C for 40 minutes and digested in a mixture of nitric acid and perchloric acid (10:1). Then the solution was centrifuged at 5000 rpm for 5 minutes and double filtered with Whatmann filter paper no.4 and the filtrate were used for analysing the concentration by Atomic Absorption Spectrometry (Shimadzu Model AA–6300), available in the Science Instrumentation Centre of Ayya Nadar Janaki Ammal College (Autonomous), Sivakasi, Tamil Nadu. The Accumulation Factor (AF) was considered to determine the quantity of nanoparticles absorbed by the plant from soil. This is an index of the plant to accumulate a particular nanoparticle with respect to its concentration in the soil and is calculated using the formula (Ghosh and Singh 2005).

$$\text{Accumulation Factor (AF)} = \frac{\text{Nanoparticle concentration in the tissue of the whole plant}}{\text{Initial concentration of metal in the substrate.}}$$

RESULTS AND DISCUSSION

Synthesis of copper nanoparticles from maize leaves: The reduction of copper sulphate using *Zea mays* L. leaf extract was viewed by the color change from dark brown to sea green (Figure 1). Due to the excitation of surface Plasmon vibration in nanoparticles, it exhibits sea green colour. Copper nanoparticles synthesized from *Zea mays* L. involve 5.0 ml of leaf extract and 5.0 ml of 0.001M aqueous copper sulphate solution. After 1 hour of incubation the colour change was found to be sea green. (Sriram and Pandidurai 2017; Plaksenkova et al. 2019; Wu et al. 2020).

Figure 1: Synthesis of copper Nanoparticles from maize leaves.

a) Control b) Copper nanoparticles

Characterization of synthesized copper nanoparticles: UV-Visible spectra analysis of copper nanoparticles: UV-Visible spectrophotometer recorded the reduction of copper sulphate in the leaf extract of *Zea mays* L. Copper nanoparticles exhibited maximum absorbance at 600 nm in various time intervals (Figure 2). The peak at 600 nm is due to Plasmon resonance of copper nanoparticles.

Fourier transform infrared spectroscopy (FTIR) of copper nanoparticles: FTIR spectrum of synthesized copper nanoparticles was shown in Figure 3. Functional groups involvement between metal particles and biomolecules is analyzed by Fourier-Transform Infrared spectroscopy. Identification of biomolecules responsible for capping, reduction and stabilization of metal nanoparticles were

carried out using FTIR. The spectrum showed the band at 1192.01cm-1 corresponds to O=C-O-C stretching of ester, which are very strong bonds. The supernatant with the active functional groups results in swift reduction of copper nanoparticles from Cu ions (Ghosh et al. 2020).

Figure 2: UV-Vis Absorption Spectrum of copper nanoparticles synthesized by using *Zea mays* L. leaf extract

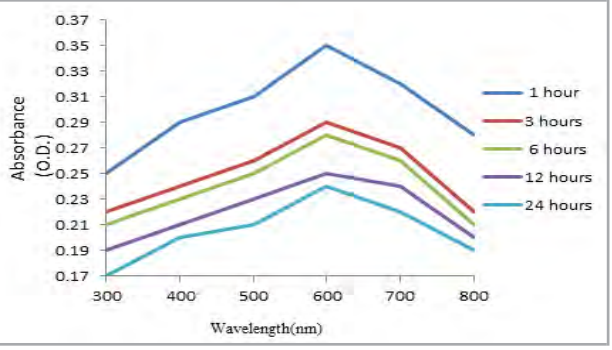
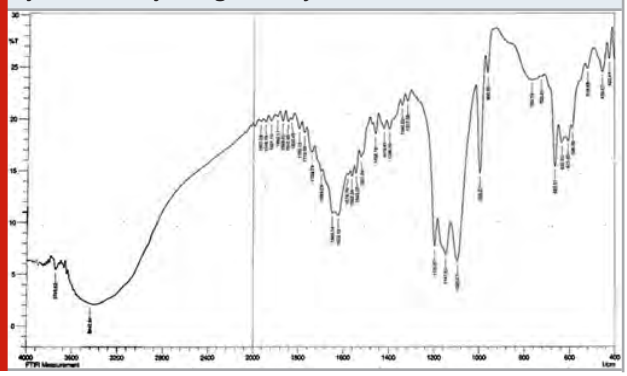
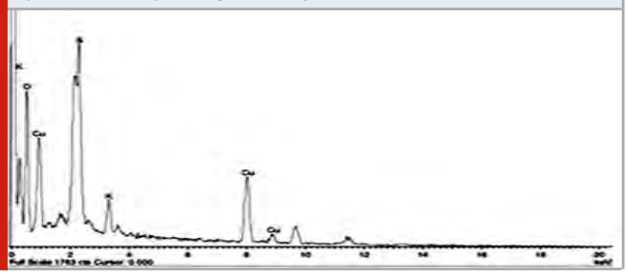


Figure 3: FTIR spectrum of copper nanoparticles synthesized by using *Zea mays* L. extract.



Energy Dispersive X-ray analysis (EDAX) of copper nanoparticles: EDAX spectra were recorded from the synthesized copper nanoparticles and showed a peak in the Cu region, confirmed the copper nanoparticle formation (Figure 5). The horizontal axis displays the energy in electron volts while the vertical axis represents the number of X-ray counts. The lines displayed with major emission energies for copper correspond with the peaks of the spectrum that ensures the identification of copper nanoparticles (Wu et al. 2020).

Figure 4: EDAX analyses of copper nanoparticles synthesized by using *Zea mays* L. leaf extract



Scanning Electron Microscope (SEM) analysis of copper nanoparticles: Copper nanoparticles size and shape were clearly visualized using Scanning Electron Microscope. Figure 6 shows the presence of copper nanoclusters in panoramic view with the size ranging between 150-200 nm. The copper nanoparticles with higher magnification reveals that these nanoparticles are in the form of small nanoclusters with average diameter of 40 nm which has good uniformity. The SEM observations reveal that the size of copper nanoparticles is about 40 to 45 nm (Wu et al. 2020).

Figure 5: SEM image of copper nanoparticles synthesized by using *Zea mays* L. leaf extract

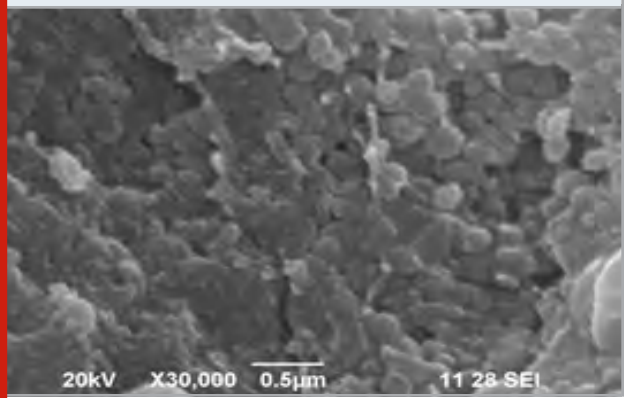
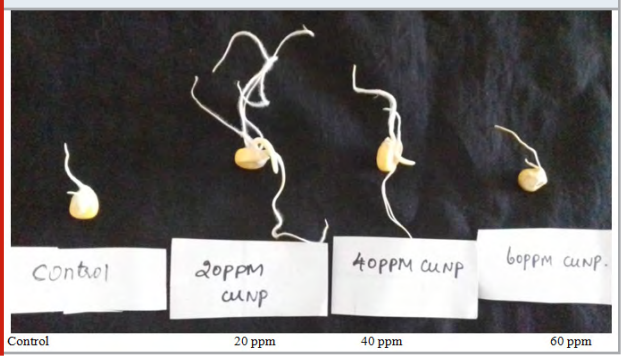


Figure 6: Seed germination analyses of *Zea mays* L. treated with copper Nanoparticles (6th day)



Effect of copper nanoparticles on root and shoot length of *Zea mays* L.: Figure 7 shows the seed germination studies of *Zea mays* L. supplemented with various concentrations of copper nanoparticles. On 6th day different concentrations (20 ppm, 40 ppm and 60 ppm) of copper nanoparticles supplemented to *Zea mays* L. showed maximum growth in root length with 20 ppm (7.30 ± 0.20 cm) (Table 1) The shoot length was higher in 20 ppm (2.17 ± 0.15 cm). On 12th day also the root length was found to be maximum with 20 ppm (14.77 ± 0.31 cm) (Table 1). The shoot length was higher in 20 ppm (4.70 ± 0.10 cm). It is similar to the results obtained by (Rajput et al. 2018). Seed germination analysis of the present study reveals those 20 ppm concentrations of copper nanoparticles increases the root and shoot length on 6th and 12th day when compared to the other concentrations (40 ppm&60 ppm) and control. Similar results were

observed by (Almutairi and Alharbi 2015; Rajput et al. 2018; Wu et al. 2020).

Effect of copper nanoparticles on fresh and dry weight of *Zea mays* L.: Total fresh weight of *Zea mays* L. on 12th day was higher in 20 ppm (3.00 ± 0.10 grams) (Table 2). Similarly, the total dry weight of *Zea mays* L. was higher in 20 ppm (2.90 ± 0.10 grams). It is similar to the results obtained in the past studies (Kolenčík et al. 2019).

***In vivo* growth analysis of *Zea mays* L. supplemented with copper nanoparticles at one time:** Figure 8 shows the *In vivo* growth of *Zea mays* L. supplemented with various concentrations (20 ppm, 40 ppm and 60 ppm) of copper nanoparticles. The root length of *Zea mays* L. was higher in 20 ppm (11.73 ± 0.21 cm), (Table 3). Similarly, the shoot length was higher in 20 ppm (12.27 ± 025 cm). The leaf length of *Zea mays* L. was higher in 20 ppm (45.23 ± 0.25 cm). Similarly, the leaf surface area was higher in 20 ppm (39.67 ± 0.21 cm2) (Rajput et al. 2018; Wu et al. 2020).

Table 1. Effect of copper nanoparticles on root and shoot length of *Zea mays* L. seeds (6th and 12th day)

Concentration of copper nanoparticles (ppm)	6th day		12th day	
	Root length(cm)	Shoot length(cm)	Root length(cm)	Shoot length(cm)
Control	2.23±0.15	0.50±0.10	4.53±0.25	1.07±0.06
20	7.30±0.20	2.17±0.15	14.77±0.31	4.70±0.10
40	4.30±0.10	1.53±0.15	8.67±0.21	3.20±0.10
60	2.90±0.20	0.73±0.06	5.87±0.15	1.80±0.10

Values represent the mean (±) standard error of three independent experiments. All the experiments were statistically analyzed by One-way Anova using SPSS interpretation. The results were significant at $p < .05$

Table 2. Effect of copper nanoparticles on fresh and dry weight of *Zea mays* L. seeds on 12th day

Concentration of copper nanoparticles	Fresh weight of *Zea mays* L.(grams)	Dry weight of *Zea mays* L. (grams)
Control	1.83± 0.06	1.27±0.06
20ppm	3.00± 0.10	2.90±0.10
40ppm	2.10± 0.06	2.07±0.10
60ppm	1.83±0.15	1.73±0.06

Values represent the mean (±) standard error of three independent experiments. All the experiments were statistically analyzed by One-way Anova using SPSS interpretation. The results were significant at $p < .05$

Biomass analyses of *Zea mays* L. supplemented with copper nanoparticles at one time: The root weight of *Zea mays* L. was higher in 20 ppm (0.636 ± 0.004 grams). Similarly, the shoot weight was higher in 20 ppm (0.802 ± 0.003 grams). The weight of the leaf was higher in 20 ppm (0.906 ± 0.003 grams). Similarly, the whole plant weight was higher in 20 ppm (2.344 ± 0.005 grams) (Table 4). In the present study we analyzed the role of copper nanoparticles in *Zea mays* L. and it was revealed that 20 ppm concentration proved best for the growth and yield of *Zea mays* L. based on the parameters like root, shoot length, leaf surface area and leaf length. Similar results are observed in past studies (Hafeez et al. 2015; Wu et al. 2020).

In vivo growth analysis of *Zea mays* L. supplemented with copper nanoparticles continuously for 15 days: Figure 9 shows the *in vivo* growth of *Zea mays* L. supplemented with various concentrations (20 ppm, 40 ppm and 60 ppm) of copper nanoparticles continuously for 15 days. The root length was higher in 20 ppm (12.37 ± 0.15 cm) (Table 5). The shoot length was higher in 20 ppm (13.23 ± 0.25 cm). Based on measuring the leaf length and leaf surface area it was revealed that the leaf length was higher in 20 ppm (46.97 ± 006 cm) (Table 5). Similarly, the leaf surface area was higher in 20 ppm (41.17 ± 0.29 cm^2). The results were similar to results obtained in past studies (Margenot et al. 2018; Wu et al. 2020).

Biomass analyses of *Zea mays* L. supplemented with copper nanoparticles continuously for 15 days: The root weight was higher in 20 ppm (0.637 ± 0.003 grams), (Table 6). The shoot weight was also higher in 20 ppm (0.804 ± 0.001 grams). The leaf weight was higher in 20 ppm (0.923±0.002 grams). Similarly, the whole plant weight was higher in 20 ppm (2.367±0.20grams). In a study the effect of silver nanoparticles on plant growth parameters such as root length, fresh weight dry weight, and germination percentage of fenugreek were analyzed. The result of this experiment showed that use of silver nanoparticles increased the germination in Fenugreek (Hojjat 2015). These results correlate with the present study as we supplemented different concentration of

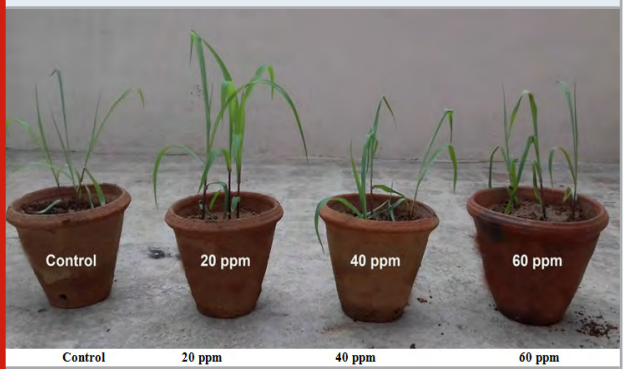
copper nanoparticles (20 ppm, 40 ppm and 60 ppm) for 15 days in *Zea mays* L .and lower concentrations i.e., 20 ppm concentration of copper nanoparticles had significant positive influence on root, shoot and leaf length, leaf surface area, root, shoot leaf and whole plant weight (Hojjat 2015; Plaksenkova et al. 2019; Wu et al. 2020).

Table 3. Root and shoot length of *Zea mays* L. supplemented with copper nanoparticles at one time

Concentration of copper nanoparticles (ppm)	Root length(cm)	Shoot length(cm)	Leaf length(cm)	Leaf surface area(cm^2)
Control	8.90±0.10	10.97±0.15	35.83±1.26	28.80±0.26
20	11.73±0.21	12.27±025	45.23±0.25	39.67±0.21
40	8.83±0.15	7.30±0.20	37.00±0.10	29.50±0.30
60	8.47±0.06	7.10±0.10	32.37±0.47	24.77±0.23

Values represent the mean (±) standard error of three independent experiments. All the experiments were statistically analyzed by One-way Anova using SPSS interpretation. The results were significant at $p < .05$

Figure 7: In vivo growth analysis of *Zea mays* supplemented with copper nanoparticles at one time.



Analysis of chlorophyll: Copper nanoparticles supplemented at one time on day 1 in *Zea mays* L. possess increase in the chlorophyll content from control (Total chlorophyll 18.5 mg) to 40 ppm (Total chlorophyll 26.2 mg) concentration of copper nanoparticles. Chlorophyll content decreased with 60 ppm (16.5 mg) concentration of copper nanoparticles. Copper nanoparticles supplemented continuously for 15 days in *Zea mays* L. possess increase in the chlorophyll content from control to 20ppm (Total chlorophyll 19.2 mg). Further increase in the concentration of copper nanoparticles 40 ppm (15.2 mg) and 60 ppm (14.5 mg) decreases the total chlorophyll content. The results reveal that 20ppm concentration of copper nanoparticles is considered as the optimum level in increasing chlorophyll content as the increase in the chlorophyll content with 40 ppm (26.2) concentration copper nanoparticles supplemented at one time in *Zea mays* L. is negligible. It is similar to the results obtained in past studies (Plaksenkova et al. 2019).

Analysis of copper nanoparticles in plant material: The results of the atomic absorption spectrometry reveal that among the various concentration (20 ppm, 40 ppm and 60 ppm) of copper nanoparticles supplemented to *Zea mays* L. at one time, 60 ppm (1.5067 ± 0.0021), followed by 40ppm (0.0114 ± 0.0006) and 20 ppm (0.0044 ± 0.0004 ppm). The copper nanoparticle in control was 0.0025 ± 0.0033. Similarly, among the various concentration (20 ppm, 40 ppm and 60 ppm) of copper nanoparticles supplemented to *Zea mays* L. continuously for 15 days, 60 ppm (1.6110 ± 0.0036) has more copper particles followed by 40 ppm (0.0212 ± 0.0003 ppm) and 20 ppm (0.0084 ± 0.0002) The copper nanoparticles in control were 0.0025 ± 0.003.

Table 4. Root, shoot, leaf and whole plant weight of *Zea mays* L. supplemented with copper nanoparticles at one time.

Concentration of copper nanoparticles (ppm)	Root weight (grams)	Shoot weight (grams)	Leaf weight(grams)	Whole plant weight(grams)
Control	0.545±0.003	0.612±0.003	0.666±0.008	1.810±0.009
20	0.636±0.004	0.802±0.003	0.906±0.003	2.344±0.005
40	0.549±0.001	0.613±0.002	0.665±0.005	1.818±0.008
60	0.540±0.001	0.607±0.003	0.662±0.004	1.807±0.005

Values represent the mean (±) standard error of three independent experiments. All the experiments were statistically analyzed by One-way Anova using SPSS interpretation. The results were significant at $p < .05$

Table 5. Root and shoot length of *Zea mays* L. supplemented with copper nanoparticles continuously for 15 days.

Concentration of copper nanoparticles (ppm)	Root length(cm)	Shoot length(cm)	Leaf length(cm)	Leaf surface area(cm^2)
Control	8.90±0.10	10.97±0.15	35.83±0.06	28.80±0.29
20	12.37±0.15	13.23±0.25	46.97±006	41.17±0.29
40	10.93±0.06	10.10±0.10	37.97±0.06	30.33±0.49
60	8.77±0.25	10.03±0.06	34.80±0.26	26.17±0.29

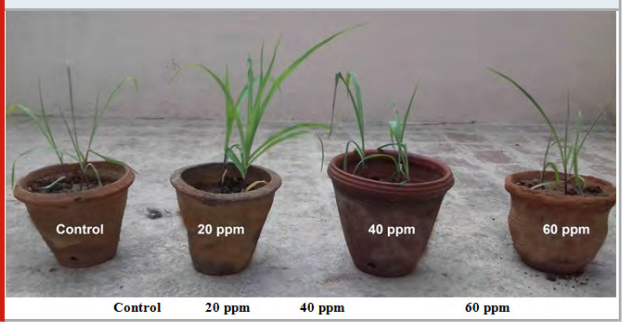
Values represent the mean (±) standard error of three independent experiments. All the experiments were statistically analyzed by One-way Anova using SPSS interpretation. The results were significant at $p < .05$

Table 6. Root, shoot, leaf and whole plant weight of *Zea mays* L. supplemented with copper nanoparticles continuously for 15 days

Concentration of copper nanoparticles (ppm)	Root weight (grams)	Shoot weight (grams)	Leaf weight(grams)	Whole plant weight(grams)
Control	0.545±0.003	0.612±0.003	0.666±0.008	1.810±0.009
20	0.637±0.003	0.804±0.001	0.923±0.002	2.367±0.20
40	0.546±0.004	0.615±0.005	0.671±0.001	1.824±0.006
60	0.541±0.002	0.605±0.003	0.661±0.001	1.805±0.006

Values represent the mean (±) standard error of three independent experiments. All the experiments were statistically analyzed by One-way Anova using SPSS interpretation. The results were significant at $p < .05$

Figure 9: *In vivo* growth of *Zea mays* L. supplemented with copper nanoparticles Continuously for 15 days



Accumulation Factor (AF): To evaluate the copper nanoparticle accumulation in the plant tissue, the accumulation factor (AF) was calculated on the effect of copper on *Zea mays* L. and tabulated in Table 7. The accumulation factor was significantly increased with the increasing concentrations of copper nanoparticles. Accordingly, the accumulation factor in *Zea mays* L. was ranging from 0.004 ppm to 1.40 ppm with copper nanoparticles supplemented at one time. Similarly, the accumulation factor in *Zea mays* L. was ranging from 0.007 ppm to 1.50 ppm with copper nanoparticles supplemented continuously for 15 days. Many studies on accumulation of nanoparticles in plants reveals that they influence crop improvement, yield, plant advancement and huge numbers have aggregated in various plant tissues (Javed et al. 2019; Melusi et al. 2021).

Table 7. Accumulation factor of copper nanoparticle

Nanoparticles	Control (ppm)	20 ppm	40 ppm	60 ppm
Copper nanoparticles supplemented at one time to *Zea mays* L.	0.002	0.004	0.0106	1.40
Copper nanoparticles supplemented continuously for 15 days to *Zea mays* L.	0.002	0.007	0.019	1.50

The accumulation factor was significantly increased with the increasing concentrations of copper nanoparticles in the present study. However, increasing concentration of nanoparticles in plants causes toxicity in molecular and cellular level. In a study by Melusi et al. (2021), accumulation of silver nanoparticles in plants were relatively higher with large sized particles compared to the small one. Since copper nanoparticles are usually

large in size accumulation could be less when compared to accumulation of small sized nanoparticles (Melusi et al. 2021).

CONCLUSION

The findings of the present study reveal that copper nanoparticles have potential to enhance the growth of *Zea mays* L. Among the various concentrations (20 ppm, 40 ppm and 60 ppm) of copper nanoparticles, 20 ppm is considered as the optimum level for the growth of *Zea mays* L. However higher concentration (40 ppm, 60 ppm and more) of nanoparticles affect the plant growth. The outcome of the present study will be useful in finding the potential of nanoparticles in crop improvement and other agricultural applications.

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

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Genetical Communication

Optimization of Cytogenetic Protocol for Chromosome Preparation in Freshwater Crabs

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ABSTRACT

Freshwater crabs are the most fascinating groups among the decapod crustaceans having great economical and ecological importance. However, the cytogenetic studies in freshwater crabs are very less on record probably due to the small size and the large number of chromosomes that pose a major hurdle in their chromosomal preparations. The research reported herein presents the method to obtain the well-spread chromosomes from freshwater crab species that belong to infra order-Brachyura and order-Decapoda. For optimizing the cytogenetic protocol, different concentrations of colchicine for various time intervals were used. Furthermore, the duration of hypotonic treatment, fixative and the type of tissue used were also investigated. Best results were procured using the testis tissue incubated with 0.1% colchicine for 48 hrs, 0.8% hypotonic solution (potassium chloride) for 50 minutes, and Carnoy's fixative (thrice after every 10 minutes). The cell suspension method was markedly better than the traditional dabbing method. The present study was undertaken with an aim to determine the ideal treatment conditions at individual steps involved in slide preparation for getting the best metaphase complement in freshwater crabs. The data obtained in the present study provides a simple and efficient procedure for preparing good quality metaphase spreads that are critical for cytogenetic analyses including chromosome counts, banding procedures, fluorescence in situ hybridization, karyotyping, and construction of ideograms. Chromosomal studies prove to be a great asset as the science of taxonomy and cytology are closely related to each other and cytological studies have been recognized as a vital tool for relative taxonomic classification.

KEY WORDS: CELL-SUSPENSION, CYTOGENETICS, FRESHWATER CRAB, METAPHASE.

INTRODUCTION

Freshwater crabs are the enormous conglomeration within the Brachyura which is the most species-rich among all the decapod crustaceans. Presently, 125 species of freshwater crabs are found in India that includes 25 genera and 2 families (Pati and Thackeray 2018; Mitra 2019). Despite being ecologically and economically important, little is known about the cytogenetics of freshwater crabs in general and Jammu and Kashmir Union Territory in particular, due to a large number of small-sized chromosomes. Chromosomes play a significant role in the heredity, alterations, divergence, and phylogenetic development of a species. Analyzing the number, size, and structure of chromosome are significant for the characterization of a species and has great applicability to resolve various taxonomic ambiguities that are encountered due to the phenotypic plasticity of characters in closely related taxa. Furthermore, cytogenetic knowledge has come to occupy an indispensable role while implementing techniques for genome modifying and manipulating. The knowledge about the karyotype of species acts as the baseline for advanced cytogenetic methods and future genome sequencing studies (Abdelrahman et al. 2017, Iannucci et al. 2020).

The general workflow for the preparation of chromosomal stages is as follows: metaphase blocking, preparation of tissue, hypotonic treatment, fixation, slide preparation, and staining. Firstly, spindle poison is used to arrest the cell spindle fibre at the metaphase stage (Rieder and Palazzo 1992; Silva et al. 2011), followed by incubation of cells in hypotonic solution so that the swelling and bursting of nuclei takes place. After this, the tissue is subjected to fixation which is the most crucial step in the entire process wherein tissue and its components are fixed selectively at a particular stage. This is followed by preparation of cell suspension, slide preparation, and lastly staining (Moore and Best 2001; Wang et al. 2010; Calado et al. 2013). The aquaculture sector is greatly benefitted from cytogenetic analysis of any culturable species owing to the fact that it

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offers basic information with respect to a species which in turn is a prerequisite for planning and implementation of breeding programs such as interspecific hybridization, induction of polyploidy, and a better understanding of the phylogeny of the organism (Bartley et al. 2001; Christopher et al. 2010).

MATERIAL AND METHODS

Collection of crabs was done from selected sites of the Jammu region, by netting and handpicking with the help of a local fisherman. The live specimens were collected in oxygen-filled polythene bags and brought to the Animal cytogenetics laboratory of the Department of Zoology, University of Jammu where they were placed in a well-aerated aquarium and fed on vegetal remains, chironomid larvae, and artificial feed. They were allowed to acclimatize for 8-10 days at 25°C in the aquarium until the experiment was conducted. In this study, the initial work was to acquire the amalgamation of colchicine level and time required for procuring good metaphase spreads. Varying colchicine levels i.e., 0.01, 0.05, and 0.1% for varying time period i.e., for 6 hrs, 24hrs, and 48hrs were used at 1µl/g of the bodyweight of the crab. After injecting colchicine, gills, hepatopancreas, and testicular tissue were taken out and the pieces of tissue were placed in a cavity block. To this 5ml of potassium chloride, sodium chloride, and sodium citrate were added for 30min, 50min, 60min (Lee et al. 2004; Okomoda et al. 2018).

After the removal of hypotonic solution, these tissues were fixed using 3-5ml of freshly prepared chilled fixative; ethanol: acetic acid: distilled water in the ratio of 3: 3: 4 and methanol: glacial acetic acid in the ratio 3:1 with an exposure time of 10 minutes and rinsed three times. Some part of fixed tissue was chopped after fixation in a centrifuge tube and the suspension was centrifuged at 1000 rpm for 5 minutes, following which supernatant was decanted leaving behind the pellet of cells containing only about 0.5ml of fixative. The pellet of cells was rinsed three times by centrifugation at 1000rpm for 5minutes. The slides were prepared by both the dabbing method and the cell suspension method. In the cell suspension method, two to three drops of cell suspension were dropped on a warm glass slide using a 200µl micropipette from a certain height so that cells burst open and facilitate proper chromosome spreading. The slides were then air-dried at room temperature.

In the dabbing method, prior to centrifugation, fixed tissue was dabbed onto pre-cleaned slides using forceps. The slides were then air-dried. After air drying, the slides were stained with 4% Giemsa phosphate buffer solution in coplin jars for 20, 30, and 40 minutes. This was again followed by rinsing of slides with distilled water to remove the excess stain and then air-drying was done at room temperature. Scanning of prepared slides was done using camera aided Olympus microscope and photomicrography was done using digital Sony SSC-DC3789 (Choudhary et al. 2013; Jasrotia and Langer 2021).

RESULTS AND DISCUSSION

The results procured exhibit that the crabs injected with 0.1% colchicine intramuscularly (third or fourth walking leg), for 48 hrs had superior chromosome spreads as compared to the results obtained by other levels of colchicine concentration and time of exposure. Treatment with 5ml of hypotonic solution (0.8% potassium chloride) for 50 minutes proved to be effective in this study. The optimum method for the chromosome preparation was the cell suspension method. The addition of Carnoy's fixative i.e., methanol and glacial acetic acid in 3:1 ratio after the treatment with KCl with three changes of fixative after every 10 minutes gave satisfactory results. The best Giemsa concentration and time for staining were 4% and 30 minutes respectively.

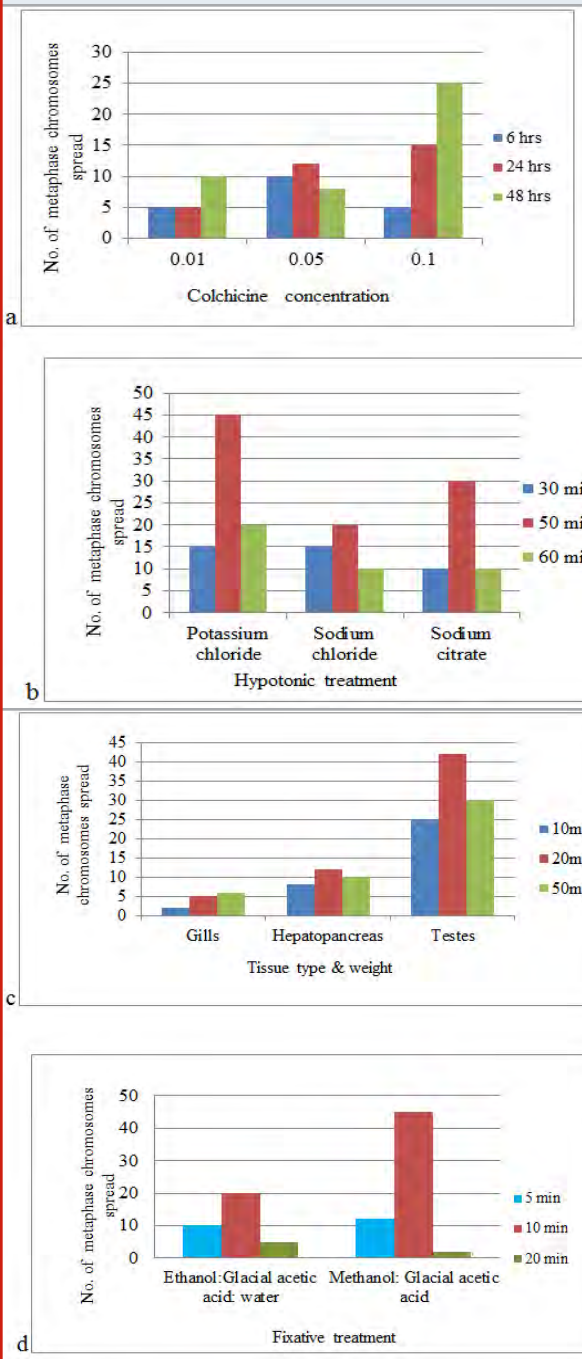
A proper method for the preparation of slides is an essential prerequisite for establishing the cytogenetic status of a species. The cell suspension method emerged to be the best when compared to the dabbing method. One need to be very careful while dropping the cell suspension on slides i.e., it should be made sure that the slide is tilted at an angle of 45° approximately and the tip of the micropipette should be held at a distance of 25-30cm from the slide so that the proper dispersion of the chromosomes occur on the slide. Moreover, the chromosome preparation should be done at 25°C temperature and 50% humidity because the temperature and humidity greatly influence the process of drying of slides.

The concentration of the mitotic inhibitor i.e., colchicine, and duration of its exposure is indeed a crucial factor as it helps to facilitate the quality of metaphase chromosomes. There is a recommendation that colchicine treatment is essential as it elevates the yield of metaphase chromosomes and a more reliable morphology of chromosomes is produced. Insufficient colchicine treatment leads to failure of arresting at metaphase stage and even extended exposure to colchicine causes condensation of chromosomes (Rieder and Palazzo 1992; Wood et al. 2001; Caperta et al. 2006). In the present study, colchicine treatment for 6 hrs gave adequate metaphase complements but the quality of the chromosomes was not good. Based on the present experimental protocol, an exposure time of 48 hrs of colchicine treatment is recommended for getting a quality spread of metaphase chromosomes (Fig. 1a) and this is more or less in line with the results recorded by Lee et al., (2004) for other crab species. From the present study, it can be inferred that if the duration of colchicine treatment is less, then there will be a decrease in the quality spread of chromosomes.

Following the inhibition of the mitotic spindle by colchicine, the hypotonic solution is utilized, so that nuclei of the mitotic cells swell up, burst and chromosomes spread out (Moore and Best 2001). There may be overlapping or loss of chromosomes if the appropriate hypotonic solution is not used or the time of exposure to the solution is not appropriate (Baksi and Means, 1988). Treatment with the hypotonic solution is the deciding phase as chromosome count is difficult if metaphases did not spread adequately.

Quality metaphase spreads were obtained using potassium chloride (Fig. 1b) over sodium citrate and sodium chloride and these observations draw support from the observations recorded by Swagatika and Kumar (2014) who have also witnessed similar findings while working with mud crab and flower crab as experimental material.

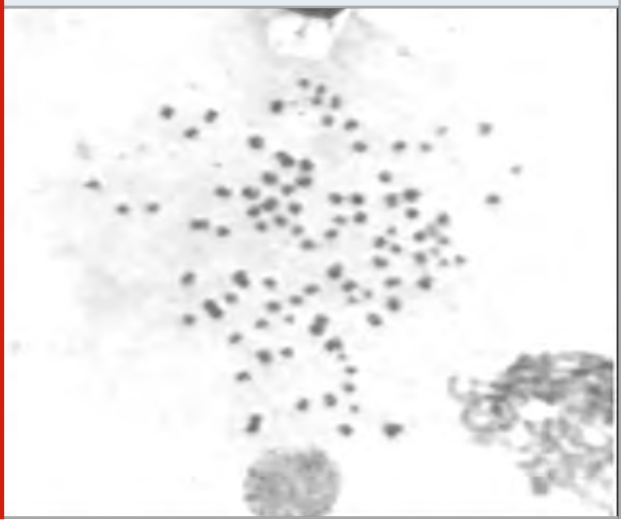
Figure 1: Effect of different procedures on the number of clear metaphase chromosome spreads in freshwater crabs
a.Concentration of colchicine X Time span of exposure
b.Hypotonic treatment X Time span of exposure
c.Type of tissue X Amount of tissue
d.Fixative treatment X Time span of exposure



The process of hypotonic swelling gets arrested by the addition of methanol and glacial acetic acid in the ratio of 3:1 and cells get preserved in a stable state. A higher number of well-spread metaphase chromosomes were observed in the testis tissue as the testicular tissue contains both mitotic cells (spermatogonia) and meiotic cells (spermatocytes). There are very few cytogenetic studies in decapods using gills or hepatopancreatic tissues. During the present study low-quality, metaphase spreads were obtained from gills and hepatopancreas (Fig. 1c) which may be due to the presence of fat tissues (Indy et al. 2010; Salvadori et al. 2012; Gonzalez –Tizon et al. 2013; Salvadori et al. 2014.

Treatment with Carnoy's fixative (methanol: glacial acetic acid, 3:1) also proved to be optimal compared to ethanol: acetic acid: distilled water, 3: 3: 4. (Fig. 1d) which is in accordance with findings reported by Gopikrishna and Shekhar (2003). This study suggests that cell suspension dropped on the clean pre-warmed glass slide with micropipette from a height of about 25-30cms and dried at room temperature before staining is the better method for slide preparation as compared to the method in which intact tissues were dabbed on slide directly with the help of forceps and then air-dried. After air-drying, the slides were stained using 4% Giemsa phosphate buffer solution for 30 minutes. A metaphase chromosome complement obtained in the present study is depicted in Fig. 2.

Figure 2: Metaphase chromosome complement of freshwater crab species



CONCLUSION

The findings of the present study construct the blueprint of the standard protocol for cytogenetic scrutinization of freshwater crabs. This protocol will be efficacious for further cytogenetic and molecular studies involving freshwater crabs which can be the focus of future research work.

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Biochemical Communication

The Impact of The Activity of a Mould Fungus Culture on The Depth of Hydrolysis of Raw Material Carbohydrates in Alcohol Production

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ABSTRACT

In the study, the authors proceeded from the need to solve the problem of improving the technological processes in the food industry to improve the quality and working conditions. For the most complete use and regulation of microorganisms, it is necessary to know their needs, living conditions, and metabolism well. The objective of this work has been to determine the activity and formation of enzymes of the amylolytic complex by mould fungi in alcohol production. The studies were carried out in 2018-2020 based on the Premium LLC (Russia) and the Kabardino-Balkarian State Agrarian University (Russia) The objects of research were the moldy fungi Aspergillus oryzae, Aspergillus niger, Aspergillus awamori, and their varieties. The study included several successive stages, namely: deep cultivation of molds, the establishment of enzymes of the amylolytic complex during their assimilation of various sources, the study of enzymes of microbial origin, and saccharification of starchy raw materials with fungal enzymes. It has been found that the formation of the amylolytic complex enzymes by mould fungi occurs when they assimilate various sources of mineral and organic nitrogen, as well as simple sugars, dextrins, and starch. As the temperature increases, the reactivity of the enzymes first grows and then, after passing a certain level, begins to decrease rapidly. It has been found that the duration of saccharification doesn't affect the final results of the fermentation. Optimal conditions for the saccharification of mash ensure the greatest amount of starch hydrolysis products in the saccharifier and maintain maximum enzyme activity.

KEYWORDS: ALCOHOL PRODUCTION, AMYLOLYTIC ACTIVITY, BOILED MASS, CULTIVATION, HYDROLYSIS, MOULD FUNGI ENZYMES, STARCH SACCHARIFICATION.

INTRODUCTION

The alcohol industry, which processes grain raw materials using biotechnology methods for alcohol and fodder products, is one of the large-scale consumers of enzyme preparations of microbial origin. About 60% of the total volume of enzyme preparations entering the Russian market is used in the production of alcohol from grain. Due to the strong competition in the Russian alcohol market, obtaining high-quality ethyl alcohol is an important task for the industry. The problem of improving the quality of alcohol is solved in two ways: technological (improving the quality of processed raw materials and water, rational choice of alcoholic yeast, reducing the duration of alcoholic fermentation, etc.) and improving the methods of hydrolysis of raw carbohydrates (regulation of enzymatic catalysis of high molecular weight polysaccharides, the amount of enzymes during the cultivation of microorganisms, etc.) (Serba et al., 2018). Microorganisms are a rich source of enzymes, the use of which opens up new prospects and opportunities for further improvement of technological processes in food production, increase in the yield and quality of products, and improvement of working conditions

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(Khokonova & Tsagoeva, 2019a; Khokonova & Tsagoeva, 2019b; Domnicheva, 2020).

For the most complete use and regulation of microorganisms, it is necessary to know well their needs, conditions of vital activity, and metabolism. Enzymes of mould fungi and bacteria get a wide application in alcohol production plants processing starchy raw materials in near future (Ashkhotov 2009; Vinarov, Kukharenko & Nikolaykina 2019). By the nature of the technological process, alcohol production is biochemical, as it is based on the action of malt enzymes that catalyze the hydrolysis of starch to form simple sugars, which are converted into ethanol by yeasts. Previously, yeast was believed to be the fermenting enzyme (Mukailov & Khokonova, 2015). The saccharification of starch by extracts from germinated grains can be regarded as the beginning of the conscious use of enzymes (Kachmazov, 2012; Vinarovet al., 2019; Domnicheva, 2020).

The objective of this work was to determine the activity and formation of amylolytic complex enzymes by moulds in alcohol production. The novelty of the research lies in the fact that for the first time in the Kabardino-Balkarian Republic, the criteria for the cultivation of mold fungi enzymes were investigated and substantiated. Their influence on the hydrolysis of carbohydrates in alcohol production was determined.

MATERIAL AND METHODS

Our research was carried out in 2018-2020 under the conditions of LLC Premium and at the Department of Production and Processing Technology of Kabardino-Balkarian State Agrarian University named after V. M. Kokov. The objects of research were microbial enzymes, mould fungi, distillery mash, and a stillage. The research methodology consisted of the following stages: deep cultivation of mould fungi, the establishment of amylolytic enzymes in their assimilation of various sources, the study of enzymes of microbial origin, saccharification of starchy raw materials by fungal enzymes.

RESULTS AND DISCUSSION

The high amylolytic activity was observed in the cultivation of mould fungus on a medium with starch, maltose, dextrins, sucrose, and glucose, and the enzyme activity on glucose is no lower than on maltose, dextrins, or starch. Starch and its hydrolysis products are considered to be the best carbon sources in the surface and deep cultivation of the fungus (Iarovenko, 1996). In deep cultivation of the fungus Asp. oryzae 153 capable of producing highly active amylase, it was found that the maximum amount of the enzyme was produced by the fungus when the medium contained 2% starch and 0.3% nitrogen in the form of ammonium sulphate (Table 1).

Table 1. Enzyme generation in deep cultivation

Sources of carbon (2%)	Final pH	Dry mycelium weight, g	The amount of amylase	
			per 100 ml	per 1 g of dry mycelium
Starch	8.0	1.46	93.70	64.20
Dextrins	7.9	2.07	69.10	33.40
Maltose	8.2	1.75	77.70	44.40
Glucose	8.2	1.79	20.60	11.50
Cellulose	7.3	not cons.	1.90	-
Inulin	7.9	1.55	5.25	3.39
Sucrose	6.8	1.89	0.53	0.28
Mannose	8.3	1.81	1.28	0.71
Xylose	8.3	1.72	2.74	1.59
Mannitol	7.2	2.00	1.92	0.96
Glycerine	7.2	2.15	0.39	0.18
Ethyl alcohol	-	no growth	-	-
Sodium acetate	8.5	0.22	1.66	7.65
Sodium malate	8.2	0.18	0.30	1.68
Ammonium succinate	7.7	0.18	0.30	2.00

The data show that the amount of amylase formed on media with a sufficiently high starch content — 2, 4, or 6% — depends more on the nitrogen concentration in the medium than on the starch concentration. However, to maximize the formation of maltose and dextrinase, the starch concentration should be increased to 4 or even 6%.

In this case, the formation of amylolytic complex enzymes — amylase, maltase, dextrinase by mould fungi occurs during their assimilation of various sources of mineral and organic nitrogen, as well as simple sugars, dextrins, and starch (Ashapkin, 2005). It is practically proved that the accumulation of amylolytic enzymes by the fungus

Aspergillus niger is stimulated by adding magnesium oxide to the stillage. According to Russian researchers, the activity of dextrinase from magnesium increases on average by 50% compared to the activity obtained by neutralization of the stillage with calcium carbon dioxide (Table 2).

Table 2. Enzyme activity in the neutralisation of the stillage

pH		Amylase		Dextrinase		Mycelium, g	Neutralising agent	Substrate
initial	final	units / 100 ml	%	units / 100 ml	%			
5.0	5.5	5.2	100	490	100	3.50	$CaCO_3$	Stillage with 3% of soluble substances
5.2	5.0	3.7	70	695	140	2.82	$MgCO_3$	
5.3	5.5	34	100	630	100	4.05	$CaCO_3$	Stillage with 5% of soluble substances + 2% of flour
5.7	4.8	34	100	1072	170	3.98	$MgCO_3$	

To determine the optimum concentration of magnesium for fungal growth, it is recommended to assume the following ratio of magnesium to phosphorus: 36 phosphorus ions are required for each magnesium ion. Sulphur is a component of glutathione, which activates proteolytic enzymes that are important for the reproduction of microorganisms. At the same time, sulphur is found in important amino acids, such as methionine, and also stimulates the reversed amylase. As the temperature increases, the reactivity of the enzymes first increases and then, after passing a certain level, begins to decrease rapidly. The temperature optimum of the amylolytic enzymes is shown in Table 3.

Table 3. Optimal conditions for the vital activity of enzymes

Enzymes	Optimal	
	pH	temperature, °C
Amylase	4.5-5.5	50-52
Maltose	4.5-4.7	58-62
Dextrinase	4.5-6.5	58-62
Total effect by the degree of saccharification	4.7-5.1	58-61

Table 4. Enzyme activity in cultures of mould fungi of the genus Aspergillus in the surface cultivation method

Fungi types	Medium composition	Dry medium enzyme activity		
		amylase	dextrinase	maltose
Aspergillus oryzae	Wheat bran	37.1	240	22
Aspergillus oryzae	Sillage grains	5.1	120	3.4
Aspergillus oryzae	Rye grits	6.6	210	17
Aspergillus oryzae		45	240	26
Aspergillus niger				
	Wheat bran	1.2	300	78
Aspergillus oryzae I-475		124	665	-
Aspergillus awamori		16	400	57
Aspergillus oryzae	Potato mash,	26	283	-
Aspergillus oryzae K. S.	bran, sprouts			
	Wheat bran	44	250	-
Aspergillus awamori		-	315	-
Asp. oryzae		63	-	-
Aspergillus awamori		11	625	103
Aspergillus oryzae 153		43	326	33
Aspergillus awamori ch		15	858	126
Aspergillus niger S-4		1	501	77

The table shows that maltose and dextrinase are relatively more stable than the enzyme amylase, which is active under less strict experimental conditions. During saccharification of starchy raw material by fungal enzymes, starch hydrolysis does not end in the saccharifier but takes place mainly during fermentation. Therefore, the completeness of starch saccharification in the saccharifier for further digestion and alcohol yield is not of fundamental importance. Table 4 shows the activity of enzymes in cultures of mould fungi of the genus Aspergillus in the surface cultivation method.

The optimal temperature for saccharification of starchy mash by mushroom enzymes ensures that amylase and dextrinase activity is maintained for a long time (Serba et al., 2018). An additional check showed that raising the saccharification temperature to 58 and even 62°C did not impair any of the fermentation indices, but in terms of the sterility of the mash, these temperatures were unequal. Low temperatures of 50 to 55° C are conducive to the development of infections and have little effect on the process of gelling of insoluble starch of raw materials; higher temperatures of 58° C may cause the inactivation of enzymes. Therefore a saccharification temperature of 57-58°C and a fermentation time of about 62 hours is accepted. A culture with such enzyme activity should be used which can hydrolyse 70-80% of the starch in the main mash. Some authors consider that amylase should be obtained to ensure that at least 20% of soluble starch is converted into hydrolysis products (Kadieva et al., 2003; Kachmazov, 2012; Belokurova, 2015; Domnicheva, 2020).

Depending on the activity, the consumption of the fungal culture can vary within certain limits. The rate and depth of hydrolysis of raw material carbohydrates are determined by the activity of the deep fungal culture. The degree of starch hydrolysis increases because it becomes more accessible for enzyme action due to the dissolution of protein and hemicellulose shells (Serba et al., 2018). The optimum external physicochemical conditions ensuring the maximum action of the enzyme complex should also be established: pH values, medium composition, temperature, and the duration of the process (Faradzheva & Fedorov 2002; Khokonova & Tsagoeva, 2019a).

Table 5. Characteristics of mash saccharified with a deep culture *Aspergillus niger*

Volume of the fungus culture, %	pH	Dry matter concentration, %	Maltose, g/100 ml	Reducing substances, g/100 ml	% of saccharification on
5	5.9	15.0	5.02	12.88	41
10	5.9	14.6	5.48	12.57	46
15	5.8	14.0	6.60	12.26	61

The composition of the medium plays an important role in protecting many enzymes from temperature-induced inactivation. For example, protein coagulation has been identified as the cause of inactivation when malt extracts are heated. If sugars or peptone are present in the medium, they prevent coagulation and, thus, protect the amylase from inactivation. Consequently, the starch saccharification process of digested raw materials containing sugars, dextrins, proteins, and polypeptides can also be carried out at elevated temperatures, which are less dangerous under these conditions for amylase inactivation than in aqueous solutions. Divalent metal ions (Mg2+, Ca2+, Ba2+, Mn2+, Ni2+) stabilise the amylase molecule and prevent its cleavage by proteolytic enzymes. However, even within one hour and at 55°C, the degree of starch saccharification does not exceed 51% and can vary according to the concentration of the added enzymes. Table 5 shows the characteristics of a mash saccharified with Aspergillus niger S-4 deep culture for 5 minutes at 55°C.

As the consumption of fungus culture increases in relation to the volume of the boiled mass, the concentration of dry matter decreases, which, in turn, also reduces the amount of reducing substances. Using 15%, the amount of maltose increases to 6.60% which is 1.58% higher than using 5% of the fungus culture. The highest percentage of saccharification is 61% when using 15% of the fungus culture. Further hydrolysis process occurs during fermentation. Consequently, the duration of saccharification has no effect on the final fermentation results (Khokonova & Tsagoeva, 2019b; Domnicheva, 2020).

Figure 1: A correlation and regression analysis, which revealed a strong direct correlation between the percentage of fungus culture and the time of saccharification.

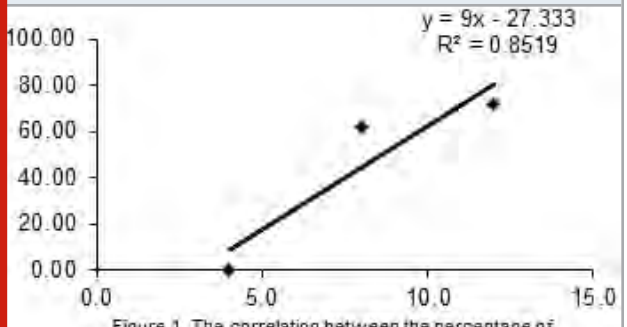

Figure 1. The correlation between the percentage of fungus culture and the duration of saccharification

Optimal conditions for the saccharification of mash are not so much necessary to ensure the greatest amount of starch hydrolysis products in the saccharifier but rather to maintain maximum enzyme activity (Mukailov & Khokonova, 2015). After the raw material has been boiled and cooled, a small

amount of deep culture is added to liquefy it (Khokonova & Tsagoeva, 2019a; Khokonova & Tsagoeva, 2019b). The remaining, largest part of the culture is transferred to a second-stage tube-type saccharifier, where the mass stays for 3-5 minutes at 65°C; the consumption of the deep culture is 15% of the volume of the mash. A correlation and regression analysis was carried out based on the results of our research (Figure 1).

CONCLUSION

Thus, the research shows that the formation of amylolytic enzymes by mould fungi occurs during the assimilation of various sources of mineral and organic nitrogen as well as simple sugars, dextrins, and starch. As the temperature increases, the reactivity of the enzymes at first increases and then, after passing a certain level, begins to decrease rapidly. The duration of saccharification was found to have no effect on the final fermentation results. Optimal conditions for the saccharification of mash ensure the greatest amount of starch hydrolysis products in the saccharifier and maintain maximum enzyme activity. Three catalysts — amylase, maltose, and dextrinase — are needed for the effectiveness and feasibility of microbial enzymes.

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Microbiological Communication

Bactericidal Activity of *Coleus forskohlii* Extract Against Multi Drug Resistant *Acinetobacter baumannii* Strains Isolated from a Hospital

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ABSTRACT

As opportunistic bacterium Acinetobacter baumannii has the capability to develop resistance against different types of antibiotics. The goal of the research was to isolate and molecularly identify antibiotic resistance pathogenic bacteria from hospital and its bio-control using *Coleus forskohlii* ethanolic extract. For this about 78 swab samples were collected from different areas of the Prince Meshari Bin Saud General Baljarshi Hospital. The pathogenic bacteria were molecularly identified using 16s rRNA sequencing. In addition, the resistance profile of the strains was checked using different antibiotics. These includes Tetracyline (T), Chloramphenicol (C), Penicillin G (PG), Streptomycin (S), Erythromycin (E), Fusidic Acid (FC), Oxacillin (OX), Novobiocin (NO), Gentamicin (GM), Ampicillin G (AG), Sulphatriad (ST), Colistin sulphate (CS), Cotrimoxazole (CM), Cephalothin (CO), Trimethoprim (TM), Sulphamethoxazole (SMX), Clindamycin (CD), and Tetracycline (T). Antibacterial activity was measured using the microtiter broth dilution method to determine the minimum inhibitory concentration (MIC), as well as the minimum bactericidal concentration (MBC). Approximately 112 bacterial strains were isolated from 78 swab samples. Out of these bacterial strains, eight were identified as pathogenic bacteria using 16s rRNA sequences. All of these eight strains were derived from the Genus Acinetobacter. Among them, two strains of Acinetobacter were resistant to 14 different antibiotics. These two bacteria were identified as *Acinetobacter baumannii* and Acinetobacter sp. These MDR strains have been used for antibacterial activity against plant extract. The results showed that the MIC of the extract against these pathogenic strains was approximately 4 mg. Therefore, it is concluded that the plant extract has the ability to kill MDR resistant Acinetobacter strains in an ecofriendly way.

KEY WORDS: ACINETOBACTER, ANTIBIOTIC RESISTANT, BACTERICIDAL, SWAB, 16S RRNA.

INTRODUCTION

The genus *Acinetobacter* contains over 30 species, the majority of which live in soil and water, but some commensal strains have been isolated from human specimens (Visca et al. 2011). *A. baumannii*, a member of this genus, is thought to be clinically significant due to its multidrug resistance and high mortality rates in people with weakened immune systems worldwide (Peleg et al. 2008; Poulikakos et al. 2014). Several studies have supported the isolation of *A. baumannii* from various environmental sources. The intensive study of clinical isolates of *A. baumannii*, on the other hand, does not reveal the species' diversity. As a result, environmental isolates are critical for understanding the diverse nature of *A. baummanii* (Berlau et al. 1999; Huys et al. 2007; Diancourt et al. 2010; Hamouda et al. 2011; Asaad et al. 2021). Actinetobacter baummanii has emerged as a secondary infectious pathogen in critically ill patients who have been hospitalized for extended periods of time with intensive procedures prior to the use of antimicrobial drugs (Gottesman et al. 2021).

This species plays a significant role in healthcare-associated infections in institutions around the world (Perez et al. 2007; Gottesman et al. 2021). Bacterial resistance to antimicrobial drugs has prompted the United Nations to address the antimicrobial resistance problem. The emergence of multidrug resistant (MDR) *A. baumannii* mutants, which cause nosocomial infections, has harmed many countries in Asia, Latin America, Europe, and the Middle East (Saudi Arabia), where endemics have been reported worldwide (Tognim et al. 2004; Kim et al. 2013; Moradi et al. 2015).

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MDR is most commonly found in adults in intensive care units, with cabapenem resistance being the most common phenotype (Mustasim 2018; Adel et al. 2020; Arta et al. 2019). Curing the majority of human and domestic animal diseases with natural products is primitive and goes hand in hand with human civilization. Traditional medicines are primarily derived from medicinal plants, and various active ingredients in medicinal plants are used to treat a variety of diseases (Woldeyes et al. 2012; Handique et al. 2016; Noha 2021; Fahim 2021).

Plant-based medicines account for nearly 25% of all prescribed medications worldwide (Rates 2001). Different parts of medicinal plants, such as flowers, leaves, barks, stems, fruits, and roots, are used to treat diseases caused by microorganisms because they have various pharmacological activities (Metra et al. 2020; Chen et al. 2021). Recently, there has been an increase in concern about plant-derived drugs, which demonstrate the significance and validity of traditional claims about the value of natural products in healthcare. When compared to synthetic drugs, the researcher's interest has been focused on the exploration of antimicrobial drugs derived from plants/microorganisms that are safe, healthy, and cost effective, with no side effects on the host (Cragg and Newman 2001; Nitha et al. 2012; Chen et al. 2021).

Pathogens are becoming increasingly resistant as a result of widespread and ineffective treatment of infectious diseases. As a result, scientists are eager to investigate plants for biologically active ingredients that are effective against infectious bacterial and other microorganisms (Shears 2000). *Coleus forskohlii* is a member of the Lamiaceae family, which has a vast number of members that are generally found in the Mediterranean region (lukhoba et al. 2006). Phytochemical examination of C. forskohlii reveals the distribution of several phytochemicals such as alkaloids, reducing sugars, flavonoids, tanins, and terpenoids. Flavonoids in phenolic compounds are well-known for their anti-allergic, anti-cancer, antioxidant, and anti-inflammatory activities (Aiyelaagbe and Osamudiamen 2009; Alasbahi et al. 2010).

According to Ammon and Muller (1985), the *C. forskohlii* plant can be used to cure a variety of maladies including insomnia, heart disease, respiratory issues, epilepsy, angina, asthma, intestinal issues, abdominal colic, and inflammation. Different bio-active chemicals, such as terpenoids, phenolics, saponin, tanins, and alkaloids, were found to scavenge free radicals created in the body in another study (Rout et al. 2012). GC-MS study of ethanolic extracts of *C. forskohlii* reveals a molecule called n-hexadecanoic acid, which has antibacterial and antifungal properties. Shanmugam and Pradeep (2019) revealed that *C. forskohlii* rhizome extract had antibacterial activity (Agoramoorthy et al. 2007; Shanmugam and Pradeep 2019; Khatun 2020). Our purpose was to examine MDR resistant bacteria at Prince Meshari Bin Saud General Baljarshi Hospital and employ plant extract to control pathogenic MDR bacterial strains.

MATERIAL AND METHODS

For sampling, swab samples were collected from Different area in Prince Meshari Bin Saud General Baljarshi Hospital. The bacterial Samples were directly taken to the laboratory and we kept in the refrigerator prior to further experimentation. For bacteria isolation, about 1 ml of collected samples were added to 100 ml freshly prepared nutrient broth medium and incubated at 30 °C and 180 r/min for 24 hours. The sample was serially diluted and were spreaded on the plates. After incubation the pure colonies were transfer 50 % glycerol. The samples were preserved in -80 °C until further experimentation.

Molecular identification and DNA isolation/Gene amplification was done according to the manufacturer's instructions. DNA was extracted using the GeneJet Genomic DNA Purification Kit (Thermo Scientific). The 16F27 and 16R1525 primers were used for 16S rDNA amplification in each extracted DNA sample (Hauben et al. 1997). The polymerase chain reaction (PCR) was set for 30 cycles following amplification at the following temperatures and times: 92 °C (2 min); 42 °C (30 seconds); and 74 °C (4 min) before incubation (4 °C) at the end of the final cycle. Following amplification, the fragments were sequenced by Macrogen (Seoul, South Korea), and a phylogenetic tree was constructed using MEGA version 4 software (Tamura et al. 2007; Kubota et al. 2008; Hanan et al. 2009). Drug susceptibility was tested using Kirby Bauer's diffusion protocols with minor modifications. Antibiotics were used in accordance with the Clinical and Laboratory Standards Institute guidelines: Tetracyline (25 µg), Chloramphenicol (25 µg), Penicillin G (1 unit), Streptomycin (S), Erythromycin (5 µg), Fusidic Acid (10 µg), Oxacillin (5 µg), Novobiocin (5µg), Gentamicin (10 µg), Ampicillin G (1 unit), Sulphatriad (5 µg), Colistin sulphate (5 µg), Cotrimoxazole (5 µg), Cephalothin (5 µg), Trimethoprim (5 µg), Sulphamethoxazole (5 µg), Clindamycin (2 µg)), and Tetracycline (10 µg).

For the preparation of ethanolic extract, the dried plant sample was treated with absolute ethanol at a 1:20 ratio on a magnetic stirrer for 48 hours before being filtered with Whattman No. 1. The supernatant was evaporated using a Rotary Evaporator until an oily extract was obtained. The crude extract was then stored in sterile universal bottles at -20 °C. For antibacterial activity determination, agar diffusion method or agar well diffusion was used to test the antibacterial activity of various Ethanolic extracts (Daoud et al. 2015). A fresh bacterial culture (1 ml) was pipetted into the center of sterile petri dishes. In the petri dish, molten cooled Muller Hington agar (MHA) was thoroughly mixed with the inoculum. A sterile cork borer was used to make 6 mm wells after the bacteria-containing agar plates solidified. These wells were filled with extracts (20% w/v) in 100 □l increments. Following a 24-hour incubation at 37 °C, the plates were chilled for 30 minutes to allow the extracts to better diffuse into the agar. The antibacterial activity is determined after the incubation period by measuring the zone of inhibition (including wells measurement). For the experiment, DMSO (10%) was used as a negative control (Daoud et al. 2015).

Minimum Inhibitory concentration (MIC) is the highest dilution of extracts that inhibits microorganism growth without killing the organism. The treatment of plant extracts in tested plates from the disc diffusion method that exhibit an inhibition zone was tested for MIC. The clinical laboratory standards institute (CLSI) protocols were followed when using the broth macrodilution method (Jorgensen and Turnidge 2015). The minimal bactericidal concentrations (MBC) are the samples' lowest concentrations at which inoculated bacteria are completely killed. The Minimal bactericidal concentration was determined by spreading 100 µl of the MIC contents that showed no bacterial growth on nutrient agar plates for 24-hour incubation at 37°C. The first well with a colony count of <5 is considered negative for growth and is reported as the MBC.

RESULTS AND DISCUSSION

Globally, the problem of *Acinetobacter* infections and drug resistance development is a major prevailing research issue that must be adequately addressed. Synthesizing biofilm is a key feature, particularly in bacterial diseases where its spread is aided by air and mechanics (Ming et al. 2014; Badave and Kulkarni 2015). In our study, we found a high prevalence of MDR strains among *A. baumannii* isolates from ICU patients. The high prevalence of MDR strains in ICUs could be attributed to the study population's regular use of antimicrobials.

Isolation and identification: About seventy-eight swab samples were collected from different areas of the Hospital. From these swab samples about 112 bacterial strains were isolated. The list of sampling having pathogenic strain is detected is shown in Table 1. Out of these bacterial strains, eight were identified as pathogenic bacteria using 16s rRNA sequences. All of these eight strains were derived from the Genus Acinetobacter. Among them, two strains of Acinetobacter were resistant to 15 different antibiotics. The resistant profile of all the pathogenic strains is shown in Table 2.

Table 1. Samples collected from hospital area having pathogenic bacterial strains

No.	Samples ID	Location/Room	Stools/Tables
1.	3-2	Operations Room	Tool table
2.	17-3	ER Male waiting Room	Chair handles, Door handle, Floor
3.	25-3	ER Triage Room	Chair, Measuring device
4.	5-1	Examination Room 2	Doctor Table
5.	1-2	Examination Room 2	Door handle
6.	7-3	ER Reception	Reception Table
7.	15-3	ER Male waiting Room	Chair handles, Door Handles
8.	15-1	ER Male waiting Room	Chair handles, Door handle, Floor

Table 2. Antibiotic profile of the isolates from local hospital

S.No	ID	T	C	E	FC	OX	NO	PG	GM	CO	CS	AG	CM	ST	S	TM	SMX	T	CD
1	3-2	15	12	16	R	R	R	R	22	15	R	R	R	R	8	R	R	R	R
2	17-3	16	13	16	R	R	R	R	21	R	R	R	R	R	R	R	R	R	R
2	25-3	20	12	15	R	R	R	R	24	14	R	R	R	R	9	R	R	R	R
4	5-1	19	R	15	R	R	R	R	20	14	R	R	R	R	R	R	R	R	R
5	1-2	22	12	14	R	R	R	R	20	16	R	R	R	R	8	R	R	R	R
6	7-3	20	14	18	R	R	R	R	24	16	R	R	R	R	R	R	R	R	R
7	15-3	18	15	16	R	R	R	R	21	R	R	R	R	R	R	R	R	R	R
8	15-1	22	20	18	R	R	R	R	20	R	R	R	R	R	9	R	R	R	R

Tetracyline (T), Chloramphenicol (C), Penicillin G (PG), Streptomycin (S), Erythromycin (E), FUSIDIC ACID (FC), Oxacillin (OX), Novobiocin (NO), Gentamicin (GM), Ampicillin G (AG), Sulphatriad (ST), Colistin sulphate (CS), Cotrimoxazole (CM), Cephalothin (CO), Trimethoprim (TM), Sulphamethoxazole (SMX), Clindamycin (CD), and Tetracycline (T)

The 16s rRNA gene was amplified in order to identify the most resistant isolates molecularly. The gene product's sequence was submitted to Genbank in order to obtain an accession number. The resistant bacteria 17.3 was identified as *Acinetobacter baumannii* (MT875278) and bacteria 15.3 was identified as Acinetobacter sp. (MT875271). Following the acquisition of the accession number, a phylogenetic tree was constructed using MegaX software, as shown in

Figure 1. Our findings show that the 16s rRNA sequences of bacteria *Acinetobacter baumannii* and Acinetobacter sp. are similar to those of many other Acinetobacter strains, as shown in Figure 1.

Figure 1. Phylogenetic tree of *Acinetobacter baumannii* strains isolated from local hospital

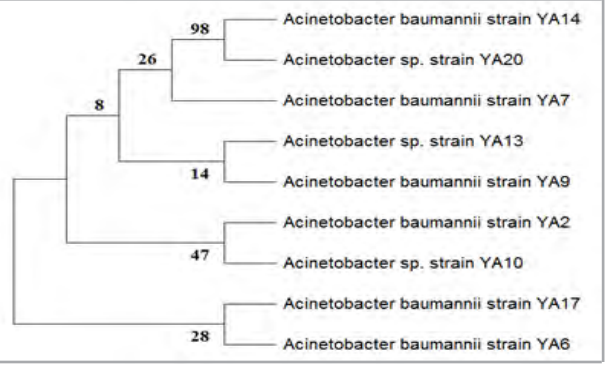
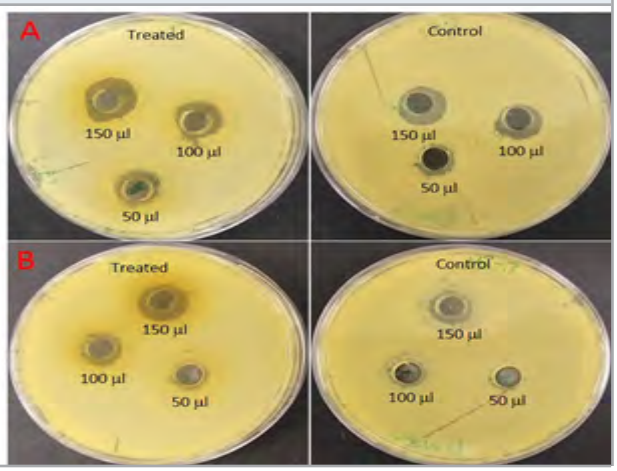


Figure 2. Zone of inhibition of *Coleus forskohlii* against A). *Acinetobacter baumannii* B). Acinetobacter sp.



Antibacterial activity of plants extract: Plant Ethanolic extract was investigated to evaluate their antibacterial activity against the infectious bacteria isolated from local hospital. The extract's antimicrobial activity was investigated to determine its efficacy against the two microorganisms being studied. *Coleus forskohlii* Ethanolic extract was quantitatively tested against two pathogenic Acinetobacter by assessing the diameter of the inhibition zones as shown in Table 3 and Figure 2. Extracts of *Coleus forskohlii* and had high activity against Acinetobacter. The results presented showed significant activity among (9.5±0.8 - 16.5 ±0.86) range against micro-organisms with inhibition zones.

Minimum inhibitory concentration (MIC): *Acinetobacter* sp. and *Acinetobacter baumannii* were found to be most sensitive pathogens which survived up to 2 mg/ml and 4 mg/ml (Table 4), thus having an MIC of 2 mg/ml and 4 mg/ml, respectively. *Acinetobacter baumannii* was found to be comparatively less sensitive as they survived up to 4mg. Minimal bactericidal concentrations (MBC) results was found 16 mg/ml of *Coleus forskohlii* Ethanolic extract against the selected pathogenic strains.

Due to its resistance to several medicines, treating Acinetobacter strains is a significant issue. So, there is a need to control Acinetobacter strains in an ecofriendly way. For this *Coleus forskohlii* Ethanolic plant extract was used in order to kill these multidrug resistant strains. According to our findings, this plant has the ability to kill multidrug-resistant Acinetobacter and can be utilized to control the bacteria. The two Acinetobacter strains selected were resistant to nearly fourteen antibiotics. But results show that *Coleus forskohlii* plant extract has the ability to kill these strains isolated for hospital. Our findings support those of Malleswari et al. (2013) who discovered significant broad spectrum antibacterial activity against *Pseudomonas fluorescence, Bacillus subtilis, Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae, Streptococcus pneumonia,* and *Staphylococcus aureus* using *C. forskohlii* roots, shoots, and leaves extracts (Malleswari et al. 2013; Mitra et al. 2020; Khatun 2020).

Table 3. Antibacterial activity of *Coleus forskohlii* against Acinetobacter strains in aerobic condition.

Sample	Inhibition zone (mm)					
	Acinetobacter baumannii			***Acinetobacter* sp.**		
Conc.	150µl	100µl	50µl	150µl	100µl	50µl
Coleus forskohlii	16.5 ±0.86	12.3±0.57	10.3±0.76	12.5±0.57	10.5±0.76	9.3±0.5

Furthermore, Lu et al. (2010) mentioned that Gallic acid have a strong antimicrobial, antioxidant and anticancer (Rice et al. 1996; Lu et al. 2010). p-hydroxybenzoic acid, has been reported to have antioxidant activity against free radicals, antimicrobial activity against pathogenic bacteria and fungi (Rice et al. 1996; Heleno et al. 2013; Mitra et al. 2020; Khatun 2020). Many researchers have identified various plant extract efficiency and active compounds as antimicrobial sources to inhibit the growth of bacteria (infectious). According to some reports, compounds found in plant extracts such as terpenes, alkaloids, and phenols affect the enzymes and proteins of the target cell membrane. These plant compounds are responsible for cell disruption caused by proton efflux to the cell's exterior causes cell senesces or disrupts enzymes required for amino acid biosynthesis (Burt 2004; Gill and Holley 2006). There are

also some reports about the hydrophobicity of plant extracts. This property of extracts enables them to interact with microbial proteins in cell membranes and mitochondria, posing a threat to cell integrity and altering permeability (Friedman et al. 2004; Tiwari et al. 2009; Mitra et al. 2020; Khatun 2020).

Table 4. Minimum inhibitory concentration (MIC) of samples against Acinetobacter strains tested.

	Bacteria strain	**Coleus forskohlii**
	MIC	MBC
Acinetobacter baumannii	2 mg/ml	16 mg/ml
Acinetobacter sp.	4 mg/ml	16 mg/ml
Bacteria strain	*Coleus forskohlii*	
	MIC	MBC
Acinetobacter baumannii	2 mg/ml	16 mg/ml
Acinetobacter sp.	4 mg/ml	16 mg/ml

CONCLUSION

The findings of the present study determines that the production of resistant strains of Acinetobacter causes hospitalizations to be prolonged, as well as high medication costs. Acinetobacter infection is significantly accelerated by factors such as improper antibiotic use, mechanical ventilation, and cross infection. There is already a high rate of resistance to common antibiotics. The Ethanolic extract of plants may play an important role in reducing Acinetobacter infections and inhibiting deadly epidemic nosocomial infections.

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

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

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Biotechnological Communication

Plant Growth Promoting Potentials of SVH1 *Bacillus* Sp. from *Hemidesmus indicus*

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ABSTRACT

Endophytes are microorganisms that can live within the inter or intracellular regions of various plant tissues without causing any harmful effects. The endophytes possess different properties which are beneficial to its host plant. They can directly or indirectly promote the plant growth. *Hemidesmus indicus* (L.) R.Br. is a laticiferous twining shrub that belongs to the family Apocynaceae. It is an important medicinal plant that is widely used in various systems of medicine and the plant has pharmacological, phytochemical and ethnobotanical importance. The tuber of the plant is widely exploited in traditional medicine. The microbial population associated with this plant is less explored. In the current study, ten different endophytic bacteria were isolated from the tuber of the plant by following standard isolation procedure. These bacteria were analysed for plant growth promoting potentials. Of all the isolates, the one designated as SVH1 showed maximum plant growth promoting properties like IAA production, Phosphate solubilization, ACC deaminase production and Siderophore production. SVH1was identified as *Bacillus* species following morphological, biochemical and molecular characterisation. IAA production from this endophyte was 23.48 µg/ml which is significantly higher than many of the previous reports. The production of IAA was also confirmed by RP- HPLC analysis. The phosphate solubilization index of SVH1 was noticed as 60 which is also a significantly higher value. The isolate SVH1 which is also positive for ACC deaminase and siderophore production can be expected to have growth promoting role in *H. indicus*. This strain can also be explored in future for the production of valuable bioactive compounds similar to that of *H. indicus*.

KEY WORDS: ACC DEAMINASE, *BACILLUS* SP, *HEMIDESMUS INDICUS*, IAA, PHOSPHATE SOLUBILIZATION, SIDEROPHORE.

INTRODUCTION

Microorganisms that are symbiotically associated with plants are called endophytes. Plants are the potential reservoir of these indigenous microbes. Endophytic bacteria provide reward to the host plant in many ways - by producing plant growth regulators, inhibiting the phytopathogens and also by helping in phytoremediation. They exist in the plant body asymptomatically. Bacterial endophytes promote plant growth either directly or indirectly. The growth of a plant can be influenced by the endophytes and they also produce phytohormones. Indole 3 Acetic Acid (IAA) is one the major auxin produced by them (Elbeltagy et al. 2000; Lodewyckx et al. 2002; Schulz and Boyle 2006; Lin and Xu 2013). Endophytes also help the plants to procure nutrients like nitrogen, phosphorus and iron (Yaish et al. 2005; Nandy et al. 2020).

Ethylene is a natural product synthesised by the plant which controls the growth and developmental responses and the response of plants in biotic and abiotic stresses. The hike in ethylene production in a plant is disadvantageous. This may even lead to death of the plant. The endophytic bacteria within the plant helps them to overcome this situation to an extent by producing an enzyme,1-aminocyclopropane-1-carboxylate deaminase (ACC deaminase). This enzyme breaks the compound ACC into α-keto butyrate and ammonia which can be further utilized by the plants for other purposes (Santoyo et al. 2016). Plant growth is promoted by the endophytic bacteria indirectly by competing with the pathogenic microbes for nutrients and other factors thereby protecting the plants from them (Gray and Smith 2005; Miethke and Marahiel 2007; Nandy et al. 2020).

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Moreover, some endophytic Plant Growth Promoting Bacteria (PGPB) also have the proficiency to solubilise unavailable phosphate to available form. Siderophores are iron-chelating compounds secreted by some microorganisms under iron-limiting conditions. This is the strongest soluble iron binding compound. Siderophore production by associated endophytes supports the plants to survive difficult environments and make iron as a limiting factor to plant pathogens (Miethke and Marahiel 2007). *H. indicus* (L.) R.Br. is an important medicinal plant that is widely used in various systems of medicine and the plant has pharmacological, phytochemical and ethnobotanical importance (Nandy et al. 2020).

It has been recently reviewed by many researchers that the endophytic bacteria within a host plant have plant growth promoting properties (Jasim et al. 2013, Puri et al. 2017, Pawlik et al. 2017; Nandy et al. 2020). They are used as bioinoculants for sustainable agriculture (Kahtani et al. 2020). The endophytic bacterial community of *H. indicus* is less explored even though it is a medicinally significant plant. In this scenario, the present study aimed at the isolation, identification and analysis of the plant growth promoting potentials of the endophytic bacterial isolate SVH1 from *H. indicus*.

MATERIAL AND METHODS

According to the procedure of Jasim et al. (2013), isolation of the endophytic bacteria from *H. indicus* were done. All the nutrient agar plates inoculated with the root of the plant including the control plate were incubated at 28 0C for 5 days and observed daily for bacterial growth. The plates were examined regularly and distinct colonies developed were selected which were further purified by repeated streaking. The pure cultures thus obtained were regularly sub cultured and maintained. The endophytic bacterial isolates obtained from the culture plates were screened for the plant growth promoting traits. Using the procedure of Rahman et al. (2010), IAA production was tested.

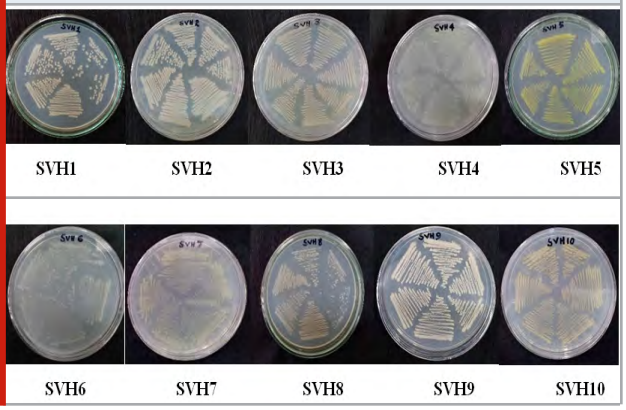
The presence of IAA was confirmed using Reverse Phase HPLC. Using the method of Jasim et al. (2013), the ACC deaminase production and phosphate solubilization of the endophytic bacterial isolates from *H. indicus* were screened. The growth of bacteria in the media after 2 days of incubation was considered as positive result for ACC Deaminase production and the formation of yellow zone around the colony indicates phosphate solubilization. Siderophore production was tested in CAS agar medium. Development of the yellowish halo around the colonies indicate the production of siderophore. The endophytic isolate which showed maximum plant growth promoting traits was subjected to morphological, biochemical and molecular characterization (Schwyn and Neilands 1987; Jasim et al. 2013).

Morphological characterisation of the colony of the endophytic bacterial isolate was done by observing the characters like colour, surface, margin, elevation, opacity, motility and Gram's staining features of the colony. According to Cappuccino and Sherman (2014), biochemical characterization of the selected endophytic isolates was done. For molecular characterization, genomic DNA was isolated from the bacterial isolates and was used as template for Polymerase Chain Reaction (PCR). The PCR product was checked by agarose gel electrophoresis, purified further and was then subjected to sequencing. The sequence data thus obtained was checked by BLAST analysis (Zhang et al. 2000; Cappuccino and Sherman 2014).

RESULTS AND DISCUSSION

Isolation of the endophytic bacteria: Isolation of the endophytic bacteria was done by using fresh and cleaned tubers of *H. indicus*. The surface sterilization of the plant material was effective as no growth appeared on the control plates. Ten endophytic bacterial isolates were obtained from the culture plates by purification through continuous sub-culturing and were named as SVH1-SVH10 (Figure 1).

Figure 1: The isolated endophytic bacteria from *H. indicus*



Screening of the endophytic bacterial isolates were done for plant growth promoting traits. The results are given in Table 1. The screening studies revealed the potential of SVH1 in having plant growth promoting traits like IAA production, phosphate solubilization, ACC deaminase production and Siderophore production.

Identification of the isolate SVH1 by morphological, biochemical and molecular characterization: Morphological characterization: The morphological characterization of the endophytic bacteria isolate SVH1 was observed. (Figure 2) and tabulated (Table 2). The isolate was Gram positive and motile.

Biochemical characterization: Biochemical characterization of the isolate was done using several tests. Indole Production test, Starch hydrolysis test, Catalase test, Voges-Proskauer's test, Methyl Red test, Nitrate Reduction test and Citrate utilisation test was positive for the isolate SVH1. The isolate was negative for Urease test.

Molecular characterization of the isolate by 16s rRNA gene sequencing: Molecular characterization of the isolate was done by using 16s rRNA sequencing. The amplification of the 16s rRNA was confirmed by agarose gel electrophoresis (Figure 3). PCR product was gel eluted and

sequenced and presented in FASTA format. The 16S rRNA sequence of the endophytic isolate was compared with that of other bacterial sequences by using BLAST. The sequence analysis of SVH1 showed maximum identity to *Bacillus* sp. The PCR amplified product of 16s rRNA gene sequence and the phylogenetic tree of SVH1 is given below.

Table 1. Screening of the endophytic bacteria for plant growth promoting traits

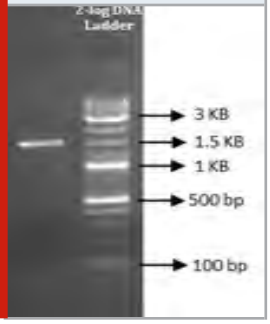
Plant growth promoting traits	Endophytic bacterial isolates									
	SVH1	SVH2	SVH3	SVH4	SVH5	SVH6	SVH7	SVH8	SVH9	SVH10
IAA Production	+++	+	+	+	+	_	+	+	+	+
Phosphate solubilization	+	+	+	_	_	_	_	_	+	+
ACC Deaminase Production	++	+	_	_	_	_	_	_	_	+
Siderophore Production	+	_	_	_	+	_	_	+	+	+

Figure 2: SVH1

Table 2. Morphological characterisation

Morphology	*Bacillus* sp.
Colour	Creamy white
Surface	Smooth
Margin	Entire
Elevation	Pulvinate
Opacity	Opaque
Gram staining	Positive

Figure 3 PCR amplified product of SVH1

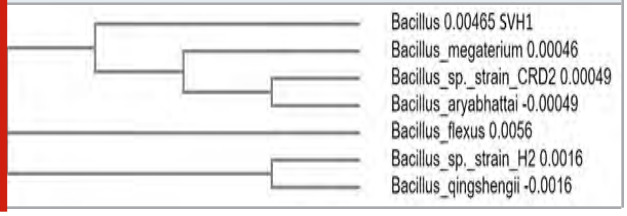


Following is the base pair sequence of SVH1.
>*Bacillus* SR1515-SVHI-RSF1_E05.ab1

GCAGTAATTCAGCTTGCTTCTATGACGTTAGCGGCGGACGGGTGAGTAACACGTG
GGCAACCTGCCTGTAAGACTGGGATAACTTCGGGAAACCGAAGCTAATACCGGAT
AGGATCTTCTCCTTCATGGGAGATGATTGAAAGATGGTTTCGGCTATCACTTACAG
ATGGGCCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGA
TGCATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCA
GACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGAC
GGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAG
GGAAGAACTAGTACGAGAGTAACTGCTCGTACCTTGACGGTACCTAACCAGAAAG
CCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATC
CGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAG
CCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAG
AGAAAAGCGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACA
CCAGTGGGCGAAGGCGGGCTT

From analysing the base pair sequence of SVH1, the phylogenetic tree of the SVH1 was constructed (Figure 4).

Figure 4: Phylogenetic tree of the endophytic isolate SVH1



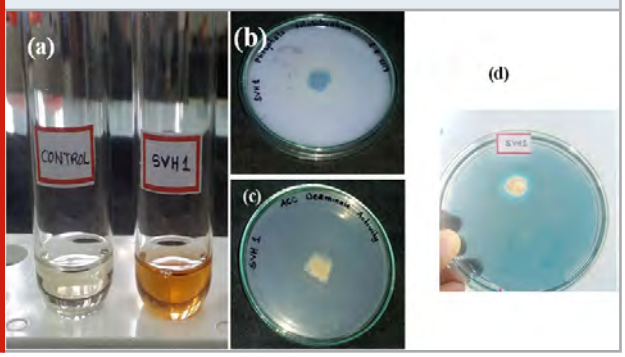
Screening of the isolates for plant growth promoting traits: Plant growth promotion by the endophytic microbes including fungi and bacteria makes them a better resource for modern agriculture (Zhao et al. 2017). In the present study we have isolated ten different endophytic bacteria from the roots of *H. indicus*. After the screening for plant growth promoting traits, SVH1 showed positive results for all the four screening tests of IAA production, Phosphate solubilization, Siderophore production and ACC Deaminase production.

IAA production: From the screening of the selected endophytic isolates for the production of IAA, it was confirmed that the strain SVH1 is positive for IAA as it showed the development of red colour (Figure 5 a). Production of IAA was confirmed by RP-HPLC. The ethyl acetate extract of SVH1 was subjected to HPLC analysis. Both the standard IAA and the extract of SVH1 showed the peak at the same retention time (Figure 6). The amount of IAA produced by each endophyte was also calculated using their absorbance at 530 nm.

Table 3. Quantification of IAA production

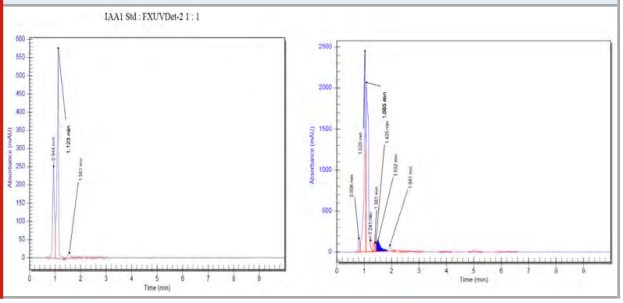
Endophytic bacteria	Amount of IAA produced (µg/mL)
SVH1	23.48
SVH2	15.24
SVH3	16.64
SVH4	16.08
SVH5	7.99
SVH7	11.44
SVH8	7.30
SVH9	1.8
SVH10	3.63

Figure 5: Plant growth promoting traits of SVH1 (a) - IAA production, (b)-phosphate solubilization, (c) - ACC deaminase production, (d) - Siderophore production.



IAA is an important phytohormone among the different auxins produced by plants. Some endophytic bacteria also have the ability to produce IAA. This may be the reason for the increased growth rate of plants when it is associated with endophytic bacteria. IAA performs different functions like stimulation of cell division, cell and tissue differentiation, cell elongation and lateral root formation. In bacterial cells, IAA has no specific function, but it can be speculated that this helps to maintain the interaction between plants and associated endophytic bacteria (Pattern and Glick 1996). Etesami et al. (2015) suggested that bacterial IAA loosens the plant cell wall and the result of this reaction is increase in the release of the root exudates. This helps the rhizosphere bacteria to acquire more nutrients and colonization of this rhizosphere bacteria in to the plant will also be promoted (Etesami et al. 2015). Although a large number of bacteria have been reported to produce IAA, this is the first report on bacterial endophytes producing IAA from *H. indicus*. *Bacillus* species isolated from the roots of *Catleya walkeriyana*, a Brazilian species of orchid, an endangered species, showed increase in all characters of the plant and prolonged the percentage of seedling survival (Galdiano et al. 2011; Chagas et al. 2015).

Figure 6: HPLC Analysis of the standard IAA and the ethyl acetate extract of the endophytic isolate, SVH1. A. Indole 3 Acetic Acid B. Ethyl acetate extract of SVH1.



The genus *Bacillus* sp. was reported as one of the main genera of Plant Growth Promoting Rhizobacteria (Chagas et al. 2015). These PGPR can later colonize in to the plants and exist as endophytes. From table 3, it was understood that SVH1 showed maximum production of IAA in the medium supplemented with L-tryptophan. Gomes et al. (2017) reported the production of 4.12 µg/mL IAA by endophytic *Bacillus* sp. isolate EB.78 from Banana plant. Comparing to this result, *Bacillus* sp. SVH1 from *H. indicus* showed the production of 23.48 µg/mL IAA in the medium supplemented with L-tryptophan which is significantly greater. Bhatt and Maheswari (2020) isolated a zinc solubilizing *Bacillus megaterium* CD25 and it showed the production of 13.8 µg/mL IAA. These bacteria also found to enhance growth in *Capsicum annuum* L. The IAA production by the endophytic bacteria in plants helps them for growth promotion and as well as it amplifies the further colonization of other beneficial bacteria. The strain SVH1 identified as *Bacillus* sp. in this study may have plant growth promoting role in *H. indicus* (Gomes et al. 2017; Bhatt and Maheswari 2020).

ACC deaminase Production: The endophytic bacterial isolate SVH1 was screened for the production of ACC deaminase on DF salt minimal medium containing 0.2% ammonium sulphate. The growth of the isolate on this media indicated that it is positive for the production of ACC deaminase (Figure 5 c). ACC deaminase is the enzyme that cleaves ACC into ammonia and α-ketobutyrate. The reduction in the level of ACC in plants resulted in the simultaneous reduction of plant ethylene levels due to the presence of ACC deaminase producing endophytic bacteria (Glick et al. 2007; Bhatt and Maheswari 2020).

The higher level of ethylene in plants is lethal to them. But the association with ACC deaminase producing endophytic bacteria enable the plants to tolerate the stressed conditions caused by ethylene. Different *Bacillus* species have been reported to produce ACC deaminase. ACC deaminase

producing *B. subtilis* was reported from tomato seedlings by Xu et al. (2014). Kothari and Vyas (2015) reported that *B. cereus* brm, an endophyte from *Vigna radiate* (Mung beans) produced ACC deaminase and the plant could overcome inhibitory influence of salt on root and shoot elongation under salinity stress. Similar results were found in the past study (Grobelak et al. 2018). The endophytic bacterial consortium with ACC deaminase production improved the growth of plants under the stress of heavy metal contaminated soil. By analysing these results, *Bacillus* sp. could be considered as a potent plant growth promoter. Thus, SVH1 could also be considered as a potent plant growth promoter (Xu et al. 2014; Kothari and Vyas 2015; Grobelak et al. 2018; Bhatt and Maheswari 2020).

Phosphate solubilization: Yellow zone around the bacterial colony indicated the phosphate solubilization efficiency of the isolate SVH1 (Figure 5 b). Phosphate solubilization index was found out using the following formula. Phosphate solubilization index =Total diameter of the colony (diameter of the colony + diameter of the yellow zone)/Diameter of the colony×100. The solubilization zone diameter of SVH1 was 2.2 cm and colony diameter were 1.6 cm. The phosphate solubilization index of SVH1 is 60. Nutrients available for plants determine the rate of growth in plants. Macronutrients and micronutrients are the essential nutrients for plants. Among the macronutrients, phosphorus is an important one. The growth in plants may be limited at some point in their lifetime due to the non-availability of this phosphorus. Different phosphorus compounds are abundant in agricultural soils, but in the insoluble form. Several reports have shown that different rhizosphere bacteria have the ability to solubilize insoluble phosphates available in the soil (Khan et al. 2020).

Rhizosphere colonizing bacteria produce low molecular weight organic acids which in turn lead to the acidification of the soil or media. This contributes primarily towards the phosphate solubilizing mechanism (Puente et al. 2004). The potential of bacterial endophytes to solubilize mineral phosphates is a promising characteristic to the agricultural field (Quecine et al. 2012). Longfei et al. (2015) reported that endophytic *Bacillus* and *Paenibacillus* strains isolated from the medicinal plant *Lonicera japonica* showed higher rate of phosphate solubilization compared to that of other endophytic bacterial strains from this plant. From the study of Joe et al. (2016), phosphate solubilizing endophytic *Bacillus* isolates from *Phyllanthus amarus* promoted plant growth and phosphorus content under salt stress. Saeid et al. (2018) reviewed from their studies that *Bacillus* species could be considered as the potent phosphate solubilizing group of microorganisms. *Bacillus* sp. SVH1 from *H. indicus* showed phosphate solubilization efficiency of 60 which is a significantly a greater value (Quecine et al. 2012; Longfei et al. 2015; Joe et al. 2016; Saeid et al. 2018; Khan et al. 2020).

Siderophore production: After 24 hours of incubation, the strain SVH1 showed the formation of yellowish hallow around the colony in the CAS agar medium. This indicated that this strain is capable of producing siderophore. Siderophores are the low molecular weight compounds that chelate iron with great affinity. The production of siderophores by the endophytic bacteria will help them to scavenge the iron from the extracellular environment. This payback the host plant directly and indirectly. They can promote the plant growth directly by making the iron in the surroundings easily available to the plant and indirectly by their biocontrol activities. The endophytic bacterial strain SVH1 showed siderophore production in CAS medium (Khan et al. 2020).

From the study of George et al. (2011) different Bacillus sp. isolated from the rhizosphere and roots of coconut showed siderophore production and they were antagonistic against *Ganoderma applanatum* and *Theilaviopsis paradoxa*. Rajkumar et al. (2009) reviewed that an endophytic siderophore producing Bacillus sp. isolated from the tissues of nickel hyperaccumulator plant *Allysum bertoloni* showed plant growth promotion under Ni stress. Siderophore producing endophytic bacteria thus can act as a promising source for plant growth promotion as well as biocontrol. Khan et al. (2020) reported the production of siderophores by endophytic bacteria from *Lilium lancifolium*. *Bacillus* SVH1 could also be considered as a potent plant growth promoter (Rajkumar et al. 2009; George et al. 2011; Khan et al. 2020).

CONCLUSION

The findings of the present study confirms that endophytic bacterial isolate SVH1 from *H. indicus* can be used as a potent plant growth promoter. The isolate SVH1 was identified as *Bacillus* sp. SVH1and was found to possess IAA production, ACC deaminase production, phosphate solubilization activity and siderophore production. The endophytic bacteria with plant growth promoting characteristics can be used as bioinoculants for sustainable agriculture. *Bacillus* sp. SVH1 along with other potent plant growth promoting endophytic bacteria could be used for the preparation of a consortium to be used as an environment friendly replacement of chemical fertilizers after the quantitative studies in future. The secondary metabolite production by the endophytic bacteria is another significant trait. Production of the similar metabolites as that of the host plant by SVH1 and other endophytic bacteria from *H.indicus* would be investigated in the future studies.

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

Ethical Statement: Ethical clearance certificate vide STUDY No : 1577/P&D-I /TNGMSSH/20 l 7/ BMS/003/07/2020 dated 22.07.2020

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Biomedical Communication

Influence of Physical Activity on the Development of Infertility in Men Aged 24-50

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ABSTRACT

Every year the number of families without children in Russia is growing. Each has its own reasons: social or the presence of pathology of the reproductive system. The number of families in which pregnancy does not occur for more than a year from the beginning of planning is growing every year. Therefore, it is becoming more urgent to establish the causes of the development of infertility, as well as develop additional and more informative methods for its diagnosis and effective treatment. The objective was to assess the reproductive health status of men of fertile age and to establish whether the type of occupational activity may be one of the causes of male infertility in Penza Oblast (Russia). Based on the Perinatal Center "Regional Children's Clinical Hospital named after N.N. Filatov" (Penza, Russia), the authors collected and analyzed archived data for 2014-2019. The data on 986 men with a diagnosis of male infertility were processed using summary methods of statistical analysis in the Statistica 7.0 program. Based on the information obtained from the patients' medical history and the results of spermograms, a database was formed. The authors determined whether age-related changes in the body could be one of the causes of infertility in the study group. Male infertility was identified predominantly in persons aged 26 to 36 years, which could be caused by low physical activity during working hours. The sedentary lifestyle of men associated with professional activity causes an increase in the temperature of the scrotal organs. As a consequence, spermatogenesis and its hormonal regulation are disturbed, which leads to the development of male reproductive health disorders. The patients were divided into two groups according to their activity during working hours. A comparison of the obtained results and the results of statistical analysis allowed the authors to conclude that the probability of male infertility is higher in men who are sitting for a long time than in those who are physically active during the working day.

KEY WORDS: ACTIVITY, MALE INFERTILITY, OCCUPATION, SPERMOGRAM, STATISTICS.

INTRODUCTION

The declining birth rate in Russia is an alarming factor for society (Demographic Yearbook of Russia 2019, 2020). Currently, special attention is paid to the problem of family infertility and methods of solving it from a medical point of view. Along with the female factor, the cause of infertility in the family can be a male or a mixed factor. Male infertility is a common condition among married couples. According to the World Health Organization, in 47% of cases, infertility is caused by impaired sexual function in men. Decreased fertility is most commonly associated with spermatogenesis. Recently, modifiable lifestyle factors have been playing an important role in the development of infertility, generating interest in this area of research. According to Rosstat data from 2014 to 2019, the coefficient reflecting the number of marriages per 1,000 people in Russia decreased from 8.4 to 6.5, while the coefficient reflecting the number of births per 1,000 people in Russia decreased from 13.3 to 10.1. This allows us to conclude that the number of families who do not have children is increasing every year in Russia. Each family has its own reasons for this: social or reproductive system abnormalities (Radzinsky et al., 2020; Ramzi , 2021).

Currently, special attention is being paid to studying the problem of family infertility and how to solve it from a medical point of view. Apart from the female factor, infertility in the family may also be caused by male or mixed factors. The number of families in which pregnancy does not occur over a year of planning is increasing every year, so it becomes more relevant to establish the causes of infertility, to develop additional and more informative methods of diagnosis and effective treatment. There is an opinion that

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one of the causes of male infertility is a sedentary lifestyle (Ilacqua, et al., 2018; Melonek et al., 2021). The objective of the study was to assess the reproductive health status of men of fertile age, using clinical and diagnostic laboratory examination data, statistical analysis, as well as to evaluate the influence of a sedentary lifestyle (occupational activity) on the development of male infertility in Penza Oblast (Russia).

MATERIAL AND METHODS

Between 2014 and 2019, N.N. Filatov Regional Children's Hospital examined 986 men diagnosed with male infertility and formed two groups of patients based on daily muscle activity. The data were provided for research in a generalized form without specified personal data. The results of the analysis included patients' medical history and spermogram results: age (24-50 years old), total sperm count, sperm concentration in 1 ml, actively motile sperm cells, inactive sperm cells, morphologically normal sperm cells, defective sperm cells. Patients' activity at work was also considered. Besides, one of the inclusion criteria was the absence of pregnancy in the spouses during the year of married life together. Of particular note is that the results of the general blood and urine tests and serological and microscopic examination of the prostate secretion in the examined patients were within the normal range.

An ultrasound examination of the prostate, scrotal and penile organs revealed no abnormalities; all the patients had no history of previous or newly diagnosed STDs; the MAR test was normal, i.e. <50% (Dolgov, 2006); and there were no changes in the hormonal status of the patients. During verification of the patients' ejaculate results, the following conditions were observed: ejaculate dilution time, viscosity, and pH were within normal limits; leucocytes, erythrocytes, and amyloid cells were present in amounts not exceeding the acceptable values; crystallization phenomenon and sperm glutination were not detected. Based on the results of data collection, a database was created, including information from patients' medical history and the results of their spermograms: age; total number of sperm cells; concentration of sperm cells in 1 ml; actively motile sperm cells; immobile sperm cells; morphologically normal sperm cells; defective sperm cells; activity at work. STATISTICA 7.0 program was used for statistical processing of archived data from 2014-2018.

RESULTS AND DISCUSSION

The following results were obtained during the analysis of the available material. During 2014-2019, the birth rate in Penza Oblast decreased by more than 40%. Thus, in 2014, 14,736 children were born in the region, and in 2019, their number did not exceed 8,782, which can be attributed to many factors, including male infertility. Considering these results, an age periodization was performed (Kochetov, et al., 2012) to establish the age category of patients predominant in the study group. As a result of statistical analysis of the data, it was found that more than 50% were patients between 26 and 36 years old. The dependence of the spermatozoa concentration in 1 ml of ejaculate on the age of the patients was determined by plotting a scatter diagram. The obtained diagram allows us to conclude that there is a dependence of spermogram indicators on age and that among the patients included in the study, a significant decrease in sperm concentration in 1 ml ejaculate is observed in men over 37 years. Normally, the concentration of spermatozoa in 1 ml of ejaculate should be greater than 20 million (Kurashvili, 2009). The scatter diagram shows that the spermogram indicator takes values below normal in patients aged 28 years and above. Similarly, the relationship between the age of the patient and the number of active spermatozoa, which should normally be >50%, is determined. The scatter diagram for this criterion indicates that a significant decrease in the analyzed index (<50%) is found in patients from the age of 25 years.

The dependence of spermogram indicators on the physical activity of the examined patients at work, which was the main objective of our study, turned out to be significant. Men in Penza Oblast are engaged in professional activities for an average of 9 hours a day, which is most of their time spent awake. If a man spends this time sedentary or sitting, the consequences for the body and the reproductive system, in particular, can be adverse. Many factors can interfere with the normal course of spermatogenesis, such as taking medication, X-rays, increased scrotal temperature, etc. The testicular temperature must not exceed 33°C for the testicular function to be most effective, which is ensured by the seven scrotal membranes with several temperature regulation mechanisms. When a man is in a sitting position for a long time, the testicles overheat. Therefore, spermatogenesis, as well as the synthesis and secretion of male sex hormones, namely 5α-dihydrotestosterone synthesis from testosterone by 5α-reductase enzyme, are inhibited (Ilacqua et al., 2018; Radzinsky et al., 2020).

The haematotesticular barrier can be impaired in inactive men, resulting in anti-sperm antibodies in sperm, which immobilize the spermatozoa and impair fertilization (Dohle et al., 2010). To establish the influence of sedentary occupational activity on the development of infertility, a new criterion "Activity at work" was introduced. The first group included men in the following positions: programmer, accountant, driver, concrete worker, dispatcher, operator, worker on duty, engineer, technologist, director, or power engineer — i.e. less active men, due to "sedentary" work. The second group included men in the following positions: car electrician, welder, mechanic, manager or salesman, laborer, electrician, handyman, locksmith, boreturner, policeman — these are men with moderate physical activity at work.

Using the STATISTICA 7.0 program, we determined the percentage of patients according to the "Activity at work" criterion in infertility. The number of less active patients in the study group was found to be slightly higher (59%). To assess the dependence of the development of infertility in the examined patients on the values of the spermogram, we built a scaling diagram for the following criteria: total sperm count, inactive spermatozoa, morphologically normal spermatozoa, and defective spermatozoa. A scatter diagram of the total sperm count in the ejaculate shows that this

figure is twice as low in the ejaculate of less active men. The number of inactive spermatozoa should not normally exceed 50% (Rebrova, 2002; Kurashvili, 2009; Radzinsky et al., 2020).

The scaling diagram for the analyzed criterion indicates that in men who work mainly in the sitting position, the number of inactive spermatozoa exceeds 70%. Statistical processing of the spermogram results for the number of morphologically normal and defective spermatozoa was also performed by constructing range diagrams. It is assumed that in healthy men, the number of morphologically normal spermatozoa should be greater than 4% (Kurashvili, 2009). Therefore, after evaluating the results obtained, it can be concluded that there are no cases of the absence of morphologically normal spermatozoa in the group of more active patients (group 2).

The degree of influence of the relationship between professional activity, sedentary lifestyle, and heat factor on the male reproductive system is currently poorly understood. Previously, experiments were carried out with the effect of heat on the organs of the scrotum, during which it was found that the heat factor has a negative effect on male fertility (Durairajanayagam et al., 2015).

CONCLUSION

A statistical analysis of clinical and diagnostic laboratory criteria of patients with infertility shows that in Penza Oblast in 2014-2019, men aged 26 to 36 years prevail among patients diagnosed with male infertility. One of the reasons for the development of the disease in patients may be an inactive lifestyle due to "sedentary" work, which causes an increase in testicular temperature, leading to a decrease in spermatogenesis. Age-related changes in the male body are not the only cause of infertility in the study groups. Reduced physical activity is one of the most important causes of male infertility.

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

Ethical Clearance Statement: The Current Research Work Was Ethically Approved by the Institutional Review Board (IRB) of Medical Institute of Penza State University, Penza, Russia.

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Biotechnological Communication

Optimization of Culture Conditions for L-Glutamic Acid Overproduction by Mutant *Corynebacterium glutamicum* X680 Using Response Surface Methodology

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ABSTRACT

L-glutamic acid is a non-essential, proteinogenic amino acid. Its large-scale industrial production using fermentation strategies was initiated in 1957. L-glutamic acid has a huge industrial demand which accounts for about more than fifty lakhs tons /annum throughout the globe. Japan, United States, South Korea, China and Europe are some major producer countries. Several trials have been made to scale-up the process by optimization of different parameters by one factor at a time. But recent studies revealed that Response Surface Methodology (RSM) is the most effective tool for process optimization as it involves multiple factors at a time. For this paper, Response Surface Methodology (RSM) was used for statistical optimization of fermentation conditions that influence the yield of L-glutamic acid by a biotin auxotrophic mutant *Corynebacterium glutamicum* X680. Response surface optimization of Box-Behnken design (BBD) was applied to evaluate the effects of culture conditions including some medium composition components. Among the variable screened, inoculum age, temperature, glucose concentration and urea concentration had significant effects on product yield. Production was significantly ($p<0.05$) increased. Inoculum age, 48.92 h; temperature, 29.99oC; glucose,11.56% and urea, 1% could result in highest L-glutamic acid production of 25.61 mg/ml by *Corynebacterium glutamicum* X680 as predicted by RSM which is very close to the actual value 25.7mg/ml that confirmed its validity. RSM appeared as a very effective tool for optimization of L-glutamic acid production by this mutant. This research has been conducted to bolster future researches with credible information and updated references. This paper can act as a primary source for future studies to begin their research from.

KEY WORDS: CORYNEBACTERIUM GLUTAMICUM X680, FERMENTATION, L-GLUTAMIC ACID, RESPONSE SURFACE METHODOLOGY, STATISTICAL OPTIMIZATION

INTRODUCTION

L-glutamic acid is a proteinogenic, non-essential amino acid. It was first discovered by Ritthausen in (1866) on hydrolysis of gliadin (Ritthausen 1866). In (1908), a Japanese scientist Kikunae Ikeda observed that glutamate was essential for a meal to taste good (Ikeda 1908). He isolated glutamic acid from kelp like sea weed broth, locally called 'konbu' (*Laminaria japonica*) in Japan. Later it was also found in asparagus, hydrolysate of wheat gluten, tomato, cheese etc. He identified L-glutamic acid as a separate food additive with a distinct taste which was a century later identified as the fifth basic taste modality 'umami' (Lindemann et al. 2002). Next, in the year (1909), monosodium salt of glutamic acid was commercialized as monosodium glutamate (MSG) by Ajinomoto co. and gained the popular trade name Ajinomoto. Since then, it was used in the wide range of products within processed industry, as a flavoring agent to influence market economy (Ault 2004; Alharbi et al. 2020).

Several trials have been made to improve the commercial production of L-glutamic acid. Initially hydrolysis of protein was used but it turned out to be a cumbersome process. Later, its chemical synthesis was also tried, however, during the course of its synthesis, racemic mixture of DL isomers was generated from which separation of metabolically active L-isomer of glutamic acid was very difficult (Sano 2009; Alharbi et al. 2020). Over and above its flavor enhancing capacity, it has several commercial values. It is a precursor of multiple amino acids such as proline and arginine. It is also a neurotransmitter. So, excessive use of MSG as

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flavor enhancer may lead to development of neurotoxicity (Xiong et al. 2003). It has got several pharmaceutical and therapeutic uses. In cosmetic industry, glutamic acid and its derivatives are widely used (Hermann 2003; Lee et al. 2014; Alharbi et al. 2020).

A new dimension has opened through the discovery of L-stereo-specific glutamic acid over producer microorganism from Japanese soil in 1955-56 by a group of scientists of Kyowas Hakko Co. Inc headed by Dr. Shigezo Udaka (Kinoshita 1987). Multiple efforts led to development of several L-glutamic acid over producers through a series of successful research through several decades. But the major constrain of its industrial production lies upon its huge production cost. So, reduction of its production cost is another motto (Joseph and Rao 1973; Kishimoto et al. 1980; Nanninga and Gottschal 1985; Tsuchida et al. 1987; Neubeck et al. 1993; Amin 1994; Das et al. 1995; Bona and Moser 1997; Gourdon and Lindley 1999; Kumagai 2000; Delaunay et al. 2002; Choi et al. 2004; Takeno et al. 2007; Engels et al. 2008; Niaz et al. 2009; Tarek and Mostafa 2010; Nadeem et al. 2011; Zareian et al. 2012; Nishio et al. 2013; Shyamkumar et al. 2014; Vuoristo et al. 2015; Wendisch et al. 2016; Hirasawa and Wachi 2017; Sgobba et al. 2018; Wen and Bao 2019; Liu 2019).

Considering its huge industrial demands (which accounts for about more than fifty lakhs tons /annum throughout the globe), Japan, United States, South Korea, China and Europe are the major the major producer countries (Liu 2019). However, the huge market demand for L-glutamic acid in the fast-food industries in India is almost totally dependent on import. Each microorganism grows well within a definite range of environmental consortium which imparts the necessity for optimization of its culture conditions. Response surface methodology (RSM) is a strong statistical tool for analyzing the effects of multiple parameters on the process (Baş and Boyaci 2007; Mansouri et al. 2012; Zafar and Mahmood 2015). Empirical optimization is a single dimensional study which requires a long time to conduct, whereas RSM reduces number of experimental trials and makes the whole process more compact and quicker. Moreover, it calculates complex interactions between the variables depending on the experimental trials which give more accuracy in the experimental results (Fahimitabar et al. 2021).

Alharbi et al. (2020) used RSM for optimization of L-glutamic acid production by *Corynebacterium glutamicum* and obtained 16.499g/L L-glutamic acid. Very recently, Fahimitabar et al. (2021) applied RSM for optimization of L-glutamic acid production using *Corynebacterium glutamicum* in batch culture (Alharbi et al. 2020; Fahimitabar et al. 2021). Present study was undertaken to investigate the influence of independent variables and their interactions on L-glutamic acid production by a biotin-auxotrophic mutant *Corynebacterium glutamicum* X680 in sub-merged fermentation process.

MATERIAL AND METHODS

Isolation, characterization and development of a biotin dependent L-glutamic acid overproducing strain *Corynebacterium glutamicum* X680 was done in our previous study (Ganguly 2019). This mutant was used throughout the present study. The growth medium for the mutant was composed of glucose, 2%; peptone, 0.05%; yeast extract, 0.1%; beef extract, 0.3%; K_2HPO_4, 0.1%; KH_2PO_4, 0.1%; $MgSO_4.7H_2O$, 0.025% and water, 1L. L-glutamic acid production was carried out in a medium composed of: glucose, 10%; urea, yeast extract, 0.2%; 0.8%; K_2HPO, 0.1%; K_2HPO, 0.1%; $MgSO_4.7H_2O$, 0.025% and water, 1L. The experiments were carried out at 30 ^{0}C for 72h incubation at pH7.0. L-glutamic acid accumulated in the fermentation broth was analyzed by Thin Layer Chromatography (TLC) method using silica gel (Merk, Germany) using n-butanol: methanol: water as mobile phase in a ratio of 5:4:3 (v/v/v) and the spots were visualized using ninhydrin (0.2%) in alcohol followed by heating. The product was confirmed as L-glutamic acid by FT-IR (Fig 2). L-glutamic acid recovered from the fermentation broth was analyzed further by agilent 1200 Infinity HPLC (using a phenyl-hexyl column and 50mM N-methylmorpholine/acetate buffer with pH 7.4 containing 12% acetonitrile as elute solvent and detected by UV absorption at 636nm with a flow rate of 1ml/min for 2.5 min and 2ml/min for 1.5 min). After filtration through a membrane filter (0.22µm), the sample was analyzed by HPLC to measure the amount of L-glutamic acid relating to the standard calibration curve.

The first step in this study was to identify the levels of process parameters which have high influence on enzyme production (response). After preliminary screening, a Box–Behnken factorial design (BBD) was employed for further optimization of the effective level of four most influencing factors for production of L-glutamic acid. An experimental design comprising 29 experimental run was created using the four parameters at three equidistance level (−1, 0 and +1) on Design Expert 12 software (Stat ease Corp, USA). The response was tannase activity (U/ml). A polynomial quadratic equation was adopted to evaluate the contribution of each independent variable in this process.

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j$$

Where, Y is the predicted response; xi and xj are the independent variables in coded values that influence the response, βo the offset term; βi represent the linier effect of xi; βij represent the interaction effect between xi and xj; βii represent the quadratic effect of xi. Regression analysis and estimation of the coefficients were performed using Design Expert software (Stat ease Corp, USA).

RESULTS AND DISCUSSION

Overproduction of desired metabolite by submerged fermentation is not only dependent on the selection of suitable strain, but also optimization of culture conditions and downstream purification technology exert significant impact (Alharbi et al. 2020). In this present investigation, after preliminary OVAT analysis it was found that four factors viz. inoculum age, temperature, glucose

concentration and urea concentration play pivotal role in L-glutamic acid production by the *Corynebacterium glutamicum* X680. Subsequently, the effects of these four factors were optimized through RSM according to BBD. A total of 29 run with different combination of the four variables (in three equi-distance levels) along with the actual and predicted responses were presented in Table 1. ANOVA of the quadratic regression models suggested that the models were significant with a computed F-value of 183.93 for L-glutamic acid production and P-value (Prob> F) lower than 0.0001 (Table 2). A lower value for the coefficient of variance ($P < 0.0001$) suggests higher reliability of the experiment. The fitness of the model was also examined by the coefficient of determination (R^2).

Table 1. RSM for optimizing L-glutamic acid production by BBD

Run	A: Inoculum age	B: Temperature	C: Glucose	D: Urea	Actual	Predicted
1	48	32	14	1	16.2	16.42083
2	48	28	14	1	19.6	19.9375
3	48	30	12	1	25.4	25.48
4	48	30	12	1	25.6	25.48
5	48	30	14	0.7	18.9	18.73333
6	60	30	10	1	22.1	21.97917
7	36	32	12	1	18.7	18.38333
8	48	32	10	1	21.9	21.52083
9	48	30	12	1	25.4	25.48
10	36	30	14	1	19.2	19.32917
11	36	30	12	0.7	20.9	20.77083
12	48	30	14	1.3	16.7	16.5
13	36	30	10	1	18.6	18.92917
14	48	30	12	1	25.7	25.48
15	60	30	12	0.7	16.4	16.22083
16	36	30	12	1.3	15.1	15.2375
17	60	28	12	1	19.1	19.45
18	48	28	12	1.3	18.1	17.84583
19	48	28	10	1	19.6	19.3375
20	36	28	12	1	18.9	18.75
21	48	30	10	1.3	20.2	20.4
22	48	28	12	0.7	19.2	19.17917
23	48	30	12	1	25.3	25.48
24	48	32	12	1.3	17.9	17.92917
25	60	30	14	1	17.4	17.07917
26	60	32	12	1	18.3	18.48333
27	48	32	12	0.7	17.5	17.7625
28	48	30	10	0.7	19.1	19.33333
29	60	30	12	1.3	20.5	20.5875

In the present study, R^2 values were found to be 0.9946 that indicated the response models can explain 99.46% of total variations for L-glutamic acid production and the rest may occur due to chance. Analysis of the quadratic effect indicated that all variables significantly contributed to these responses ($P < 0.05$). The models also showed statistically insignificant lack-of-fit of 4.70 for L-glutamic acid. All the statistical indices suggested that the models were suitable to represent the real relationship among the selected factors for L-glutamic acid production by *Corynebacterium glutamicum* X680. The mutual effects of any two factors on L-glutamic acid were also found encouraging. The most significant ($P < 0.05$) mutual interactions for L-glutamic acid production was in between inoculum age and urea concentration followed by temperature and glucose concentration which are revealed by AVOVA data (Table 2) and response surface-contour plots (Fig. 1). Using Design Expert 12 numerical optimization subroutine design space was explored with the fitted quadratic model to arrive at an optimum fermentation condition. The optimized variables were found using a desirability objective function that assigns relative importance to the responses.

Table 2. Statistical analysis of the BBD model

Source	Sum of squares	df	Mean square	F-value	p-value	Comments
Model	253.3	14	18.09	183.93	< 0.0001	Significant
A-Inoculum age	0.48	1	0.48	4.88	0.0443	
B-Temperature	1.33	1	1.33	13.55	0.0025	
C-Glucose	15.19	1	15.19	154.39	< 0.0001	
D-Urea	1.02	1	1.02	10.38	0.0062	
AB	0.09	1	0.09	0.9149	0.355	
AC	7.02	1	7.02	71.39	< 0.0001	
AD	24.5	1	24.5	249.09	< 0.0001	
BC	8.12	1	8.12	82.57	< 0.0001	
BD	0.5625	1	0.5625	5.72	0.0314	
CD	2.72	1	2.72	27.68	0.0001	
A^2	72.54	1	72.54	737.44	< 0.0001	
B^2	73.63	1	73.63	748.51	< 0.0001	
C^2	51.1	1	51.1	519.44	< 0.0001	
D^2	100.27	1	100.27	1019.31	< 0.0001	
Residual	1.38	14	0.0984			
Lack of Fit	1.27	10	0.1269	4.7	0.0746	not significant
Pure Error	0.108	4	0.027			
Cor Total	254.67	28				

Factor coding is Coded.

Sum of squares is Type III – Partial

Figure 1: Three-dimensional Response Surface and contour plots showing most effective interactions among selected variables influencing L-glutamic acid production by Corynebacterium glutamicum X680 (a)Temperature vs inoculum age; (b) glucose vs inoculum age; (c) (c) Urea vs inoculum age; (d) Glucose vs temperature; (e) Urea vs temperature; (f) Urea vs glucose

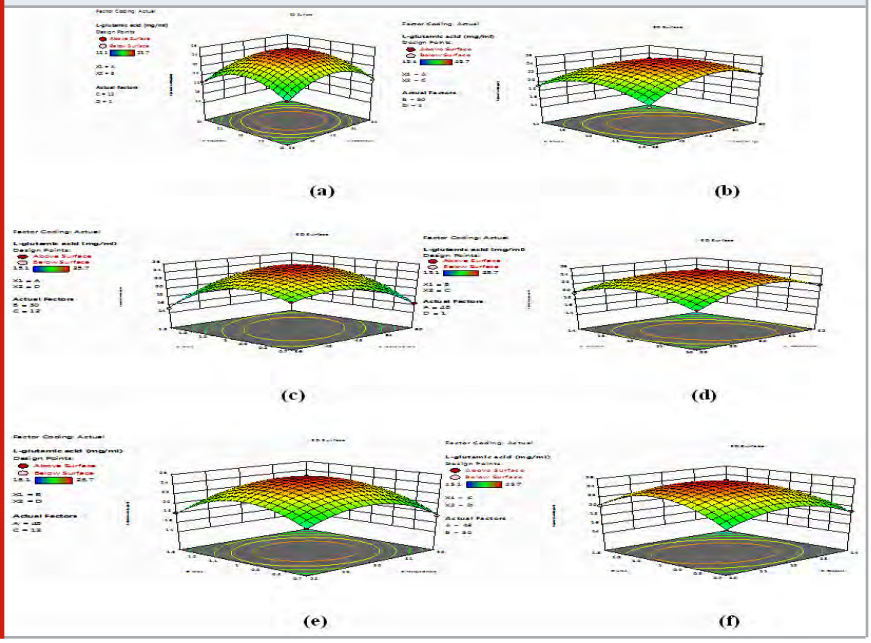
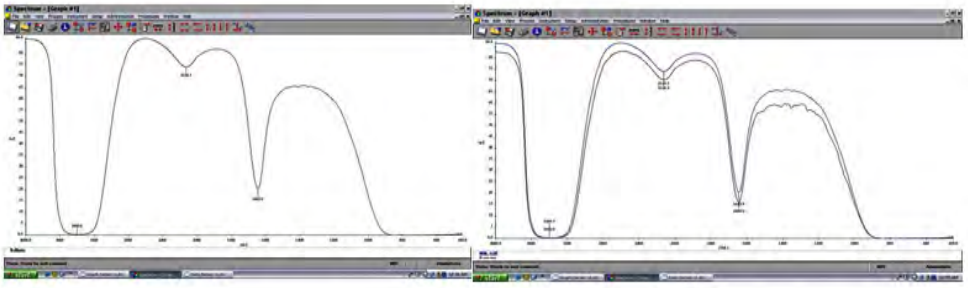
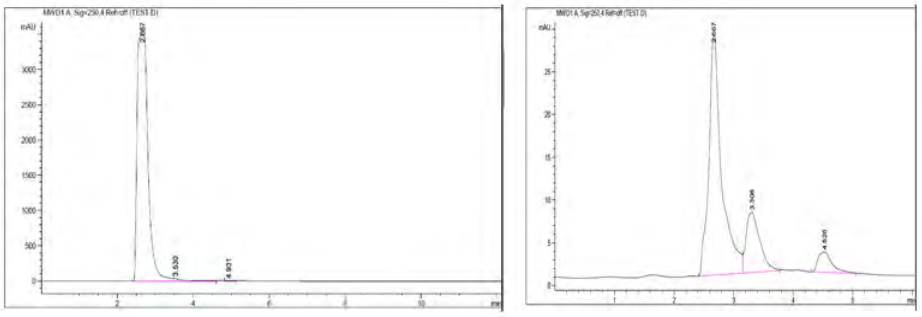


Figure 2: FT-IR for L-glutamic acid (a) Control; (b) Sample



(a) (b)

Figure 3: HPLC for L-glutamic acid. (a) Control, (b) Sample



(a) (b)

Solutions with higher desirability suggested inoculum age of 48.92 h, temperature of 29.99oC, glucose concentration of 11.56% and urea concentration of 1% could resulted in highest L-glutamic acid production of 25.25.61 mg/ml by *Corynebacterium glutamicum* X680. Under these conditions, confirmation experiments were conducted in three replicates and the result have good agreement with the predicted model which validate the suitability of the model (Guo et al. 2017; Fahimitabar et al. 2021).

The Model F-value of 183.93 implies the model is significant. There is only a 0.01% 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 A, B, C, D, AC, AD, BC, BD, CD, A^2, B^2, C^2, D^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The Lack of Fit F-value of 4.70 implies there is a 7.46% chance that a Lack of Fit F-value this large could occur due to noise. Lack of fit is bad -- we want the model to fit. This relatively low probability (<10%) is troubling. Yang et al. (2014) optimized fermentation conditions using Streptomyces albus Y07 as a high yielding strain through RSM. Nor et al. (2017) made a comparative analysis on medium optimization for L-lysine-methionine production by a newly isolated strain Pendiococcus pentosaceus RF1 using one factor at a time, Response Surface Methodology and artificial neural network (Nor et al. 2017).

RSM appeared as a very effective tool for this experiment. Medium optimization for ε-poly-L-lysine production by *Streptomyces diastatochromogenes* was carried out by Guo et al. (2017) using RSM. Very recently, Alharbi et al. (2020) noted RSM as a very effective statistical tool for optimizing culture conditions for L-glutamic acid production by *Corynebacterium glutamicum* NCIM2168, however the production was 16.499g/L. Very recently, Fahimitabar et al. (2021) also applied RSM for optimization of L-glutamic acid by *Corynebacterium glutamicum* in batch fermentation. In our present study, it has been found that RSM appeared to be a very effective tool so far as the optimization of L-glutamic acid production by the mutant was concerned considering multifactorial interactions at a time (Fahimitabar et al. 2021).

CONCLUSION

The findings of the present study targets RSM that helps to point out the causal relationship between different factors and response using a reliable and robust mathematical model. For the validation of this model, statistical and

regression analysis was done. It was found that among different variables involved, most effective variables are inoculum age, 48.92h; temperature, 29.990C; glucose, 11.6% and urea, 1%. Under theses optimum conditions, the predicted response for L-glutamic acid was to be 25.61mg/ml and the actual value was found 25.7mg/ml under the same conditions. These results implied the validity of the predicted value by RSM and experimental value was quite close.

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Physiological Communication

Synchronous Stimulation of Reproductive Cycle in Cows and Heifers

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ABSTRACT

Estrous synchronization allows monitoring the estruation onset and ovulation in the cows or heifers with normal estrous cycles. For dairy cattle, grouping the cows with regular estrous cycles facilitates artificial insemination and accelerates genetic improvement. The number of days needed for artificial insemination is significantly reduced. Therefore, the authors studied the efficacy of the combined use of the gonadotropin-releasing hormone, prostaglandin, and progesterone preparations for stimulating estruation has been studied. The combined use of hormones and prostaglandins is effective in stimulating the arousal stage in cows and heifers. The authors developed a complex scheme of induction (stimulation) and synchronization of reproductive function in cows and heifers. Experimental testing of this technique was carried out in 2017-2019 based on the "Russia" CJSC in the Kanevsky district (Russia). 106 animals were examined. The analysis of literary sources shows that the yellow body of ovary hormones has a specific effect on the genitals. The results of this study were showed that during inflammatory processes in the genitals of cows, the follicles undergo luteinization and can transform into the persistent yellow body, thus causing the absence of the estrous cycles. The studied was confirmed that one-time diagnostics during rectal examination does not provide grounds for distinguishing the persistent yellow body from the yellow body of the cycle. For determining the persistent yellow body, a two-time study in the intervals of three to four weeks is required. Prolonged infertility caused by the progesterone hormone leads to a shortfall in calves and milk. Therefore, the persistent yellow body treatment should be performed in time.

KEY WORDS: ANOVULATION, CYSTS, ESTRUATION, FOLLICLES, PROSTAGLANDINS, PROGESTERONE, PERSISTENT YELLOW BODY, REPRODUCTION.

INTRODUCTION

Increasing the production of milk and beef is impossible without the constant introduction of new technologies and innovative solutions. Therefore, the creation of large dairy farms and specialized complexes on an industrial basis has led to the emergence of the cattle reproduction problem (Koba et al. 2017; Koshchaev et al. 2018; Troshin et al. 2018b). In particular, the changing conditions of livestock keeping, feeding, and care cause metabolic disorders, disrupt the functions of the pituitary-hypothalamic system and the neurohormonal regulation in the organism and, therefore, cause dysfunction of the genital organs of the animals, which entails a decrease of their fertility and milk productivity (Troshin et al. 2018a; Semenov et al. 2019).

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Among the factors influencing the increase in fertility are the conditions for keeping livestock and the observance of the synchronization of sexual heat. The correct approach allows one to control the time of arrival of cows or heifers with normal cycles in heat and ovulation. Also, the time required for identifying the symptoms of estruation is significantly reduced, which simplifies the work schedule by shortening the period of estruation identification and artificial insemination (Anisimova et al. 2018, 2019; Kulikova et al. 2019). In solving the problem of estrous cycles induction and synchronization in cows, two approaches have been established: 1. Suppression of follicular growth in the ovaries through the use of gonadotropic drugs. 2. Induction of the yellow body lysis, followed by follicular growth stimulation. For suppressing the ovaries' gonadotropic function, domestic and foreign science suggests using natural and synthetic prostaglandins (progesterone and its derivatives) for a period equal to the duration of the luteal period of the estrous cycle (Troshin et al., 2018, Nazarov et al., 2021).

For female cattle, this period is 15 – 36 days, while the estrus and estruation in the animals occur on the fifth to the sixth day, and ovulation occurs on the seventh day after the treatment. Progesterone and its derivatives are taken orally (with food or water), intravaginally, intramuscularly, and subcutaneously. It is very important to use the correct dosage for obtaining good results from using prostaglandin. The dosage and frequency of injections depend on the type of product used. Prostaglandin may be used for all nonpregnant cows. If an injection is made to a pregnant cow or a heifer, it may cause an abortion, depending on the stage of pregnancy, as a result of the influence on the formed yellow body. When injected in a cow that has already formed a yellow body (usually, on the sixth through 17th day of the estrous cycle), prostaglandin will cause its premature resorption (Troshin et al., 2018, Sarsadskikh & Abramov, 2020, Nazarov et al., 2021).

At the beginning of the cycle, before the yellow body forms (days one through five), prostaglandin will have no effect. Likewise, when the yellow body regresses at the end of the cycle (days 18 through 21), prostaglandin will not be effective either, but cows at this stage of the cycle should reach estruation at about the same time as the treated animals. The joint use of the gonadotropin-releasing hormone (GnRH) with prostaglandins for good estruation synchronization and monitoring the time of ovulation, according to the analysis of the literature data, proved to be effective and harmless to animals. Certain synchronization drugs were not approved for unlimited use. Therefore, one had to read the attached instructions from the standpoint of the restrictions of their use (Sarsadskikh & Abramov, 2020, Nazarov et al., 2021).

However, in the production conditions, serious shortcomings were revealed in the use of these methods, in particular, the laboriousness of their use, maintaining the dosage of progestogens, and the fertilization rate of the animals did not exceed 42 %. This work aims to develop a system for hormonal synchronization of the stages of the sexual cycle in cows and heifers. Following the objective, the following tasks were set: 1. Developing a system for synchronizing the arousal stage of estrous cycle that would be capable of fruitfully inseminating large numbers of cows and heifers in a short time. 2. Preventing the period of mass calving in dairy cattle breeding for economic purposes and reducing the postpartum period. 3. Substantiating the use of hormones and prostaglandins for arousal stage synchronization in cows, when the identification of estruation was difficult or impossible due to some production reasons.

MATERIAL AND METHODS

Given the above, and due to the adopted program for increasing the cattle population at farms, the staff of the Department of Anatomy, Veterinary Obstetrics, and Surgery of the Kuban State Agrarian University performed scientific research on developing methods for accelerated (extended) reproduction of a cattle herd. As a result of these studies, a complex scheme of induction (stimulation) and synchronization of the reproductive function in cows and heifers was developed, which included two stages:

1. Induction of the reproductive function using a complex preparation (patent for the invention of the Russian Federation No. 2707193, dated November 24, 2019, authors: Nazarov, Kazarinov, et al).
2. Estrous (estrus, estruation) synchronization with the use of drugs from the group of progestogens and prostaglandins.

The experimental testing of this method was performed in 2017 – 2019 under the aegis of ZAO Russia in the Kanevskoy district. For developing a system of hormonal synchronization of the arousal stage of estrous cycles, the cows and heifers were subjected to clinical and obstetric-gynecological examinations. The anamnestic data, entries in the insemination and calving books were considered, and the feeding, keeping, use, and the animal insemination technologies were analyzed. All the cows that had not reached estruation within 25 – 30 days after calving, the heifers – one month after reaching the physiological maturity (that reached 70 % of the live weight of adult cows of the same breed in 14 – 17 months), and the cows repeatedly (three and more times) reaching estruation were studied. During the gynecological examination, attention was paid to the state of the vagina, uterus, and the ovaries; and their size, shape, consistency, uterus response to massage, the presence or absence of ovarian hypofunction, follicles, yellow bodies, and cysts were determined (Nazarov et al. 2016; Skvortsova et al. 2018).

Table 1. The content of sex hormones in the blood of the cows with ovarian hypofunction (nmol/l)

No.	Hormones	Without considering the ovarian cyclicity	Follicular growth wave	Follicular atresia
1	Progesterone	1.68 ± 0.269	0.51 ± 0.096	2.97 ± 0.458
2	Estradiol-17β	0.69 ± 0.032	0.63 ± 0.056	0.77 ± 0.028
3	The P/E ratio	2.44	0.81	3.86

The functioning persistent yellow body was diagnosed by a double rectal examination of the cows with an interval of three to four weeks. The amount of progesterone in the blood of the cows with the persistent yellow body was quite sufficient for inhibiting the function of the pituitary-hypothalamic system with the termination of follicular maturation. The clinical symptoms of a persistent yellow body in cows are associated with disrupted sexual cyclicity

due to the changes in its hormonal function. This violation is most often manifested in a complete depression of the ovarian function, and less often — in the inferiority in the form of anovulatory estrous cycles. In the case of anovulatory estrous cycles, cows have estrus and estruation, but insemination is ineffective (Varenikov et al. 2014; Ratoshny et al. 2018; Tuzov et al. 2018;).

In the endometrial cells, progesterone induces 17β-hydroxysteroid dehydrogenase, which is a key enzyme in the metabolism of estradiol, and transforms it into inactive estrone. It actively reduces the content of prostaglandins in the myometrium by decreasing the synthesis and increasing the activity of the enzymes responsible for their decomposition. Gestagens reduce the sensitivity of the myometrium to the contractile action of serotonin and histamine and increase the expression of β-adrenergic receptors in the myometrium, which have an inhibitory and uterine-relaxing effect. In the endometrium, progesterone causes proliferative changes that create the conditions for embryo implantation. High concentrations of progesterone block the release of pituitary gonadotropic hormones (FSH and LH), which play a fundamental role in the regulation of folliculogenesis, ovum development, and the manifestations of the symptoms of estruation. FSH leads to the synthesis of estrogens in the follicle, which, by increasing the number of FSH receptors, contribute to FSH accumulation, further follicles maturation, and an increase in the estradiol secretion; other follicles undergo atresia at this time (Kryukov et al. 2018).

RESULTS AND DISCUSSION

Based on the analysis of the literature data and practical observations, a system for the synchronous stimulation of estruation in cows and heifers was developed. The system was experimentally tested in 2017 – 2019. Of the 106 cows subjected to synchronous hormonal stimulation of the arousal stage, 65.1 % showed full-fledged estruation. Sixty-nine out of the 106 cows subjected to the induction and synchronization with subsequent fruitful insemination calved and 65 live calves were born, i.e., the calf crop was 61.3 % per 100 cows. It should be noted that before the studies, the gross calf crop was 51 % per 100 cows.

Table 2. The effectiveness of the hormonal induction of sexual cyclicity in the cows with ovarian hypofunction, given the functional state of the gonads

Groups of the cows	The number of the cows subjected to hormonal stimulation	The number of the cows that showed estruation		Calved	
		animals	%	animals	%
Experimental 1	106	69	65.1	65	61.3
Experimental 2	186	158	84.9	119	63.9

In 2018 – 2019, the staff of the Department performed a similar production experiment in the induction and synchronization of the arousal stage in 186 cows. Out of the 186 cows subjected to the induction and synchronization of the arousal stage followed by artificial insemination, 158 animals showed full-fledged estruation and were fertilized. In April to May 2019, 119 animals calved; the calf crop was 63.9 % per 100 cows. Earlier, this farm had obtained 47 calves per 100 cows on average; therefore, this method allowed increasing the calf crop by 21.1 %. Out of the 186 cows subjected to induction and synchronization, 28 cows did not show full-fledged estrous cycles, which was associated with the effect of the dairy dominant on the reproduction function. Alimentary abortions were diagnosed in two cows.

The obtained positive results of using the system (method) of the arousal stage induction and synchronization in cows provide grounds for recommending it for wide practical use in the accelerated (expanded) cattle reproduction in cattle breeding. It allows to promptly perform total artificial insemination of the animals, compacted (catch-up) calving in the most favorable and profitable for farm periods (seasons), and to significantly increase the calf crop. For the widespread introduction of this method at dairy farms, it is necessary to have a sufficient number of full-fledged animals with a normal reproductive capacity. In forming groups of cows for the induction and synchronization of their sexual function, it is advisable to use the animals of medium nutrition state and above two months after calving. A high therapeutic effect for the functional disorders of the ovaries in cows may be obtained only with the differentiated use of the gonadotropic, progestogenic, and other drugs. The dosages, the frequency, and the schemes of use depend on the specific state of the sexual function. In this regard, veterinarians must be proficient in the methods of gynecological examination of animals. Depending on the diagnosis, the following methods of using hormonal and other drugs for regulating the sexual function in the cows with functional ovarian disorders were studied during the gynecological examination of the animals (Nazarov et al., 2021).

The cows with functioning persistent yellow bodies were parenterally injected with 100 ml of the complex drug: on the first day, 2 ml of ovarelin, on the second day, 1.55 g of natural progesterone one time vaginally (the PRID Delta device) per animal for seven days. The PRID Delta was introduced with a special device (applicator), and two days after the removal of the device, 0.5 % dinoprost was used in a dosage of 5 ml per animal. The cows were inseminated 48 hours after the dinoprost administration.

The exogenous progesterone, acting on the hypothalamic-pituitary system, inhibited or stopped the functional activity of the yellow body, while the prostaglandins introduced on this background activated the growth, maturation, and ovulation of the follicles. This joint use of progesterone and prostaglandins makes it possible to fertilize 70 – 75 % of the animals in a month, compared to 5 – 7 % in the reference.

The introduction of progesterone alone in optimal dosages on the background of functioning yellow bodies did not ensure maturation and ovulation of the follicles. They were subjected to cystic atresia. The joint use of hormones and prostaglandin is effective for estrous synchronization, regardless of the stage of the estrous cycle that may be observed in healthy animals at the time of their introduction. The use of these drugs at the initial stage of the estrous cycle (days one through seven) suppresses the forming yellow body, which results in a rapid decrease in progesterone production. The injected progesterone prevents the ovaries from starting a new cycle until it is stopped. The use of progesterone after seven days of the estrous cycle will not cause yellow body regression. However, by the time of the termination of its administration in the animals in the second half of the estrous cycle, a natural regression of the yellow body will occur, and in those in the first half of the estrous cycle, it will regress in response to the introduction of prostaglandin. Therefore, the injected progesterone will again delay estruation of the animal (Sarsadskikh & Abramov, 2020, Nazarov et al., 2021).

Progesterone inhibits estrus, general arousal, heat, as well as the growth and maturation of follicles in the ovaries and ovulation. It ensures the transfer of the uterine glands from the proliferation phase to the secretion phase and prepares the endometrium for the nidation of the zygote. It also blocks the contractile function of the uterine muscles, maintaining normal conditions for the development of the embryo and fetus, preventing their death, and promoting the development of the secretory tissue of the mammary gland. In terms of structure and direction of action for obstetric and gynecological practice, of particular interest is PHF2 alpha, formed in the membranes of the epithelial cells of the endometrium. According to the principle of feedback, it regulates the function of the corpus luteum. The effectiveness of hormonal programs for synchronization of sexual cycling and ovulation in cows depends on the presence or absence of an inflammatory process in the uterus and corpus luteum in the ovary at the time of their inclusion in the synchronization program. When cows were inseminated after 67-73 days, the fertilization rate of clinically healthy animals was 30.7%, with a history of subclinical endometritis – 29.1%, and clinically pronounced endometritis – only 23.3% (Lobodin, 2006, Nazarov et al., 2021).

All animals with cycles can be treated using the proposed program, regardless of the stage they were in at the moment of its start. This program provides the possibility to perform insemination based on the symptoms of estruation, or use it for synchronized artificial insemination.

CONCLUSION

Thus, the intensification of reproduction and the prevention of infertility in cattle, along with the natural factors of sexual function regulation and stimulation (full-fledged feeding, active exercise) include the use of prostaglandins and hormonal drugs, which are highly effective stimulants for the treatment of sexual dysfunction caused by functional disorders of the ovaries in the form of persistence or decreased function of the yellow body. The hormone and prostaglandin synchronization program may be started at any stage of the estrous cycle. The combination of natural progesterone, gonadotropin-releasing hormone, and prostaglandin allows a shortened estrous synchronization and ovulation program.

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

Ethical Clearance Statement: The Current Research Work Was Ethically Approved by the Institutional Review Board (IRB) of Agrarian University, Krasnodar, Russian Federation, 350004 Russia.

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Biotechnological Communication

Bioactivity, Chemical Profiling and 16S rRNA-based Phylogeny of Haloalkaliphilic *Nocardiopsis* sp. GhM-HA-6 Isolated from the Gulf of Khambhat, Gujarat, India

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ABSTRACT

Actinomycetes are well known sources of antibiotics, however; recently the focus of antimicrobial research has been turning towards actinomycetes of extreme environments. Therefore, present work would highlight the isolation, identification and characterization of antimicrobial metabolites produced by marine haloalkaliphilic actinomycetes. Saline soil sample was collected from Ghogha coast (Gulf of Khambhat), Bhavnagar, Western India. Isolation was carried out using selective media while identification was done based on morphological, cultural and molecular characterization. The antimicrobial potential was checked by spot inoculation method. Optimization was carried out by the one variable at a time (OVAT) method. The antimicrobial compounds were extracted using ethyl acetate and characterized by GC-MS. The haloalkaliphilic actinomycetes *Nocardiopsis* sp. GhM-HA-6 was isolated from saline soil of Ghogha coast using starch agar with 10% w/v NaCl and pH 9 and was identified as *Nocardiopsis* sp. based on morphology, cultural characteristics and 16S rRNA phylogenetic analysis (NCBI Genbank Accession number: KF384492). The organism showed antimicrobial activity against five Gram positive and three Gram negative bacteria while the isolate didn't show any antifungal activity. Results of optimization showed that the highest production of antimicrobial compounds was obtained using starch broth with 0.5% w/v starch, 1% w/v yeast extract, 10% w/v NaCl and pH 9. GC-MS analysis of ethyl acetate extract of the isolate showed presence of a total 18 compounds including various antimicrobial compounds like 2, 4-bis (1, 1-dimethylethyl)-Phenol, various types of alkanes and their derivatives. Haloalkaliphilic actinomycete *Nocardiopsis* sp. *GhM-HA-6*, from a rarely explored marine habitat, can be a source of antimicrobial compounds with the novel biotechnological applications.

KEY WORDS: ANTIMICROBIAL ACTIVITY; HALOALKALIPHILIC; GAS CHROMATOGRAPHY-MASS SPECTROSCOPY; MARINE ACTINOMYCETES; 2, 4-BIS (1, 1-DIMETHYLETHYL)-PHENOL.

INTRODUCTION

Actinobacteria are well known for their ability to produce valuable secondary metabolites such as antibiotics and antimicrobial substances. Amongst genera of actinobacteria, *Nocardiopsis* genus was primarily described by Meyer in 1976 and was placed in the class of Actinobacteria; subclass Actinobacteridae; order Actinomycetales and family Nocardiopsaceae (Ibrahim et al. 2018). Rainey et al. (1996) defined a new family referred as Nocardiopsaceae considering phylogenetic position, morphological features, and chemotaxonomic properties of *Nocardiopsis* sp. This genus includes Gram positive, aerobes, non-acid fast with catalase positive properties (Cook and Meyers 2003; Bennur et al. 2015; Ibrahim et al. 2018). Studies by Kroppenstedt and Evtushenko (2006) showed various features of genus *Nocardiopsis* which includes the presence of meso-2, 6-diaminopimelic acid, but lack of diagnostically important carbohydrates in cell wall structure, absence of madurose or nocardomycolic acids in whole cell hydrolysates and high GC content in their genomes (Dhakal et al. 2017; Subramani and Sipkema 2019).

Actinobacteria are widely found in soil and physiologically extreme environments such as desert, saline, hyper saline, and alkaline origins. Particularly, *Nocardiopsis species* are often

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found from hyper saline environments such as soda lakes and salterns along with Streptomyces species (Mwirichia et al. 2010; Jose and Jebakumar 2012; Kamjam et al. 2017). It is believed that *Nocardiopsis* genus might be playing a mediating role in breakdown of naturally occurring complex polymers, these species mainly produce extremozymes, compatible solutes, and surfactants (Bennur et al. 2015). Genus *Nocardiopsis* has been reported to possess gene clusters for polyketide synthases (PKSs) and non-ribosomal peptide synthetases (NRPSs) and additionally many of the natural isolates have also demonstrated capability antibiotic production (Zitouni et al. 2005; Meklat et al. 2011; Becerril-Espinosa et al. 2013; Romanenko et al. 2013; Schorn et al. 2016; Subramani and Sipkema 2019).

Nocardiopsis sp. often secretes important antimicrobials including polyketides, phenzines, quinoline alkaloids, terphenyls, proteins, thiopeptides, and amines (Bennur et al. 2015). Marine isolates of *Nocardiopsis* sp. are often reported to produce cyclic hexapeptides which render antimicrobial activity (Wu et al. 2013). Dolak et al. (1980) found the production of a novel antibiotic 3-trehalosamine was reported from soil-derived N. trehalosei. The study also reported production of dopsisamine and thiopeptide antibiotics by *N. mutabilis* and *Nocardiopsis* sp. TFS65-07, respectively (Takahashi et al. 1986; Engelhardt et al. 2010a). Among the nine maritime states in the Indian peninsula, only a few states have been extensively covered for the study of marine actinobacteria for antagonistic properties against different pathogens (Sivakumar et al. 2007). The Gulf of Khambhat is a coastal region situated in the Bhavnagar District of the Indian State Gujarat, which covers approximately 3120 km^2 area of mudflat with some rocky (sandstones) intertidal area and a volume of 62,400 million m^3 (Ramnathan et al. 2002; Subramani and Sipkema 2019).

Alang and Ghogha coasts of Gulf of Khambhat, India is relatively less explored with respect to antagonistic properties of actinomycetes. Ghogha is a small town situated at the mid-western shore of the Gulf of Khambhat, covering about 4 km long area (21°40'32" to 21°41'18" N and 72°17'5" to 72°16'48 E) in Bhavnagar, Gujarat with unique characteristics of having sandy supratidal zone, rocky-muddy middle intertidal zone and highly muddy lower intertidal zone (Solanki et al. 2016; Subramani and Sipkema 2019). The uniqueness of this region is its salinity and alkalinity, which harbors various unidentified, unique haloalkaliphilic bacterial species that can potentially produce secondary metabolites. This highlights the urgent need for exploration of untapped locations for novel antimicrobial antagonist producers. In the light of this knowledge, the present study was intended to isolate, screen and characterize the antimicrobial metabolite producing haloalkaliphilic actinomycetes from the Gulf of Khambhat.

MATERIAL AND METHODS

The haloalkaliphilic actinomycete, *Nocardiopsis* sp. GhM-HA-6 was isolated from saline soil of Ghogha coasts (Gulf of Khambhat), Bhavnagar (Longitude: 72° 11' E and latitude: 21° 46' N), India and treated with calcium carbonate and Ringer's solution. Approximately 10-15g of wet soil sample was mixed with a pinch of calcium carbonate and was dried at 75°C overnight. Next day, 1g of soil sample was added to 10ml of Ringer's solution. From the supernatant, 0.1 ml of the sample was spread separately on starch agar (Hi-Media, India), containing 10% w/v NaCl and pH 9. The plates were incubated at 30°C; on the sixth day a typical chalky white colony was appeared, the colony characteristics were noted and Gram's staining was performed.

On the seventh day, colony was picked up and re-streaked on starch agar slants supplemented with 10% w/v NaCl and pH 9 to ensure the purity of the colony (Chakrabarti 1998). The culture was maintained at 4°C. The molecular identification of the isolate was carried out by 16S rRNA gene sequencing. The 16S rRNA gene was amplified using universal primers 518F 5'ccagcagccgcggtaatacg3' and 800R 5'taccagggtatctaatc3'. PCR products were purified and sequenced. The resultant sequences aligned within the NCBI database (National Centre for Biotechnology Information) using BLASTN. The phylogenetic tree was constructed using neighbor-joining with Kimura 2-state parameter and pairwise-deletion model analysis implemented in the program MEGA software version X and also evaluated further in a bootstrap analysis of 1,000 replicates. The antimicrobial potential of *Nocardiopsis* sp. GhM-HA-6 was checked by spot inoculation method using starch agar (10% w/v NaCl, pH 9) (Kumar et al. 2010; Kumar et al. 2018).

The spore suspension of the isolate was spotted on the medium and incubated at 28°C until sporulation. The test organisms, procured from MTCC, Chandigarh (ATCC equivalent), were used to check antimicrobial activity. These included Gram-positive organisms: *Bacillus subtilis* (MTCC 441), *Staphylococcus aureus* (MTCC 737), *Micrococcus luteus* (MTCC 106), *Staphylococcus epidermidis* (MTCC 3615), *Bacillus megaterium* (MTCC 428), *Bacillus cereus* (MTCC 430); Gram-negative organisms: *Pseudomonas aeruginosa* (MTCC 424), *Escherichia coli* (MTCC 443), *Enterobacter aerogenes* (MTCC 8560), *Serratia marcescens* (MTCC 97), *Shigella flexneri* (MTCC 1457), *Salmonella enterica typhimurium* (MTCC 98), *Proteus vulgaris* (MTCC 1771), *Klebsiella pneumonia* (MTCC 3384) and three fungi: *Aspergillus niger* (MTCC 282), *Fusarium oxysporum* (MTCC 284), *Candida albicans* (MTCC 227).

The test organisms were grown in nutrient broth at 37°C for 24 hours. The molten nutrient agar, with 1% activated test culture, was poured on the sporulated *Nocardiopsis* sp. GhM-HA-6. After the incubation of 24 hours at 37°C, the zone of inhibition was measured for each test organism. To achieve the highest production of antimicrobial compounds, effects of various growth conditions were studied by the one variable at a time (OVAT) method. The effect of various media (starch broth, starch casein broth, actinomycetes broth, glucose asparagine broth, glucose glycine broth, soybean malt broth and complete broth with 10% w/v NaCl and pH 9), effect of starch as a carbon source (0-1.5% w/v), effect of yeast extract as a nitrogenous source (0-1.5% w/v), effect of NaCl (0-15% w/v) and effect of pH (8-11)

on the production of antimicrobial compound was checked. All the flasks were incubated at 30^{0}C for 7-8 days. Then the cell free filtrates of the culture were collected from all the flasks separately and were tested against the actively growing sensitive culture of *S. flexneri* by agar well diffusion method.

The isolate was cultivated in starch broth with 10% w/v NaCl and pH 9 on a rotary shaker (120 rpm) at 37^{0}C for 8 days. The cell-free extract was obtained by filtration of broth culture using Whatman No. 1 filter paper. Equal volume of ethyl acetate was added to the culture filtrate for the extraction of the bioactive compounds. The mixture was added to 250 ml glass flask, sealed with a cotton plug, followed by aluminum foil to reduce evaporation of the organic solvent and placed on a shaker for 2 hours at 150 rpm. Post agitation the mixture was transferred to a separating funnel to generate different layers; the organic layer that contained the secondary metabolites and the aqueous layer. The crude extract was obtained by concentrating the solvent by evaporation and stored at 4°C for further use. Identification of the chemical compounds present in the crude ethyl acetate extract of *Nocardiopsis* sp. *GhM-HA-6* was carried out by Gas Chromatography-Mass Spectrometry (GC-MS) analysis.

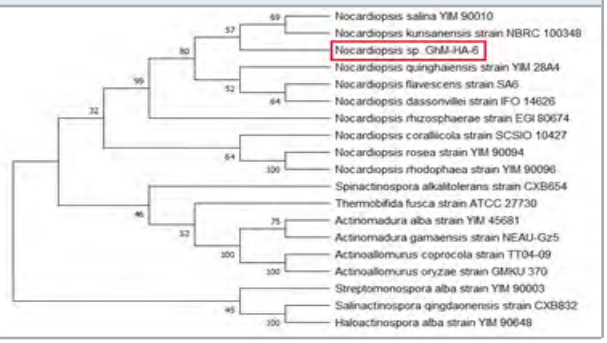
Analysis was conducted on a capillary column (Rxi-5ms, 30m, 0.25 mm id, 0.25 µm film thickness) with the following conditions: constant flow of Helium, 1.0 ml min-1; the fixed inlet temperature, 285°C throughout the analysis; injection volume, 3 µl in the linear with an open purge valve (30:1 split ratio); Linear velocity: 36.8 cm/second; Pressure: 65.0 kPa; Purge flow: 3.5 ml/min; Column flow: 1 ml/min; Oven ramp: 80°C holds for 2.0 min, 18°C/min to 260°C holds for 6.0 min, 4°C/min to 285°C holds for 6.0 min; Total run time: 30.25 min. The MS instrument with Ion source temperature: 200°C; Interface temperature: 300°C; Solvent cut time: 5.0 min; Detector voltage: 1 kV; Acquisition mode: Scan mode; Scan speed: 909; Event time: 0.78 second; starting m/z: 40 to 700 m/z. The peaks were identified by comparing the mass spectra with the National Institute of Standards and Technology (NIST, USA) library.

RESULTS AND DISCUSSION

Isolation and Morphology of the Organism: *Nocardiopsis* sp. GhM-HA-6, a haloalkaliphilic actinomycete, was isolated from the Gulf of Khambhat, Western India. The isolate was characterized on the basis of its cell and colony morphology and Gram's reaction. The colonies were small sized, round shaped, regular, slightly raised, rough, and opaque. It was Gram-positive, having a filamentous, long thread-like structure. It started sporulation on starch agar after 3 days of incubation with a fluffy mass of spores (Kumar et al. 2018).

Molecular Identification of *Nocardiopsis* sp. GhM-HA-6 through 16S rRNA Sequencing: The 16S rRNA gene sequencing of the strain *Nocardiopsis* sp. GhM-HA-6 showed the presence of 1456 bp long 16S rRNA gene in the genomic sequence. The sequence was submitted to NCBI, GenBank, Maryland, USA with accession number (KF384492). The phylogenetic tree was constructed using the neighbor-joining with Kimura 2-state parameter and pairwise-deletion model analysis implemented in the program MEGA software version X and also evaluated in a bootstrap analysis of 1,000 replicates (Figure 1). The molecular characterization through 16S rRNA gene sequencing revealed that the strain belonged to *Nocardiopsis* sp. (Kumar et al. 2018).

Figure 1: The phylogenetic tree of Nocardiopsis sp. GhM-HA-6



The Antimicrobial Potential: The isolate *Nocardiopsis* sp. GhM-HA-6 inhibited the growth of five Gram-positive bacteria *Bacillus subtilis, Staphylococcus aureus, Micrococcus luteus, Staphylococcus epidermidis, Bacillus cereus,* and three Gram-negative bacteria *Shigella flexneri, Pseudomonas aeruginosa, Serratia marcescens* (Figure 2). The highest inhibition was recorded against *Shigella flexneri*, however the isolate didn't show any inhibitory effect against fungi.

Optimization of Growth Conditions for Antimicrobial Compound Production: Optimum production of the antimicrobial compound, against *S. flexneri* was obtained in starch broth followed by soybean malt broth, starch casein broth and actinomycetes broth. However, *Nocardiopsis* sp. GhM-HA-6 wasn't able to produce any kind of antimicrobial compounds in complete broth, glucose asparagine broth and glucose glycine broth (Figure 3). Optimization of various growth conditions showed that the highest antimicrobial compound production was obtained in the presence of 0.5 % w/v starch, 1% w/v Yeast extract, 10% w/v NaCl and pH 9 (Figure 4-7).

Figure 2: The antibacterial potential of *Nocardiopsis* sp. GhM-HA-6

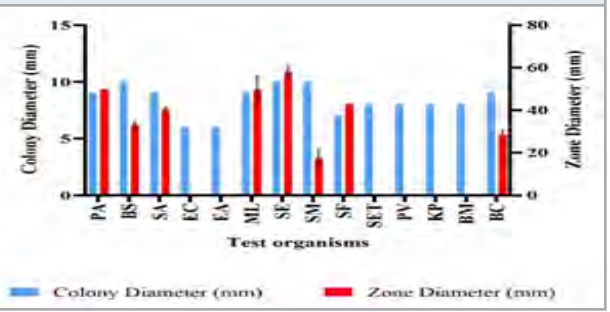


Figure 3: Effect of various media on growth and antimicrobial compounds production by *Nocardiopsis* sp. GhM-HA-6 against *S. flexneri*

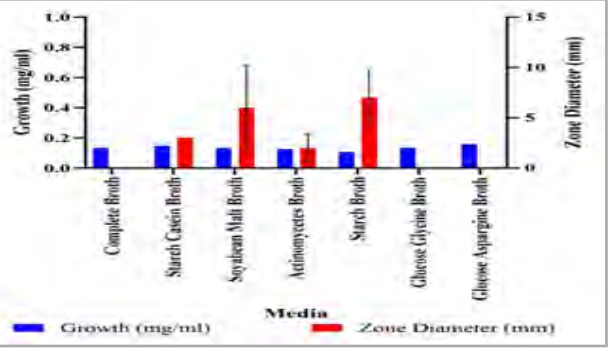


Figure 4: Effect of starch concentrations on antimicrobial compounds production by *Nocardiopsis* sp. GhM-HA-6 against *S. flexneri*

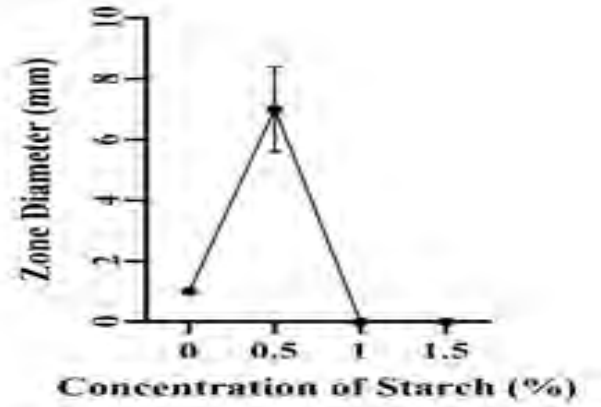
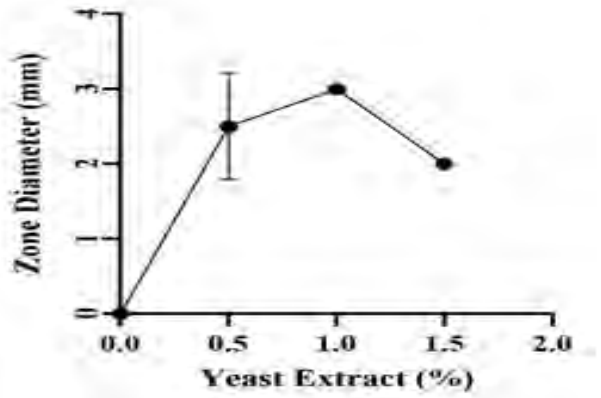


Figure 5: Effect of yeast extract on antimicrobial compounds production by *Nocardiopsis* sp. GhM-HA-6 against *S. flexneri*



Identification of Bioactive Compounds by GC-MS Analysis of Ethyl Acetate Extract: Identification of the bioactive compounds, present in ethyl acetate extract, was carried out using GC-MS analysis. The GC-MS chromatogram of the *Nocardiopsis* sp. GhM-HA-6 crude extract showed a total of 18 peaks (Figure 8). When compared with the NIST database, the nearest compound hits for those peaks were found (Table 1).

Figure 6: Effect of salt concentrations on antimicrobial compounds production by *Nocardiopsis* sp. GhM-HA-6 against *S. flexneri*

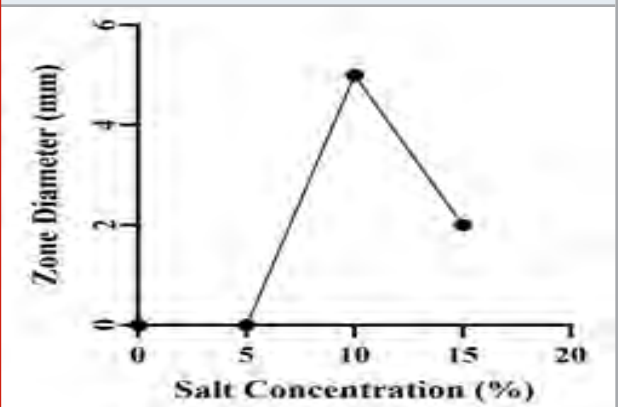


Figure 7: Effect of pH on antimicrobial compounds production by *Nocardiopsis* sp. GhM-HA-6 against *S. flexneri*

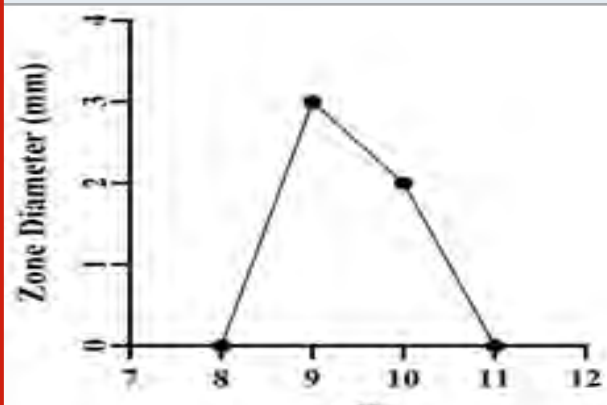
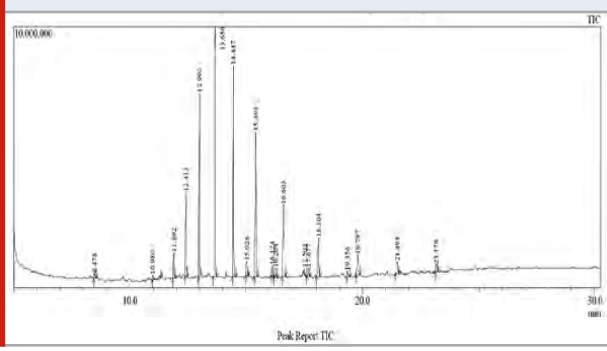


Figure 8: GC-MS chromatogram of *Nocardiopsis* sp. GhM-HA-6 ethyl acetate extract



The declining trend in the discovery of new antimicrobial compounds and the enigmatic development of antibiotic resistance in bacteria has increased the need for scientists to search for novel antimicrobial compounds (Genilloud

2017; Durand et al. 2019). Microbial life can be found in the most diverse conditions, including extremes of temperature, pressure, salinity, pH, nutrient concentrations, water availability and presence of high levels of radiations, harmful heavy metals and toxic compounds such as organic solvents and hydrocarbons (Thumar and Singh 2008; Durand et al. 2019Subramani and Sipkema 2019). Ability to sustain under extremity makes them an interesting system to study the biochemical and molecular basis of adaptations, besides biotechnological aspects (Sharma et al. 2012). In the light of this knowledge, we should explore new habitats in order to identify novel actinobacterial isolates and bioactive compounds. Recently, extremophilic actinobacteria have attracted the researchers with the hope that these organisms would add an inventive dimension to naturally available antimicrobial products research (Zitouni et al. 2004; Vijayakumar et al. 2012; Dhanasekaran et al. 2014; Sharma et al. 2020; Rathore et al. 2021).

Table 1. GC-MS analysis of *Nocardiopsis* sp. GhM-HA-6 ethyl acetate extract

Peak No.	Retention Time	Name of the Compound	Molecular Formula	Molecular Weight	Peak Area %
1	8.478	Phenol, 2,4-bis (1,1-dimethylethyl)-	$C_{14}H_{22}O$	206.32	0.52
2	10.980	Heptadecane	$C_{17}H_{36}$	240.46	1.09
3	11.892	Heneicosane	$C_{21}H_{44}$	296.6	1.76
4	12.413	Docosane	$C_{22}H_{46}$	310	5.85
5	12.990	Tricosane	$C_{23}H_{48}$	324	13.77
6	13.656	Tetracosane	$C_{24}H_{50}$	338	20.94
7	14.447	Pentacosane	$C_{25}H_{52}$	352	18.26
8	15.026	Hexacosane	$C_{26}H_{54}$	366	1.35
9	15.408	2-methylpentacosane	$C_{26}H_{54}$	366.7	14.08
10	16.124	Heptacosane	$C_{27}H_{56}$	380	0.95
11	16.264	Tetratetracontane	$C_{44}H_{90}$	619.2	1.05
12	16.603	2-methylhexacosane	$C_{27}H_{56}$	380.7	8.28
13	17.503	2,24-dimethylpentacosane	$C_{27}H_{56}$	380.7	0.56
14	18.104	2-methylheptacosane	$C_{28}H_{58}$	394.8	4.78
15	19.356	Octacosane	$C_{28}H_{58}$	394	0.69
16	19.797	Nonacosane	$C_{29}H_{60}$	408	2.52
17	21.498	Triacontane	$C_{30}H_{62}$	422	1.52
18	23.178	Hentriacontane	$C_{31}H_{64}$	436.85	1.01

Significant studies have not been conducted so far in the Gulf of Khambhat. Therefore, the present study was intended to isolate, screen and characterize antibiotics producing haloalkaliphilic actinomycetes from the Gulf of Khambhat. *Nocardiopsis* sp. GhM-HA-6, a haloalkaliphilic actinomycete was isolated from the Ghogha site of Bhavnagar district. The isolate was identified as Gram-positive, filamentous, long thread-like structure containing, spore forming actinomycete based on cell and colony morphology and Gram's staining while molecular characterization confirmed the isolate as *Nocardiopsis* sp. GhM-HA-6 (Rathore et al. 2021).

The isolate showed the highest similarity with *Nocardiopsis salina* YIM 90010 (98.46%) and the lowest similarity with *Actinomadura gamaensis* (90.61%). The isolate was able to inhibit the growth of total eight test organisms. The antimicrobial compounds produced by the isolate were more effective against Gram-positive bacteria as compared to Gram-negative bacteria while the isolate didn't show any inhibitory effect against fungi. Among all selected test organisms, the highest sensitivity was observed against *Shigella flexneri*, the intestinal tract pathogen. Sharma et al. (2016) checked the broad-spectrum antimicrobial activity of forest derived soil Actinomycete, *Nocardia* sp. PB-52 and this study results were quite comparable with it (Sharma et al. 2016; Nithya et al. 2018). Nithya et al. (2018) isolated 134 morphologically distinct actinobacteria from various soil samples collected from the Saudi Arabian desert.

They also used the same methods for isolation, identification, and characterization of antimicrobial compounds from the actinomycete isolates. Range of parameters can affect the production of antimicrobial compounds, so to increase the yield, the effect of growth media, carbon source, nitrogen source, NaCl and pH was checked on the production of antimicrobial compounds by isolate. Optimization of media showed that the highest antimicrobial compound production and the highest antimicrobial activity against *S. flexneri* was found in starch broth followed by soybean malt broth,

starch casein broth and actinomycetes broth. Optimization showed that the higher production of antimicrobial compounds was observed in media supplemented with complex carbon sources such as starch and malt while production of antimicrobial compounds wasn't found in media supplemented with glucose like simple carbon source. So, for further production processes starch was used as a carbon source (Elsayed et al. 2020).

The optimization of starch concentration indicated that 0.5% w/v is the optimum concentration required for the highest production of antimicrobial compounds, further increase or decrease in concentration of starch inhibits the production of antimicrobial compounds. Yeast extract was provided as the nitrogen source, initial increase in concentration of yeast extract increased the production of antimicrobial compounds up to 1% w/v, further increase in yeast extract concentration decreased the production. The isolate showed the highest production of antimicrobial compounds at 10% w/v NaCl followed by 15 % w/v NaCl. The isolate wasn't able to produce antimicrobial compounds in absence or at lower concentrations of NaCl. Optimization of pH showed that the isolate was able to produce antimicrobial compounds at pH 9 and 10 but the highest activity was observed at pH 9 (Kumar et al. 2019).

The GC-MS chromatogram of the *Nocardiopsis* sp. GhM-HA-6 ethyl acetate extract indicated the presence of 18 compounds, from which 15 compounds have already been reported to have antimicrobial potential against variety of pathogens including Phenol, 2, 4-bis (1, 1-dimethylethyl); Heptadecane; Heneicosane; Docosane, Tricosane, Tetracosane, Pentacosane, Hexacosane; Heptacosane; Tetratetracontane; 2-methylhexacosane; Octacosane; Nonacosane; Triacontane; Hentriacontane while methyl derivatives of alkanes identified from ethyl acetate extract haven't been reported for antimicrobial activity (Padmavathi et al. 2014; Nandhini et al. 2015; Begum et al. 2016; El-Naggar et al. 2017; Kumar et al. 2019; Elsayed et al. 2020).

CONCLUSION

The findings of the present study indicated that *Nocardiopsis* sp. GhM-HA-6, isolated from the Ghogha site, inhibited the growth of various test organisms including *Staphylococcus epidermidis, Pseudomonas aeruginosa, Shigella flexneri,* and *Staphylococcus aureus*. The ethyl acetate extract of *Nocardiopsis* sp. GhM-HA-6, indicated the presence of potent antimicrobial compounds such as Phenol-2, 4-bis (1, 1-dimethylethyl) - which can further be purified and used for the treatment of various known and unknown infections. In nutshell, our findings in this field will surely be a significant contribution to the knowledge of compounds unique from marine bacteria as potential sources of new drugs in the pharmacological industry.

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

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Biotechnological Communication

Antioxidant Potential of Fucose Isolated from the Marine Macroalgae *Padina gymnospora*

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ABSTRACT

The marine source variety allows the selection of polysaccharides isolated from seaweeds with specific characteristics that are completely absent in polysaccharides from terrestrial plants. Algal polysaccharides and their structural diversity constitute a source of several biological capacities that may represent an interesting tool for novel therapeutic benefits and industrial applications, including nutraceuticals, pharmaceuticals, and functional foods. Currently, sulfated polysaccharides are found principally as recipients in feed, food and pharmaceutical formulations, but the discovery of surprising biological capacities makes these polymers a very exciting research field. For a vision towards the future, the use of algal polysaccharides in medicine is expected to considerably progress. Padina is a widely available brown alga in the marine coastal region and gained great attention of researchers all over the world. Padina can be used as food, fodder, plant growth promoter and bio-fertilizer. The brown alga is well known and is utilized for its various pharmacological properties like antimicrobial, insecticidal, antioxidants, antibiotics, anti-inflammatory, hypo-allergenic, hepatoprotective and antidiabetic activities. However, the current work aimed to evaluate in vitro antioxidant activity of L-Fucose-potent polysaccharide isolated from *Padina gymnospora*. L-Fucose was extracted with ethanol and acetone from brown algae *Padina gymnospora*, followed by isolation and purification process. The polysaccharide composition was assessed using high-performance liquid chromatography. Free radical scavenging activity of purified L-Fucose was evaluated using different in vitro systems such as DPPH radical scavenging assay, Hydrogen peroxide, Nitric oxide, Ferric reducing antioxidant power, Deoxyribose Radical scavenging assay, ABTS Radical cation scavenging assay, Superoxide radical scavenging assay, Superoxide Dismutase scavenging assay. Based on the results obtained, we conclude that L-Fucose isolated from *Padina gymnospora* have potential radical scavenging activity.

KEYWORDS: ANTIOXIDANT ACTIVITY, DPPH, L-FUCOSE, LIPID PEROXIDASE (LPO), RADICAL SCAVENGING ACTIVITY.

INTRODUCTION

Brown seaweed (Phaeophyceae) is the largest and most complex type of algae, having brown, olive or yellowish–brown in color. There are about 1800 species of brown seaweed, broadly distributed from tropical to polar zones of ocean in the world (Harris et al. 2011). Marine plants have long been recognized as producers of biologically active substances. Potential activities of some marine plants like mangroves, seaweeds, sea grasses and lichens have been reported from India. Marine secondary metabolites are secretory products of marine microbial species, sponges, seaweeds, and another marine biota. Due to increasing demand shown as an output of the research towards search of therapeutic molecules from natural sources, greater interest is growing on marine organisms especially seaweeds (Maheswaran et al. 2013; Kumar et al. 2021).

Seaweeds are extraordinary sustainable resources found within the marine ecosystem which have been explored as a source of food and feed and about 50% of the global photosynthesis is being contributed through marine algae (Neelam 2005). Seaweeds have been used in traditional medicine for many centuries, and are of potential interest for the pharmaceutical and food industries (Guaratini et al.

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2012). Seaweed is a preferable bioactive compound as it comprises stable antioxidants as compared to terrestrial plants and helps in preventing oxidative stress and other mammalian diseases (Kumar et al. 2021). Seaweeds or marine macroalgae are primitive non-flowering plants with absence of true root, stem and leaves in their structural system. There are a lot of reports proving on antibacterial activity of solvent extracts from different marine algae. Several bioactive compounds from the seaweeds have shown pharmacological properties, primarily for treating deadly diseases like tumor, Acquired Immuno Deficiency Syndrome (AIDS), rheumatic arthiritis etc (Guaratini et al. 2012; Kumar et al. 2021).

Brown algae contains a broad spectrum of acid polysaccharides which constitutes alginic acids, comprising of uronic acid; the homo fucans, consisting of sulfated fucan and the heterofucans, that contain portions of mixed neutral sugars and uronic acids in addition to sulfated fucose. In all these polysaccharides, difference in branched structures, a varied distribution of sulfate and occasionally acetyl groups may be observed (Castro et al. 2016). Free radicals are highly reactive molecules with an unpaired electron that are produced by radiations or as by-products of metabolic products of metabolic processes. The free radicals in excited state initiate chain reaction within biological system which lead to disintegration of cell membrane and cell compound, including lipids, protein and nucleic acids. Antioxidants are defensive compounds released by the cell during such oxidative stress to scavenge free radicals such as peroxide, hydro peroxide or lipid peroxyl and decreases the levels of oxidative stress and prevent the development of complication associated with oxidative stress related disease. Thus, naturally occurring antioxidants are non-toxic without any deleterious side effects when compared to the chemically synthesised (Wu et al. 2008; Castro et al. 2016; (Kumar et al. 2021).

Sulfated polysaccharides (SP) of marine resources are the anionic polymers that occurs abundantly in most of the macroalgal community. Fucoidan represents the SPs exclusively of marine brown algae whereas agar and carrageenan are present mainly in those SPs that occur in the red algae. Green algae have a heterogeneous class of SPs with different sugar residues such as glucuronoxylorhamnans, glucuronoxylor rhamnogalactans, and xyloarabinogalactans (Na et al. 2010; Costa et al. 2010). Fucoidan refers to structural polysaccharide composed mainly on sulfated L-fucose, and consists of less than 10% of othercontributing monosaccharides. The term sulfated fucan can be used to define heterofucans containing sulfated fucose and neutral sugars. However, fucans and fucoidans are often interchangeably used to describe the SPs of seaweeds. Fucoidans are extracted from brown seaweeds such as *Ecklonia cava, Saccharina longicruris, Fucus vesiculosus, Ascophyllum nodusum*, and *Undaria pinnatifida* (Ramli et al. 2020; Kumar et al. 2021).

Fucoidan is a sulphated polysaccharide containing important biological activities due to having a different amount of sulphate group in its chemical structure. Fucoidans are a series of sulphated polysaccharides that occurs widely in the cell walls of brown macroalgae. Fucoidans are reported to exhibit diverse physiological and biologically therapeutic properties. In addition, the pharmacological potential of fucoidans tends to increase with their degree of sulfation and they can be easily extracted from the source using either by percolation in hot water or an acid solution. These polymers generally occur in the intercellular tissues or mucilaginous matrix of brown algae. However, the structure of algal fucans varies among species and sometimes also among different parts of the seaweed of same species (Rocha et al. 2005; Kumar et al. 2021).

Thus, each purified sulfated L-fucose is a unique novel compound and thus can be explored as a potential lead compound or a prodrug. Many research has proved the anti-inflammatory activity of a fucoidan from the alga *Fucus vesiculosus*, called sulphated fucan. During inflammatory response, fucoidans are potent inhibitor of migration of leucocytesto the site of inflammation, which is contributed by its interaction with P and L-selectin (Zang et al. 2001; Klintman et al. 2002; Cardoso et al. 2010). Sulfated fucans from the Fucales and Laminariales orders has been reported to prevent recruitment of leucocytes in an inflammation model studied in rats (Cardoso et al. 2010; Paiva et al. 2011; Kumar et al. 2021). However, the present study was an attempt to determine the antioxidant potential of the L-Fucose purified from *Padina gymnospora*.

MATERIAL AND METHODS

Padina gymnospora was collected from coastal water bodies in Rameshwaram. These algae were washed using tap water, dried under sunlight and then dried in an oven at 60 °C. Finally, they were crushed into a fine powder and stored in a 4 °C refrigerator for further analysis. The method for extraction of fucoidan from brown macroalgae as described in the past studies, were followed with some modification (Yang et al. 2008; Rodriguez-Jasso et al. 2011; Foley et al. 2011).Ten grams of algal powder was mixed with 100 mL of 85% ethanol and incubated in shaker for around 12 h at room temperature to remove lipids and pigments. The solutions were then subjected to centrifugation for 10mins at 3273g to remove the supernatant. The remaining sediment was repeatedly washed with acetone to remove any contaminants and left to dry at room temperature overnight. Five grams of the sediment of was extracted with 200 mL of deionized water for 1 h upon hot plate at 65 °C and stirred occasionally. The mixture was again subjected to centrifugation at 3273 × g for 10 min.

1% CaCl2 was added to the supernatant to precipitate the alginate and the solution was subsequently added to 95% ethanol to obtain a final ethanol concentration of 30% (v/v). Finally, the fucoidan was recovered after centrifuging at 3273 × g for 10 min. The fucoidan extract obtained was lyophilised and stored at 4°C. The commercial Fucoidan from Padina gymnospora (Sigma, USA) were used as a reference to check the purity of the experimentally recovered fucoidan. 200 mg of extracted fucoidan was separately dissolved in 20 ml of distilled water and heated at reflux with 0.75 ml of 3.0M HCl for 3 h. After cooling, the mixture was centrifuged at 3000 rpm and 1.0 M NaOH

was added to neutralise the supernatant solution and poured over 100 ml of ethanol. Then the precipitate was redissolved in distilled water and freeze dried.

The polysaccharide content was assessed using an HPLC system comprising a pump, injection valve with a 20-µL sample loop, PL Hi-Plex H column and refractive index detector. 10 mg of the sample was treated with 2 mL of 2 M trifluoroacetic acid at 121°Cfor 1 h. After trifluoroacetic acid hydrolysis, the reaction medium was dried with a vacuum concentrator, and distilled water was added to redissolve the sample. The resultant mixture was neutralized to approximately pH 7 by using 1N NaOH. One milligram per millilitre of polysaccharide sample was injected into the HPLC system. The column was kept in a 65°C column oven (COLBOX), and distilled water was used as the mobile phase at a flow rate of 0.6 mL/min. The data were analyzed using the software Chromera Perkin Elmer system. The antioxidant activity such as DPPH radical scavenging assay, Hydrogen peroxide scavenging activity, Nitric oxide scavenging activity, Ferric reducing antioxidant Power (FRAP), Deoxyribose Radical Scavenging Activity, ABTS [2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonicacid)] Radical Cation Scavenging Assay, Superoxide radical scavenging activity (SO), Superoxide Dismutase Scavenging Assay (SOD) (Suganya et al. 2017).

The statistical analyses for all the experiments were done using Excel 2013 through statistical formula. Experimental data were expressed as mean ± SD and IC 50 values were calculated. The experiment was performed in triplicates for all the test samples (Suganya et al. 2017).

RESULTS AND DISCUSSION

Chemical composition of L-Fucose result: Collected algae was first cleaned with filtered seawater to remove contamination and then dipped in tap water to remove salt. The fucose content of sample was found to be12% and HPLC chromatogram of L-Fucose standard and L-Fucose extracted from was depicted in Figure 1 and figure 2 respectively. In recent years, a broad series of polysaccharides from edible seaweeds have emerged as an important class of bioactive natural products, possessing many important properties of pharmacological relevance. Fucose, sulfate, and L-fucose can be used to represent the quality of the fucoidan. Cho et al. (2010) reported that the bioactivity of fucoidan was positively correlated with sulfate content (Yangthong et al. 2009; Cho et al. 2010). Fucoidan is a sulphated polysaccharide containing important biological activities due to having a different amount of sulphate group in its chemical structure. It has anticoagulant, immunomodulation, anticancer, antiviral, anticomplement, antithrombotic, and antiproliferative activity (Hentai et al. 2019).

DPPH radical scavenging assay: DPPH is extensively utilized stable free radical mediator used to evaluate the radical scavenging efficacy of plant extracts. In the presence of hydrogen donating antioxidant, stable DPPH radical is converted into a non-radical component (DPPH-H), due to this reaction that the colour of the DPPH solution is converted from purple to yellow. In this assay, all the tested polysaccharide samples showed high DPPH radical scavenging capacities. The present study indicates DPPH scavenging activity for L-Fucose in Padina gymnospora as 15.70 ± 1.012 to69.31±2.08 (Fig. 3), which is high level that of L-Fucose in the present study. DPPH is a compound that possesses a nitrogen free radial and is readily destroyed by a free radical scavenger. This assay was carried out to assess the anti-oxidative potential of compounds functioning as proton radical scavengers or hydrogen donors in an *in vitro* system (Singh et al. 2004). Moreover, Kumar et al. (2021) reported that, DPPH is a stable free radical appears in purple color in methanol/ethanol turns colorless by reduction in the presence of hydrogen donating antioxidants (Kumar et al. 2021).

Figure 1: Standard L-Fucose

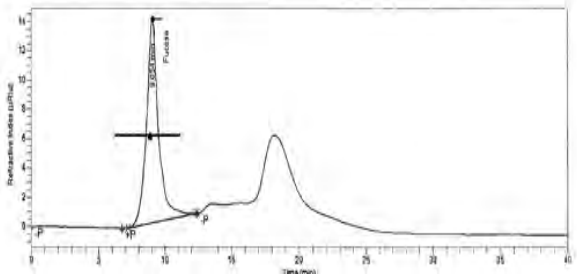


Figure 2 L-Fucose in *Padina gymnospora*

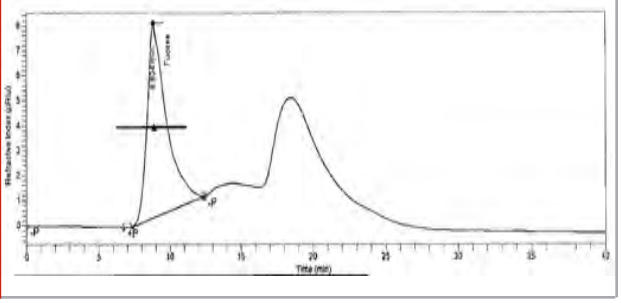
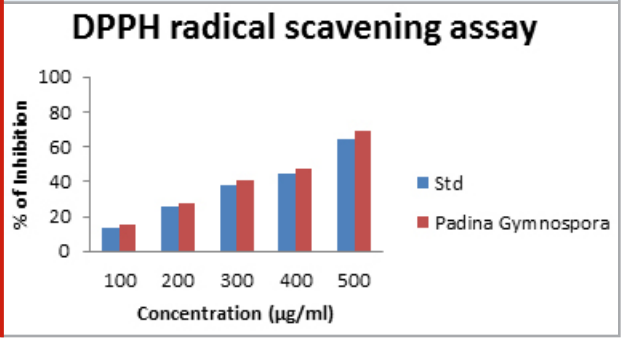


Figure 3: DPPH radical scavenging assay



Hydrogen peroxide scavenging activity: Hydrogen peroxide itself being very unreactive, becomes toxic to cells as a consequence of increase in concentration of hydroxyl radicals in the cells (Halliwell 1991). Hence, compounds with good hydrogen peroxide scavenging ability are considered physiologically important. The

commercial fucoidan sample exhibited the most potent hydrogen peroxide scavenging activity in contrast the other polysaccharide samples showed 22.26-81.71% inhibition Fig 4. The measurement of H_2O_2 scavenging activity is one amongst the potent methods for determining the ability of antioxidants to decrease the level of pro-oxidants such as H_2O_2 (Czochra et al. 2002). However, it can cross biomembranes and can slowly oxidize a number of reactive compounds leading to cell death (Kumar et al. 2021).

Figure 4: Hydrogen Peroxide scavenging assay

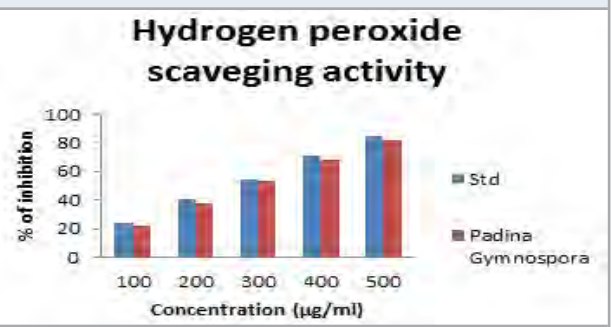


Figure 5: Nitric Oxide scavenging assay

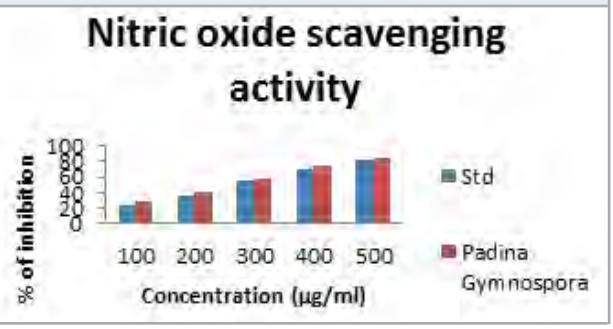
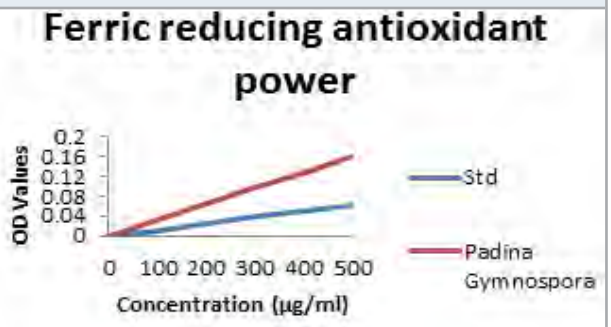


Figure 6: FRAP scavenging assay



Nitric oxide scavenging assay: Nitric oxide radical is an important signalling molecule in human body; however, the accumulation of this radical creates adverse side effects. Therefore, compounds with high nitric oxide radical scavenging activities are important, but fewer compounds have been documented for their NO scavenging ability (Kim et al. 2007; Kumar et al. 2021). The nitric oxide scavenging assay was performed with L-Fucose samples along with the standard. Based on percentage of inhibition and different concentration ranges 100 – 500 µg/ml the result was given in Figure5.The inhibition was founded in L-Fucose with percentage of 28.71% to 84.69%. The low inhibition was recorded in standard (23% to 82.45%). All the test samples possess higher percentage of inhibition when compared with standard ascorbic acid which produced (Kumar et al. 2021).

Ferric reducing antioxidant Power (FRAP): In ferric reducing Antioxidant power (FRAP), the antioxidant activity was determined based on the ability of the components in the samples to reduce Ferric (III) to Ferrous in a redox linked colorimetric reaction that involves single electron transfer. The L-Fucose which is usually present in brown seaweeds is potent antioxidant. In the present study results showed increased level (26.48-88.67%) which is shown in Fig 6. Oxygen derived free radicals or reactive oxygen species (ROS) formed in the within biological system during energy producing metabolic process, plays an important role in pathophysiology of a number of diseases (Cuzzocrea et al. 2001; Li et al. 2006; Bhuyar et al. 2021).

Figure 7: Deoxyribose scavenging assay

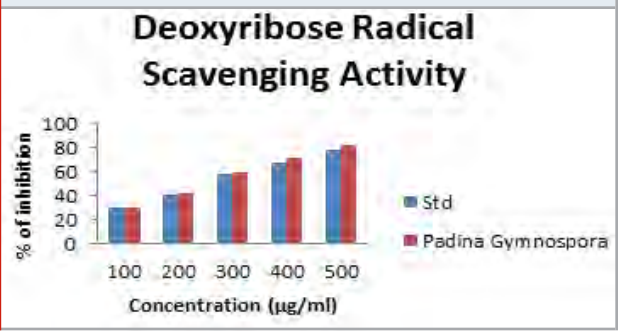
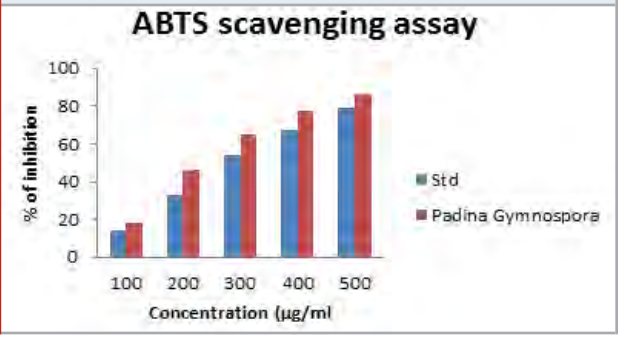


Figure 8: ABTS scavenging assay



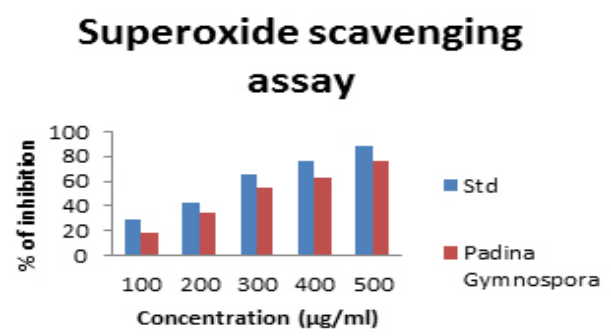
Deoxyribose Radical Scavenging Activity: Oxidative stress induced by the free radicals has been gained a vital importance as it forms the root cause of about 200 human diseases. Free radicals are highly reactive molecules with unpaired electrons and are generated during various cellular processes. They represent an essential part of metabolism and aerobic life. Many of the reactive oxygen species (ROS) and reactive nitrogen species (RNS) are grouped under free radicals. The Deoxyribose radical assay was expressed as percentage activity of ascorbic acid control at 100 to 500 µg/mL and the data are shown in Fig.7. The polysaccharides are arranged from the highest activity, which ranged from

30.78 to 81.94% (Hybertson et al. 2011; Finosh et al.2013; Bhuyar et al. 2021).

ABTS Scavenging Activity: The ABTS radical reactions involves the transfer of electrons and the process take place faster rate when compared with that of DPPH radicals. In the present study the ABTS radical scavenging activity from *Padina gymnospora* (18.56 to 86.19) was given in fig 8. There are numerous reports in the literature on the antioxidant capacity of algae. Bhuyar et al. (2021) reported that, antioxidant potential in which the Padina gymnospora showed 15.56 to be the best antioxidants and scavenging among all the polysaccharides studied. The hydroalcoholic and aqueous extracts of many seaweeds have been studied and reported for antioxidant activity by their inhibitory activity on lipoxygenase activity, DPPH radical and deoxyribose assays (Jimenez-Excriget al. 2001; Lekameera et al. 2007; Lekameera et al. 2008; Bhuyar et al. 2021).

Recently, several marine alginate derivatives like those of sulphated fucoidans from *Laminaria japonica* the brown seaweed, agar-like sulfated galactans from Nori, the red seaweed and sulphated polysaccharides from *Fucus vesiculosus*, have been reported to possess antioxidant activity. This property is contributed by the presence of reductones that are reported to be terminators of free radical chain reaction (Duh 1998; Xue et al. 2001; Ruperez et al. 2002; Duan et al. 2006; Bhuyar et al. 2021).

Figure 9: Superoxide scavenging assay



Superoxide radical scavenging activity (SO): Superoxide radicals are a highly toxic species generated by many biological and photochemical reactions. Although, the superoxide radical was a weak oxidant in most organisms, it could produce hydrogen peroxide and hydroxyl radicals through dismutation and other reactions and is the major source of free radicals formed *in vivo*. Moreover, superoxide radical and its derivatives are cell-damaging through causing damage to the DNA and membrane of the cell (MacDonald et al. 2003; Yuan et al. 2005). The SO antioxidant assay was found to be 18.33±75.92 %at 100µg/ml which gradually increases with increase in concentration (Fig. 9). The reducing properties are generally associated with the presence of reductions. The relation between polysaccharide structure and function was also analysed (Yuan et al. 2005; Bhuyar et al. 2021).

Superoxide Dismutase radical scavenging activity (SOD): The radical-scavenging activity of L-Fucose extracted from Padina gymnospora varied in a range from 18.50 to 84.64 % on SOD radicals at a concentration of 100 to 500 mg/ml. Antioxidants can grant protection from oxidative damages and prevent the onset of many chronic diseases. They are naturally present in our body (endogenous) and the additional supplementation can be done through the diet (exogenous). Natural antioxidants like ascorbic acid (vitamin C), α-tocopherol, and carotenoids are readily absorbed through diet (Padayattyet al. 2003; Bhaskar 2013). Butyl hydroxyanisole (BHA) and butyl hydroxytoluene (BHT) are commercial synthetic antioxidants which are proven to cause major side effects leading to cancer. Hence natural antioxidants always gained importance as safe, cost effective food supplements (Bhuyar et al. 2021).

CONCLUSION

The finding of the present study states that, the total sulfated polysaccharide in Padina gymnospora showed a potential free radical scavenging ability responsible for the antioxidant activity. The results showed an equally good radical scavenging activity when compared to the standard. Several Preparative approach and analytical techniques in isolation and purification of the compound may be thus helpful in obtaining sufficient quality of the bioactive principle and determination of its radical inhibiting capacity along with its median inhibitory concentration will be helpful to assess the drug potentiality of compounds from the natural source. Further studies are required to assess antioxidant based therapeutic potential of Padina gymnospora.

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Physiological Communication

Cellulosolytic Activity of Gastrointestinal Microflora and Energy Metabolism in Yakutian Horses During Winter

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ABSTRACT

The article presents a study to improve the technology for winter feeding of horses of the Yakut breed. Feeding technology affects digestion and assimilation of the carbohydrate complex of plant feed since the supply of horses with energy at negative temperatures, the physical and technical properties of wool, and the quality of meat products depend on the absorption of nutrients by the body. The article presents a comparative study of the cellulolytic activity of the microflora of the gastrointestinal tract and energy metabolism of adult horses and foals at the age of 9 months and the efficiency of including the probiotic "Sakhabactisubtil" into the compound starter feed for foals under one year of age. As a result of the research, it has been found that during the winter, when consuming the hay and oat diet, foals at the age of 9 months consume considerably more metabolic energy per kilogram of metabolic weight than mares. A mare used 0.99 MJ of energy per kilogram of metabolic weight (LW+0.75), while foals used 1.39 MJ, which was 40% more. In the experiment to establish the efficiency of using compound starter feed with the probiotic "Sakhabactisubtil", young horses of the experimental groups used the nutrients of the feed better.

KEY WORDS: COMPOUND STARTER FEED, FOALS, HAY AND OAT RATION, MARES, PROBIOTIC "SAKHABACTISUBTIL", YAKUTIAN HORSE.

INTRODUCTION

Yakutian horses are pastured all year round, consuming pasture fodder, with only 10-15% of their annual ration being hay with a small amount of oats. However, for 7 months of the year, horses consume the haylage of winter pasture which represents grassland litter or aftergrass. The chemical composition of the aftergrass is dominated by crude fibre (35%) and nitrogen-free extractive substances (44.7%). Crude fat is 4.2%, crude ash is 7.8%, and only crude protein is 8.8%. With ageing, lignin accumulates in plants, which creates a solid carbohydrate-lignin complex, which reduces the digestibility of fibre and non-natural extractive substances in farm animals. The accumulation of knowledge on the digestibility and assimilation of carbohydrate complex of plant fodder is important to improve the technology of winter feeding of Yakutian horses because it affects the energy supply of horses at subzero temperatures. A horse consumes fodder firstly using a maximum of easily accessible nutrients in the stomach and intestine: soluble proteins, starches, sugars, minerals, vitamins, and in the large intestine, with the help of residual enzymes of the small intestine and microorganisms, utilizes hardly accessible fibre, residues of protein, carbohydrates, and minerals (Novakovskaya, 2015; Khompodoeva et al., 2020; Ivanov et al., 2020).

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The horse's small intestine digests 60 to 70% protein, 65 to 75% soluble carbohydrates, and only 15 to 25% fibre. A large part of the diet, 75-85% fibre, 30-40% protein, and 25-35% soluble carbohydrates, is digested in the large intestine. Fibre digestion is carried out by cellulosolytic bacteria. These processes are as active as rumen digestion in corresponding cattle. According to the currently adopted technology, foals are weaned for sale for meat and for repair at the age of 6-7 months in November at a lower air temperature of 15-20°C, when the carcasses of slaughtered foals are rapidly cooled. In this case, the fatness of foals left for repair is reduced in the first days and the animals are poorly nourished due to the stress of weaning and the unfamiliar hay and oat ration. A slowdown in the rate of growth and development of foals occurs during the whole winter period with an almost complete halt in mid-winter. Meanwhile, it is known in the Yakutian horse breeding practice that foals left on a suckling with mothers without weaning are also growing well in winter. At the same time, foals' growth and development depend on the mother's milk yield (Gabyshev, 2002; Vinokurov, 2012; Sharaskina, 2019).

This peculiarity of foals' growth and development during the first winter shows how important the quality of foals' nutrition is. It seems to us that the cellulosolytic activity of the microflora of the gastrointestinal tract and the energy metabolism of the foals are important as well. At this time, the foals are transitioning from a diet of mother's milk and green pasture grass to a hay and oat diet, i.e. a transition from an easily digestible carbohydrate diet to the digestion of feed with hard-to-digest fibre, with a high content of almost indigestible lignin. We also suggest that in young horses aged 7-11 months, the cellulosolytic activity of the gastrointestinal tract is less active than in adult animals. The important thing in the nutrition of young foals under 1 year of age is energy supply. Both the integument and, most importantly, the subcutaneous and intra-abdominal fat insulate the body less from the cold than in adult animals because the coat in foals is shorter and fat deposits are thinner than in adult animals. Equally important is the duration of feed intake, as it generates heat, defined as a specific dynamic action of food or incremental heat production (Novakovskaya, 2015; Ivanov, 2018; Ivanov et al., 2021).

This heat under conditions of stress from freezing helps to maintain the body temperature of the animal. Based on the results of many years of research, it has been established that bacteria of the genus Bacillus dominate in the microbiocenosis of frozen soils in Yakutia. The bacterial strains B. subtilis TNP-3 and B. subtilis TNP-5 isolated from perennially frozen soils possess a wide range of unique biological properties: pronounced antagonistic action against context-dependent pathogenic and pathogenic microorganisms (bacteria, fungi, and viruses), interferon-inducing activity, immunostimulating effect, antibiotic resistance, ability to stimulate growth and development of beneficial intestinal microflora, in particular, bacteria of Lactobacillus and Bifidobacterium. The unique biologically active properties of strains B. subtilis TNP-3 and B. subtilis TNP-5 have led to the development of the biopreparation "Sakhabactisubtil". The preparation is widely used in northern animal breeding: in respiratory diseases, digestion, reproduction, dysbacteriosis, and mycotoxicosis, also as a part of hygienic and polishing agents, is effective for silage and haying of forages, for rehabilitation of oil-polluted soils, besides, it is a part of inactivated vaccines against horse infectious diseases (Vladimirov et al., 2020; Neustroev et al., 2020b, Neustroev and Tarabukina, 2021).

The studied strains of B. subtilis TNP-3, TNP-5, and their combination were found to possess cellulase activity. According to Russian and foreign scientists, bacteria of the genus Bacillus are characterized by polyenzymatic properties. Bacillus cells include a set of enzymes of different classes, which provides them the ability to exist in different substrates (Laktionov, 2012; Skryabina et al., 2020; Abdelfattah et al., 2015; Murad and Azzaz, 2010). These enzymes can significantly affect feed digestibility and nutrient uptake, improve chemical composition, physical and technical properties of wool, and enhance the quality of meat products. In this respect, our task was to compare the cellulosolytic activity of the microflora of the gastrointestinal tract and energy metabolism of adult horses and young animals at the age of 9 months and to determine the efficiency of including the probiotic "Sakhabactisubtil" into the compound starter feed for young animals up to one year of age.

MATERIAL AND METHODS

The work was carried out at the research and production farm of the Horse Selection and Breeding Laboratory "Olbuordaakh", Amga ulus, of the M.G. Safronov Yakut Scientific Research Institute of Agriculture during the winter of 2019 and 2020. The studies were carried out based on a permit to conduct research work issued by a commission consisting of the head of the nasleg I.V. Semenov and the commission members P.P. Arsentieva and I.V. Kuzmina No. 1-002 dated 16.02.2019. During the experiments, humane research methods were used in relation to animals. Selection and chemical analysis of winter pasture fodder and hay and oat rations were carried out according to the methods of "Modern Biological and Biochemical Research Methods in Zootechnics" (Burtseva and Rudishin, 2014). Laboratory research was carried out on an infrared analyser NIR SCANNER model 4250 in the laboratory of agricultural product processing and biochemical analyses of the Yakut Scientific Research Institute of Agriculture. The formulation of compound starter feed for foals was developed based on establishing energy, nutrient, and mineral deficiencies and following the foals' needs for basic nutrients (Draganov et al., 2010). The experiment on the digestibility of nutrients in the hay and oat ration was conducted on 4 mares of Yakutian breed aged 10 years with approximately the same live weight. The horses consumed 14 kg of hay and 2 kg of oats per day per head. The duration of the preparatory period was 4 days, the accounting period 6 days.

The experiment on the determination of the efficiency of mixed fodders was carried out on 45 foals at the age of 9 months during stationary winter feeding. Three groups of animals were formed, selected on the principle of analogue

pairs by live weight, age, and body type. Differences between the pairs in body weight and age did not exceed 10%. The control group received a diet consisting of 5 kg of hay and 2 kg of oats. In the first experimental group, the oats were replaced by compound starter feed. The second experimental group received compound starter feed with the inclusion of the probiotic "Sakhabactisubtil". The experimental and control groups of animals were kept in separate barns and the duration of the experiment was 60 days. At the beginning and end of the experiments, animals were weighed and blood samples were taken for analysis. At the end of the feeding experiments, tests were carried out on the digestibility of feed nutrients. To conduct digestibility experiments, 6 horses from each group were placed in special barns to collect faeces and record the feed fed. The duration of the preliminary period was 3 days and the accounting period was 6 days. Fodder and faeces sampling and storage were conducted according to the methods of "Modern Biological and Biochemical Research Methods in Zootechnics" (Burtseva and Rudishin, 2014). Energy value of fodder – by the regression equation of the All-Russian Research Institute of Horse Breeding (Kosharov et al., 1983).

ME = 19.46dP+35.43dF+15.95dFb+15.95dNfES, where
ME – metabolic energy of fodder (MJ)
dP – digestible protein, g
dF – digestible fat, g
dFb – digestible fibre, g
dNfES – digestible nitrogen-free extractive substances, g

The content of total energy in diets was calculated according to the equations developed by L.K. Ernst Federal Research Center for Animal Husbandry and Institute of Animal Physiology, Biochemistry and Nutrition:

TE = 24.24cP+38.87cF+18.39cFb+17.14cNfES;
DE = 23.93dP+32.66dF+18.5dFb+17dNfES;

where cP, cF, cFb, cNfES are "crude" nutrients, g; dP, dF, dFb, dNfES are digestible nutrients, g (Nadalyak et al., 1986; Shcheglov, 1991).

Biochemical analyses of fodder, faeces, blood were carried out in the biochemistry laboratory of the M.G. Safronov Yakut Scientific Research Institute of Agriculture. The preparation Sakhabactisubtil was developed from an equal combination of strains of *Bacillus subtilis* TNP-3 and *Bacillus subtilis* TNP-5 with B. subtilis content of 5x109 CFU in 1 ml. The strains of *Bacillus subtilis* TNP-3 and *Bacillus subtilis* TNP-5 were extracted from frozen soils of Yakutia and deposited in the All-Russian collection of microorganisms used in animal husbandry and veterinary medicine (The Russian State Center for Animal Feed and Drug Standardization and Quality, Moscow) (Vladimirov et al., 2020). The drug was given at a dose of 50x109 CFU/ml at the rate of 10 ml per 1 head daily in combination with hay and mixed fodder for 10 days. The preparation Sakhabactisubtil was manufactured by the Scientific and Production Center HOTU-BACT according to TU 9384-003-00670203-06 and was used according to the instructions approved by the Rosselkhoznadzor on 06.07.2012.

Samples of faeces from foals for microbiological studies were taken from three groups – before the experiment and after the experiment. Microbiological studies were carried out in the laboratory for the development of microbial preparations at M.G. Safronov Yakut Scientific Research Institute of Agriculture – Division of Federal Research Centre "The Yakut Scientific Centre of the Siberian Branch of the Russian Academy of Sciences. The following media were used to study the intestinal microbiota of young horses and for the quantitative accounting of isolation and quantification of bacteria: lactobacagar – for lactic acid microorganisms, bifidum medium – for bifidobacteria, azide medium – for enterococci, MAP (after heating to 85ºC for 15 minutes) – for spore-forming aerobic bacteria, Endo – for Escherichia, Baird-Parker – for staphylococci, medium with bromothymol blue – for yersinia, Chapek – for microscopic fungi. Microbial growth was recorded after 18, 24, and 48 hours for bacteria and 5 days for fungi. The number of microorganisms was determined in colony-forming units (CFU) per 1 g. Genus and species identification of microorganisms was carried out according to the "Handbook on microbiological and virological research methods" (1982), "Identification of zoopathogenic microorganisms" (1995), and "Bergi's bacteria identifier" (1997). The main numerical data obtained in the studies were processed by a biometric method using the Microsoft Excel computer program.

RESULTS AND DISCUSSION

Table 1 compares the digestibility of the nutrients in the hay and oats diet in mare and foal experiments.

Table 1. Comparison of the digestibility of nutrients in the hay and oat diet of mares and foals, %

Indicator	Group Mares	Foals
Dry substance	64.04 ± 1.71	63.1±0.06
Organic substance	66.8 ± 4.41	68.2±0.17
Crude protein	69.2 ± 0.17**	73.0±0.08**
Crude fat	60.9 ± 0.48*	62.01±0.32*
Crude fibre	52.5 ± 0.71**	49.5±0.25**
NfES	72.7 ± 1.42	70.14±0.60

Note: * – P ≥ 0.95; **- P ≥ 0.99; *** – P ≥ 0.999

The table shows that digestibility ratios for all components were higher in foals, except crude fibre and nitrogen-free extractable substances. Comparatively high ratios of crude protein and fat in foals, probably, were connected with the fact that their diet contained relatively more oats than the diet of mares (foals had 2 kg of oats in 5 kg of hay, mares had 2 kg of oats in 14 kg of hay). The chemical composition of oats in both experiments was practically the same. Epy composition of hay in the experiments is given in Table 2.

Table 2. Chemical content of hay in experiments on mares and foals in winter (percentage of dry substance)

Indicator	Mares	Foals
Indicator	Mares	Foals
Water	5.45 ± 0.13	5.26±0.11
Percentage per substance:		
Crude protein	14.42 ± 0.61**	17.68±0.09**
Crude fat	3.16 ± 0.64	2.72±0.06
Crude fibre	32.85 ± 0.32**	37.02±0.50**
NfES	27.24 ± 0.84*	30.32±0.12*
Crude ash	6.72 ± 0.18	7.00±0.09

Note: * – P ≥ 0.95; **- P ≥ 0.99;

Table 2 shows that the hay consumed by the foals had more crude protein, fibre, and nitrogen-free extractive substances. This is why the digestibility of protein was higher, while fibre and NfES were lower in the foals. The carbohydrate-lignin complex of foals' hay was less accessible to the cellulosolytic bacteria in the colostrum of foals compared to mares. The relatively low digestibility of the carbohydrate part of the diet can also be explained by the insufficient establishment of cellulosolytic activity of the gastrointestinal tract microflora of foals at 9 months of age, or simply by the insufficient number of cellulosolytic bacteria in the large intestine of foals compared to the intestines of adult horses. In the conditions of the Yakut winter at minus 45-55 degrees C, the knowledge of the amount of metabolic energy for life sustenance and super-support energy, not only theoretical but also practical, is of great importance.

Table 3. Energy consumption and utilisation by mares and foals, MJ

Indicator	Group	
	Mares	Foals
Energy consumed	170.24 ± 5.12	135.2 ± 1.12
Digestible	107.48 ± 2.41	82.1 ± 0.41
Faeces	62.75 ± 2.84	53.1+0.64
Urine and methane	11.16 ± 9.32	9.6+0.35
Metabolic	96.31 ± 1.17	72.5 ± 1.15
Incl. for life sustenance	50.95 ± 1.41	42.25+0.83
Super-support energy	45.36 ± 0.71	30.25+
The concentration of metabolic energy in 1 kg of dry substance	9.0 ± 0.12	9.56+0.24
Total energy metabolism, %	56.57	53.5

The quantitative characteristic of energy of urine and methane was determined according to the reference book "Farm animals. Physiological and biochemical parameters of the organism" (Reshetov, 2002). The live weight of the mares was on average 425kg, the foals were 208kg, i.e. the mares weighed more than twice as much as the foals. However, metabolic energy intake was only 33% higher. This can probably be explained by the fact that the foals also had a comparatively higher energy expenditure to sustain life. According to our calculations, the difference in metabolic energy expenditure for life sustenance in mares and foals was only 20%. This can be explained by the fact that foals expend more sustainable energy in winter than adult animals. This is due to the fact that they seem to have worse thermal insulation than adult animals. Foals have shorter coats, and their skin and subcutaneous fat are thinner than in mares. They also have much less body fat and internal fat in the abdomen. Therefore, energy losses in foals are higher than in mares, and there is more expenditure on heat production as well. It should be noted that the expenditure of super-support energy already differed by 50%, which is probably due to the fact that at this time (March), intense fetal growth begins in mares, while the growth and development of foals are almost stopped. Per kilogram of metabolic weight (LW+0.75), mares use 0.99 MJ of energy, while foals use 1.39 MJ, or 40% more.

Table 4. Deficiency of energy, protein, fibre, macro-microelements, and vitamins in the hay and oat diet of Yakutian foals

Indicator	Fodder			
	Norm	Oat	Hay	Deficit
Crude protein, g	912.8	216	425	271.8
Fibre, g	1,152.1	194	1,214	+
Metabolic energy, MJ	60.7	18.4	30.05	12.25
Calcium, g	47.6	3.0	36.0	8.6
Phosphorus, g	34.2	6.8	12.5	14.9
Magnesium, g	9.07	2.4	10.5	+
Iron, g	680.4	82.0	49.1	549.3
Copper, g	60.78	9.8	10.5	40.48
Zinc, g	215.4	45	91	79.4
Cobalt, mg	3.62	0.14	0.95	2.53
Manganese, mg	272.1	113	280	+
Iodine, mg	4.08	0.2	1.45	2.43
Selenium, mg	2.04	-	0.095	1.945
Carotene, mg	68.04	-	12.5	55.54
Vitamins: A, 1000 IU	18.14	2.0	-	16.14
D, 1000 IU	1.81	-	-	1.81
E, mg	204.2	26.0	96.0	82.2
B1, mg	19.9	14.6	6.5	+
B2, mg	19.95	2.2	3.5	14.25
B3, mg	33.56	26.0	6.0	1.56
B4, mg	453.6	1.8	300.0	151.8
B5, mg	6.8	0.26	0.8	5.74
B6, mg	9.9	-	-	9.9
B12, µg	40.8	-	-	40.8
BC, mg	13.6	-	-	13.6
H, mg	453.6	-	-	453.6
C, mg	450.6	-	-	450.6

Table 5. The formula of compound starter feed for young horses up to one year of age

Indicator	Compound starter feed	
	%	RUB/kg
Oats	30	5.25
Barley	7.0	1.31
Wheat bran	14	2.8
Soybean grist (lysine source)	7.0	3.5
Dry hop pellets	31.5	1.57
Sunflower seed cake	8.0	2.48
Kempendyay salt	0.3	0.075
Felucene with biotin, Se, Mg	1.5	1.05
Premix for horses, Classic	0.69	2.34
Probiotic, ml	0.01	0.35
Total	100	20.72

It seems to us that one of the reasons why the growth and development of foals after weaning at the age of 6-7 months is reduced is that at this time, the diet is changing from a milk ration combined with green grass to hay and oats. The microbiota of the gastrointestinal tract must be adapted to digest fodder with high fibre content. The gastrointestinal tract of foals, including the large intestine where fibre digestion by cellulosolytic bacteria occurs, is small compared to that of adult horses. Apparently, for this reason, the fibre digestion of the hay and oat ration is not high enough, resulting in a low energy intake, which leads to a delay in the growth and development of the foals in the first wintering period. Therefore, we set ourselves the task of developing a formula for compound starter feed to increase the growth and development of foals up to a year old in winter stationary feeding. Besides, considering probiotic, cellulosolytic activity, the preparation "Sakhabactisubtil" was included in the recipe of the second experimental group of foals. To compose the formula for compound starter feed, the deficit in energy, protein, fibre, minerals, and vitamins in young horses under 1 year of age was calculated (Table 4).

Table 4 shows that when consuming 7.58-7.64 kg of dry substance of the hay and oat ration, young horses up to one year of age have a deficit of almost all basic nutrients, macro- and microelements, and vitamins, except for crude fibre, magnesium, and manganese. To compensate for the deficiency of the main nutrients, macro-, and microelements for young horses up to one year old, we worked out a formula of compound starter feed (Table 5). Weighing of foals at the beginning and the end of the experiment showed that during the period of the experiment, the live weight increased from 201.44 kg to 217.0 kg (+15.56 kg) in animals of the first experimental group, the increase was from 200.5 kg to 220.3 kg (+19.8 kg) in animals of the second experimental group, and from 202.53 kg to 208.46 kg (+5.93 kg) in the control group. The difference in live weight between the animals of the control and experimental groups at the time of setting of the experiment was minimal and amounted to 1090 and 2030 grams, but at the end of the experiment, it was 8.54 and 11.8 kg or an absolute gain in the animals of experimental groups at the end of the experiment was 2.6 and 3.3 times more than the control group (Table 6).

Table 6. Live weight of young Yakutian horses before one year of age

Indicator	Group Control		Experimental I		Experimental II	
	Beginning	End	Beginning	End	Beginning	End
Live weight, kg	202.53±2.67	208.46±2.46	201.44±2.96	217.0±3.88**	200.5±1.78	220.3±3.02**
Growth rate	2.93	7.72	9.88			
Absolute growth rate	5.93	15.56	19.8			
**P ≥ 0.999						

Table 7. Measurements of young horses under 1 year of age for the period of the experiment

Indicator	Group Control		Experimental I		Experimental II	
	Beginning	End	Beginning	End	Beginning	End
Height at withers, cm	123.5±2.32	123.7±2.45	121.9±2.3	126.1±2.43**	125.3±2.75	128.7±2.82**
Oblique length of torso, cm	123.7±3.80	123.9±4.18	121.8±2.71	124.9±2.88*	123.7±3.74	132.5±4.76*
Chest circumference, cm	143.1±3.98	143.3±1.58	140.4±4.45	143.9±4.55**	139.0±3.29	145.1±3.50**
*P ≥ 0.95; **P ≥ 0.999						

The average daily gain of the young Yakutian horses during the period of the experiment, in animals of experimental groups was 280 and 360 g, in the control group – 108 g. Thus, a reliable increase in live weight was observed in the animals of both experimental groups 7,17 and 8,99 % (P ≥ 0,999) respectively, the control group animals showed a non-significant difference in growth (2,84 %). Table 7 represents the data of the experiment on the measurements of young horses for the period of the experiment.

Table 8. Amount of nutrients consumed by young animals, g (average per animal per day)

Indicator	Group		
	Control	Experimental I*	Experimental II**
Dry substance	7,584.6±31.39	7,621.89,±44.39	7,638.29±47.42
Organic substance	6,789.94±28.30	6,903.91±41.68	6,901.89±42.95
Crude protein	1,409.34±17.60	1,303.96±41.68	1,315.30±17.60
Crude fat	230.77±7.10	266.15±41.68	263.61±10.40
Crude fibre	3,096.533±88.70	2,392.13±41.68	2,383.27±22.70
NfES	2,053.3±92.30	2,941.67±41.68	2,939.71±52.70

* without a probiotic culture
** with a probiotic culture

Table 9. Digestibility coefficients of the main nutrients in the diet of young animals during the experiment period, %

Indicator	Group		
	Control	Experimental I*	Experimental II**
Dry substance	63.1±0.06	63.7±0.±0.19	66.5±0.08*
Organic substance	68.2±0.17	68.8±0.11	70.1±0.20*
Crude protein	73.0±0.08	74.1±0.08	74.8±0.20
Crude fat	62.01±0.32	63.56±0.26	65.64±0.50
Crude fibre	49.5±0.25	50.3±0.25	52.66±0.28*
NfES	70.14±0.60	72.1±0.60	73.48±1.10*

*P ≥ 0.95

It was found that the height at withers in young animals of the first experimental group was increased by 3.3 % and was 126.1±2.43 cm (P ≥ 0.999), chest circumference was increased by 2.4 % and was 143.9±4.55 cm (P ≥ 0.95), in young animals of the second experimental group the height at withers increased by 2.6% to 128.7±2.82 cm (P ≥ 0.999), chest circumference increased by 4.2% to 145.1±3.50 cm (P ≥ 0.95), in the control group – 123.7±2.45 cm and 143.3±1.58 cm respectively. When studying the effect of the feed factor on the animal's organism, the assessment and analysis of the indices of digestibility and use of nutrients in the feed is of great importance. We calculated the amount of nutrients and energy ingested by the experimental animals during the day based on the chemical composition and the amount of feed that was eaten. Table 8 shows that the amount of dry substance, organic substance, and crude protein consumed in all groups was approximately equal. The daily amount of nutrients ingested with the diet is not fully absorbed by the animals, and a certain part of them is excreted with the faeces. The difference between the amount of nutrients entering the gastrointestinal tract of the animals and the amount excreted in the faeces characterizes the amount of digested nutrients.

The data we obtained during the scientific and economic experiment show that the greatest amount of nutrients in the diets was digested by the animals fed with compound feed with the probiotic culture. At the same time, young animals of the first experimental group surpassed their control group counterparts by 0.6% in the digestion of dry substance, organic substance also by 0.6%, protein by 1.1%, fat by 1.55%, and fibre by 0.8%. The analyzed data show superiority in digestibility of young animals of experimental groups over their counterparts from the control group. As for the youngsters of the second experimental group that consumed compound feed with the probiotic culture,

they surpassed their counterparts of the control and first experimental group on all indicators, namely, by 2.8% on dry matter of the first experimental group and by 3.4% of the control, organic matter by 1.3% of the first experimental group and by 1.9% of the control, protein by 0.7% and 1.8% respectively, fat by 2.08% and by 3.63%, and fibre by 2.36% and 3.16% – the control group.

A relatively high intake of nutrients throughout the experiment provided the young horses of the experimental groups with higher coefficients of digestibility of the main nutrients. Remarkably, young horses of the control group were inferior to their counterparts of experimental group I in terms of NfES digestibility coefficient by 1.96% ($P \geq 0.95$), and those of experimental group II by 3.34%. Inclusion of compound feed into the winter ration at stationary feeding of foals had a positive effect on feed intake, digestibility and assimilability of nutrients in the ration, while compound feed with the addition of the probiotic "Sakhabactisubtil" promotes even more successful assimilation of fodder in the ration of horses.

When studying the metabolism of substances and energy in the body, as well as evaluating the nutritional value of feed and rationing of animals, the following types of energy are distinguished: total, digestible, metabolic (or physiological), the energy of heat production, and energy released during production. The transformation of feed energy into animal products is significantly affected by the level of feeding, the structure of the ration, the concentration of energy per unit of dry substance, as well as the balance of the ration on mineral elements and biologically active substances (Kalashnikov et al, 2011, Ivanov et al, 2020, Ivanov, et al, 2021). The experimental data we obtained in the physiological experiment testify to the positive effect of compound starter feed when including it instead of oats in the oat and hay ration during winter stationary feeding of young horses under one year of age on energy intake and utilization.

Table 10. Energy intake and utilization by young Yakutian horses in winter experiments, MJ

Indicator	Group		
	Control	Experimental I	Experimental II
Energy: Total	135.2 ± 1.12	136.3 ± 0.17	136.3± 2.14
digestible	82.1 ± 0.41	86.9± 0.35*	89.1 ± 0.51*
faeces	53.1±0.64	49.4±0.72	47.2±0.54
urine and methane	9.6±0.35	9.1±0.43	9.4±0.48
metabolic	72.5± 1.15	77.8± 1.25*	79.7 ± 1.19*
Incl. life-sustaining energy	42.25±0.83	42.59±1.09	42.6±0.96
Super-support energy	30.25	35.21	37.1
The concentration of metabolic energy in 1 kg of dry substance	9.56±0.24	10.21±0.11	10.43±0.23
Total energy metabolism, %	53.5	57.0	58.4
*$P \geq 0.95$			

The amount of total energy consumed by young animals of experimental and control groups was comparatively equal, the indicators of experimental group I and II exceeded the control group counterparts in this indicator by only 1.1 MJ (0.80%). At the same time, the young animals of experimental group I exceeded their control group counterparts in digestible energy by – 5.52% and metabolizable energy by – 6.81%. Experimental group II outperformed the control group in digestible energy by – 7.85%, metabolizable energy by – 9.03%. As a result, the metabolism total energy in young animals of experimental group I that consumed compound starter feed was higher – by 6.14% than in the control group counterparts. As for the young animals of the experimental group II, which consumed compound feed with probiotic culture, they surpassed their counterparts from the control group by 8.39%. The analyzed data show that the metabolism rate of the total energy of the experimental group II, which consumed the compound feed with the probiotic culture, is the highest. Thus, the obtained data indicate that feeding young animals with compound feed with the addition of the probiotic culture "Sakhabactisubtil" in the spring-winter period contributed to a significant increase in the productive use of energy (Koilybaeva et al., 2018; Neustroev et al., 2020a; Vinokurova et al., 2021).

Currently, spore-forming bacteria of the genus Bacillus are widely used as probiotic additives, as well as feed additives for farm animals (Koilybaeva et al., 2018; Vinokurova et al., 2021, Neustroev et al., 2020b). Such qualities of bacilli as high and varied biological activity, the ability to survive in the gastrointestinal tract of animals, and the thermal resistance of spores make these bacteria attractive as probiotics and appealing for further search for active strains and the creation of new highly effective drugs. As a result of the studies carried out by Neustroev, it was found that when using the drug Sakhabactisubtil (single intragastric administration to mice of the DM 1 line), mortality or

any signs of intoxication in animals were absent when the drug was administered to them in the maximum allowable volumes for animals of this type. Therefore, the drug does not have a potential toxic effect (Neustroev et al., 2020a).

CONCLUSION

Experimental data show that in winter when consuming a hay and oats diet, foals at 9 months of age consume significantly more metabolic energy per kilogram of metabolic weight than mares. Per kilo of metabolic weight (LW+0.75) mares have 0.99 MJ of energy consumption, while foals have 1.39 MJ, which is 40% more. In the experiment on establishing the efficiency of using the compound feed starter with the probiotic "Sakhabactisubtil", young horses of the experimental groups used the nutrients in the feed better. Reliably high coefficients of digestibility were observed in the experimental group II on dry matter by 5.1% (66.5±0.08%), on organic matter by 2.7% (70.1±0.20%), on crude fibre by 6.0% (52.66±0.28%), and on BEB by 4.5% (73.48±1.10%). Young animals of the experimental groups I and II outperformed their counterparts in digestible energy by – 5.52 and 7.85% (136.3 ± 0.17 and 136.3 ± 2.14 MJ), metabolism energy by – 6.81 and 9.03% (77.8 ± 1.25 and 79.7 ± 1.19 MJ), respectively ($P \geq 0.95$). The metabolism of total energy in young animals of experimental groups I and II was higher, by – 6.14 and 8.39% than in the control group counterparts. The obtained data indicate that replacement of oats in the hay and oat ration during winter stationary feeding of young animals with the compound starter feed offered by us contributed to a significant increase in the growth and live weight of foals of experimental groups due to a significant increase in the productive use of energy.

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Pharmaceutical Communication

Influence of Water Hyacinth-Based Organic Manures on Yield and Phytochemical Composition of Cultivated *Cassia angustifolia*

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ABSTRACT

The importance of *Cassia augustifolia* for pharmaceutical industries can not be over-emphasized and ignored. It is imperative to increase yield and bioactivity without the use of harmful chemicals as fertilizers. Thus, this study was undertaken to evaluate the effect of water hyacinth-based organic manure on the growth, yield, and chemical composition of *Cassia augustifolia*. The study employed eight different treatments, each with various water hyacinth and animal waste ratios. Shoot and root length, leaf number, and area were examined to study the morphological growth features. The yield was quantified in terms of the plant's fresh and dry weight recorded at 30, 60, and 90 days after sowing. Standard techniques were used to determine the chemical composition and DPPH radical scavenging activity. The most effective treatment was 25% water hyacinth + 75% chicken droppings, although there was no significant difference in the results compared to 50% water hyacinth + 50% chicken droppings. However, there was no significant difference in using 50% water hyacinth + 50% chicken droppings and 25% water hyacinth + 75% chicken droppings and are the most effective manures. *C. augustifolia* grown on manure with 50% water hyacinth and 50% chicken droppings recorded the highest TPC and TFC (60.82±1.96 mg/g GAE and 135.62±1.99 mg/g QE, respectively). The highest DPPH radical scavenging inhibition (47.83%) was exhibited by C. anugifolia grown on 25% water hyacinth + 75% cow dung manure. Our finding has given scientific insight into the use of water hyacinth-based organic manure to cultivate medicinal plants for optimum yield.

KEY WORDS: CASSIA ANGUSTIFOLIA, DPPH, ORGANIC FARMING, SUSTAINABLE AGRICULTURE, WATER HYACINTH.

INTRODUCTION

The continuous demand and supply has made it necessary to raise the yield of mass produce. Even though conventional farming has led to an increase in the yield of plants but the excessive use of chemical fertilizers has a damaging effect on environment, soil and water (Tsvetkov et al. 2018). Organic manures improve soil fertility, soil structure, water holding capacity, physical and chemical qualities of soil, microbial movement, and supplement accessibility without causing climate change. They likewise upgrade the overall development of the plant. Generally, poultry compost provides the cultivated plants more nitrogen and phosphorus than other natural manures (Garg and Bahl 2008; Vali et al. 2020).

Natural manure upgrades the accessibility of NPK and other fundamental supplements and plays a significant role in the development and advancement of a plant. Natural manure upgrades the vegetative and regenerative development of the plant, for example, Plant tallness, shoots plant, number of leaves, new biomass, and dry biomass (Vidya and Girish 2014; Reddy et al. 2017). Natural compost ensures a significant part in supplement accessibility without having a bothersome impact on Earth. Moreover, organic manures undertake a significant job to upgrade the physical properties of the soil, for example, mass thickness, improve microbial exercises, water retention, and supplement accessibility to the plant (Njoroge et al. 2017; Li et al. 2018; Vali et al. 2020).

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Organically grown medicinal plants are accepted without hesitation and draw a superior value than those grown with conventional farming. The existing data shows organically produced *Cassia angustifolia* leaves are double in price than conventionally grown *Cassia* and pods are as high as forty percent in comparison. Organic manures prepared from water hyacinth have shown to increase soil fertility, yield of the plant and texture of the soil. *Cassia angustifolia* (Senna) belongs to the genus *Cassia* which comprises many other herbs and shrubs. It is a perennial plant with 60 to 80 cm tallness (Jafari 2010; Săvulescu et al. 2018). The plant can grow well in red loamy and alluvial soil with proper drainage of water (Jnanesha et al. 2018; Aishwath and Tarafdar 2020).

It has been used since the early traditional times. Senna leaves are utilized in different conventional frameworks to treat obstruction, loss of craving, hepatomegaly, splenomegaly, acid reflux, and sickliness (Fatima and Girdharilal 2016). Hence, it is necessary to cultivate this plant as an essential medicinal resource which is free from inorganic fertilizers. Inorganic manure stays on the outside of the soil after vast precipitation bringing about draining. It unfavorably influences human health conditions and severely affects individuals' soundness (Sharma and Singhvi 2017). The present study was undertaken to add information to current and limited information on effect of water hyacinth-based organic manures on the yield and chemical composition of cultivated *Cassia angustifolia* as it is meant for direct consumption of humans and also for standard drug preparation.

MATERIAL AND METHODS

For the collection and cultivation of *Cassia augustifolia* seeds, the Zandu Foundation of Health Care in Valsad, Gujarat, provided the *Cassia angustifolia* seeds.

Pot culture experiments were conducted during the 2019 and 2020 monsoon seasons. The experiment was carried out under shade to control excessive exposure to sunlight and rainfall. The soil used in the experiment was a mixture of loamy and sandy soil (3:1 proportion). The experiment was carried out in randomized block design (RBD) with eight treatments and three replicates. The treatments comprised of T1- control (No organic manure); T2- decomposed water hyacinth; T3 (50% Water hyacinth + 50% chicken droppings); T4- (25% Water hyacinth +75% Chicken droppings); T5- (50% Water hyacinth + 50% Goat manure); T6- (25% Water hyacinth + 75% Goat manure); T7- (50% Water hyacinth + 50% Cow dung); T8- (25% Water hyacinth + 75% Cow dung). The organic manures were thoroughly mixed into the pots at a 10t/ha rate and allowed to rest for a few days. *Cassia angustifolia* seeds were planted directly into the pots and allowed to germinate and establish themselves in the soil.

Table 1. Effect of water hyacinth based organic manures on shoot length (cm) of *Cassia angustifolia* during two years

Treatment	Shoot length (cm)					
	Monsoon 2019			Monsoon 2020		
	30 days	60 days	90 days	30 days	60 days	90 days
T1	12.23±0.09^{a}*	15.30±1.65^{a}	28.16±0.60^{a}	9.60±0.70^{a}	12.70±0.94^{a}	26.20±1.54^{a}
T2	12.50±0.81^{a}	15.20±0.43^{a}	32.33±1.59^{ab}	11.43±0.84^{ab}	19.23±1.21^{bc}	30.60±1.46^{b}
T3	12.87±0.65^{ab}	16.86±1.07^{a}	34.67±1.20^{bc}	14.87±1.08^{bc}	23.87±1.05^{cd}	34.27±1.27^{b}
T4	15.10±0.61^{bc}	19.30±1.32^{a}	30.00±1.26^{a}	17.20±0.55^{c}	23.37±1.02^{cd}	30.83±1.07^{b}
T5	15.27±0.12^{bc}	18.00±1.25^{a}	34.67±0.17^{bc}	14.33±0.73^{bc}	24.67±1.02^{d}	33.33±0.44^{b}
T6	15.67±0.88^{c}	19.03±2.51^{a}	37.17±0.44^{c}	15.07±0.52^{c}	17.63±1.91^{b}	32.57±1.49^{b}
T7	14.10±1.16^{abc}	16.43±0.92^{a}	30.17±0.73^{a}	15.10±1.68^{c}	19.90±1.79^{bcd}	33.40±0.93^{b}
T8	13.27±1.12^{abc}	20.33±2.89^{a}	35.50±2.75^{bc}	14.03±1.65^{c}	20.27±0.84^{bcd}	33.53±2.29^{b}

*Values are mean of three replicates ± Standard error of mean
Same letter superscripts down the column denote no significant difference ($p \leq 0.05$)

After emergence, the plant population was reduced to one plant per pot. The growth and yield attributes (root length, shoot length, the number of leaves, and leaf area) and yield attributes (wet weight of the plant and dry weight of the plants) were measured at 30, 60, and 90 days after sowing (DAS). The mature plants from the various treatments were harvested and prepared for further research. For the extraction of Cultivated *Cassia augustifolia* from various treatments, the harvested, washed, air-dried harvested samples were extracted using ethanol via Soxhlelation. The resulting extracts were filtered, dried, and stored for further use. For the phytochemical Composition of *C. augustifolia* from various treatments, the carbohydrate content in *Cassia angustifolia* was estimated using the phenol-sulfuric acid method. The protein rotein content was estimated by the Lowry method (Masuko et al. 2005; Shen 2019). Total phenolic content (TPC) was determined by the Folin-Ciocalteau method, and total flavonoid content (TFC) was estimated by the Aluminum Chloride method (Vijayasekhar et al. 2016; Shen 2019).

Chlorophyll content was estimated using an established protocol. 2, 2-Diphenyl-1-Picryl hydrazyl (DPPH) radical scavenging assay was evaluated using standard procedure (Qaiyum Ansari et al. 2013; Rajalakshmi and Banu 2015; Vijayasekhar et al. 2016). For statistical analysis all the data obtained were subjected to one-way ANOVA, and the means of the treatments were separated and compared using Duncan's Multiple Range Test (DMRT).

Table 2. Effect of Water hyacinth based organic manures on root length (cm) of *Cassia angustifolia* during two years

Treatment	Monsoon 2019			Monsoon 2020		
	30 days	60 days	90 days	30 days	60 days	90 days
T1	4.90±1.35^{a*}	5.46±0.08^{a}	12.90±1.81^{a}	3.00±0.58^{ab}	6.10±0.83^{a}	9.20±0.52^{a}
T2	5.83±0.43^{ab}	7.76±1.64^{ab}	12.07±0.58^{a}	2.40±0.40^{a}	6.03±0.98^{a}	12.23±1.25^{ab}
T3	5.60±0.06^{ab}	9.90±0.30^{acd}	13.13±1.57^{a}	3.76±0.21^{abc}	6.03±1.15^{a}	14.10±0.96^{b}
T4	7.10±0.38^{b}	9.13±0.81^{ac}	15.27±4.11^{a}	5.30±0.53^{bc}	8.53±0.38^{a}	15.67±2.80^{b}
T5	7.50±0.21^{b}	11.17±1.17^{cd}	12.00±1.96^{a}	6.13±0.62^{c}	7.47±0.72^{a}	12.53±1.60^{ab}
T6	7.20±0.46^{b}	11.93±0.52^{cd}	13.03±1.21^{a}	5.40±0.76^{bc}	7.17±0.78^{a}	12.37±0.54^{ab}
T7	5.77±0.24^{ab}	12.03±0.87^{cd}	13.33±0.29^{a}	4.37±0.59^{abc}	6.23±0.58^{a}	12.90±0.49^{ab}
T8	6.03±0.68^{ab}	11.90±0.10^{cd}	12.33±1.13^{a}	4.97±1.57^{bc}	7.33±0.58^{a}	12.93±0.64^{ab}

*Values are mean of three replicates ± Standard error of mean
Same letter superscripts down the column denote no significant difference ($p \leq 0.05$)

Table 3. Effect of Water hyacinth based organic manures on number of leaves of *Cassia angustifolia* during two years

Treatment	Monsoon 2019			Monsoon 2020		
	30 days	60 days	90 days	30 days	60 days	90 days
T1	12.67±1.33^{a*}	36.00±1.00^{a}	72.00±9.23^{a}	6.67±0.67^{a}	17.33±3.17^{a}	43.67±8.09^{a}
T2	14.00±2.31^{ab}	64.67±6.96^{ab}	89.67±9.86^{a}	8.00±1.15^{a}	30.33±2.18^{bc}	62.67±3.76^{a}
T3	15.33±1.76^{ab}	80.33±2.40^{b}	106.33±6.84^{a}	10.67±0.67^{ab}	36.33±0.88^{bc}	97.00±8.33^{b}
T4	13.33±1.33^{ab}	85.33±3.33^{b}	152.67±35.5^{b}	13.67±0.88^{bc}	32.00±4.35^{bc}	157.67±14.17^{c}
T5	19.00±1.00^{b}	71.00±0.00^{ab}	98.33±7.79^{a}	15.00±1.53^{c}	28.33±0.88^{bc}	94.67±6.98^{b}
T6	14.67±2.40^{ab}	75.00±2.00^{ab}	99.67±5.20^{a}	13.67±0.88^{bc}	25.33±3.52^{bc}	101.67±5.55^{b}
T7	16.67±1.76^{ab}	69.67±4.63^{ab}	81.00±5.13^{a}	15.67±1.45^{c}	28.67±2.73^{bc}	91.67±4.48^{b}
T8	13.33±1.76^{ab}	52.00±2.64^{a}	99.00±15.57^{a}	16.00±2.31^{c}	29.67±0.88^{bc}	90.67±4.70^{b}

*Values are mean of three replicates ± Standard error of mean
Same letter superscripts down the column denote no significant difference ($p \leq 0.05$)

RESULTS AND DISCUSSION

The present study results revealed varying morphological characters exhibited by *Cassia augustifolia* under various treatments at 30, 60, and 90 days after sowing (DAS). The shoot length was not much affected by using various water hyacinth-based organic manures (Table 1). However, in the 2019 monsoon season, the highest shoot length was found in treatment T6 (37.17±0.44 cm) 90 DAS. In the 2020 season, T3 (34.27±1.27 cm) had the most extended shoot length, 90 DAS. Conversely, the shortest shoot length for the two monsoon seasons was recorded by T1. Table 2 shows that at the end of 90 days after sowing, treatment T4 (water hyacinth and chicken litter 1:3) shows the highest root length i.e. 15.27 and 15.67 in 2019 and 2020 respectively.

Data in Table 3 shows the effect of organic manures on the number of leaves of *Cassia angustifolia* at 30, 60, and 90 days after sowing. The highest number of leaves were found in treatment T4 (water hyacinth and chicken litter 1:3) during both the harvests i.e. 152.67 leaves/plant and 157.67 leaves/plant in 2019 and 2020 respectively followed

by treatment T3 (water hyacinth and chicken litter 1:1) i.e. 106.33 during 2019 and T6 (Water hyacinth and Goat manure 1:3) during 2020. Table 4 shows data of the leaf area of *Cassia angustifolia* for 2019 and 2020 at 30, 60, and 90 days. Data shows that the highest leaf area was observed in treatment T3 (water hyacinth and chicken litter 1:1) during 2019 and T4 (water hyacinth and chicken litter 1:3) during 2020 but the difference between T3 and T4 was not very high in 2019.

Table 4. Effect of Water hyacinth based organic manures on leaf area (cm^2) of *Cassia angustifolia* during two years

Treatment	Leaf Area (cm^2)					
	Monsoon 2019			Monsoon 2020		
	30 days	60 days	90 days	30 days	60 days	90 days
T1	0.90±0.06^{a*}	2.42±0.40^{a}	4.17±0.80^{a}	0.76±0.14^{a}	3.02±0.32^{a}	3.25±0.37^{a}
T2	0.90±0.06^{a}	2.57±0.12^{a}	4.53±1.59^{ab}	2.07±0.68^{ab}	3.82±0.34^{a}	4.81±0.34^{bc}
T3	0.97±0.03^{ab}	3.32±0.34^{a}	6.45±0.78^{b}	4.11±0.34^{cd}	4.28±0.52^{bc}	4.95±0.21^{b}
T4	1.03±0.03^{abc}	3.04±0.43^{a}	5.75±0.20^{ab}	2.75±0.31^{d}	4.24±0.13^{bc}	6.93±0.06^{c}
T5	1.13±0.12^{bc}	3.27±0.10^{a}	5.94±0.45^{ab}	1.99±0.31^{abd}	4.03±0.16^{ab}	5.76±0.23^{b}
T6	1.07±0.07^{abc}	2.60±0.08^{a}	6.09±0.62^{ab}	2.13±0.19^{ab}	3.96±0.25^{ab}	5.19±0.40^{b}
T7	1.20±0.06^{c}	2.68±0.41^{a}	4.85±0.38^{ab}	1.25±0.41^{ad}	4.03±0.22^{ab}	5.30±0.37^{b}
T8	1.07±0.03^{abc}	3.08±0.34^{a}	4.41±0.50^{ab}	1.84±0.25^{abd}	3.82±0.41^{a}	4.93±0.20^{bc}

*Values are mean of three replicates ± Standard error of mean
Same letter superscripts down the column denote no significant difference ($p \leq 0.05$)

Table 5. Effect of Water hyacinth based organic manures on fresh weight of the plant (g) of *Cassia angustifolia* during two years

Treatment	Fresh weight of the plants (g)					
	Monsoon 2019			Monsoon 2020		
	30 days	60 days	90 days	30 days	60 days	90 days
T1	0.86±0.05^{a*}	1.83±1.17^{a}	4.00±0.58^{a}	0.34±0.05^{a}	1.70±0.20^{a}	2.50±0.29^{a}
T2	0.93±0.21^{ac}	3.67±0.67^{ab}	5.67±0.67^{ab}	0.39±0.13^{a}	2.34±0.07^{ab}	3.50±0.29^{ab}
T3	1.13±0.11^{abc}	6.00±0.58^{cd}	10.00±1.15^{c}	2.03±1.68^{b}	5.47±0.14^{c}	6.00±0.58^{cd}
T4	1.44±0.06^{bd}	6.33±0.88^{d}	13.33±1.85^{d}	1.68±0.18^{bc}	3.58±0.41^{d}	7.00±0.58^{d}
T5	1.75±0.13^{d}	4.33±0.88^{bc}	8.33±0.33^{bc}	1.55±0.09^{cd}	2.66±0.32^{b}	5.67±0.33^{c}
T6	1.38±0.03^{bcd}	4.67±0.33^{bcd}	10.33±0.33^{c}	1.32±0.07^{cd}	3.11±0.27^{b}	6.33±0.33^{cd}
T7	1.33±0.20^{abcd}	4.17±0.44^{bc}	5.67±0.33^{ab}	1.49±0.09^{cd}	3.65±0.34^{d}	4.17±0.17^{b}
T8	1.12±0.24^{abc}	4.00±0.58^{bc}	6.33±1.33^{ab}	1.15±0.25^{d}	3.76±0.32^{d}	3.50±0.29^{ab}

*Values are mean of three replicates ± Standard error of mean
Same letter superscripts down the column denote no significant difference ($p \leq 0.05$)

This research shows that treatment T4 (water hyacinth and chicken litter 1:3) showed the highest fresh weight in gram per plant at 90 DAS during both 2019 and 2020, followed by T6. The results of T6 were comparable to that of T3 with proximity. The least effective treatment was T2 which shows that only organic matter supply in the form of water hyacinth was proved to be ineffective when compared with other treatments with a combination of water hyacinth and animal litter in different proportions. Table 6 shows data of the effect of different water hyacinth based manures on the dry weight of *Cassia angustifolia.* Treatment 4(water hyacinth and chicken litter 1:3) shows the best results as far as biomass is concerned, among other treatments. The least dry weight was found in the case of T2 among all other treatments.

The current study was also carried out to determine the quantitative presence of various phytochemicals in *Cassia angustifolia*. The data obtained as mentioned in Figure 1 indicated that carbohydrate and protein content in treatment T7 was the highest among all the treatments (620.52 mg/g DW and 140.78 mg/g DW), while the phenol

and flavonoid content (mentioned in Figure 2) was found highest in treatment T3 among others (60.82±1.96 mg/g DW and 135.62±1.99 mg/g DW). Treatment T6 showed the highest chlorophyll a, b, and total chlorophyll content when compared to other treatments as shown in Figure 3 (1.05±0.01 mg/g FW, 0.61±0.06 mg/g FW and 1.67±0.07 mg/g FW respectively). Figure 4 shows that plants treated with treatment T8 showed the best radical scavenging activity (47.83 % at 100 ug/ml concentration of extract).

Table 6. Effect of Water hyacinth based organic manures on dry weight of the plant (g) of *Cassia angustifolia* during two years

Treatment	Dry weight of the plants (g)					
	Monsoon 2019			Monsoon 2020		
	30 days	60 days	90 days	30 days	60 days	90 days
T1	0.26±0.03^{a*}	0.43±0.07^{a}	1.07±0.23^{a}	0.07±0.01^{a}	0.32±0.09^{a}	0.60±0.06^{a}
T2	0.30±0.05^{aab}	1.05±0.22^{ab}	1.29±0.09^{ab}	0.07±0.02^{a}	0.79±0.02^{b}	1.40±0.21^{a}
T3	0.30±0.06^{ab}	2.00±0.29^{c}	3.00±0.58^{c}	0.60±0.01^{c}	0.61±0.18^{b}	2.83±0.73^{bc}
T4	0.42±0.02^{abc}	2.08±0.39^{c}	3.33±0.33^{c}	0.38±0.07^{c}	1.07±0.05^{bd}	3.00±0.58^{bc}
T5	0.57±0.06^{c}	1.27±0.37^{bc}	2.07±0.07^{b}	0.29±0.03^{ac}	0.91±0.14^{bd}	3.17±0.17^{c}
T6	0.46±0.06^{bc}	1.33±0.17^{bc}	3.30±0.15^{c}	0.24±0.11^{ac}	1.53±0.20^{c}	2.67±0.17^{bcd}
T7	0.43±0.06^{abc}	1.13±0.18^{ab}	1.73±0.09^{ab}	0.29±0.03^{ac}	1.25±0.03^{cd}	1.83±0.12^{ad}
T8	0.38±0.09^{ab}	1.10±0.21^{ab}	1.57±0.33^{ab}	0.31±0.26^{c}	1.01±0.25^{bd}	1.57±0.47^{ad}

*Values are mean of three replicates ± Standard error of mean
Same letter superscripts down the column denote no significant difference (p≤0.05)

Figure 1: Total Carbohydrate (A) and Total Protein contents (B) of *Cassia angustifolia* cultivated with water hyacinth-based manures.

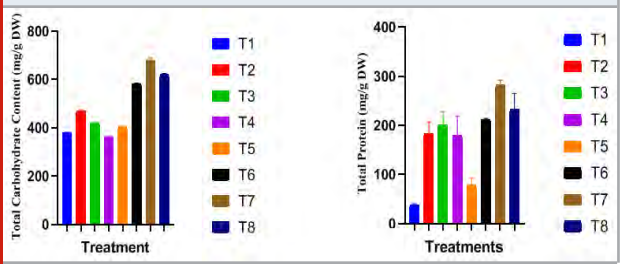
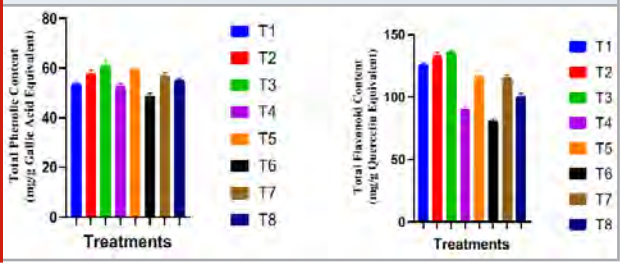
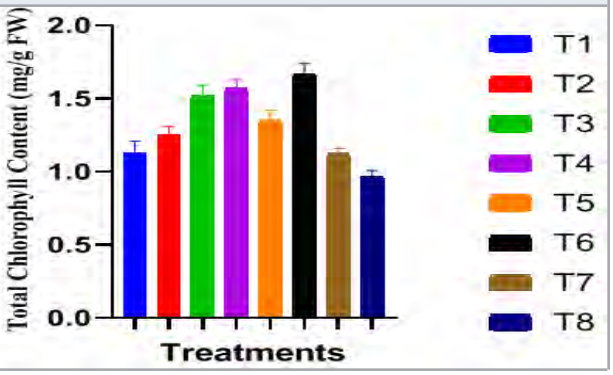


Figure 2: Total Phenolics (A) and Total Flavonoid contents (B) of *Cassia angustifolia* cultivated with water hyacinth-based manures.



Effect of water hyacinth based manures on growth and yield of *Cassia angustifolia*: It was noted that all the treatments produced higher result than control in case of the shoot and root length. Chicken manure and water hyacinth is rich in nitrogen and phosphorus (Amanullah et al. 2007). Hence the combination of the two in equal amounts lead to increased root and shoot length in treatments T3 and T4 as phosphorus is an essential element for root growth and nitrogen is a crucial element for shoot growth as it increases photosynthesis and protein formation which inturn leads to an increased number of leaves as well as leaf area in the plant (Hawkesford et al. 2011; Awad et al. 2012).

Figure 3: Total Chlorophyll Estimation of *Cassia angustifolia* cultivated with water hyacinth based manures

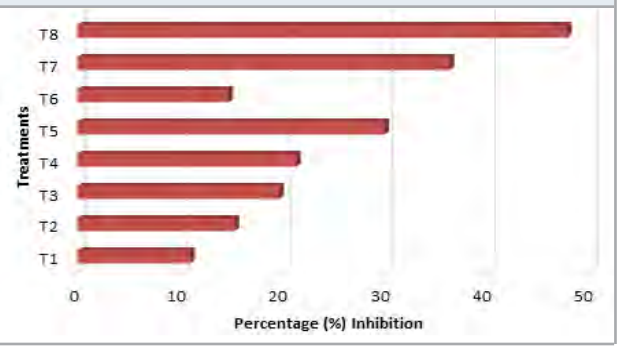


The increase in these parameters in the case of other treatments was attributed to the factor that the remaining treatments were able to provide nutrients just in a proper amount not exceedingly resulting in no toxicity in any way to the plant. The yield as shown in case of fresh and dry weight of leaves of *Cassia angustifolia* is least in the case of control. Treatment T3 significantly increased the leaf number hence increasing the fresh weight of the plant as well as the dry weight of the plant. Another research study also concluded that the use of water hyacinth-based manures

suggestively amplified leaf number. The results of the plant root length in reaction to poultry compost alone or joined with fertilizer is in close concurrence with those revealed by Awad. The primary benefit of using organic manures was to provide plants with nutrients released gradually as the plant grows (Myint et al. 2011; Awad et al. 2012; Moi 2015).

Effect of water hyacinth based manures on phytochemicals of *Cassia angustifolia*: Water hyacinth is utilized as a prospective plant development regulator. These controllers carry out changes in the plant leading to increased production of primary and secondary metabolites. A significant increase in the primary metabolites namely carbohydrate and protein suggest the stable nourishment received by the plants in the form of composts.

Figure 4: DPPH Radical scavenging inhibitory activity of *C. angustifolia* extracts cultivated with water hyacinth-based manures



The secondary metabolites produced by the plants hold a great significance in making medicine from medicinal plants. Higher chlorophyll in treated plants shows balanced nourishment received by the plants. Phenols are commonly found in plants resulting in anti-oxidant properties. The phenolic components of *C. angustifolia* in this study are similar to the reports of Maria (Rani and Usha 2013). Phenols are also considered to have antimicrobial properties. It is evident from the research that *Cassia angustifolia* can be used for its phytochemicals as well as for its potent anti oxidant properties. Flavonoid results agree with the results of past studies (Onofrei et al. 2017). *Cassia angustifolia* has antibacterial, hypo-cholesterolaemic, hepato-protective, anti-diabetic, anti-inflammatory and anti-oxidant actions (Veerabahu and Dec 2010; Silva et al. 2018).

CONCLUSION

The findings of the present study were carried out to explore the potential of different organic manures on the growth and yield parameters of *Cassia angustifolia* M. Vahl. Keeping in view the value of organically grown medicinal plants and due to the increasing use of fertilizers to grow food and medicinal plants, various health issues are reported in humans. Hence, the current study opens up a wide range of possibilities to increase growth, yield and phytochemical components of the plant organically as a part of sustainable agriculture without using chemical fertilizers for the growth of medicinally important plants.

ACKNOWLEDGEMENTS

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

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Biotechnological Communication

Molecular Identification of Some Filamentous Bacteria Isolated from Contaminated soil for Poly Hydroxyl Butyrate Degradation

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ABSTRACT

When some bacteria grown in increasing concentrations of carbon, a polymer called Poly β- hydroxyl butrate (PHB) was produced and accumulated inside the bacterial cell up to 70% of the cell dry weight. This material can be used safely in different modern application to replace plastic which has negative effects on man, animals and environments. This study aimed to isolate some actinomycetes for PHB degradation and optimization the growth conditions for maximum degradation. Contaminated soil samples were collected from industrial area of Jeddah and used for actinomycete isolations. The isolated bacteria were screened on medium containing PHB as a carbon source and presence of cleat zone on solid agar medium around the bacterial colonies mean PHB degradation. Out of thirty isolates, eight isolates were the most active in PHB degradation. After growth in liquid medium, these isolates were identified and characterized using morphological, physiological and chemical methods. Using molecular methods, they were belonging to different genera of actinobacteria. The most active isolate was Streptomyces sp. MM21.The effects of some growth factors on growth and PHB degradation was determined. Growth was measured by dry weight (g/l) while PHB degradation was detected by depolmerase assay (U/ml). It was clear that addition of 2.5 g/l of yeast extract and 1 g/l glucose enhanced both growth and PHB degradation. Similarly, adjusting medium at pH 7.0 and incubation temperature at 25°C for 7 days lead to maximum PHB degradation. The maximum growth was in medium containing 0.5 g/l of PHB. In conclusion, PHB degradation was detected by actinobacteria and was affected by some physical and biochemical factors. It was noticed that optimization of growth conditions enhanced both growth and degradation process by the selected *Streptomyces* isolate.

KEY WORDS: CONTAMINATION, DEGRADATION, IDENTIFICATION, PHB, STREPTOMYCES, 16SR RNA.

INTRODUCTION

Different used materials in our homes and factories are from petro-based plastics which have a variety of serious harmful effects on human health and aquatic environment due CO2 releases, ingestion of plastic and bis-phenol accumulation (Gervet, 2007, Halden, 2010). The worldwide annual production of synthetic polymers was many billion tons and now the disposal of these materials was of big concern. The decrease of plastic materials is urgent and developing biodegradable plastics as an alternative material is the solution (Shimao 2001, Sabapathy et al., 2021, Müller-Santos et al., 2021).

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Pranamuda et al. (1995) reported that poly hydroxyalkanoic acids (PHA) and poly β-hydroxybutyrate (PHB) are biodegradable materials with properties similar to conventional plastics and can be used safely in our life. Biodegradable alternative polymers are environmentally-friendly obtained from renewable resources and most ecological studies were focused on PHB biodegradation. The distribution of bioplastic degrading bacteria in the environment has been investigated and the diversity or abundance of these microorganisms were recorded. The most active microbial population must be transferred from the lab. to the environment to enhance degradation process (Sabapathy et al., 2021).

Time is needed to utilize biodegradable plastics and maintain the health of the environment. Looking for microorganisms convert bio- polymers to monomers using effective eco-friendly methods is urged. Poly β- hydroxyl butrate is a

polymer produced by many genera of bacteria up to 75% of the cell dry weight. These polymers were used by bacteria as reserved food but can be extracted, characterized and used in many countries as safe and eco-polymer. Biodegradation of these polymers has been investigated in soils, composts and natural waters by bacteria and fungi which contained PHB depolymerases (Mergaert et al., 1994, 1995, Oda et al., 1997, Sharma et al., 2019) and rate of degradation depend on the properties of the polymers. PHB depolymerases are responsible for extracellular PHB degradation (Jendrossek and Handrick, 2002, Alhazmi et al., 2018, Alves et al., 2020).

Recently from a dumping yard rich in plastic wastes, Stenotrophomonas sp. RZS7 was isolated, characterized and grown for PHB depolymerase production during its growth. This enzyme was extracted and purified by ammonium salt precipitation, column chromatography and solvent purification (Sayyed et al., 2020, Alves et al., 2020). This study aimed to isolation and identification of actinobacteria from contaminated soil for degradation of PHB and optimization the degradation conditions for maximum activities.

MATERIAL AND METHODS

Sample collection and bacterial isolation and selection: Contaminated soil samples (10) from the industrial zone, Jeddah, Saudi Arabia were collected in sterile plastic bags, air dried and sieved. Different bacterial isolates were obtained on Starch nitrate agar after growing for 5 days at 25◦C and the pure isolates were maintained on the same medium in the refrigerator at 4oC until used. All isolated bacteria were screened on mineral salt agar medium containing PHB as carbon source and the most active isolates that showed the highest clear zones were selected and identified.

Bacterial identification: The locally isolated bacteria used in this study were from contaminated soil samples. The used medium for isolation was starch-nitrate agar. This medium contained (g/l): starch, 20; KNO_3, 2; K_2HPO_4, 1; KH_2PO4, 0.7; $MgSO_4 \cdot 7H_2O$, 0.7; agar, 20. The medium pH was adjusted to 7. All isolate were purified and preserved on the same medium on slants at 4◦C. The selected isolates were identified according to International *Streptomyces* Project (Shirling and Gottlieb 1966). DNA was extracted and 16SrDNA genes of the active isolates were purified and sequenced. The sequence was analyzed and compared to 16SrDNA genes in the Ribosomal Data Project and EMBL-GenBank databases.

The used polymer: The homo- polymer of PHB was obtained as powder from from Biomer Inc., Germany. The suspension of PHB (1.0 g/ 100 ml distilled water) was sonicated for 15 min (Ultrasonic Homogenizer 4710 series ColeParmer Instrument Co. Chicago, Illinois 60648). The sonicated solution was autoclaved separately and added aseptically as a carbon source to 900 ml of liquefied sterile agar medium. The obtained medium was mixed well and used.

Screening of the different bacteria for PHB degradation: Agar plates were prepared from Mineral salts agar medium and each plate contained 10 ml of the previous medium as a bottom layer and 6 ml of the medium, contained 0.1% of the polymer suspension as the sole carbon source (the upper layer). At first all plates had an opaque appearance due to the presence of polymer in the top layer. The plate was inoculated with 1 ml of the bacterial suspension (6x106 CFU/ml), previously grown for one week in starch nitrate broth medium at 120 rpm for 4 days. The inoculum was spread over the surface of the medium. Incubation was then carried out at 25°C. Assessment of degradation activity was detected by measuring the mean value of the clear zone diameter and the mean diameter of the bacterial colony.

Detection of PHB degradation on solid and in liquid medium: All isolated bacteria were screened on Mineral salt agar medium supplemented with (0.10 g/l) as a sole carbon source. After incubation for 5 days, clear zone diameter was measured for each isolate (Alhazmi et al., 2018). Also, PHB metabolizing in shake flask experiments was conducted in 250 ml Erlenmeyer flasks containing 50 ml of the basal Mineral salt PHB medium which contained (g/l): PHB (0.5), KH_2PO_4 (0.7), K_2HPO_4 (0.7), MgSO4 (0.7), Yeast extract (2.5), Glucose (1), NaCl (0.005), $FeSO_4$ (0.002) and $ZnSO_4$ (0.007). The medium pH was adjusted to pH 7. Each flask was inoculated with 2 ml of fresh prepared bacterial suspension, containing 4x106cfu/ml and incubated in shaking incubator at 25oC and 120 rpm for 5 days. At the end of the growth period, the growth was collected by centrifugation at 4,000 rpm for 20 minutes, dried at 50°C for 2 days in an oven. The bacterial dry weight was determined as g/l. PHB degradation by depolymerase enzyme was measured quantitatively in the culture filtrate by the decrease in the absorbance using spectrophotometric method, according to the method of Kobayashi et al. (1999). All experiments were made in triplicate and averages were calculated.

Enzyme assay: Depolymerase enzyme was responsible about PHB degradation and its activity was predictable from the decrease in turbidity of PHB at 37°C (Klingbeil et al. 1996). The best conditions for maximum PHB depolymerase production: For maximum production of PHB depolymerase, growth conditions were optimized. The degradation of PHB was performed using 50 ml of Mineral salt medium (pH 7.0) with 0.1 % PHB as a carbon source in250 ml Erlenmeyer flasks. All flasks were incubated at 120 rpm for 5 days. The effect of different concentrations of yeast extract (0.5, 1.0, 1.5, 2.0) and 2.5 g/l or different concentrations of glucose (0.5, 1.0, 1.5, 2.0 and 2.5 g/l) on growth and depolymerase activity were determined. Similarly, the effects of different pH values (6, 6.5, 7.0, 7.5and 8.0), incubation temperature (20, 25, 30. 35, 40 and 45°C), incubation period (3, 4, 5, 6, 7, 8, 9 and 10 days) and different concentrations of PHB (0.25, 0.5, 1.0, 1.5, 2.0 and 2.5 g/l) were determined. At the end of growth period, growth and depolymerase activity were assayed. After incubation, bacterial growth (dry weight) and depolymerase production was detected as described before.

Statistical analysis: Statistical analyses were performed using the statistical Package for Social Science (SPSS for windows, version 16) (SPSS Inc., Chicago, IL, USA). The result was expressed as mean ± standard deviation and significant difference between samples was determined using t-test. Significant results were recorded when $P<0.05$.

RESULTS AND DISCUSSION

Soil samples were collected from contaminated area in the industrial zone in Jeddah, Saudi Arabia and different bacteria were isolated on Starch Nitrate Agar using dilution plate method. All plates were incubated at 25°C for 7 days. About 30 isolates with different color were obtained and isolates were Gram positive. All the isolates were screened on MSA medium containing 0.5% PHB. The degree of growth was determined, and diameter of the clear zone was measured. Out of thirty isolates, eight isolates were the most active in PHB degradation on solid medium (Table 1). Moreover, after growing in liquid medium, growth and PHB were determined for the eight active isolates which have different colonies characters and colors (Figure 1). All Bacterial isolate degrades PHB in liquid and solid media with different percentages (Table 2). The percentage of PHB degradation was ranged from 20 to 50 %. The isolates MM7, MM14 and MM21showed the best growth (++++) using PHB as carbon source and the biggest clear zones which were ranged from 20 to 25 mm. The lowest growth and the smallest clear zone were recorded for isolates MM1, MM5, MM11, MM27 and MM30.

Table 1. The isolated Gram-positive bacteria, color of aerial and substrate mycelia, growth and diffusible pigment production

Bacterial isolate	Bacterial shape	Aerial mycelia	Substrate mycelia	Growth	Diffusible pigment
MM 1	Filamentous	White	Gray	+++	+ve
MM 5	Filamentous	Brown	Yellow	++	+ve
MM 7	Filamentous	Blue	Yellow	++	+ve
MM 11	Filamentous	Black	Gray	++	+ve
MM 14	Filamentous	White	Yellow	++	+ve
MM 21	Filamentous	Pink	Brown	+++	+ve
MM 27	Filamentous	Gray	Black	++	+ve
MM 30	Filamentous	Gray	Yellow	+	+ve

+++: high growth, ++: moderate growth, +: little growth, +ve: positive result

Table 2. The detection of PBP by the selected actinomycete isolates

Bacterial isolate	Clear zone diameter (mm)	Growth (Dry weight, g/ l)	PHB Degradation		
			A235	(U/ml)	% Of degradation
MM 1	17± 1.8	0.12±0.04	0.064	0.161±0. 06	32
MM 5	21± 3.8	0.13 ±0.06	0.064	0.160±0. 08	32
MM 7	29± 3.7	0.13 ±0.07	0.088	0.229±0. 07	44
MM 11	21± 3.0	0.12 ±0.07	0.044	0.118±0. 09	22
MM 14	33± 2.1	0. 19±0.09	0.080	0.205±0.01	40
MM 21	38± 2.9	0. 15±0.09	0.104	0.257±0.04	50
MM 27	20± 3.3	0. 11±0.02	0.058	0.145±0. 08	28
MM 30	22± 3.1	0. 15±0.07	0.056	0.140±0. 03	28

These isolates were identified and characterized using morphological, physiological and chemical methods. Taxonomic identification by morphology of each isolate was mainly based on many identification keys. Using molecular methods, they were belonging to different genera of actinobacteria. The selected isolates were belonging to two genera and many species. They were identified as *Amycolatopsis sp., Sreptomyces mutabilis, Sreptomyces coenleonbidus* and the least 5 isolates belong to genus *Streptomyces* and identified as *Streptomyces* sp., (Table 3, Figure 2). In case of growing the 8 isolates in MSB medium contained 0.5% PHB as carbon source, they will grow and

degrade PHB by secretion of depolmerase. All isolates grow well in the previous medium but with different degrees. It was found that the isolate *Streptomyces* sp. MM21 with accession number GU980133.1 grow strongly after hydrolyzing PHB by producing depolymerase enzyme. The enzyme concentration for this isolate was measured asU/ml. The highest growth and PHB degradation were recorded for *Streptomyces* sp. MM21.

Figure 1: Some Gram-positive bacterial isolates grown on starch nitrate agar for 14 days at 25◦C

Isolate MM21 was the most active isolates for PHB degradation, thus it was selected for more detail studied. It was grown in medium containing PHB as a carbon source and at the end of incubation period, growth and PHB degradation were measured. Addition of yeast extract at different concentration from 0.0 to 2.5 g/l to medium containing PHB as a carbon source was studied. After 5 days, the growth and PHB degradation were measured. From the results, it was clear that presence of yeast extract in growth medium enhanced both growth and PHB degradation. Maximum growth measured by dry weight (g/l) was in medium containing 2.5 g/l of yeast extract. Similarly, the highest PHB degradation, measured by enzyme activity (U/ml) was obtained in MSB medium containing 2.5 g/l of yeast extract. The lowest growth and PHB degradation were observed at control which contained 0.0% yeast extract (Figure 3).

Table 3. The identified actinomycetes selected for PHB degradation

Isolate no.	Accession Number (AC)	Closest genus or species	Similarity (%)
MM 1	EU570625.1	*Streptomyces mutabilis*	99
MM 5	KF991647.1	*Streptomyces coeruleorubidus*	99
MM 7	KP262516.1	*Streptomyces* sp.	99
MM 11	KP262516.1	*Streptomyces* sp.	97
MM 14	KF479186.1	*Amycolatopsis* sp.	99
MM 21	GU980133.1	*Streptomyces* sp.	98
MM 27	GU980133.1	*Streptomyces* sp.	99
MM 30	GU980133.1	*Streptomyces* sp.	100

Figure 2: Phylogenetic tree of the eight bacterial isolates that degrade PHB and most related genera.

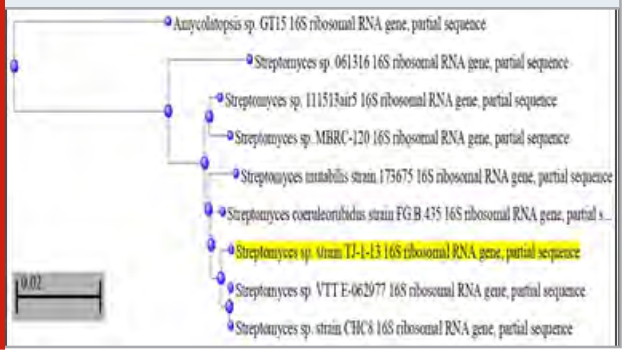
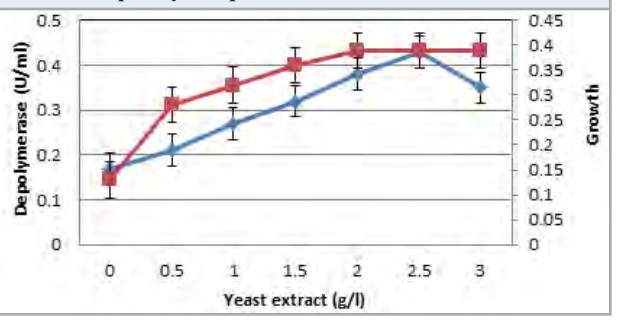


Figure 3: Effect of different concentrations of yeast extract on growth and PHB degradation (U/ml) by the selected isolate Streptomyces sp. MM21.



Similarly, the isolate MM21 was grown in medium containing PHB as a carbon source in addition to different concentration of glucose, 0.5 to 2.5 g/l to enhance the growth. After 5 days, the growth and PHB degradation were measured. From the results, it was clear that presence of glucose in growth medium enhanced both growth and PHB degradation and the maximum growth measured by dry weight (g/l) was in medium containing 1.0 g/l glucose. Similarly, the highest PHB degradation, measured by enzyme activity (U/ml) was obtained in MSB medium containing glucose (1.0 g/l). The lowest growth and PHB degradation were observed at control which had no glucose (Figure 4). From the previous results, maximum growth and PHB degradation by the bacterium isolate MM21 was recorded in medium containing PHB as a carbon source in addition to 2.5 g/l yeast extracts and 1.0 g/l glucose.

After 5 days of incubation at different temperatures, 20 to 45°C, the growth and PHB degradation were measured. From the results, it was clear that increasing temperature enhanced both growth and PHB degradation up 25°C, and then the growth and PHB degradation were decreased by increasing incubation temperatures. From the results, maximum growth and PHB degradation was recorded at

25°C after 5 days of incubation whereas the lowest growth and PHB degradations were at incubation temperature 20 and 45°C (Figure 5). The medium was prepared at different pH values, pH 6.0- pH 8.0. The inoculated flasks were incubated at 25°C for 5 days and growth and PHB degradation were measured. From the results, it was clear that adjusting the initial pH of the medium at 7.0 enhanced the growth, but maximum PHB degradation (U/ml) was recorded at pH 7.5as shown in Figure 6.

Figure 4: Effect of different concentrations of glucose on growth and PHB degradation (U/ml) by the selected isolate Streptomyces sp. MM21.

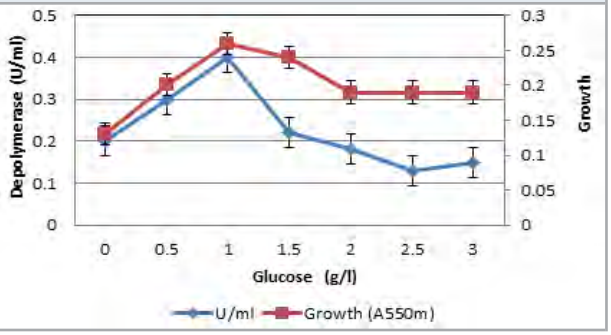


Figure 5: Effect of different temperature on growth and PHB degradation (U/ml) by the selected isolate *Streptomyces* sp. MM21.

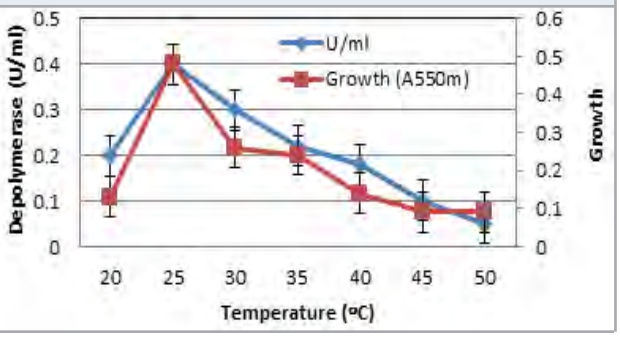
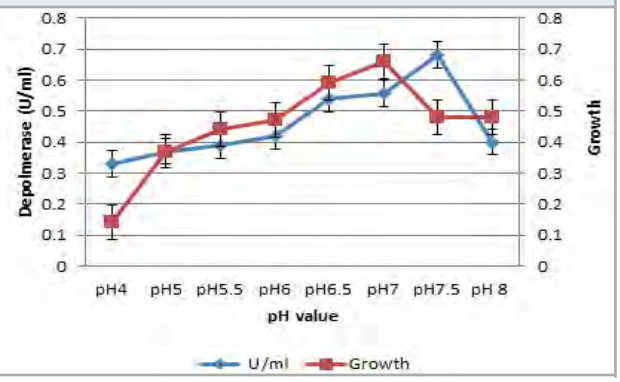


Figure 6: Effect of different pH value on growth and PHB degradation (U/ml) by the selected isolate Streptomyces sp. MM21.



Also, MM21 was grown in medium containing PHB as a carbon source, yeast extracts and glucose. All flasks were incubated at 25°C for 3, 4, 5 ,6, 7, and 8 days. At the end of the growth period, the growth and PHB degradation were measured. From the results, it was clear that incubation at 6 days enhanced growth but maximum PHB degradation was measured in the broth medium after 3 days of growth at 25°C. The lowest growth and PHB degradation were observed after1 and 2 days of growth (Figure 7). From the results, it was clear that presence of PHB in growth medium enhanced both bacterial growth and PHB degradation and the maximum growth was in medium containing 0.5 g/l of PHB. Similarly, the highest PHB degradation, measured by enzyme activity (U/ml) was obtained in MSB medium containing 0.5 g/l of PHB. Increasing the PHB concentrations showed no clear effect on both growth and PHB degradation. The lowest growth and PHB degradation were observed 2.0- 3.0 g/l of PHB as carbon source (Figure 8).

Figure 7: Effect of different incubation period on growth and PHB degradation (U/ml) by the selected isolate *Streptomyces* sp. MM21.

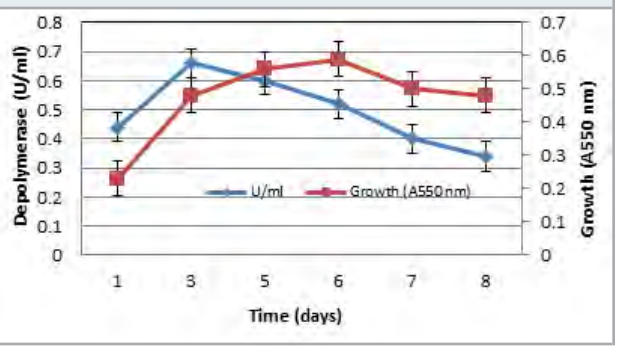
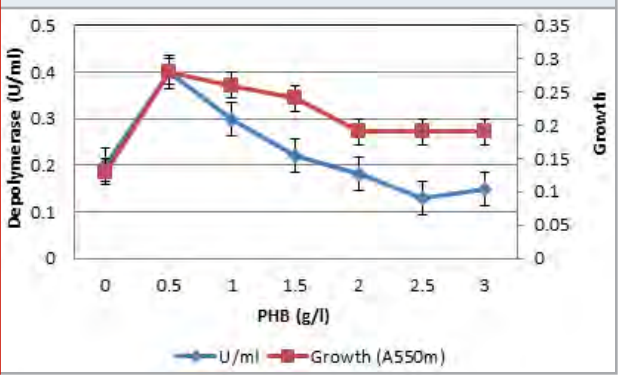


Figure 8: Effect of different concentrations of PHB on growth and PHB degradation (U/ml). by the selected isolate *Streptomyces* sp. MM21.



In this study, out of 30 *Actinomycete* isolates, 8 isolate (27%) were able to degrade PHB in solid and liquid medium. Higher percentage of actinomycetes that can degrade PHB were recorded. Hoang et al., (2007) isolated Actinomycetes from Touchien River in Taiwan and screened them for the ability to degrade poly (ethylene succinate), poly (caprolactone) and/or poly(b-hydroxybutyrate) (PHB) by the clear-zone method. About 135 isolates out of 305

were PHB-degraders (44.2%), 83 isolates were PCL degraders (27.2%), and 64 isolates (21.0%) were PES degraders. Furthermore, 46 isolates could degrade both PHB and PCL (15%), 39 isolates could degrade both PHB and PES (12.8%). Based on the appearance of isolates, the most important isolates (91.9%) were identified as *Streptomyces* sp. while *Micromonospora* genus was represented by 8.1%. In this study, we have evaluated the degradation of poly-hydroxybutyrate by genus *Streptomyces* (87%) and *Amycolatopsis* (13%). The isolate MM21, obtained from contaminated soil with oil wastes was the most active isolate in PHB degradation and was identified as *Streptomyces* sp. MM21.

The maximum ability of the previous bacterium to degrade PHB was observed on solid and in liquid media containing the polymer as the sole carbon source. Degradation process was increased with increasing period of incubation and maximum degradation was obtained after 7 days. The degradation of the polymer was determined on solid medium by measuring the clear zone diameter and in liquid medium by measuring the decrease in the absorbance. On contrast, Mabrouka and Sabryb (2001) used two other methods to detect degradation. The over layer plate method was applied as an approach to determine the PHB degradation by the tested strain. In contrast to bacteria, fast colonial growth of the *Streptomyces* strain covered the medium in plates and interfered with reading the clear zone formed. Therefore, the use of test tubes proved to be more advantageous for assaying degradation activity. The synthesis of *Streptomyces* sp. MM21 depolymerase seems to be highly regulated.

The presence of readily utilizable carbon sources in addition to the polymer in the medium inhibited the degradation process compared to the control (medium containing the polymer only). This indicates that PHB depolymerase expression is repressed in the presence of a soluble carbon source that permits high growth rates. However, after exhaustion of the nutrients, the synthesis of PHB depolymerase is decreased. This regulation has been reported in many bacteria (Klingbeil et al. 1996). This is consistent with observations of Molitoris et al. (1996) on degradation of polyhydroxyalkanoate by *Comamonas* sp. but different from those observed with other bacterial depolymerases (Nishida and Tokiwa, 1993). Examination of polymer sheets incubated in sterile medium showed that PHB has a mainly smooth surface with many irregular pits and lesions. It is assumed that the mycelia growth of the bacterium penetrates the lesions and depolymerases are secreted. Thus, the hydrolysis products increase. As a consequence, the surface area of the polymer accessible to degradation increases. It has been demonstrated that streptomycetes offer excellent potential for degradation of biopolymers in the marine environment (Moran et al., 1995, Alhazmi et al., 2018, Alves et al., 2020).

The degradation of biopolymers helps to overcome some of the pollution problems associated with the use of petroleum polymers. PHB has been investigated for its degradation in many terrestrial and aquatic environments (Jendrossek and Handrick, 2002). Scientist have traditionally used morphology for identifying bacterial species, and even nowadays it is still the potential means of species identification and the newly emerged DNA sequence-based methods have shown higher potentiality for identifying species, thus, molecular techniques were used to confirm the classification. Many different PHB biodegrading members of *Bacillus* and *Streptomyces* have been isolated from soil (Mergaert et al., 1993). Polyhydroxyalkanoic acids are biodegrable plastics that have been extensively studied, because of their similarity to conventional plastics, complete biodegradability and current market domination (Verlinden et al., 2007, Sabapathy et al., 2021).

The biodegradation of plastics proceeds actively under different conditions according to microbe properties and each microbe has its own optimal growth conditions (Lucas el al., 2008). All isolates were screened for depolymerase production on medium containing 0.5% PHB as inducer. Induction and expression are subjected to a complex regulation and depolymerase enzyme is not required for balanced bacterial growth and may be synthesized in response to energy or nutrient limitation (Lodhi et al., 2011). In this work, the tested bacteria (40% of the tested bacteria) grow on medium containing PHB as carbon source are producer for depolymerase while Aly et al. (2015) reported that more than 50% of the screened actinobacteria were depolymerase producing. Also, Aly et al. (2017) reported that out of 16 fungal isolates, 8 (50%) gave clear zone on solid medium containing PHB as carbon source (Sabapathy et al., 2021).

Depolymerase enzyme detection was obtained either using plate-clearing technique and/or measuring the enzyme activity in liquid medium. Plate-clearing technique was very simple method to detect polymer degradations in agar medium and used to detect the PHB degradation as a clear halo zone around the bacterial colonies (Lodhi et al., 2011). For bacteria, growth on agar medium is usually easier than in broth medium (Choi et al., 2001), thus, no further assessment was conducted for isolates that showed weakly activity on solid agar medium containing PHB as carbon source. The weak or lack of activity in some isolates may be due to a loss of trait in the isolate or the used culture conditions was not effective in inducing the depolymerase enzyme which are extracellular in fungi whereas the enzymes are either extracellular or intracellular in bacteria. Enzyme assay for these isolates indicated the optimal environmental conditions required for depolymerase enzyme to induce the highest level of biopolymer degradation (Sabapathy et al., 2021).

Among 20 isolates, 8 isolates were proven to biodegrade PHB. The isolates were basically characterized at the morphological level. The knowledge on the participation and role of actinomycetes in hydrolysis of PHB are extremely limited and need to be elucidated in further studies. This study is important in isolation of some bacterial isolates which produce excellent depolymerase enzyme in the culture supernatant after growth on PHB as carbon source. Although, this enzyme has a wide distribution in bacterial cells and in some algal genera, bacterial depolymerase have

considerable potential in commercial applications due to substrate specificity, resistance to proteolysis and catalytic efficiency (Shah et al., 2008, Sabapathy et al., 2021).

Temperature is one of the most critical parameters to be controlled in any bioprocess. A number of mesophilic microbes have been found to be responsible for biodegrading PHB in soil and aquatic environments and many the thermo -tolerant strains are capable of biodegrading PHB at high temperatures ≥ 40°C from soil and compost (Mergaert et al., 1994, Kim, 2000). Im this study, maximum depolymerase production was at 25°C while Bacillus strain TT96 (Tansengco and Tokiwa, 1998) and *Streptomyces* strain MG (Tokiwa and Calabia, 2004, Pirttilä et al., 2021) were capable of degradation at higher temperatures. *Aspergillus fumigates* was able to biodegrade PHB better at 45°C after 24 h of incubation in liquid medium and little information on microbial degradation of PHB at high temperatures was available (Lodhi et al., 2011). Maximum degradation was after 5 days and there was a gradual decrease in production of enzyme after that. As it is well known, degradation in liquid medium was affected with time. On contrast, Papaneophytou et al. (2009) reported maximum enzyme production by *Thermus thermophilus* HB8 after 24 h of incubation. The gradual decrease in the production of enzyme after 5 days was as a result of utilization of substrate and other nutrients (Shivam et al., 2009, Kouřilová et al., 2021).

Moreover, maximum enzyme production was at pH 7.5 while Aly et al. (2015) used pH 7 for maximum degradation of PHB by *Streptomyces*. Increasing pH more than 7.5 affected the charges on the amino acids within the enzyme active site. Similarly, in liquid medium maximum production of PHB depolymerase by *A. fumigates* was observed at pH 7 after 24 h of incubation (Lodhi et al., 2011) and at pH 9.0 at 65ºC by B*acillus megaterium* (Takaku et al., 2006) and at pH 7.5–8.0 when sewage sludge was used as inoculum (Briese et al., 1994). In the present study, the maximum production of PHB depolymerase was found at 0.5% of PHB concentration while it decreased with further increase in polymer concentration which might be due to substrate level inhibition (Manna and Paul, 2000). In case of *Arthrobacter* sp., the optimal concentration of PHB was 0.1% (Asano and Watanabi, 2001).

Production of extracellular PHB depolymerase by *T. thermophilus* was reported after 24 hr of incubation using 0.1% PHB (Papaneophytou et al., 2009). In the current study, presence of glucose at 1 g/l and yeast extract at 2.5 g/l in the growth medium along with PHB enhanced activity of PHB depolymerases. On contrast, lactose enhanced PHB degradation by A. fumigates (Lodhi et al., 2011). According to Manna and Paul (2000), degradation of PHB by bacterial strains was affected significantly when the PHB containing medium was supplemented with easily consumable carbon sources. Glucose, fructose and arabinose supplementation lowered the extent of degradation. However, after exhaustion of the nutrients, the synthesis of PHB depolymerase is depressed (Jendrossek and Handrick, 2002, Kouřilová et al., 2021, Kamnev et al., 2021). Inoculum size affect growth and PHB degradation and increasing the quantity increased the growth until certain limit at which there is no increase in PHB degradation (Kamnev et al., 2021).

Degradation increased with increasing period of incubation (Mabrouk and Sabry, 2001, Hidalgo et al, 2013). In accordance with our results are those reported for *Pseudomonas lemoignei*, (Steinbuchel and Hein, 2001). This result is in good agreement with a previous investigation which demonstrated the importance of carbon sources in the growth medium for enzyme production as the rate of polymer degradation was influenced by the degree and availability of secondary carbon and by the initial carbon source (Shivakumar, 2013, Bhagowati et al., 2015). Moreover, a PHB biodegrading bacterium *Stenotrophomonas* sp. RZS 7 was isolated from Indian soil samples of municipal area on minimal salt medium containing PHB as the only source of nutrient. An optimum yield of enzyme was obtained after 5 days of incubation at 37°C and at pH 6.0 (Wani et al., 2016, Kouřilová et al., 2021).

CONCLUSION

Maximum PHB degradation was obtained after growing the selected *Streptomyces* isolate in medium containing 2.5 g/l yeast extracts, 1 g/l glucose and 0.5 g/l PHB as a carbon source at pH 7 and incubation at 25◦C for 3 days. Many studied are needed for detecting the important characters of depolymerase enzymes which have the main roles in PHB degradation.

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Conflict of Interests: The authors declare no conflict of interest exist.

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Dental Communication

Palatal Bone Thickness in Different Growth Patterns of Dravidian Population Using Cone-Beam Computed Tomography Systems

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ABSTRACT

Facial type of any individual comprises variations in the skeleton structures and is pertinent to genetic and environmental factors. This may influence the palatal bone thickness, for instance, palate can be deep and narrow for vertical growers while wide and shallow for horizontal growers. Therefore, the aim of the present study was to evaluate palatal bone thickness in different growth patterns in the Dravidian population. 30 CBCTs were retrieved and classified into three groups according to the growth patterns- 10 horizontal growth pattern (<30°), 10 average growth pattern $(\simeq 32 \mp 2°)$ and vertical growth pattern (>30°). The growth pattern was determined based on the SN.GoGn angle. The measurements were made at the midline of the palate and 4 mm sequentially posterior from the standardized landmark. The measurements were made in the anterior, middle and posterior region of the palate. These measurements in the CBCT were made with the help of CS 3D imaging software. For bone height assessment, linear measurements were performed in a sagittal plane using the ruler tool of the software. One-way ANOVA was performed to evaluate the mean palatal thickness of bone in horizontal growth pattern, average growth pattern and vertical growth pattern in IBM SPSS statistics 23. On performing one-way ANOVA, it was observed that there was a statistically significant difference in the mean of the palatal bone thickness in the anterior of vertical growers. Within the limitations of the present study, it can be concluded that the placement of palatal implants in vertical growers should be done with caution.

KEY WORDS: GROWTH PATTERNS, MINISCREWS, PALATAL BONE THICKNESS.

INTRODUCTION

The development of mini-implants has gained widespread recognition in the orthodontic field due to their temporary absolute skeletal anchorage without impeding the movement of the orthodontic tooth, as well as their small diameter, which allows easy placement in narrow interdental spaces (Mo et al. 2010). Mini-implants are used in numerous regions, such as, interradicular, infrazygomatic crest, buccal shelf, ramus and palate. Although mini-implants can be placed buccally or palatally, palatal mini-implants are preferred due to their superior stability and have good quality as well as quantity of bone (Baumgaertel 2009; Mohammed et al. 2018; Park and Shin 2020). Mineral content of bone matrix and heterogeneity of mineralization of paramount importance for quality of bone as well as stability of mini-implants. Moreover, the quality of bone is influenced by several factors, such as heredity, race, environment, nutrition and lifestyle (Frost 1990; Sommerfeldt and Rubin 2002; Borgen et al. 2020).

The success of any mini-implant depends on the degree to which it integrates mechanically and biologically with the host bone. They are commonly inserted in the anterior region of palate, mid-palatal area, and posterior region of palate (Arcuri et al. 2007; Yadav et al. 2018). The advantage of placing miniscrew in these areas, have no significant anatomical structures, such as nerves, blood vessels, or roots, that interfere with the placement. Additionally, the palatal region is keratinized thereby causing less irritation to the tissue (Ryu et al. 2012; Burhan 2013; Kinzinger et al., 2021). Karagkiolidou et al. (2013) reported that success rate of 98% is observed in the anterior region of the palate. Even so, Manni et al. (2011) revealed that failure rate was higher in females as compared to males (Deguchi et al. 2006;

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Gracco et al. 2008; Manni et al. 2011; Karagkiolidou et al. 2013; Uribe et al. 2015; Kinzinger et al., 2021).

These mini-implants are widely used for molar intrusion, molar protraction, anterior segment protraction and retraction, and en-masse retraction (Deguchi et al. 2006; Gracco et al. 2008; Uribe et al. 2015). Facial type of an individual any individual comprises variations in the skeleton structures and it is pertinent to genetic and environmental factors. They have direct association with craniofacial growth and are divided into three groups- brachyfacial, mesofacial and dolichofacial. In brachyfacial, there is a tendency of horizontal growth pattern. Mesofacial has balanced growth of all facial thirds and dolichofacial has predisposition to vertical growth patterns (McNamara 1981; Cassidy et al. 1998; Kageyama et al. 2006). This may influence the palatal bone thickness, for instance, palate can be deep and narrow for vertical growers while wide and shallow for horizontal growers (Linder-Aronson 1983; Chen 2021). The amount of available bone for insertion of miniscrews is a limiting factor for orthodontic treatment requiring skeletal anchorage. Taking these factors into account, the aim of the present study was to evaluate palatal bone thickness in different growth patterns in the Dravidian population.

MATERIAL AND METHODS

This retrospective study retrieved CBCTs of subjects who were referred for orthodontic treatment. The CBCTs was obtained using orthophos SL 3D dentsply sirona. Inclusion criteria was as follows: no previous orthodontic treatment, angle's class I malocclusion; permanent dentition; mild to moderate crowding. Patients having carious, decayed tooth, deciduous dentition periodontal disease with alveolar bone loss, traumatic dental injury, impacted teeth and systemic diseases was excluded from the study. After applying the inclusion and exclusion criteria, 30 CBCTs were retrieved and classified into three groups according to the growth patterns- 10 horizontal growth pattern (<30°), 10 average growth pattern $(\simeq 32 \mp 2°)$ and vertical growth pattern (>30°). The growth pattern was determined based on the SN.GoGn angle.

The region of interest was determined using the orientation lines of the plane. In the axial view, the midsagittal plane and the maxillary first premolar region was defined, superimposing the sagittal and coronal lines, respectively (Fig 1). Followed by, line joining the middle of the distal bony margin of the incisive foramen and the posterior nasal spine (PNS), midsagittal view (Fig 2). This was taken as the standardized landmark to locate the centre of palate. Subsequently, the measurements were made at the midline of the palate and 4 mm sequentially posterior from the standardized landmark (Fig 3). The measurements were made in the anterior, middle and posterior region of the palate.

These measurements in the CBCT were made with the help of CS 3D imaging software. For bone height assessment, linear measurements were performed in a sagittal plane using the ruler tool of the software. For statistical analysis, one-way ANOVA was performed to evaluate the mean palatal thickness of bone in horizontal growth pattern, average growth pattern and vertical growth pattern in IBM SPSS statistics 23.

Figure 1: Represents the axial view wherein the midsagittal plane and the maxillary first premolar region was defined, superimposing the sagittal and coronal lines, respectively. (Image obtained from CS-3D imaging software)

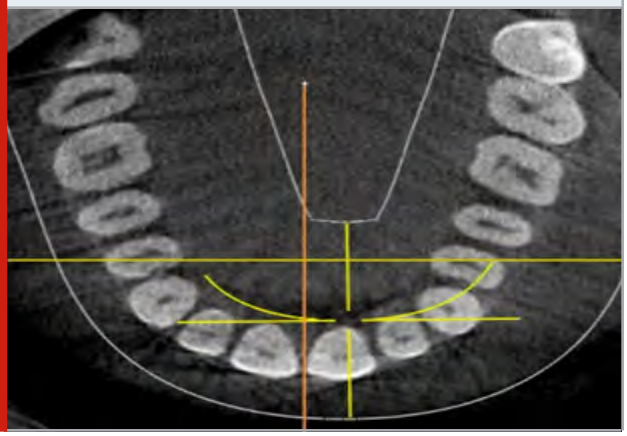


Figure 2: Represents the sagittal view wherein the line joining the middle of the distal bony margin of the incisive foramen and the posterior nasal spine (PNS) was taken as the standardized landmark to locate the centre of palate. (Image obtained from CS-3D imaging software)

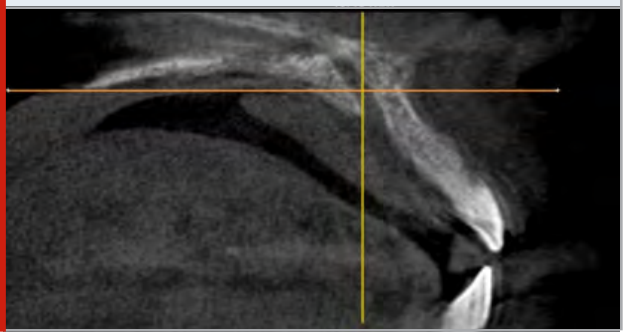
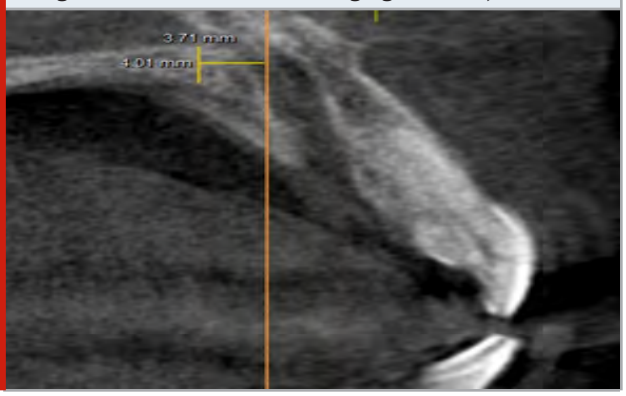


Figure 3: Represents the measurements made at the midline of the palate and 4 mm sequentially posterior from the standardized landmark.
Image obtained from CS-3D imaging software)



RESULTS AND DISCUSSION

On performing one-way ANOVA, it was observed that there was a statistically significant difference in the mean of the palatal bone thickness in the anterior of vertical growers (Table 1).

Table 1. Represents the mean difference of the palatal bone thickness in different growth patterns in anterior, middle and posterior region.

Region	Growth pattern	Sum of squares	df	Sig
Anterior	Vertical Average Horizontal	2.47	2	0.03
Middle	Vertical Average Horizontal	0.15	2	0.28
Posterior	Vertical Average Horizontal	2.31	2	0.08

Introduction of skeletal anchorage in orthodontics has enabled orthodontists to facilitate tooth movement in all the three planes efficiently. Above all, palate is the desirable region for mini-implants placement for skeletal anchorage. Several previous studies have attempted to examine the thickness of the palatal bone using various direct and radiological measurements (Henriksen et al. 2003; Jung et al. 2011; Kim 2014). In our study, the thickness of the palatal bone was assessed using CBCT. This information is helpful during orthodontic treatment to determine the appropriate length of the mini screw implant and to identify sites for mini screw implant placement with sufficient bone available, especially in the palatal areas of patients. Information pertaining to palatal bone thickness supports the selection of the ideal miniscrew implant placement sites and miniscrew implant length to ensure adequate retention and to avoid damaging vital structures. Information on the thickness of the palatal bone will help in choosing the ideal locations for mini screw placement and the length of the implant to ensure adequate retention and avoid damage to vital structures (Awadhi et al. 2018; Fallahi et al. 2021).

The growth trend of the one-third face determines the patient's face type and the direction of its development, which can be more horizontal, more balanced, or vertical. In this sense, the shape of palate is one of the individual characteristics of the face typology and can have different morphologies (Fallahi et al. 2021). In the present study, there was a statistically significant difference observed in the mean palatal bone thickness in the anterior region of the vertical growers. This finding is in partial agreement with the study conducted by Esteves and Bommarito (2007), wherein it was observed that patients with vertical growth patterns have narrow and deep palate but they did not mention about the palatal thickness as well as the region (Esteves and Bommarito 2007). Similar finding was reported by Poon et al. (2005) wherein it was observed that hyperdivergent patients had bone availability is lower in the anterior and middle region (Poon et al. 2015; Fallahi et al. 2021). Limitations of the present study was limited sample size.

CONCLUSION

The findings of the present study conclude that the placement of palatal implants in vertical growers should be done with caution. These results could be helpful for clinicians to improve the successful use of temporary palatal anchors.

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Microbiological Communication

On the Functional Diversity of Plant Growth-Promoting Rhizobacteria in Bharuch, Gujarat, India

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ABSTRACT

The study of PGPR diversity is important for acquainting the knowledge about the unique characteristics of microorganisms and for discovering novel PGPR strains in the rhizosphere that may be used in inoculant formulation to boost the crop production. In this regards, present study was aimed at investigation of functional diversity of plant growth promoting rhizobacteria (PGPR) from Bharuch district using diversity indices and species evenness concept. The study was also focused on correlation of functional trait of microbes with soil characteristics and host type. To accomplish the objective, present study was designed to target nineteen different location of Bharuch district of Gujarat state (India). These sites were scrutinized and studied for PGPR diversity by isolating nitrogen fixers, phosphate solubilizers, potash solubilizers and zinc solubilizers. The location Panoli (L17) was found to be more diverse in PGPR trait followed by Motali (L1). Locations, Safipura (L2), Kharod (L8), Dhamrod (L9), Kosmadi (L10) and Nandav (L19) shows the relative abundance for nitrogen fixers whereas Bakrol (L18) shows the relative abundance for phosphate solubilizers. The principal component analysis (PCA) and hierarchical cluster analysis (HCA) was used to establish the statistical correlation of functional diversity with soil characteristic, host type and locations. The combinatorial assessment of diversity and correlational study with soil and host type may provide an information on the distribution and roles of PGPR in particular habitat. Such an approach can throw a light on absence, presence and abundance of beneficial and plant growth promoting micro-organisms at a selected locality and give us an idea on need of enrichment of the land with beneficial microbes. The study can also be useful in selecting the site according to the need and requirement of particular type of PGPR.

KEY WORDS: BHARUCH, DIVERSITY INDICES, HIERARCHICAL CLUSTER ANALYSIS (HCA), PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR), PRINCIPAL COMPONENT ANALYSIS (PCA).

INTRODUCTION

Chemical fertilizers are currently utilized extensively by farmers as a supplement of basic nutritional requirement. However, extensive use of these fertilizers causes various hazardous environmental issues. Therefore, there is an urgent need to reduce or replace the utilization of chemical fertilizers without compromising plant yield and soil quality (Vejan et al. 2016; Kaneriya and Pattani 2021). The application of bioinoculants or plant growth promoting rhizobacteria (PGPR) as a biofertilizers is promising and alternative approach which has been employed in the field of agriculture (Santos et al. 2019; Lebrazi et al. 2020).

Different strategies have been utilized by PGPR to promote plant growth, like improvement of phosphate, zinc, potash uptake by solubilizing these complexes into plant usable and absorbable forms, fixation of atmospheric nitrogen, production of vitamins or phytohormones, suppression of plant diseases through competitive colonization etc. It is also necessary that farmers of both developing and developed countries have easy access of biofertilizers. Therefore, efforts are required to explore more diverse PGPR for their utilization as potent bio-fertilizers to improve the yield of different economically important crops (Tchakounté et al. 2018; Nosheen et al. 2021).

The study on PGPR diversity is an important aspect to acquaint knowledge about the unique feature of microorganisms that can be utilized for crop production (Costa et al. 2018). The study on PGPR diversity is also important in finding the availability and feasibility of the novel PGPR strain in the rhizosphere that can be exploited in inoculant formulation commercially (Shukla 2019). Diversity indexes like the Simpson index, Shannon index (H') and the equitability index (J') have generally been used

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to describes the diversity of microbial communities as a mathematical point of view (Simpson 1949; Shannon and Weaver 1949; Pielou 1975). The rhizospheric zone of plants possess different types of PGPR and the trait of PGPR may vary according to the type of host. Therefore, it is necessary to correlate the PGPR trait with the type of host (Verma et al. 2019; Basu et al. 2021).

Overall, understanding of PGPR microbial diversity map for any region, is useful to arrive at assessment that can act as indicators for enhanced plant productivity and soil quality by formulating biofertilizers. An understanding of PGPR diversity is also useful in designing the customised biological inputs or inoculum for crops, depending on the scarcity of the specific functional macro or micro nutrient solubilizers. Very less information is available on PGPR diversity of agricultural land from Bharuch district. In this context, the present study was aimed at isolation of PGPR from different locations of Bharuch district, estimation of diversity by calculating the diversity indices, correlational study of functional diversity with soil characteristics, host type and locations using PCA and HCA.

MATERIAL AND METHODS

All the reagents and media used under study were of analytical grade. Pikovskaya's agar, Aleksandrow agar and Luria Bertani agar were purchased from Himedia, India. Jensen's agar and Zinc solubilizing medium were prepared in laboratory. Jensen's agar was comprised of (g/L): sucrose, 20.0; $Fe_2(SO_4)3•H_2O$, 0.1; K_2HPO_4, 0.5; $MgSO_4•7H_2O$, 0.5; NaCl, 0.5; $CaCO_3$, 2.0; $Na_2MoO_4•2H_2O$, 0.005; agar, 2.0 and pH, 7.2. Zinc solubilizing medium was comprised of (g/L): glucose, 10; ZnO, 1.0; (NH4)2SO4, 0.5; KCl, 0.2; yeast extract, 0.5; $FeSO_4•7H_2O$, 0.01; $MnSO_4$, 0.01; K_2HPO_4, 0.5; agar, 2.0 and pH, 7.2. For sample collection and processing, rhizosphere soil samples were collected from nineteen different locations of Bharuch district, Gujarat (India).

These locations were Motali (L1), Safipura (L2), Sanjali (L3), Alonj (L4), Amboli (L5), Umarwada (L6), Samor (L7), Kharod (L8), Dhamrod (L9), Kosamdi (L10), Boridara (L11), Boidra (L12), Kondh (L13), Mahuvej (L14), Moti Pardi (L15), Narmada River (L16), Panoli (L17), Bakrol (L18) and Nandav (L19). Soil samples were collected by digging with intact root system and necessary information was recorded. The samples were kept in plastic bags and stored at 4°C until use. Soil electric conductivity (EC) and pH were measured before processing of soil samples.

For the isolation of PGPR, ten gram of soil sample was suspended in 90 ml of sterile normal saline in 250 ml flask and shaken at 150 rpm for 1 h at 37°C. The supernatant was then serially diluted and inoculated in selective media for the isolation and screening of desired PGPR. Using spread plate technique 0.1 ml of soil suspensions were spread on differential media viz. Jensen's medium, Pikovskaya's agar, Aleksandrow agar and Zinc solubilizing medium for the isolation of nitrogen fixers, phosphate solubilizers, potash solubilizers and zinc solubilizers, respectively. The plates were incubated at 28°C for 5-6 d. Different types of isolates were then selected based on morphological characteristics and solubilization. Isolates were further purified by sub-culturing on respective medium. Positive isolates were maintained on Luria Bertani agar slants. To determine the functional diversity and data analysis, the results obtained after isolation of PGPR were used to calculate diversity indices, Berger-Parker index and evenness metrics (Simpson 1949; Shannon and Weaver 1949; Sheldon 1969; Berger and Parker 1970; Pielou 1975).

Table 1. The formula used to calculate biodiversity indices.

Index	Formula	Explanation
Dominance	$D = \sum_{i=1}^{n} Pi^2$	*Pi*: the ratio of species *i* *n*: the total number of species
Shannon Index	$H' = -\sum_{i=1}^{n} Pi\ ln(Pi)$	*Pi*: the ratio of species *i* *n*: the total number of species
Berger-Parker index	$D = n_{max} / N$	*n*: the maximum number of identified species N: the total number of individuals
Shannon (Pielou) evenness index	$J' = \frac{H'}{H'_{max}} = \frac{H'}{\ln S}$	*H'*: the value of Shannon Index *H'max*: the maximum value of Shannon Index
Simpson evenness index	$E_{1/D} = \left(\frac{1}{D}\right)/n$	*D*: the value of dominance *n*: the total number of species
Sheldon index	$S = e^{H'}/n$	*H'*: the value of Shannon Index *n*: the total number of species

The formula used for the calculations of biodiversity indices has been presented in Table 1. Principal component analysis (PCA) was used to determine the statistical correlation between soil characteristics and population diversity and, between the host family and population diversity. Hierarchical cluster analysis (HCA) was used to cluster the

locations according to population diversity. PCA and HCA were performed using XLSTAT 8.0, Add ins of statistical software.

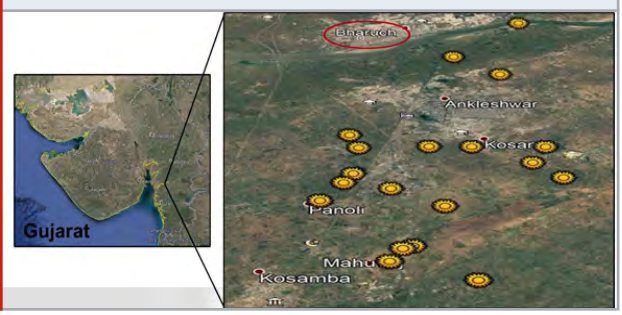
RESULTS AND DISCUSSION

Bharuch district is located at 21.7°N 72.97°E with an average elevation of 15 meters near the banks of the river Narmada. Bharuch has tropical savanna climate and its weather is strongly moderated by the Arabian Sea. Monsoon season falls between the month of June to September and has about 800 millimeters of rain. The average maximum temperature during these months is about 32°C (90°F). The winter remains between the month of December to February with average temperatures of 23°C (73°F).

Major crops grown in this area are maize (*Zea mays*), sugarcane (*Saccharum officinarum*), pigeon pea (Cajanus cajan), wheat (*Triticum aestivum*), cotton (*Gossypium hirsutum*) and green gram (*Vigna radiata*). The natural vegetation of the area consisted of *Prosopis juliflora*, *Prosopis cineraria*, *Azadirachta indica*, *Ziziphus mauritiana* and *Eucalyptus* sp. (Jangir et al. 2018).

The present study was attempted to give information about functional diversity of PGPR from Bharuch district, Gujarat (India). Total 427 samples were collected from different nineteen locations of Bharuch district (Figure 1). The isolates showing differentiation in their colony morphology were selected, purified and tested for zone of solubilization (Figure 2). Total 3, 351 isolates were screened for PGPR trait. The number of PGPR isolated from different locations, showed maximum microbial population of nitrogen fixers (93.6%) followed by phosphate solubilizers (3.51%), zinc solubilizers (2.24%) and potash solubilizes (0.64%) (Figure 3). The diversity indices were evaluated to study the species evenness of PGPR in different locations of Bharuch district.

Figure 1: Map showing the sampling sites of Bharuch district (Source: District Profile 2021; https://bharuch.nic.in/district-profile/)



The high value of Shannon index (H') is an indication of more diversity. The zero value of H' indicates that only one species or the same number of individuals represent the diversity (Kanieski et al. 2018). In the present study, L17 shows the maximum H' which clearly indicated that location L17 shows maximum diversity of PGPR followed by L1, L5 and L6. For the locations, L8, L9, L10 and L18, the value of H' is 0.000 which indicates that these locations do not show any diversity. The value of H' is generally observed between 1.5 and 3.5 and rarely exceed above 4.0 (Margalef 1972). In the present study, the low value of H' may be due to smaller sample unit size. Sometimes, interpretation becomes very much difficult due to narrowly constrained value of Shannon index (H').

Figure 2: Photographs of different types of bacteria on specific medium. (A) nitrogen fixing bacteria, (B) phosphate solubilizing bacteria, (C) potash solubilizing bacteria and (D) zinc solubilizing bacteria with hollow zone of solubilization around the colonies

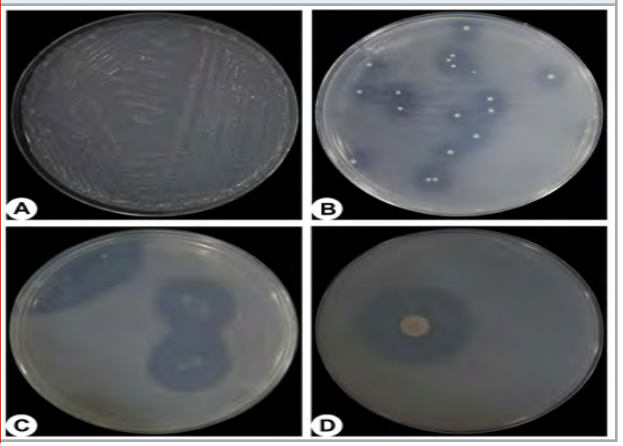
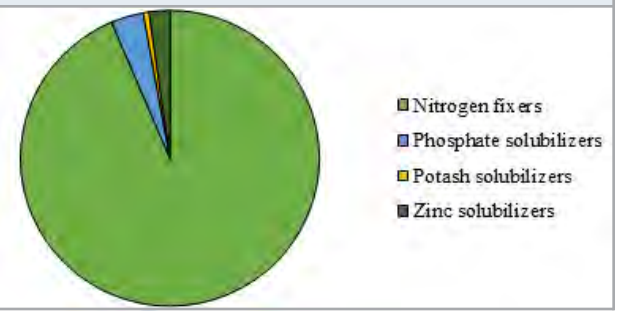


Figure 3: Diversity of PGPR from different locations of Bharuch district



For example, in L11 and L12, the values of Shannon index (H') are 271 and 227, respectively. Here, it is difficult to interpret that whether these two locations have similar kind of diversity or have substantial variation. Several researchers have overcome this problem by using eH' as a replacement of H' (Kaiser et al. 2000; Jha et al. 2010). Thus, the values of eH' for the locations L11 and L12 becomes 1.255 and 1.569, respectively (Table 2). High value of Shannon evenness J' signifies the equal distribution of individuals in all species. The value of J' ranges from 0 to 1 and, if the value of J' reaches to 1 indicates that all species are equally distributed (Kanieski et al. 2018). In present study, L17 shows higher value of J' reflects the more uniform distribution of PGPR followed by L13. The locations L2, L8, L9 and L10 do not show any uniform distribution (Table 3).

The Berger-Parker index is just a measurement of dominance (Berger and Parker 1970). High value of Berger-Parker index is an indication of maximum dominance and least

diversity. In present study maximum values are observed for L2, L8, L9, L10 and L18. These locations are dominant in nitrogen fixers except L18 which has shown dominant trait for phosphate solubilizers. However, these locations were not diverse for different types of PGPR (Table 2). The Simpson index gives an idea about the difference in species abundance distribution. The dominance of one species to that of other species can be calculated by D = ΣPi2.

Table 2. Diversity indices of PGPR from different locations of Bharuch district.

Locations	Diversity indices					Mean	SD
	Shannon H'	eH'	Simpson 1/D	Simpson 1-D	Berger-Parker index		
L1	0.675	1.964	1.490	0.329	0.811	1.054	0.747
L2	0.000	1.000	1.000	0.000	1.000	0.600	0.577
L3	0.163	1.177	1.080	0.074	0.962	0.691	0.586
L4	0.232	1.263	1.133	0.117	0.938	0.737	0.595
L5	0.637	1.891	1.514	0.340	0.800	1.036	0.728
L6	0.566	1.761	1.412	0.292	0.833	0.973	0.692
L7	0.077	1.080	1.030	0.029	0.990	0.641	0.579
L8	0.000	1.000	1.000	0.000	1.000	0.600	0.577
L9	0.000	1.000	1.000	0.000	1.000	0.600	0.577
L10	0.000	1.000	1.000	0.000	1.000	0.600	0.577
L11	0.271	1.312	1.166	0.142	0.923	0.763	0.601
L12	0.227	1.255	1.127	0.113	0.940	0.732	0.594
L13	0.451	1.569	1.385	0.278	0.833	0.903	0.651
L14	0.077	1.080	1.030	0.029	0.985	0.640	0.579
L15	0.543	1.722	1.385	0.278	0.842	0.954	0.683
L16	0.229	1.257	1.104	0.094	0.951	0.727	0.594
L17	0.959	2.610	2.329	0.571	0.579	1.410	1.003
L18	0.000	1.000	1.000	0.000	1.000	0.600	0.577
L19	0.232	1.261	1.107	0.097	0.950	0.729	0.595

The diversity decreased as the value of dominance D increased. Therefore, Simpson index is generally stated as 1/D of 1-D and the value of which can be rise as the grouping becomes more even. In present study, the value of Simpson index was found to be highest in L17 representing the highest diversity followed by L5. Simpson index is not truly talking about absolute evenness although it highlights the dominance in diversity. Therefore, a separate measure called Simpson evenness, E1/D can be taken into consideration which measures the relative species abundance. In present study, the value of E1/D was higher in L2, L8, L9, L10, L18 and L19 (Table 3).

Out of these locations, L2, L8, L9, L10 and L19 showed the relative abundance for nitrogen fixers whereas L18 shows the relative abundance of phosphate solubilizers. Sheldon index is another diversity index which reflects a perfectly even abundance distribution and generally varies between 0 to 1 (Sheldon 1969). According to Sheldon index, L2, L8, L9, L10 and L18 were evenly abundantly distributed. In present study, these locations reflected the abundant distribution of either nitrogen fixers or phosphate solubilizes only. PCA was studied to investigate the statistical correlation of PGPR diversity with soil characteristics (soil pH and EC) and host type (host family). The PCA biplot presenting the interaction of Shannon diversity (H') with soil pH and EC has been shown in Figure 4.

Total 69.57% of variation was accounted by principal components (PC). Out of which, 37.24% and 32.34% of the total variation was accounted by PC1 and PC2, respectively. Soil pH did not show any correlation with Shannon diversity (H') whereas soil EC had influenced the PGPR diversity by 5.5%. This can be ascribed by the reason that the almost uniform soil characteristics of targeted locations explored in present study.

The PCA biplot displaying the interaction of PGPR diversity with host family has been shown in Figure 5. Total 94.15% of variation was accounted by principal components (PC). Out of which, 69.22% and 24.93% of the total variation was accounted by PC1 and PC2, respectively. The maximum diversities of nitrogen fixers, phosphate solubilizers and zinc solubilizers were obtained from the host plants belonging to family Leguminosae followed by Poaceae and Malvaceae. The highest diversity of potash solubilizers were obtained from the host plants belonging to the families Rutaceae, Anacardiaceae, Lythraceae and Cucurbitaceae. This kind of correlation study may helpful in selecting the host plant for achievement of maximum diversity of PGPR or particular type of PGPR.

Table 3. Evenness of PGPR from different locations of Bharuch district.

Locations	Value index				
	Shannon evenness J'	Simpson evenness E1/D	Sheldon S	Mean	SD
L1	0.487	0.372	0.491	0.450	0.068
L2	0.000	1.000	1.000	1.000	0.000
L3	0.235	0.540	0.589	0.455	0.192
L4	0.337	0.566	0.632	0.512	0.155
L5	0.580	0.505	0.603	0.572	0.063
L6	0.515	0.471	0.587	0.524	0.059
L7	0.112	0.515	0.540	0.389	0.240
L8	0.000	1.000	1.000	1.000	0.000
L9	0.000	1.000	1.000	1.000	0.000
L10	0.000	1.000	1.000	1.000	0.000
L11	0.391	0.583	0.656	0.543	0.137
L12	0.327	0.564	0.627	0.506	0.158
L13	0.650	0.692	0.785	0.709	0.070
L14	0.112	0.515	0.540	0.389	0.240
L15	0.495	0.462	0.574	0.510	0.058
L16	0.208	0.368	0.419	0.332	0.110
L17	0.873	0.776	0.870	0.840	0.055
L18	0.000	1.000	1.000	1.000	0.000
L19	0.211	1.000	0.420	0.544	0.409

Figure 4: PCA biplot showing the statistical interactions of soil characteristics (soil pH and soil EC) with diversity index (H')

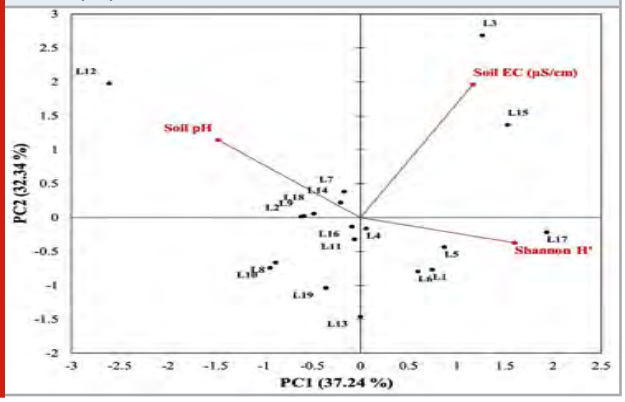
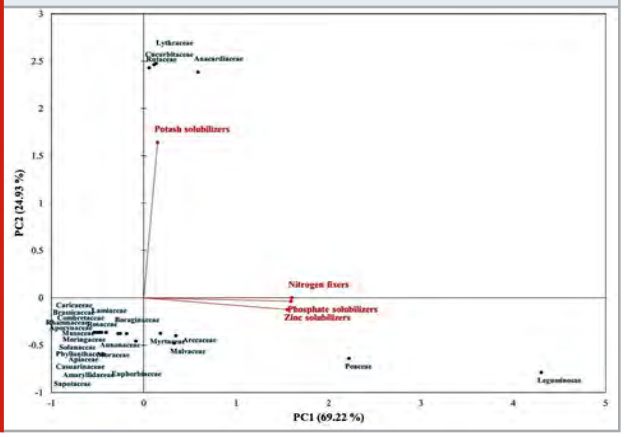


Figure 5: PCA biplot showing the statistical interactions of PGPR with host



In addition to PCA, HCA was also studied to cluster the different locations of Bharuch district according to maximal diversity of PGPR. The HCA dendrogram showed the clustering of different locations of Bharuch district according to PGPR diversity and has been presented in Figure 6. The locations have been grouped into three different clusters. Cluster 1 includes L1, L3, L5, L6, L15 and L17; Cluster 2 includes L2, L4, L7, L8, L9, L10, L11, L13, L14, L16, L18 and L19; and Cluster 3 includes only L12. Cluster 1 was diverse from Cluster 2 which in turn diverse from Cluster 3. Moreover, Cluster 1 showed the maximum diversity of all four types of PGPR. So, the locations fall in this cluster can be explored for in depth study. This information may helpful in selecting the locations according to PGPR variations.

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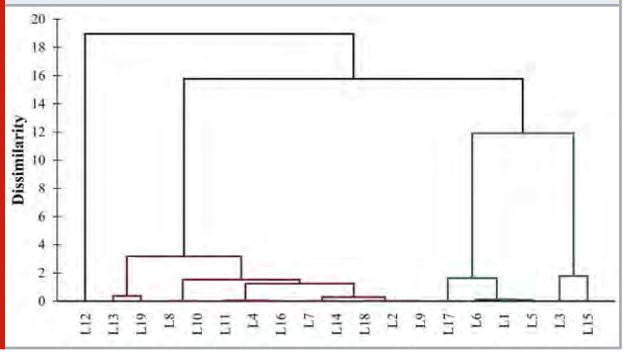
Figure 6: HCA dendrogram showing the clustering of locations according to PGPR diversity.



Overall, it was observed that the soil of Bharuch district is deficient in potash solubilizing microorganism (Figure 3). So, the focus should be much more on amendments of potash solubilizing microorganisms throughout the Bharuch district. Similarly, some clusters of Bharuch district were deficient in phosphate solubilizer where as others were deficient in zinc solubilizers. Therefore, PGPR diversity according to cluster may helpful in getting the knowledge about the PGPR deficiency of soil. Additionally, the type of PGPR may vary with the type of host. For example, in present study, the potash solubilizers were obtained from the plants belonging to Rutaceae, Anacardiaceae, Lythraceae and Cucurbitaceae family. Host specific PGPR knowledge may helpful in improving the soil health and microflora by selective crop rotation according to the need and deficiency of soil. So, in depth studies are also required on host specific PGPR (Kanieski et al. 2018).

Currently, farm land soil analysis is promoted by the government agencies in many countries to ascertain the nutrient deficiency for specific reasons depending on the type of soil. This data is used to recommend the specific micronutrients which can be added to overcome the deficiency in an economical way rather than applying a mixture of multiple nutrients which are actually not needed. If similar approach is applied to recommend function specific PGPR depending on the functional deficiency can effectively reduce the input cost in sustainable cultivation technique using PGPR. The PGPR diversity data can be prepared according to clusters. Recommendation can be given based on the deficiency of PGPR in a particular cluster which can lead to higher cost to benefit ratio of biological inputs. In India, massive projects are going on for preparing the soil health card of agriculture land by the government where periodic soil analysis is carried out. If the functional diversity parameters are added in existing system it can generate a detailed data base on the basis of which recommendation can be made for sustainable agriculture promoted by PGPR (Kanieski et al. 2018; Basu et al. 2021).

On the basis of the study, microbial fertilizers manufacturing industries can build a capacity of mass multiplication of functional inoculum and organised distribution network can be established cluster wise. Moreover, it is also important for farmers to know the type of PGPR present in particular locations/clusters, so that farmers can know the quantity and type of biofertilizers they should add to optimize the crop production and yield. The farmers can choose the PGPR products on the basis of the functional requirement rather than using consortium of multiple PGPR. If farmers start getting visible and consistent solution by proper use of PGPR, it can reduce the use of chemical fertilizers which in turns can benefit the soil and nature at great extent (Basu et al. 2021).

CONCLUSION

The finding of the present study gives an idea about the distinctive location of Bharuch district for highest diversity of PGPR. Looking toward the diversity data, Panoli (L17) followed by Motali (L1) seems more diverse and even for all types of PGPR. The soil collected from rhizosphere of host plants belonging to family Leguminosae, Poaceae and Malvaceae has shown maximum diversity. Therefore, future investigation will focus on detail diversity study of PGPR collected from rhizosphere of host plants belonging to family Leguminosae, Poaceae and Malvaceae from Panoli and Motali locations of Bharuch district. The functional diversity of PGPR from Bharuch district is reported for the first time. The study will helpful to other researcher for development of biofertilizer.

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

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Biotechnological Communication

Screening and Molecular Characterization of Ligninolytic Enzyme-Producing Strain *Alternaria alternata* from soil

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ABSTRACT

Laccases are biotechnologically relevant enzymes used in different industries including textile industry, food processing industry, pharmaceutical industry, wood processing industry and chemical industry. Recently they are used in biosensors for detection of phenolic compounds, in biofuel cells for the generation of eco-friendly energy and also as a medical diagnostics tool. Because of wide range of applications, laccases have received so much of attention of scientists and researchers in the last few decades. They are extracellular, metalloenzymes containing copper. It has ability to catalyses the oxidation of polyphenolic compounds. Laccase activity was reported in different range of fungi including white-rot fungi basidiomycetes, deuteromycetes and ascomycetes where its main role is lignin-degrading. The searching of new microbial sources for laccase production is great demand. Present study was focused to search a new microbial strain that produces laccase from different soil samples. The strains isolated from soil enabled the laccase production and was qualitatively analyzed using 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonate) (ABTS). It is an indicator compound used for the rapid visual of laccase positives strain. Laccase isolates were identified with the change in color from dark green to purple on ABTS containing Potato dextrose agar (PDA) plates. The fungal isolates were identified by conventional methods and molecular characterization techniques using DNA extraction, polymerase chain reaction (PCR) amplification and sequence analysis. Genomic DNA was isolated, internal transcribed spacer ribosomal DNA (ITS-rDNA) amplified by PCR and ITS regions were sequenced by Sanger sequencing method. Based on conventional methods and molecular characterization, the laccase producing isolate was identified as *Alternaria alternata* LF9_CPD_NRRI MK855476.1. Genetic distance and neighbor-joining algorithm was analyzed by using MEGA 6.0 software and phylogenetic tree was constructed to study the evolutionary history.

KEY WORDS: ABTS, LACCASE, LIGNOCELLULOLYTIC, OXIDOREDUCTASE, WHITE ROOT FUNGUS.

INTRODUCTION

Laccases [benzenediol: oxidoreductase, enzyme commission (EC) 1.10.3.2] are most promising lignin-degrading enzymes also called lignocelluloses. These multifunctional enzymes oxidize phenolic compounds, including aromatic amines, by reducing molecular oxygen into water. Laccase possesses a wide-range of substrate specificity also known as extracellular globular proteins with 50-130 kilo-Dalton (kDa) molecular weight. Optimum pH at which laccase shows maximum activity is below 7, but a few studies in the past have reported that laccase can work at higher pH (Viswanath et al. 2014; Teerapatsakul et al. 2016). Because of their significant utilization in various biological processes, the enormous demand of laccases is increasing day by day in many industrial fields of bioremediation and xenobiotic degradation, dye degradation, biosensor, and food and bakery. Various improvements are undergoing to better the formation of lignocellulose-based products in nanotechnology application (Margot et al. 2013; Rao et al. 2019).

Laccases are widespread in nature and was first reported from *Rhus vernicifera* in Japanese lacquer tree. Laccase is obtained from wide variety of organisms including bacteria to higher organism such as fungi, plants and insects. Laccases expressed by such microorganisms includes majorly lignin peroxidase (LiP), manganese peroxidase (MnP) and laccases (Dwivedi et al. 2011; Xu et al. 2017). Several

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prominent lignin-degrading enzymes have been classified from fungal groups and those belonging to ascomycetes, basidiomycetes and deuteromycetes (Gedikli et al. 2010). Basidiomycetes are great producers of laccase enzyme such as *Cerena maxima, Coriolopsis polyzona, Lentinus tigrinus, Trametes versicolor, Trametes hirsute, Trametes ochracea, Trametes villosa, Pycnoporus sanguineus*, and *Pleurotus eryngii* etc (Shleev et al. 2004; Gedikli et al. 2010; Deepa et al. 2020).

The laccase producing fungi isolated from new microbial systems would be considered, extremely useful for achieving several biotechnological applications. These fungal laccases are very efficient in conversion of plastic, fuel, paint and wood components into nutrients. Currently researchers are mainly focusing on enhancing laccase production from several strains of Basidiomycetes predominantly wood-root-fungi by optimizing culture conditions suitable for production. Couple of studies have reported ascomycetes on their lignin degrading ability but very less reported on *Alternaria alternate* for laccase production. Recently laccase was reported in Bacillus species isolated from industrial waste (Glaeser et al. 2010; Gassara et al. 2010; Yadav et al. 2019; Deepa et al. 2020).

Fungi known as biological controllers illustrates the huge fungal diversity and some vital fungal groups are identified at global extent (Delgado-Baquerizo et al. 2017; Irfan et al. 2018). The objectives of the present work were to isolate laccase producing fungi from microbial system in natural habitat, screen for the presence of lignin-modifying enzyme using specific substrate containing ABTS 2,2'-azinobis-(3 ethylbenzthiazoline-6-sulfonate) as color indicator compound to identify potential fungal strains that are able to produce laccase using molecular analysis and construct phylogenetic tree.

MATERIAL AND METHODS

ABTS (2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonicacid) was purchased from Hi-Media Pvt. Ltd Lucknow, India. Other chemicals used were of analytical grade and were purchased from local markets. Soil samples were collected from the campus of Shri Ramswaroop Memorial University, Lucknow Deva-road, Barabanki, Uttar Pradesh, India located at approximately 123 meters (404 ft) above sea level coordinates: latitude: 26°50′21″N and longitude: 80°55′23″ E. Moderate rain fall region with the temperature ranges maximum 47.5^{0}C to 2.5^{0}C minimum (Hannula et al., 2017).The sample collecting site covered with various trees and shrubs, turnover of the organic substance in such soil expresses rapidly. The soil sample was collected in sterile plastic bags aseptically from a depth of 10–15 cm below the earth's surface as shown in Fig. 1.

For the isolation of microbes (ascomycetes and fungi), the soil samples were first serially diluted and then were spread evenly over the surface of potato dextrose agar plates to isolate fungi (Kanaujia et al. 2014). Further, the plates were incubated at 35^{o}C and monitored after 5th days. Fungal screening was carried out by using ABTS 2,2'-azinobis-(3 ethylbenzthiazoline-6-sulfonate) as indicator compound. Positive laccase isolates were visualized as dark green to purple color on ABTS containing Potato dextrose agar (PDA) plates which indicates the presence of laccase being produced by the fungus, as previously reported (Pointing et al., 2000). Screening of laccase producing organisms was employed on PDA plates supplemented with 0.1% and 0.01% ABTS 2,2'-azinobis-(3 ethylbenzthiazoline-6-sulfonate) respectively and incubated at 30 ^{o}C for 7 days. Culture plates indicate that the definite color changes were considered for laccase producing strain and employed further for consequent studies (Pointing et al., 2000).

Figure 1: Site for the collection of soil sample (Source: Google map, https://goo.gl/maps/kHkWMFV362NoDcGD6)

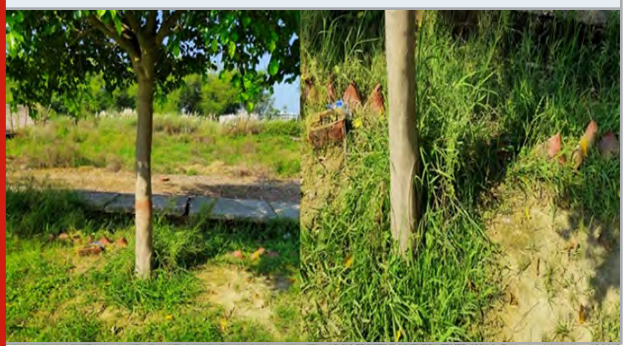
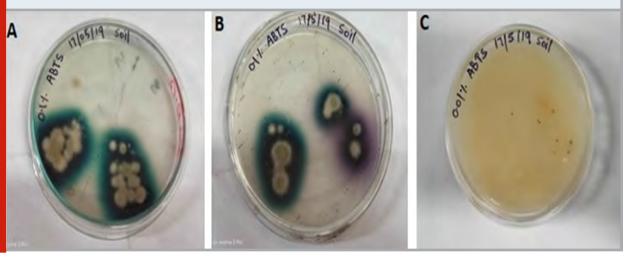


Figure 2: (A) Screening of Laccase producing *Alternaria alternate* isolate LF9_CPD_NRRI on PDA plates containing 0.1% ABTS substrate showing intense dark green color halos around the colony. (B) PDA plate containing the 0.1% ABTS substrates showing intense dark green to purple color halo around the colony. (C) PDA plates containing the 0.01% ABTS substrates showing no reaction.



Total genomic DNA was extracted from the fungal strain by genomic DNA extraction kit method. Comparison of the internal transcribed spacer (ITS) ribosomal DNA (rDNA) gene sequence was employed for molecular characterization. The gene encoding ITS region was amplified by the polymerase chain reaction (PCR) using forward (CTGGTGCCAGCAGCCGCGGYAA) and reverse primer (CKRAGGGCATYACWGACCTGTTAT3). All PCR amplifications were carried out in a Thermal Cycler

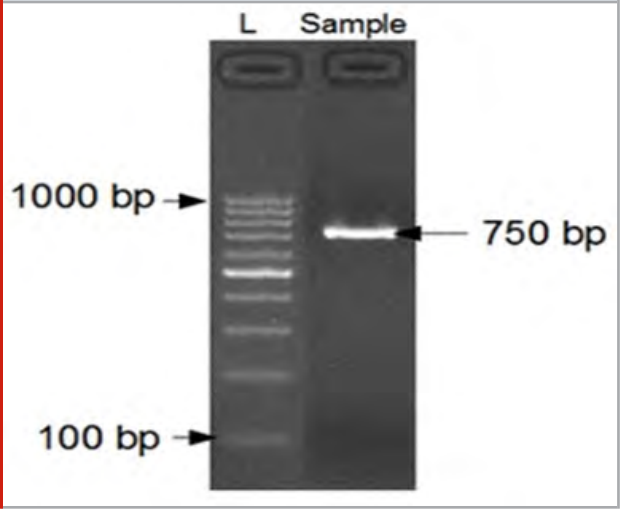
(Model No. 9902). A hot-start procedure (94 °C for 3 min) was performed and enzyme was added earlier to avoid non-specific annealing of primers. DNA was amplified during 30 cycles of 94 °C for 30 sec, 48 °C for 30 sec, 72 °C for 1 min and 72 °C for 7 min.

After purification of amplified products by agarose gel electrophoresis, the amplified products were sent for sequencing to Xcelris Labs Limited Ahmedabad, India. The most homologous sequence was determined by comparison to Gene Bank database using BLAST software. Genetic distance and neighbor-joining algorithm was analyzed by using MEGA 6.0 software and the phylogenetic tree was constructed to study the evolutionary relation between species.

RESULTS AND DISCUSSION

Screening For Laccase Activity Using Abts Assay: The main purpose of screening is to select fungi with desired characteristics proposed for miscellaneous applications of food industries, paper and pulp industry, bioremediation of environmental pollutants, textile industries, biosensor application, organic synthesis and biofuel formation, bioremediation of toxic chemical wastes, pharmaceutical and cosmetics industries, and nanobiotechnology applications (Devasia et al. 2016; Zeng et al. 2017; Zdarta et al. 2018; Veraet al. 2019).

Figure 3: 1.2% Agarose gel showing 1000bp amplicon (SSU region) of 18S rDNA.
Lane 1: 1000bp DNA Ladder and Lane 2: 750bp amplicon (SSU region) of 18S rDNA.

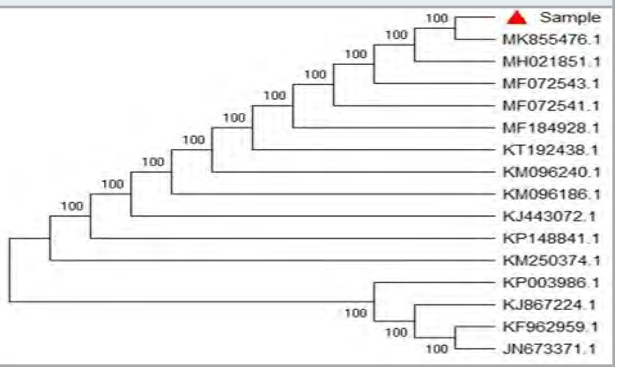


All the isolated and subculture fungal colonies were inoculated on PDA plates supplemented with specific substrate for laccase. Five days old culture was placed on PDA plates containing ABTS and incubated at 30°C for 7 days (Antecka et al. 2018). After 8th days the culture plate was able to develop dark green color on 0.1% ABTS 2,2'-azinobis-(3 ethylbenzthiazoline-6-sulfonate) containing PDA plate as shown in (Fig. 2. A) and purple color on 0.1% ABTS containing PDA medium petri-plate indicates the presence of laccase being produced by the fungus as shown in Fig. 2. B (Vantamuri and Kaliwal 2015). The 0.01% ABTS containing PDA plate shows no reaction as shown in Fig. 2. C.

Pcr Amplification And Identification Of The Fungal Strain: The amplified DNA quality was evaluated on 1.2% agarose gel, a single discrete high-molecular weight PCR amplicon band of 750 bp has been observed as shown in Fig. 3.

Sequence Analysis: Consensus sequence of 750 bp of 18S gene in SSU region was generated from forward and reverse sequence data using aligner software. The 18S gene in SSU region sequence was used to carry out BLAST alignment search tool of NCBI GenBank database. Based on maximum identity score first fifteen sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 6.0 as shown in Fig. 4.

Figure 4: Evolutionary Relationship



The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed (Saitou et al. 1987; Phatake et al. 2015). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) were shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method and were in the units of the number of base substitutions per site (Kimura 1980; Felsenstein 1985). This analysis involved 16 nucleotide sequences. Codon positions included were 1st+2nd+3rd+non-coding. All ambiguous positions were removed for each sequence pair (pair wise deletion option). There was a total of 785 positions in the final dataset. Evolutionary analyses were conducted in MEGA

6.0 (Tamura et al. 2011; Phatake et al. 2015; Senthivelan et al. 2019).

The extracellular laccase producing fungus was isolated from a new habitat and used for enzyme production. Based on the screening results using lignolytic substrates of ABTS and identified by dark green and purple color oxidation zone was developed around the colonies (positive ABTS-substrate oxidation) which were taken for the laccase production (Wang et al. 2018; Senthivelan et al. 2019). Depending upon the observations one predominant strain of fungus was selected for further study. The screening results showed the formation of color under the medium with ABTS (substrate) confirmed that the isolated fungus has ability to produce lignin-degrading enzyme (Das et al. 2016). The newly isolated fungal strain was further subjected to their morphological analysis. By virtue of their morphological identification, the isolated fungus belonged to the class of Alternaria fungal species. Further the fungus was identified as *Alternaria alternata* isolate LF9_CPD_NRRI (Accession Number: MK855476.1) by molecular identification of 18s rRNA sequencing method. The morphological characteristics similarly reported previously; the colonies of *Alternaria fungus* were olive-black in color with white margins (Abeer et al. 2015; Sharma et al. 2016; Thakkar et al. 2020).

Table 1. Alignment view table showing sequences producing significant alignments from NCBI GenBank

Accession	Description	Max score	Total score	Query coverage	E-value	Max ident
MK855476.1	*Alternaria alternata*LF9_CPD_NRRI	1424	1424	100%	0.0	99.49%
MH021851.1	*Alternaria alternata* isolate PCPDI	1424	1424	100%	0.0	99.49%
MF072543.1	*Alternaria alternata* strain 013	1424	1424	100%	0.0	99.49%
MF072541.1	*Alternaria alternata* strain 003	1424	1424	100%	0.0	99.49%
MF184928.1	*Alternaria* sp. Isolate QDFBLJ-1	1424	1424	100%	0.0	99.49%
KT192438.1	*Alternaria* sp.PMK2	1424	1424	100%	0.0	99.49%
KM096240.1	*Alternaria* sp.MF382	1424	1424	100%	0.0	99.49%
KM096186.1	*Alternaria* sp. LF255	1424	1424	100%	0.0	99.49%
KJ443072.1	*Alternaria alternata* strain G408	1424	1424	100%	0.0	99.49%
KP148841.1	*Fungal* sp.S1 ZM-2014	1424	1424	100%	0.0	99.49%
KM250374.1	*Alternaria* sp. SPS-04	1424	1424	100%	0.0	99.49%
KP003986.1	*Alternaria alternata* strain J14	1424	1424	100%	0.0	99.49%
KJ867224.1	*Alternaria* sp. DBC-AD	1424	1424	100%	0.0	99.49%
KF962959.1	*Alternaria alternata* strain HA4087	1424	1424	100%	0.0	99.49%
JN67337.1	*Alternaria alternata* strain HDJZ-zwm-34	1424	1424	100%	0.0	99.49%

Conidiophore septum sometimes demonstrated as branched or simple straight or curved shaped medium brown and sleek barred, Conidia like branched chains, short conical or cylindrical beak and they had been obscured in many previous studies (Abeer et al. 2015; Sharma et al. 2016). Similar investigations were also reported in the ABTS containing plate showed the green and purple-color in the presence of laccase producing fungi defined the positive reaction for the laccase production (Vantamuri and Kaliwal 2015). The efficiency of laccase producing strain *Alternaria alternata* was reported at 30OC on 7thday of incubation period (Tapwal et al., 2014). Recently new laccase producing fungal strain *Amesia atrobrunnea* was screened from rotted wood samples and agro waste using ABTS method (Thakkar et al. 2020).

CONCLUSION

In the present study, a fungal stain Alternaria alternata was screened and investigated. The results showed that the fungal isolate *Alternaria* sp. MK855476.1 exhibited an ability to oxidize ABTS. Ahalo of dark green and purple was formed around the fungal colonies (positive for ABTS oxidation), indicating the presence of ligninolytic enzymes laccase. Due to their broad substrate rang and a large number industrial and biotechnological application of these multicopper enzymes has led to a terrible increase in the demand for this biocatalyst. Optimizing the condition for increasing laccase production and decolourization of dyes by *Alternaria alternata* will be further investigated in the future.

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Biomedical Communication

Effects of *Murraya koenigii* Leaves and *Brassica juncea* Seeds on Hyperglycemic Rats

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ABSTRACT

The aim of this study was to investigate the effects of daily oral feeding 8% and 15% of powdered leaves of *Murraya koenigii* leaves (MKL) (commonly knoun as curry) and *Brassica juncea* seeds (BJS) (commonly knoun as mustard) for 45 days on serum glucose concentration, serum lipids, liver and kidney functions in diabetic rats. A total of 36 adult male albino rats (Sprague Dawley strain) weighting 159□2.4g each were used in this investigation. Non- diabetic control (-) (6 rats) were fed basal diet ,while diabetic control (+) main group (30) rats divided into five groups after injected with alloxan (150mg/kg), at the end of the experiment, weight gain was calculated. Liver of each rat were removed rapidly then weighted separately. Blood samples were used for estimation of fasting serum glucose, ALT, AST, ALP, triglycerides, total cholesterol, high density lipoprotein (HDLc), low density lipoprotein (LDLc), very low density lipoprotein (VLDLc). Data showed that serum AST and ALT levels declined significantly ($p< 0.05$) in all treated groups fed on 7% and 15% curry and mustard compared with diabetic positive control. Moreover, both spices resulted in reduction of serum total cholesterol and LDLc + VLDLc a companied with an increase in the HDLc and significantly lowering of serum glucose levels. Thus, these plants can be best utilized by promoting them as preferable food for diabetic patients.

KEY WORDS: DIABETES MELLITUS, M. KOENIGII, CURRY, BRASSICA JUNCEA, MUSTARD, TRIGLYCERIDES, CHOLESTEROL, GLUCOSE.

INTRODUCTION

The World Health Organization (WHO) projected that 80% of the population relies on traditional medicine, which was elucidated by the 19.4 billion USD global revenue for herbal remedies in 2010 (Ujowundu et al., 2010). Moreover, the demand for traditional medicinal plants is increasing; for instance, the market for medicinal plants is expanding at an annual rate of 20% in India. Likewise, in China, 30% to 50% of the total medicinal consumption and around 90% of the German population uses natural remedies for certain health issues (Kang et al., 2018; Phumthum and Balslev, 2018; Raghu, 2020).

Therefore, medicinal plants are used in both developing and industrialized countries. Curry leave (*Murraya koenigii* (L.) Spreng) is an aromatic, tropical, and sub-tropical plant with several culinary, nutraceutical, medicinal, therapeutic values (Wojdyło et al., 2007; Reddy et al., 2018). Though curry leave is an ancient crop native to India, its nutritive and medicinal values are not enough known yet (Raghu, 2020). The medicinal properties of *M. koenigii* have been recorded to several chemical constituents of different carbazole alkaloids and other metabolites, like terpenoids, flavonoids, phenolics, carbohydrates, carotenoids, vitamins, and nicotinic acid from different parts of the *M. koenigii* tree (Balakrishnan et al., 2020).

In recent years, limited studies have been conducted for evaluating the pharmacological and medicinal efficacy of *M. koenigii* in promoting health benefits and curing disease. *M. koenigii* has numerous disease remedial activities, for instance, different parts of the plant, such as the leaves, roots, and bark, can be prepared as tonics for inducing digestion and flatulence or as antiemetics (Mandal et al., 2010; Adebajo et al., 2006). The leaves and roots are also useful in managing blood disorders (Sim and Teh., 2011; Dar et al., 2017; Zang et al., 2017; Balakrishnan et al., 2018; Balakrishnan et al., 2020).

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Balakrishnan et al., (2020) review described the pharmacological activities of the major components of *M. koenigii* against different pathological conditions. Moreover, *M. koenigii* showed significantly decreased glycemic levels and protected the animals against the development of diabetic neuropathy (Tembhurne and Sakarkar., 2010). In addition, *M. koenigii* showed a significant decrease in blood glucose, HbA1C, and altered lipid profile. *M. koenigii* was reported to extend a protective effect in liver impairments in chronic alcoholism and was proved effective in maintaining the enzymatic oxidant status (Shah et al., 2015; Husna et al., 2018, Suman et al., 2019). When Gul et al. (2012) tested *M. koenigii*, they found it was inhibited α glycosidase.

These Alpha-glucosidase inhibitors are widely used in the treatment of patients with type 2 diabetes. In most developing countries, medicinal plants play a helpful role in managing diabetes mellitus due to their cost effectiveness. Diabetes mellitus, a metabolic disorder, is becoming a serious threat to human health. During the past few years, many phytochemicals responsible for anti-diabetic effects have been isolated from plants. Alkaloids present in the leaves of *M. koenigii* have been explored and reported to have inhibitory effects on the aldose reductase enzyme, glucose utilization, and other enzyme systems for extending anti-diabetic effects (Patel et al., 2012).

Recently, it has been reported that the *M. koenigii* significantly reduced the glycosylated hemoglobin in the treated group compared with the Control group (Suman et al., 2019). In addition, *M. koenigii* exhibited a profound antioxidant effect by reducing the malondialdehyde (MDA) level, increasing the GSH level, and significantly decreasing the homeostatic model assessment (HOMA)-insulin resistance index. Overall, it is evident that *M. koenigii* possesses antidiabetic activity and has antioxidant effects in rats (Husna et al., 2018; Bhatt et al., 2020). The aqueous seed extract of the *Brassica juncea* medicinally valued plant clearly envisaged the hypoglycemic effect. This might be due to the time taken for the intestinal absorption of the aqueous seed extract of *B. juncea* (Ahad et al., 2010; Mohammad et al., 2010). The hypoglycemic effect of the seed extract of *B. juncea* was attributed to stimulation of glycogen synthesis leading to an increase in hepatic glycogen content and suppression of glycogen phosphorylase and other gluconeogenic enzymes (Khan et al., 1995 & Xu et al., 2011).

Previous reports have demonstrated that the leaves, roots, and bark of the plant are rich sources of carbazole alkaloids, which produce potent biological activities and pharmacological effects. The present study provides insight into the major components of *M. koenigii* (leaves and seed) and their pharmacological activities in the management of serum glucose concentration, serum lipids, and liver functions in alloxan-induced diabetic rats.

MATERIAL AND METHODS

The studied samples of *Murraya koenigii* (MKL) (curry leaves powder) and *Brassica juncea* (BJS) (mustard seeds) were obtained from the local market of Al-Taif region, Makkah province, KSA.

Animals: A total of 36 adult male albino rats (Sprague Dawley strain) were used in the investigation. Animals were obtained from Laboratory Animal Centre, Department of Biochemistry, Faculty of Medicine, Umm Al-Qura University, Makkah, KSA. Each rat was housed in a special cage under controlled condition. All rats were fed for 7 days on the control diet before the beginning of the experiment. Rats were weighed after 7 days separately then were weighed once a week for 6 weeks. The diet was presented to rats in special covered cups to avoid food loss, water was provided. At the end of the experiment rat were killed and organs weight was recorded.

Induction of diabetic rate and experimental design: Rats were divided into two main groups the first groups (6 rats) fed on basal diet as a negative control (-). For the second group (30 diabetic rats), diabetes mellitus was induced in overnight fasted rats by a single intraperitoneal Streptocytocin (STZ) injection (65 mg/kg b.w.) (Ravi et al., 2004). After 3 days, fasting blood glucose levels were measured and the animals showing blood glucose level ≥225 mg/dL were used for the study (Ewart et al., 1975). Rats having fasting serum glucose 190 mg/dl were considered diabetic (NDDG, 1994). Diabetic rats were divided into 5 groups, 6 rats each, and fed experimental diets for 45 days as follows: Group 1: Diabetic standard group; positive group (+). Group 2: Fed on basal diet + 7% MKL powder. Group 3: Fed on basal diet + 15% MKL powder. Group 4: Fed on basal diet + 7% MKS powder. Group 5: Fed on basal diet + 15% MKS powder.

Diets: The basal diet consists of casein (12 %), corn oil (10 %), choline chloride (0.2 %), cellulose (5%), vitamin mixture (1 %) (Bunce and Bloomer, 1972), salt mixture (4 %) (Hegested et al., 1941) and corn starch (up to 100 %).

Blood sampling: At the end of the experiment, rats were fasted overnight and anesthetized with chloroform. Blood samples were collected in clean dry centrifuge tubes from hepatic portal vein. Blood was centrifuged for 10 minutes at 3000 rpm to separate serum, which was kept in tubes at– 18°C until analysis. Organs were taken, washed with saline solution (10% Nacl) and dried with filter paper, then weighed and kept in freezer until analysis.

Biochemical analysis: Serum blood glucose was determined according to the method of (Trinder, 1969). Serum aspartate and alanine amino transferees (AST, ALT) and alkaline phosphatase (ALP) were determined by using enzymatic colorimetric method after (Reitman and Frankel, 1957; and Haussement, 1977), respectively.Serum total cholesterol, triglyceride (TG) and high-density lipoprotein cholesterol (HDLc) were determined by using enzymatic colorimetric method (NIHP, 1987; Young and Pestaner, 1975; Fendewaid, 1972; & Grodon and Amer, 1977), respectively. The determination of low-density lipoprotein cholesterol (LDLc) and very low-density lipoprotein cholesterol (VLDLc) were carried out according to the method of (Lee and Nieman, 1996) as follows: VLDLc

= TG /5 and LDLc =Total cholesterol – HDLc – VLDLc. Atherogenic indices were calculated as HDLc /T. cholesterol % and LDLc / HDLc (Castelli and levitar, 1977).

Histopathological examination of some internal organs: Specimens from liver were collected from rats of all experimental groups at the end of the experimental period, fixed in 10% neutral buffered formalin (pH=7.0), dehydrated in ethyl alcohol, then cleared in xylol and embedded in paraffin; 4-6 microns thickness sections prepared and stained with heamtoxylin and eosin for examining both for and glandular parts of the stomach (Bancroft and Gamble, 2008).

Statistical Analysis: Statistical analyses were performed by using computer program, statistical package for social science version 24 for windows. Data were expressed as mean standard deviation (SD). Paired-sample t-test was used to compare the parameters between controls positive group and diabetic rats groups. A P-value less than 0.05 was considered statistically significant.

Table 1. Fasting Serum Glucose (mg/dl) for Diabetic Rats Fed on Curry Leaves and Mustard Seeds for 45 Days.

Groups Variables	Control (-)	Control (+)	MKL		MKS	
			7%	15%	7%	15%
glucose	101±2.1***	205.3±8.8	116.1±4.2*	108.1±1.9**	120±5.1*	117.5±2.1**

Data are expressed as Mean±SD of six experiments. A P-value less than 0.05 was considered statistically significant. Parameter of positive group were compared to negative group, and treated groups. *(P≤ 0.05) significant change; **(P≤ 0.01) high significant change. ***(P≤ 0.01) very high significant change.

Table 2. Fasting Serum AST, ALT and ALP (IU/L) for Diabetic Rats Fed on Curry Leaves and Mustard Seeds for 45 Days.

Groups Variables	Control (-)	Control (+)	MKL		MKS	
			7%	15%	7%	15%
AST	22.8±1.5***	48.9±2.1	34.1±2.4*	31.3±2.2*	40.2±1.4*	33.6±1.8*
ALT	27.1±3.5**	49.1±2.1	29.3±3.2**	27.2±1.1**	37.8±1.4	31.6±1.1*
ALP	148.3±22.7***	393.6±51.3	201±43.5**	208±12.3**	301 ±15.1	249.2±5.1*

Data are expressed as Mean±SD of six experiments. A P-value less than 0.05 was considered statistically significant. Parameter of positive group were compared to negative group, and treated groups. *(P≤ 0.05) significant change; **(P≤ 0.01) high significant change. ***(P≤ 0.01) very high significant change.

Table 3. Fasting Serum Lipid Fraction (mg/dl) for Diabetic Rats Fed on Curry Leaves and Mustard Seeds for 45 Days.

Groups Variables	Control (-)	Control (+)	MKL		MKS	
			7%	15%	7%	15%
Triglyceride	45.7±12.2**	92.3±6.7	50.3±5.9**	43.3±1.9**	51.7±9.3**	47.7±8.8**
Cholesterol	79.4±3.4**	135.7±15.1	121.6±12.2	90.7±16.1*	103.2±12.6*	89.3±2.3**
HDL-C	46.3±6.4*	24.7±1.4	29.3±2.3	31.3±2.2*	29.2±4.8*	36.7±1.8*
VLDL-C	9.14±2.4*	18.46±1.4	10.1±1.2*	8.7±0.4**	10.34±1.9*	9.54±1.8*
LDL-C	23.96±9.5***	92.54±9.8	82.2±5.4	50.7±10.4**	63.7±11.8*	43.1±4.1**

Data are expressed as Mean±SD of six experiments. A P-value less than 0.05 was considered statistically significant. Parameter of positive group were compared to negative group, and treated groups. *(P≤ 0.05) significant change; **(P≤ 0.01) high significant change. ***(P≤ 0.01) very high significant change.

Table 4. Atherogenic Indices for Diabetic Rats Fed on Curry Leaves and Mustard Seeds for 45 Days.

Groups Variables	Control (-)	Control (+)	MKL		MKS	
			7%	15%	7%	15%
LDL/HDL Ratio	0.51±0.021***	3.75±0.47	2.8±0.29	1.61±0.004*	2.18±0.028	1.17±0.06**
HDL /T.C % Ratio	58.3±3.7**	18.2±2.8	24.1±3.1	34.5±2.9*	28.29±3.5*	41.1±4.7**

Data are expressed as Mean±SD of six experiments. A P-value less than 0.05 was considered statistically significant. Parameter of positive group were compared to negative group, and treated groups. *(P≤ 0.05) significant change; **(P≤ 0.01) high significant change. ***(P≤ 0.01) very high significant change.

RESULTS AND DISCUSSION

Histopathological Results: Examined liver of control, untreated rat revealed the normal histology of hepatic lobule, which consists of central vein and around it arranged highly specialized cells (hepatocytes) (Fig. 1). Concerning liver of diabetic rat, it showed vacuolar degeneration of hepatocytes as well as focal hepatic haemorrhage (Fig. 2). Examined liver sections of diabetic rat treated with 5% curry showed vacuolations of hepatocytes especially around the central vein (Fig. 3). However, apparent normal hepatocytes associated with slight activation of kupffer cells (Fig. 4) were noticed in liver of diabetic rat treated with 10% curry. Examined liver of diabetic rat treated with 5% mustard showed portal infiltration with few leucocytic cells (Fig. 5). Moreover, no histopathological changes were observed in examined liver of diabetic rat treated with 10% mustard (Fig. 6).

Figure 2: Liver of diabetic rat showing vacuolar degeneration of hepatocytes as well as focal hepatic haemorrhage (H and E X 200).

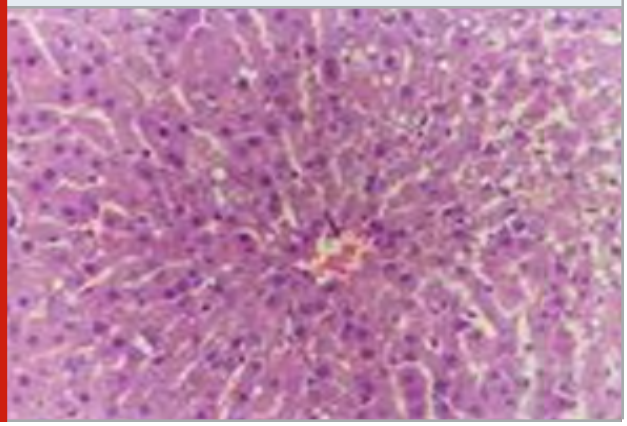


Figure 1: Liver of control untreated rat showing the normal histology of hepatic lobule (H and E X 200).

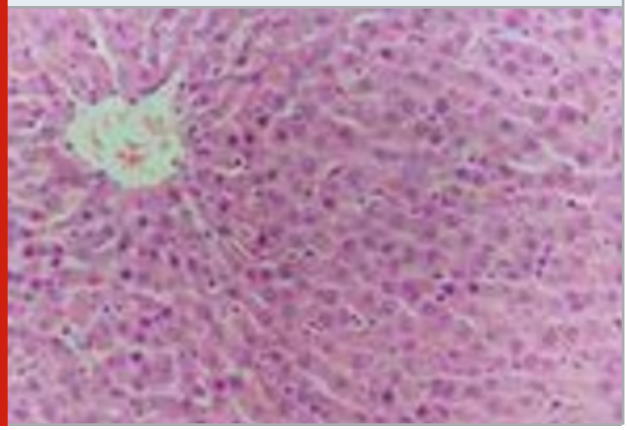
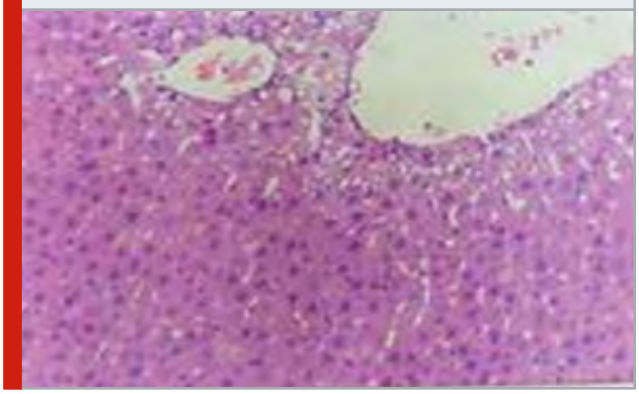


Figure 3: Liver of diabetic rat treated with 7% curry showing vacuolation of hepatocytes around the central vein (H and E X 200).



The current study was performed to evaluate the hypoglycemic effect of *Murraya koenigii* leaves and *B. juncea* seeds in alloxan induced diabetic rats. Diabetes is considered one of the major causes of morbidity and mortality affecting the elder and middle-aged population (Guariguata et al., 2014). The long-term use of present oral hypoglycemic tablets or insulin is associated with the development of resistance and various side effects. The traditional herbal options may help in fulfilling these unmet needs (Fatima et al., 2012). There are various herbs having proven antidiabetic effect such as Memordica charantia, *Eugenia jambolana*, *Trigonella foenum graecum, Embilca officinalis, Azadirachta indica, Phaseolus vulgaris*, and *Gymnema sylvestere* and *Murraya koenigii* (Fatima et al.,

2012; Husna et al., 2018; Balakrishnan et al., 2020; Bhatt et al., 2020).

Figure 4: Liver of diabetic rat treated with 15% curry showing apparent normal hepatocytes associated with kupffer cells activation (H and E X 200).

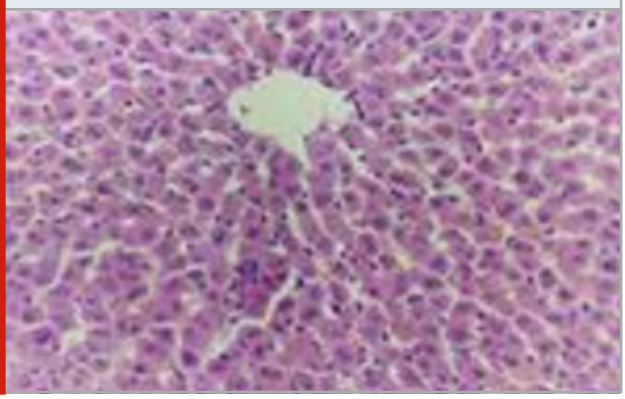


Figure 5: Liver of diabetic rat treated with 7% mustard showing portal infiltration with few leucocytic cells (H and E X200).

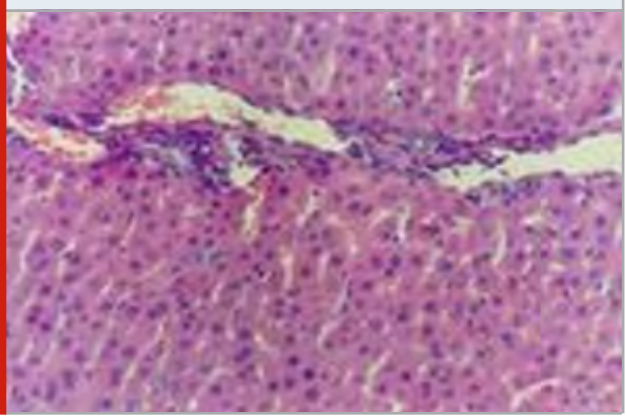
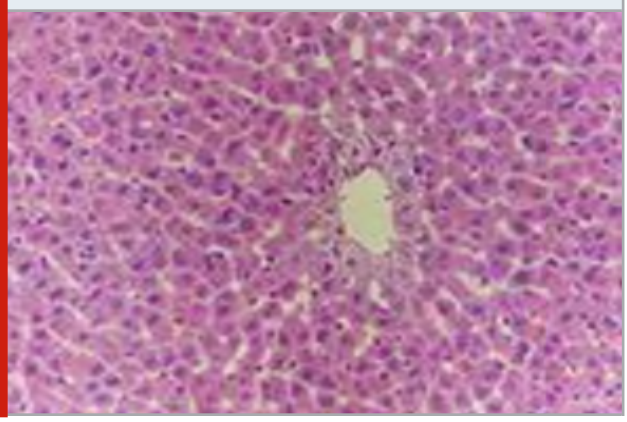


Figure 6: Liver of diabetic rat treated with 15% mustard showing no histopathological alterations (H and E X 200).



Murraya koenigii is a well-known curry leave tree. Its leaves and seeds are used as a spice in food recipe in India. Its related antidiabetic activity is attributed to alpha glucosidase activity of carbazole alkaloids contribute to its hyperglycemic activity through antioxidant effect and preservation of β-cell function. Its Alpha glucosidase inhibitory activity prevents digestion of carbohydrates and thereby reduces glucose absorption (Kesari et al., 2007; Lawal et al., 2008; Mangesh et al., 2018). Alloxan causes partial destruction of pancreatic β-cells, which leads to reduced levels of insulin and consequently resulting into hyperglycemia (Szkudelski, 2001; Lenzen, 2008). In our results, fasting serum glucose (mg/dl) for diabetic rats fed on curry leaves and mustard seeds for 45 days showed a significant decline. The hypoglycemic activity of curry leaves and mustard seeds could be due to the presence of carbazole alkaloids, which possess alpha-glucosidase inhibitory property (Duraisamy et al., 2012). Alpha-glucosidases are enzymes in the digestive tract that hydrolyze carbohydrates into glucose. One strategy that has been developed to treat type-2 diabetes is inhibition of the activity of alpha-glucosidases using synthetic drugs or natural drug candidates for the treatment of type-2 diabetes mellitus. Other possible mechanism of action of curry-leave-treated group could be potentiating insulin secretion from β cells of islets, which leads to reduced blood glucose levels (Vinuthan et al., 2004; Samuel et al., 2020).

Moreover, both spices resulted in reduction of serum total cholesterol and LDLc + VLDLc accompanied with an increase in the HDLc (Virdi et al., 2003). Administration of the extracts significantly decreased cholesterol level to near normalcy and therefore may reduce the risk of diabetes-associated cardiovascular diseases.In the present study, the *B. juncea* seed extract augmented the serum insulin levels suggesting an improved state of availability of serum insulin to control blood sugar. In addition, the present study showed that insulin serum augmenting effect was recorded highest at the dose of 7% suggesting that the serum insulin effect of the seed extract is dose dependent. This might be due to the inability of the β cells to recoup from the alloxan effect in these (Iftikhar et al., 2020). Our data showed that serum AST and ALT levels declined significantly ($p< 0.05$) in all treated groups fed on 7% and 15% curry and mustard compared with diabetic positive control. Damage to the structural integrity of the liver is reflected by an increase in the activity of this enzyme in the serum, probably because of leakage from altered cell membrane structure (Akanji et al., 1993; Rahman et al., 2001; Iftikhar et al., 2020).

Therefore, increase ALP in serum of the untreated diabetic rats confirms damage to the plasma membrane. The combination treatment attenuated the elevated activity of ALP enzyme in diabetic rats as compared with the normal controls. Our results illustrated that *B. juncea* seeds consumption significantly lowered the risk of atherosclerosis by bringing fall in concentration of plasma total cholesterol, LDL cholesterol as well as an improvement in HDL-cholesterol levels that is in full agreement with other studies described by (Khan et al., 1996; Rusdi et al., 2021). This lipid lowering property of *B. juncea* may be due to its emulsification properties that were contained in its water-soluble portion of proteins as reported by (Cui, 1997).

Reduced plasma cholesterol concentration is also affected by improved function of LDL receptor, which accelerates LDL uptake from plasma (Ness et al., 1996). These findings are in favour of former studies, showing that plant has cholesterol reducing capacity (O'Brien and Reiser, 1979). Histopathological examination of liver of control, untreated rat revealed the normal histology of hepatic lobule, however liver of diabetic rat showed vacuolar degeneration of hepatocytes as well as focal hepatic haemorrhage especially around the central vein. Examined liver of diabetic rat treated with 5% mustard showed portal infiltration with few leucocytic cells thus indicating that the extract of leaves and seeds exhibits inhibitory effect against hepatotoxicity. These results are in conformity with the previous findings (Bhatt et al., 2020; Hend et al., 2021).

CONCLUSION

From the current study, it is concluded that M. koenigii leaves and *B. juncea* seeds were found to show antihyperglycemic and hypolipedimic activity. Hence, this compound could be used as an oral hypoglycemic agent in diabetes. However further studies need to be done to confirm this activity in animal models as well as human trials. The consumption of the leave and seeds may be potentially beneficial against atherogenesis hence protective against cardiovascular disease as it possesses quality of lowering the plasma cholesterol, triglycerides, LDL-C and improving HDLC. Vacuolation of hepatocytes and portal infiltration in liver treated with the leave and seed extract respectively further indicates a need to evaluate the isolated phytochemicals from of the plant for the benefit of mankind. It can be achieved by using scientific experimental animal models and clinical trials to get the information about their action mechanism on the molecular level.

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Biotechnological Communication

Pretreatment, Saccharification and Bioethanol Production from Lignocellulosic waste using *Aspergillus* spp.

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ABSTRACT

The use of lignocellulosic material is the second-generation process of Bioethanol production and there is a sufficient supply of lignocellulosic materials in agricultural industries such as wheat bran, cotton ginning waste and sugarcane bagasse. The huge amount of waste generated from cotton ginning industry is a burning issue to dispose off, so in order to curb that issue, fermentative bioethanol production from cotton ginning waste using *Aspergillus* spp. is the best possible options to overcome it. It is also helpful in alleviating the global problem of greenhouse gases and decreasing the use of non-renewable energy with the generation of bio fuel using cotton ginning waste. The key stages involved in Bioethanol production from lignocellulosic biomass consist of pretreatment of biomass, saccharification, fermentation, and Ethanol Production. In current research work, the combination of three different methods for pretreatment of cotton ginning wastes: acid, alkaline and acid-alkaline were evaluated for the conversion of cellulose and getting higher ethanol yield. After Pre-treatment, sugar i.e., cellulose was further exposed for enzymatic hydrolysis by the cellulase enzyme produced from Aspergillus Spp. using Solid State Fermentation (SSF). The saccharified substrate was then utilized as C source for the fermentation and production of Bioethanol. The results of pre-treatment succeeded that acid pre-treated, alkaline pre-treated and acid-alkaline pre-treated substrates Cotton ginning wastes (CGW) released 172.8 µg/ml, 256 µg/ml and 140.8 µg/ml of sugar, respectively. The sugar released were then fermented with *Saccharomyces cerevisiae* using SHF method (Separate Hydrolysis and Fermentation). The amount of ethanol produced from Acid pre-treated, Alkaline pre-treated and Acid-Alkaline pre-treated Cotton ginning wastes (CGW) was 48 mg/ml, 48 mg/ml and 60 mg/ml, respectively. Thus, based on current experimental work it can be stated that the lignocellulosic biomass Cotton Ginning Wastes (CGW) can be converted into biofuel (bioethanol) using Separate Hydrolysis and fermentation process which is the best alternative source of fuel.

KEY WORDS: BIOETHANOL, COTTON GINNING WASTES, FERMENTATION, HYDROLYSIS, LIGNOCELLULOSE

INTRODUCTION

The cotton mills and cotton ginning industries worldwide are facing the biggest problem for the waste disposal and in maintaining environmental regulation, as enormous amount of cotton gin waste is created every day. The cotton crop cultivation generates two major types of residues: cotton plant trash (CPT) and cotton gin trash (CGT). Out of these two forms, CGT is very significant due to its higher production and difficulties in its disposal (Agblevor et al. 2006; Sharma and Chen 2008; Pla´cido et al. 2013; Chaudhary and Padhiar 2020).

Biological conversion of these cellulosic wastes into energy, food and chemicals at economic rate is beneficial to mankind (Arthe 2008). With the growth in population there is increasing demand of fuel, so converting such non feed material into bioethanol is of prime importance. As per the earlier research, the production of bioethanol was carried out from sugarcane and starchy feed stocks such as molasses, corn and potato etc. These substrates are measured as the first-generation process but these processes require pointedly

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increased amount of cultivable land and increase in food prices leads to its unavailability. This raises the concern since the first-generation process for bioethanol production could not meet the global energy need (Yang et al. 2007; Mitchell 2008; Chaudhary and Padhiar 2020).

Thus, in the coming years focus is shifted towards the production of bioethanol from the non-food plant sources which are considered as the second-generation process. In this process majorly lignocellulosic biomass is used, which consist of major share of agricultural and forest wastes which are the most plentiful renewable biological resources (Splomon et al. 1999; Lin et al. 2006). In the production of Biofuel using second generation process, the major steps involved are preparation of feedstock, pretreatment, saccharification, fermentation and bioethanol recovery (Saha 2004; Gamage et al. 2010). Feedstock is prepared by washing and complete drying of cotton ginning waste and further Acid Treatment, Alkaline Treatment and Acid-Alkaline Treatment were evaluated for cellulose conversion and yield of ethanol (Tyagi et al. 2019; Darwesh et al. 2020).

The current study is concerned with the production of cellulase using low-cost agro-industrial residue (CGW) as a substrate using SSF. Aspergillus spp. is selected for saccharification because of its rapid growth rate, easy preservation and high metabolic activity in production of cellulase enzyme. Pretreatment of CGW using different methods were carried out and *Saccharomyces cerevisiae* (MTCC No.172) was used for (SHF) separate hydrolysis and fermentation process for bio ethanol production. The estimation of substrate was carried out using Dinitro salicyclic acid (DNSA) method and Ethanol Estimation is carried out using Iodometric Titration (Miller 1959; Breuil and Saddler 1985; Darwesh et al. 2020). So far, there are very few such study is conducted to demonstrate the production of bioethanol from cotton ginning waste. Thus, this work significantly represents that saccharified substrate cotton ginning waste is the finest alternate way for the bioethanol making using separate hydrolysis and fermentation method to overcome the increased use of non-renewable energy source and global warming.

MATERIAL AND METHODS

Isolated Aspergillus species was used for present study. It was grown and maintained at 28˚C on Rose Bengal agar plate. The culture was maintained on Rose Bengal agar slant and were stored at 4˚C for long term preservation (Chaudhary and Padhiar 2020).

For collection of the sample, CGW (Cotton ginning waste) was collected from sites of cotton ginning mills from kadi district Mehsana, India. The cotton ginning waste was initially washed with normal tap water in order to remove the soil and other impurities and placed in oven for complete drying (Chaudhary and Padhiar 2020). For the acid pretreatment of cotton ginning waste, 700 ml of 3% sulphuric acid was prepared in 2000 ml Erlenmeyer flask. The flask was added with 54 gm of processed cotton waste and further autoclave carried out at 121˚C for 15 minutes. The flasks containing the pre-treated substrate were then neutralized by washing with tap water till the pH of pre-treated substrate and tap water equalizes. The acid pre-treated samples were then dried separately for further analysis (Chaudhary and Padhiar 2020).

For the alkaline pretreatment of cotton ginning waste, 700 ml of 5% sodium hydroxide was prepared in 2000 ml Erlenmeyer flask. The flask was added with 54 gm of processed cotton waste and further autoclave carried out at 121˚C for 15 minutes. The flasks containing the pre-treated substrate were then neutralized by washing with tap water till the pH of pre-treated substrate and tap water became similar. The alkaline pre-treated samples were then dried separately for further analysis (Dimos et al. 2019). For the acid and alkaline pretreatment of cotton ginning waste, 700 ml of 3% Sulphuric acid and 5% sodium hydroxide both were prepared in 2000 ml Erlenmeyer flaks. The flasks were added with 54 gm of processed cotton waste and further autoclave carried out at 121˚C for 15 minutes.

The pre-treated substrate was then neutralized by washing with tap water till the pH of pre-treated substrate and tap water equalizes. The pre-treated samples were then dried separately for further analysis (Dimos et al. 2019; Chaudhary and Padhiar 2020). For the experimental design of solid-state fermentation (SSF) for enzyme production, preparation of Inoculum was carried out by inoculating previously preserved culture on fresh Rose Bengal agar slant and incubating at 30˚C for 3-7 days. The activated spores were harvested and suspended in sterile D/W (100 ml). 4.5 ml of spore suspension was then further inoculated into substrate flasks for enzyme production (Chaudhary and Padhiar 2016).

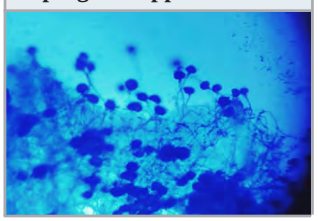
Extraction of enzyme from substrate flask is done by adding 5 ml of cold 0.05 M acetate buffer (pH 4.8). The homogenate was filtered using muslin cloth and then centrifuged at 5000 rpm at 4˚C for 15 minutes. The supernatant is used as a crude enzyme source and analyzed for carboxyl methyl cellulase (CMCase), filter paper activity (FPase) and β- glucosidase (Chaudhary and Padhiar 2016). For the saccharification of pre-treated substrates, about 5 gm of acid, alkaline and acid-alkaline pretreated cotton ginning waste taken into 100 ml Czapek Dox medium and 5 ml of the cellulase enzyme was inoculated which was extracted by SSF. Flasks were incubated at 45˚C up to 10 days in shaking condition. Sugar content was analyzed using DNSA method after every 24 hours of interval till 10 days. An enzyme blank was prepared without adding any substrate (Venkatachalapathy et al. 2014). For the estimation of cellulase activity, cellulase activity was measured by increasing level of sugar using DNSA method. After 24 hrs., 5ml of broth was harvested from each cotton pretreated flasks. The broth was then filtered with Whatman filter paper no.1 and the filtrate were used for further sugar estimation (Chaudhary and Padhiar 2016).

For the isolation of yeast strain, the yeast strain of *Saccharomyces cerevisiae* (MTCC No.172) was used for this work. It was grown and maintained at 30˚C and 4˚C on Yeast Extract Potato agar plate and slants respectively

(Guilherme et al. 2019). For ethanol Production by *Saccharomyces cerevisiae* using Separate Hydrolysis and Fermentation Method, fermentation was carried out in 100 ml volume with saccharified substrates (CGW). Enzymatic hydrolysate obtained after saccharification of acid, alkaline and acid-alkaline treated substrates (CGW) was decontaminated at 121˚C for 15 min. After disinfection, 9% inoculums of *S. cerevisiae* were added for Bioethanol production. The process was carried out at 30˚C for 72 hrs., along with shaking condition at 100 rpm. After 24 hours of incubation, samples were retrieved to determine the concentration of ethanol produced through fermentation (Chandel et al. 2007). The substrate consumption was estimated using the Dinitro salicylic acid (DNSA) method and estimation of bio ethanol was carried out after 48 hrs. using Iodometric Titration method (Miller 1959; Breuil and Saddler 1985; Chaudhary and Padhiar 2020).

Figure 1: Colonial Characteristics of Aspergillus. Spp.

Figure 2: Morphological Characteristics of Aspergillus Spp.



For the estimation of Ethanol, ethanol concentration was determined by Iodometric titration method. In this method K2Cr2O7 reacts with concentrated H2SO4 to liberate nascent oxygen which in turn oxidizes ethanol to acetic acid. Remaining amount of potassium dichromate reacted with potassium iodide to liberate iodine which was measured by titrating with 20% sodium thiosulphate using 1% starch as an indicator. The titration results were further used to calculate the ethanol concentration after fermentation process (Chaudhary and Padhiar 2020).

RESULTS AND DISCUSSION

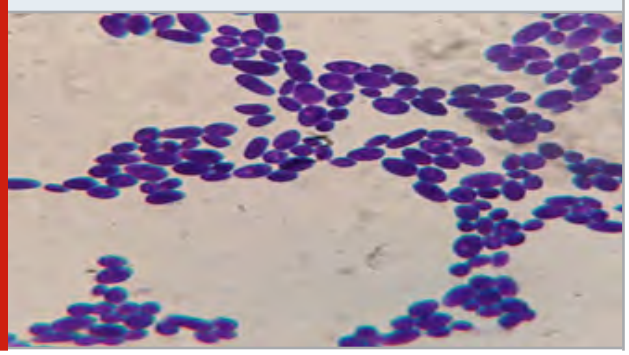
Isolation of *Aspergillus* spp.: Isolation of fungus was carried out using RBA media. Morphological observation was done and Light green to dark green appearance of colony was observed after 3 to 4 days of incubation. Microscopic observation was done after mounting with lacto-phenol cotton blue dye using high power. Light violet-colored hyphae with dark violet color spores were observed after maturation (Chaudhary and Padhiar 2016; Sahu and Pramanik 2018).

Cultural and morphological characteristics of yeast: The colony characteristics of the yeast culture were studied on Yeast extract potato dextrose agar plate which was shown in table:1. Microscopic observation of yeast under oil immersion lens was shown in fig:3. Yeast was further inoculated at 30˚C for 3 days to check ethanol production (Sahu and Pramanik 2018).

Table 1

Size	Shape	Margin	Elevation	Surface	Consistency	Opacity	Pigmentation
Medium	Round	Entire	Convex	Smooth	Moist	Opaque	White

Figure 3: Morphological characteristics of isolated Yeast



Pre-treatment of CGW: The cotton ginning wastes was used as lignocellulosic wastes in the present study (Fig:4). Impurities of CGW was removed by water washing which was shown in fig:5. Acid pre-treatment was undertaken using 3% H2SO4 and alkaline pre- treatment was undertaken using 5% NaOH. Acid and Alkaline pretreatment of cotton ginning waste carried out using mixture of 3% H2SO4 and 5% NaOH. The resultant effect of pre-treatment on cotton ginning wastes was shown in figure 6,7 and 8 after pretreatment with acid, alkali and mixture of acid-alkali respectively (Dimos et al. 2019; Chaudhary and Padhiar 2020).

Saccharification of Pretreated CGW: Saccharification of treated CGW was carried out using *Aspergillus* spp. After every 24 hours, samples were harvested and analyzed for release of sugar up to 10 days. Results of the effect of time duration on the enzymatic saccharification of the acid, alkaline, and acid-alkaline pre-treated CGW were shown in Fig: 9. The result shown that up to 3 days of incubation enzyme was not able to secret sugar from the substrate. There was gradual increase in saccharification of CGW with respect to time, on 8thday maximum saccharification was observed from substrate. When prolonged to 9th day, the yields of reducing sugar had decreased. The sugar released on 8th day from acid treated, alkaline treated and acid-alkaline treated CGW was 182.4 µg/ml, 216µg/ml and 238.4 µg/ml, respectively (Chaudhary and Padhiar 2016). The rate of sugar production decreased with increase in time after 8th day. Among different pretreatment combine acid –alkali treatment released maximum sugar after saccharification (Dimos et al. 2019; Chaudhary and Padhiar 2020).

Figure 4: Raw Cotton

Figure 5: Water Washed Cotton

Figure 6: Acid treated Cotton

Figure 7: Alkaline treated Cotton

Figure 8: Acid and Alkaline treated Cotton

Separate Hydrolysis and Fermentation (SHF) of cotton ginning waste (CGW): In SHF of CGW, first the enzymatic hydrolysis was carried out for 8 days at 45˚C temperature. On 8thday maximum reducing sugar was obtained from CGW through saccharification using *Aspergillus* spp. Enzymatic hydrolysate obtained after saccharification of acid, alkaline and acid-alkaline treated substrates (CGW) was inoculated with *S. cerevisiae* for fermentation. The process was conducted at 30˚C temperature using 100 rpm in shake flask method up to 48 hrs. After 24 hours incubation, samples were retrieved to determine the concentration of ethanol produced. Figures 10 to 12 shows the sugar utilization pattern and ethanol accumulation in the SHF inoculated with *S. cerevisiae* using cotton ginning waste (Sahu and Pramanik 2018). At 48 hrs. maximum content of ethanol were achieved by using separate hydrolysis and fermentation (SHF) method (Chaudhary and Padhiar 2016; Sahu and Pramanik 2018; Chaudhary and Padhiar 2020).

Figure 9: Sugar released from saccharified substrate of CGW

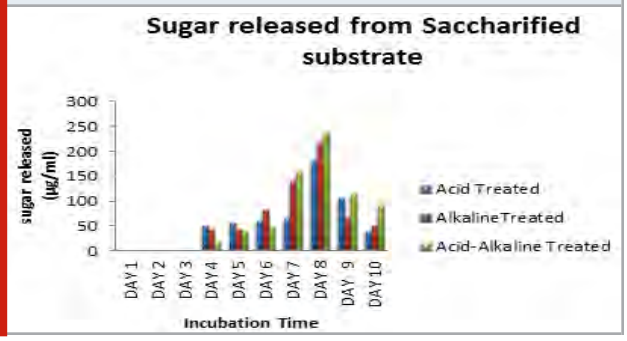


Figure 10: Sugar Utilization and Ethanol production from Acid treated CGW.

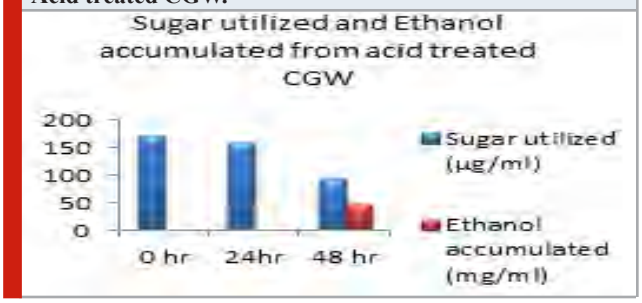


Figure 11: Sugar Utilization and Ethanol production from Alkaline treated CGW.

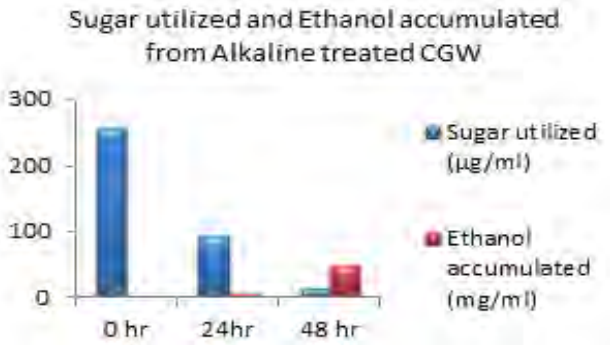
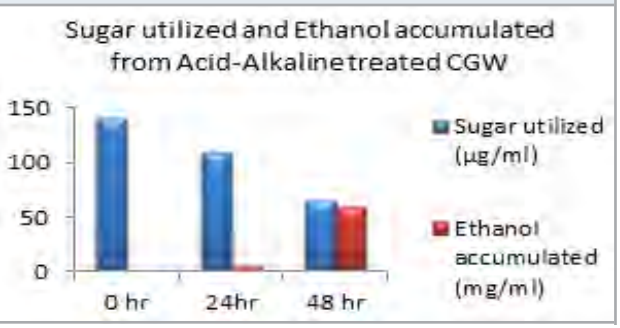


Figure 12: Sugar Utilization and Ethanol production from Acid-Alkaline treated CGW.



The amount of ethanol produced from Acid-pre-treated, Alkaline-pre-treated and Acid-Alkaline pre-treated Cotton ginning wastes (CGW) was 48 mg/ml, 48 mg/ml and 60 mg/ml respectively after 48 hours which was shown in the Fig: 10 to 12. After 24 hours amount of ethanol produced was very less as sugar utilization was also less.

After 48 hours of incubation sugar will be exhausted to negligible amount in Alkali pretreated sample which was shown in fig: 11. While in case of acid pretreated CGW available residual sugar was higher compared to alkali pretreated CGW which indicates that acid pretreated samples may releases high amount of 5 C sugar which may not be able to get converted into ethanol. Same result was observed in Acid-Alkaline pre-treated CGW shown in fig: 12. The fermentation process can be economical if it can convert both the pentose and hexose sugars in the hydrolysate to bioethanol. *Saccharomyces cerevisiae* can ferment only hexose sugars into ethanol. In present study the quantity of residual sugar was higher and the concentration of ethanol obtained in SHF is lower due to temperature difference between Saccharification and Ethanol Production. *Aspergillus* culture gave maximum Saccharification at 50^{o}C and *S. cerevisiae* gave maximum ethanol yield at 30^{o}C.

Previous studies on fermentation using *Saccharomyces cerevisiae* for substrates like, groundnut hull and rice husk, the yields of 0.142g per gram from groundnut oil and 0.108g per gram from rice husk were stated (Srivastava et al. 1997). Similarly, the sugarcane leaf litter as feedstock produced 0.130 mg/L (alkaline pre-treated) and 0.335 mg/L (acid pre-treated) of ethanol. For substrate bagasse pith hydrolyzed by cellulase enzyme the bioethanol produced was 7.7% (v/v) (Iuliana 2009). In one more research, 0.218g per gram yield of cellulosic waste mixture (office paper, newspaper and cardboard in 1:1:1 ratio) was testified (Iuliana et al. 2010). Acid-Alkaline pre-treated Cotton ginning wastes (CGW) proves better compare to only acidic or alkali pretreatment and these can be used for further scale up for bioethanol production (Chaudhary and Padhiar 2020).

Previously various research has been undertaken for the bioethanol production from diverse lignocellulosic wastes such as molasses, sugar cane bagasse, starchy biomass etc. and also various different pretreatment process such as hydrothermal process, hydrogen peroxide process etc. were used but such treatment process are very costly and also release harmful byproducts as well use of starchy feed stock increase its price and unavailability (Lin and Tanaka 2006; Pla´cido J et al. 2013; Dimos et al. 2019). Thus, the results of the present work clearly revealed that the cellulosic Cotton Ginning Wastes (CGW) could be converted into bio ethanol with enzymatic hydrolysis followed by fermentation. Thus, through appropriate use of a pretreatment process and a renewable feedstock like lignocellulosic biomass for production of a biofuel (ethanol) it is likely to serve as a sustainable substitute for energy production and reducing the requirement for crude oil (Sahu and Pramanik 2018; Chaudhary and Padhiar 2020).

CONCLUSION

The finding of the present study clearly flourished that the lignocellulosic Cotton Ginning Wastes (CGW) can be used for bio ethanol production with enzymatic hydrolysis followed by fermentation process. Among all the pretreatments, Acid-alkaline pre-treated substrate can be used to produced more amount of ethanol i.e., 60 mg/ml in comparison to acid and alkaline pre-treated CGW substrate. Result of this preliminary work can be exploited for pilot scale production and must be scale up for efficient bio ethanol production. Therefore, using a suitable pretreatment process and a renewable feedstock like lignocelluloses biomass for production of bio ethanol with Separate hydrolysis and fermentation method is very successful and can be used as an energy efficient process for renewable fuel generation.

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

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Biotechnological Communication

Glucose Uptake and α-Glucosidase Inhibition Activities of Secoisolariciresinol Diglucoside Isolated from *Linum usitatissimum*: An *In vitro* Study

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ABSTRACT

The objective of this study was to determine the uptake of glucose in L6 cell lines and alpha glucosidase inhibition activity of Secoisolariciresinol diglucoside (SDG) involved in glucose utilization. Diabetes mellitus is a clinical disorder characterized by hyperglycemia, an elevated amount of glucose circulates in the blood plasma. Different concentrations of SDG were analyzed for glucose uptake activity and alpha glucosidase inhibition activity. The results found to be significantly comparable with Metformin, the highest value was 68.41 ± 0.80 at 100 µg/ml dose for SDG and 85.50 ± 0.83 value for Metformin at the same concentration. The alpha glucosidase inhibition activity for SDG was found to be 79.67 ± 0.40 % and for Voglibose it was 96.33 ± 0.40 % at 2000µg/mL concentration. The results of the current work therefore clearly indicate the potential of SDG to manage hyperglycemia. Elevation of the glucose uptake by SDG in association with glucose transport supported the up regulation of glucose uptake, through alpha glucosidase inhibition activity it delays the carbohydrate digestion which leads to decreases the post prandial blood glucose levels. The present study shows that SDG activate glucose uptake in L-6 cell line of skeletal muscles, which can correlate to that of standard Metformin used by diabetic patients. Currently increasing usage of Nutraceuticals is more evident in order to stay away from the synthetic drugs and its side effects. Our research can be considered as a step towards a more profound understanding of hypoglycemic activity of SDG as there is no much data available.

KEY WORDS: ALPHA GLUCOSIDASE, GLUCOSE UPTAKE, HYPERGLYCEMIA, L-6 CELLS, SDG.

INTRODUCTION

Diabetes mellitus is a metabolic disease categorized by hyperglycemia, occurs when defects in insulin secretion, insulin action, or both. The diabetes associated with the consequences of long-term damage, malfunction and failure of the various organs, specifically the eyes, kidneys, nerves, heart, and blood vessels (Diabetic care 2007). It is a serious metabolic disorder affecting the reasonable number of people globally, in which the effected person experiences high blood sugar, either may be because of the pancreas does not produce enough insulin or the produced insulin will not be effectively used by the body cells. Causes for enhancing the growth of its epidemic are the variances in social structure, intellectual stress, obesity, hormonal imbalance and heredity (Amos et al. 2010; Mukhtar et al. 2019).

Diabetes is the most common endocrine disorder and by the year 2010, also it is estimated that, more than 200 million people will comprise diabetes and 300 million will subsequently have the disease worldwide by 2025 (King et al. 1998; Amos et al. 2010). The percentage of the population is expected to increase by 55% with diagnosed diabetes between 2015 and 2040, may reaches to 642 million people by the majority of cases in low and middle-income countries (Eddouks and Maghrani 2004). Cumulative epidemiological studies have suggested that, the consumption of fruits, vegetables and few medicinal herbs to decrease the incidence of diabetes. The plant kingdom expresses a large pool of biologically active compounds which has not been explored yet (Amos et al. 2010; Mukhtar et al. 2019).

According to bygone literature, more than 800 plants are reported to have anti-diabetic properties (Kesari et

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al. 2007). More than 1200 plants are used in traditional medicine for their alleged hypoglycemic activity as per ethno pharmacological surveys (Fang etal. 2008). Inhibition of the carbohydrate digestive enzymes (α-glucosidase and α-amylase), improved peripheral uptake of glucose by hemidiaphragm are the potential preliminary mechanisms that are involved in hypoglycemic activity. Phenolic compounds or polyphenols are a group of chemicals that are extensively distributed through the plant kingdom and thus form an essential part of the human diet. The α-glucosidase inhibition in our experiments could be due to the presence of these compounds. These compounds have been found to be responsible for blood glucose reducing activity and have also been reported to activate GLUT1-mediated glucose uptake (Kim et al. 2001; Eid et al. 2010; Ali et al. 2019).

Skeletal muscle is the major site for the disposal of ingested glucose in a healthy normal glucose tolerance (NGT) individual, Impaired glucose uptake in skeletal muscle is present in insulin resistance diabetes (Defronzo et al. 1988; Zisman et al. 2000). In skeletal muscle the glucose uptake is decreased by Insulin by increasing functional glucose transport molecules (GLUT-4) in the plasma membrane (Dachani et al. 2012). Insulin resistance and chronic hyperglycemia are caused by the reduction of GLUT4 (Zierath et al. 1996; Kim et al. 2001; Raghad et al. 2019). This triggers a lot of signaling cascades, inducing biological responses like glucose uptake into the cell, and glycogen synthesis (Binhthi et al. 2017). Skeletal muscle is the major tissue accountable for insulin stimulated glucose disposal and the main site of peripheral insulin resistance (Defronzo et al. 1981; Defronzo et al. 1985; Chadt and Al-Hasani 2020).

L6 cell lines are derived from the skeletal muscle, used in antidiabetic research, they are good model for the glucose uptake because they have been used extensively to elucidate the mechanism of glucose uptake in the muscle, have an intact insulin signalling pathway and express the insulin sensitive GLUT-4 (Ruddich et al. 1998). Secoisolariciresinol diglucoside (SDG), is a known antioxidant found in flaxseed (Prasad 2000; Kezimana et al. 2018). The present study was carried out, to study the hyperglycemic activity of Secoisolariciresinol diglucoside (SDG) through in vitro α-glucosidase activity and glucose uptake activity as there are no ample experimental studies have been carried so far.

MATERIAL AND METHODS

Samples of flax seeds (whole grains) were obtained from a local store in Chennai, India. p-nitrophenyl glucopyranoside (pNPG) procured from Sigma Aldrich, α-glucosidase enzyme from Sigma Aldrich. L-6 cell lines purchased from MCCS Pune. All other chemicals /reagents and solvents used in this study were purchased from Loba Chem, Merck, India Pvt. Ltd as analytical reagent grade materials and applied without subsequent purification. Isolation and purification of SDG described in, the extract of *L. usitassimum* was prepared by 80 % Aqueous methanol (Daddala et al. 2018).

For the α-glucosidase inhibition assay, the effect of SDG and its extract on α-glucosidase activity was determined as per the method described by Apostolid using α-glucosidase enzyme (Apostolid et al. 2007). The p-nitrophenyl glucopyranoside (pNPG) as a substrate solution was prepared in 100mM phosphate buffer (pH 6.8). 200 µL of the α-glucosidase was pre-incubated with different concentrations (10,20,40,80,160 and 320) of the test samples for 10min. Then, dissolved 400µL of 5.0mM (pNPG) in 100mM phosphate buffer (pH 6.8) was added to start the reaction.

The reaction mixture was incubated at 37°C for 20min and the reaction will be stopped by adding 1 mL of Na_2CO_3 (0.1M). The yellow-colored reaction mixture, 4-nitrophenol, released from pNPG was measured at 405nm using UV - VIS spectrophotometer. A blank and the sample blanks were also prepared by adding 5 µL of deionized water instead of the plant extract and 20 µL of deionized water instead of the enzyme, respectively. Voglibose was used as a positive control and the inhibitory activity of α-glucosidase was calculated using the following formula,

% Inhibition = [(Abs Control - Abs Sample) / Abs Control] x 100

Table 1. *In vitro* α-Glucosidase Inhibition assay

Concentration (µg)	Voglibose	Extract	SDG
50	52.01±0.31	21.65 ± 0.32	31.94 ± 0.58
100	67.40 ± 0.38	24.66 ± 0.40	47.27 ± 0.15
250	73.15 ± 0.47	30.67 ± 0.44	60.93 ± 0.23
500	79.47 ± 0.47	53.39 ± 0.61	68.31 ± 0.62
1000	91.90 ± 0.31	59.30 ± 0.84	69.69 ± 0.47
2000	96.33 ± 0.46	68.72 ± 0.23	79.67 ± 0.40

For the glucose utilization experimental procedure on L-6 cell lines, the glucose utilization was determined by the minor modification method defined by van de Venter et al. (2008). The L-6 cells were dislodged by the brief exposure to 0.25% Trypsin in phosphate-buffered saline, counted and suspended in the new growth medium. Then seeded at a density of 6000 cells per well into a 96-well culture plate and allowed to adhere and grow in a humidified incubator with 5% CO_2 at 37°C for three days. Two cell-free rows also included to serve as blanks. On day three after seeding, without changing the medium, different concentrations (3.125, 6.25, 12.5, 25, 50 and 100 µg) of test samples and standard Metformin were added to each well. After 48 h incubation, the spent culture medium was removed by aspiration and replaced with a 25 µl incubation buffer (DMEM medium diluted with PBS, 0.1% BSA and 8 mm of glucose) and further incubated for an additional time of 3 h at 37°C. The negative control (untreated) which contains only the incubation buffer without samples. After incubation, 10 µl of the incubation medium was removed from each well and transferred into a new 96-well plate, to

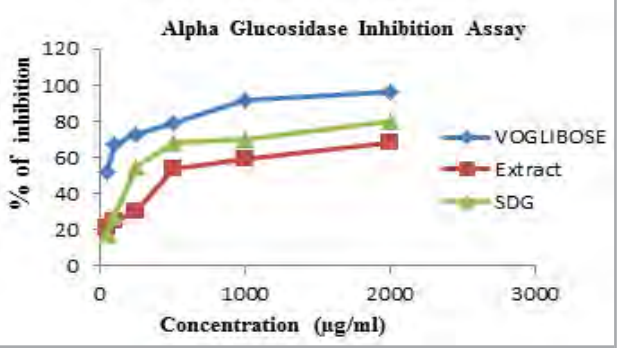
this added 200 µl of glucose oxidase reagent to determine the concentration of glucose in the medium. After 15min of incubation at 37°C, measured the absorbance at 492 nm using a microtitre plate reader. The amount of glucose utilized was calculated by using the below mentioned formula, the difference between the untreated control and treated wells.

% Glucose uptake = 100 x [Absorbance control untreated cells - Absorbance sample treated cells /Absorbance control untreated cells]

RESULTS AND DISCUSSION

α-Glucosidase Inhibition Assay: In this study, the results indicated that SDG exhibited significant effect on alpha-glucosidase than extract at all the tested concentrations, illustrated in Table -1. At the highest concentration (2000µg/ml) investigated, the SDG, extracts displayed appreciable effect on alpha-glucosidase by 68.7% and 79.7 % respectively figure 1. However, voglibose as positive controls, was more effective in the respective assays than the extract and SDG, exhibiting percentage inhibitory activity of 96.3% against alpha-glucosidase.

Figure 1: The effect of SDG, extract of *L. usitassimum* and Voglibose on alpha-glucosidase activity. Indicates a significant increase relative to the control. Data expressed as mean ± SD (n = 3).



Currently, there are numerous antidiabetic drugs available to deal with diabetes and the mechanisms of action may be the inhibition of alpha-amylase, alpha-glucosidase, lipase, and DPP-IV enzyme. Our results indicated that the SDG demonstrated significant inhibition on alpha-glucosidase. However, the mild inhibition observed by the extract of *L. usitassimum*. Whereas alpha-amylase inhibition has been reported in our previous publication. This suggests that the antidiabetic mechanism of SDG may also through the inhibition of these enzymes (Daddala et al. 2020).

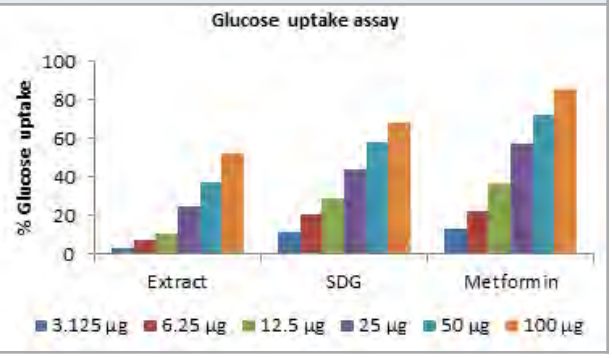
Glucose Utiliastion In L6 Cell Lines: The results obtained for glucose uptake in L6 cells in the presence of the SDG, extract of *L. usitassimum* at 3.125µg to 100 µg/ml are presented in table 2. The SDG caused a significant higher increase in glucose uptake in both L6 cells at all the concentration tested in a concentration-dependent manner when compared to the un treated control and metformin.

On the other hand, the crude extract of *L. usitassimum* also exhibited glucose uptake at 100 µg/ml but lower than observed for SDG. The L6 cell lines, the extract of *L. usitassimum* showed some significant potential in lowering blood glucose levels at 50, 100 µg/ml concentrations tested (Figure 2). However, at all the concentrations the extract also caused a slight increase in glucose uptake in L6 cells but less significant than SDG. The highest activity of glucose uptake was for extract 52.16 ± 0.41 and for SDG 68.41 ± 0.80 at 100 µg/ml concentration. Whereas slightly lower than metformin 85.50 ± 0.83. These results were nearly correlated with metformin, which were used as the standard antidiabetic drugs.

Table 2. *In vitro* Glucose uptake studies in L6 cells

Concentration (µg)	Extract	SDG	Metformin
3.125	2.86 ±0.40	11.33 ± 0.84	13.54 ± 0.35
6.25	7.52 ± 0.57	20.45 ± 0.61	22.21± 0.76
12.5	10.99 ± 0.63	29.07 ± 0.84	36.25 ± 0.86
25	25.07 ± 0.84	44.26 ± 0.52	57.54 ± 0.86
50	37.70 ± 0.89	57.84 ± 0.40	71.92 ± 0.46
100	52.16 ± 0.41	68.41 ± 0.80	85.50 ± 0.83

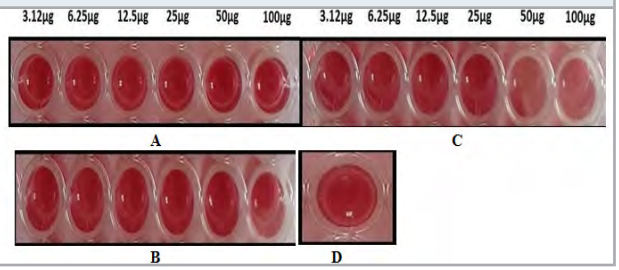
Figure 2: The effect of SDG, extract of *L. usitassimum* and Metforminon glucose utilization inL6cells. Cells were treated for 48 h in the presence or absence of varying concentration of the SDG and extract. Data expressed as mean ± SD (n = 3).



The present study has employed to identify the potential mechanism of probable antidiabetic actions of SDG. The result obtained in this study on glucose uptake using L6 cells demonstrated that SDG increased glucose uptake in L6 cells when compared to extract but somewhat lesser than Metformin (Figure 3). Metformin lowers glucose, sensitizes insulin by reducing gluconeogenesis and opposing glucagon-mediated signalling in the liver and in a lesser extent by increasing glucose uptake in skeletal muscle. It exerts its hypoglycemic effect through activation of the AMP activated protein kinase (AMPK) in the liver (Viollet et al. 2012). Most abundant tissue in the whole body is skeletal muscle. Hence, proper function of skeletal tissue is important to maintain normal blood glucose level

(Koncic et al. 2010; Chadt and Al-Hasani 2020). Insulin increases the glucose uptake in the skeletal muscle by increasing functional glucose transport molecules in the plasma membrane. Common pathological condition in non-insulin dependent diabetes mellitus is, the defect in insulin stimulated skeletal muscle glucose uptake. SDG, a lignan of flaxseed upregulated GLUT4 protein expression in the skeletal muscle (Yanwen et al. 2015; Chadt and Al-Hasani 2020).

Figure 3: A- Extract of *L. usitassimum*; B- SDG, C-Metformin, D-Control



Our experimental outcome suggested that the SDG, therefore, masquerade as metformin by increasing glucose uptake in the skeletal muscle. SDG is a phytochemical lignan, the presence of phytochemicals suppresses glucose release and also enhances glucose uptake. Therefore, it may be hypothesized SDG could be linked to activation of the insulin signalling cascade, resulting in stimulation of GLUT4 that facilitates the translocation of glucose into the cell (Hanhineva et al. 2010; Rosenzweig and Sampson 2021).

The toxicity assay revealed that *L. usitassimum* extract, SDG were not toxic to L-6 cells, producing less than 10% cell death at the concentrations investigated. However, metformin also displayed no significant toxicity but rather proliferated the cells. Furthermore, the low level of cell death exhibited by this extract, SDG and the positive controls most likely explains the significant reduction in glucose uptake in the cells (Rosenzweig and Sampson 2021). To our knowledge no prior studies have examined the *in vitro* glucose uptake assay, α-glucosidase inhibitory activity of SDG. Therefore, this will be a foremost *in vitro* approach on cell lines.

CONCLUSION

The findings of the present study suggests that SDG enhances the glucose uptake under *in vitro* conditions, exerts its hypoglycemic activity by reducing the post prandial glucose levels by possessing alpha-glucosidase inhibition activity. The antidiabetic activity might be due to the phyto constituents of *L. usitassimum*. Moreover, SDG itself is a phytochemical lignin. It is also concluded that based on the toxicity assay SDG found to be nontoxic and safe to the L6 cells. However, in vivo studies have to be carried out to validate the *in vitro* results by employing different in vivo models and clinical trials for their effective utilization as therapeutic agents.

Conflit of Interests: Authors declare no conflict of interests.

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Biomedical Communication

Biochemical and Histological Remodeling of Adrenal Glands Associated with Estradiol Valerate-Induced Polycystic Ovary in Rats: Protective Effects of *Matricaria chamomilla*

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ABSTRACT

Polycystic ovary (PCO) is a condition in which the secretion of specific hormones is disrupted. The adrenal glands are essential for the manufacture of certain hormones and the monitoring of steroid concentration, and they may be impacted in PCO situations. In a PCO-induced rat model, the researcher was aimed to investigate how administering exogenous estrogen, estradiol valerate affected on adrenal gland secretions and histology. The effects of *Matricaria chamomilla* flowers extract, and metformin will also be evaluated. 24 rats were divided into four equal groups: 1- control group, 2- PCO group, 3- metformin group, 4- *Matricaria chamomilla* group. PCO was induced by injecting 2 doses of estradiol valerate. The findings revealed a considerable increase in estrogen and ACTH levels in the PCO rats. The concentrations of cortisol and aldosterone were likewise higher in the PCO rats. These findings were corroborated by histological investigation of adrenal sections (both H&E and PCNA staining). *Matricaria chamomilla* flowers extract reduced all the hormonal alterations linked to PCO, including estrogen, ACTH, cortisol, and aldosterone. Metformin similarly reduced estrogen and ACTH. On the other hand, Metformin did not affect the hormonal changes associated with this model in terms of adrenal gland hormones (cortisol and aldosterone). In conclusion both treatments are improved that the histological investigation of adrenal secretions (both H&E and PCNA staining). The antiestrogen activity of *Matricaria chamomilla* could clarify that all the hormonal modulation occurs in this PCO model.

KEY WORDS: POLYCYSTIC OVARY; ADRENAL GLANDS; CORTISOL; ALDOSTERONE, ESTROGEN; ACTH; PCNA.

INTRODUCTION

Polycystic ovary (PCO) is a condition in which the secretion of specific hormones is disrupted, which causing reproductive issues in women. Irregular menstruation, the development of ovarian cysts, and insulin intolerance are all common PCO problems. Estrogen is a hormone generated chiefly by the ovaries but also in minor amounts by the adrenal glands. It's in charge of the formation of feminine sex traits. Estrogen is necessary for the normal functioning of sexual organs, skeletal system preservation, menstrual cycle control, and pregnancy maintenance (Reed and Carr, 2000; Clarke and Khosla, 2010; Ndefo et al., 2013; Witchel et al., 2019; Delgado and Lopez-Ojeda, 2021).

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Although estrogen levels vary naturally throughout the monthly cycle throughout a woman's life, several women may experience estrogen dominance, in which their estrogen levels are more significant than average. Estrogen dominance, or raised estrogen concentrations, can occur in PCO ladies. PCO is the reasonable explanation of oligo-ovulation, and it's supposed that estrogen dominance exerts a role. The absence of ovulation results in perpetual extraordinary estrogen levels and lacking progesterone (Hambridge et al., 2013; Dennett and Simon, 2015; Leon et al., 2021).

The adrenal glands are essential for the manufacture of certain hormones as well as the monitoring of steroid concentration in the body, and they may be impacted in PCO patients. Recently, a PCO model was established in our lab via injecting estradiol valerate in rats. The model was characterized by an increase in blood estrogen levels. Women with PCO have widespread hypersecretion of adrenal gland cortical hormones, including pregnenolone,

17-hydroxypregnenolone, DHEA, androstenedione, 11-deoxycortisol, and cortisol both at basically and after stimulation with adrenocorticotropic hormone (ACTH) (Azziz et al., 1998; Dumitrescu et al., 2015; Rosenfield and Ehrmann, 2016; Alahmadi et al., 2020).

There are also subtle systemic steroid metabolic problems in PCO women, such as tendencies favoring increased estrogen and cortisol output. Oral estrogen replacement medication has already been demonstrated to raise total cortisol levels in women in various trials. Some reports, on the other hand, did not reveal similar disparities. Most studies haven't looked at cortisol levels after estrogen replacement medication (Tsilchorozidou et al., 2003; Tock et al., 2014). It's uncertain whether the use of exogenous estrogen therapy will affect the levels of various adrenal hormones. Chamomile (*Matricaria chamomilla* L.) is a widespread herbal medicine in Southern and Eastern Europe. It is a member of the Asteraceae family. Chamomile's active components include flavonoids responsible for the pharmacological attributes of chamomile, mainly the anti-inflammatory and antioxidant benefits. *Matricaria chamomilla* extract has recently been shown to lower PCO-related estrogen increase (Heidary et al., 2018; Alahmadi et al., 2020; Sotiropoulou et al., 2020).

The purpose of this study was to look at the effects of injecting exogenous estrogen, estradiol valerate, on adrenal gland secretions and histology in a PCO-induced rat model. Furthermore, the impact of *Matricaria chamomilla* flowers extract, and metformin were assessed.

MATERIALS AND METHODS

Chemicals: In this research, *Matricaria chamomilla* flowers was purchased from World of Herbs, Egypt, estradiol valerate purchased from Abcam Inc, USA, and metformin purchased from Sigma-Aldrich Co, USA.

Preparation of *Matricaria chamomilla* flowers extract: The extract was made by extracting the crushed *Matricaria chamomilla* flowers with ethyl alcohol (70 percent), then drying the extract under vacuum.

Animals: In the current study, twenty-four adult, virgin female Wistar rats were used. They obtained from the King Fahad Research Centre at King Abdullah University in Jeddah, Saudi Arabia. Their body weight varies between 186 and 208 grams. The research was conducted out under a conventional laboratory environment of temperature, humidity, and a 12:12 h light/dark cycle after a one-week acclimatization period. The animals were not subjected to any water or food restrictions. The research procedures were accepted by the Biomedical Ethics Research Council, Faculty of Medicine, KAU, Jeddah, SA, acceptance number, 168-19.

Induction of PCO: PCO was induced by administering two doses of estradiol valerate (0.2 mg each), one dose at the start and the other after six weeks of the experiment (Alzahrani et al., 2019; Farideh et al., 2010).

Experimental groups: The rats were divided into four equal groups (n = 6). Control group: corn oil-injected group, PCO group: estradiol valerate injected group, Metformin group: PCO rats treated with metformin (500 mg/kg) (Alahmadi et al., 2020), *Matricaria chamomilla: PCO rats treated with Matricaria chamomilla* flower extract (75 mg/kg) (Alahmadi et al., 2020). Corn oil, metformin, and *Matricaria chamomilla* were injected every day into the animals starting at the beginning of the experiment and remained for one month following PCO initiation.

Collection of serum and adrenal glands samples: A cardiac puncture was used to gather blood samples. Adrenal hormones (cortisol and aldosterone), anterior pituitary hormone (ACTH), and estrogen levels were measured after the serum was extracted and kept frozen at -80°C. Both adrenal glands were removed and preserved in 10% buffered formalin for histopathological evaluations and immunohistochemical proliferating cell nuclear antigen (PCNA) expression.

Measurement of serum cortisol, aldosterone, ACTH, and estrogen: Serum cortisol, aldosterone, ACTH, and estrogen were quantified in El-Safwa Laboratory, Tanta, Egypt, utilizing ADVIA Centaur automated competitive chemiluminescence immunoassay (Bayer HealthCare).

Evaluation of histopathological changes (light microscope): Adrenal glands were preserved in formalin, fixed in paraffin wax, sectioned at 3–5 mm, and stained with hematoxylin and eosin (H&E).

Evaluation of PCNA immune expression (light microscope): Immunohistochemical staining was performed employing the immune peroxidase (PAP, peroxidase/anti peroxidase) procedure utilizing anti PCNA antibodies from Lab Vision (Fremont, CA. USA) diluted at 1/100.

Table 1. Effect of *Matricaria chamomilla* flowers extract on serum ACTH and estrogen levels measured in estradiol valerate-induced PCO in rats.

Experimental group	ACTH (pmol/L)	Estrogen (ng/mL)
Control	55.33 ± 1.15	99.47 ± 1.13
PCO	89.83 ± 4.29[a]	339.27 ± 20.33[a]
Metformin	70.50 ± 3.55[b]	92.42 ± 2.98[b]
Matricaria chamomilla	58.83 ± 2.41[b]	95.80 ± 2.36[b]

Results are expressed as mean ± SE (n=6). [a]significant difference compared to the control group. [b]significant difference compared to the PCO group.

Statistical interpretation of findings: GraphPad Prism version 5 was used to analyse the results, which included an ANOVA test and a Tukey's multiple comparison test. p

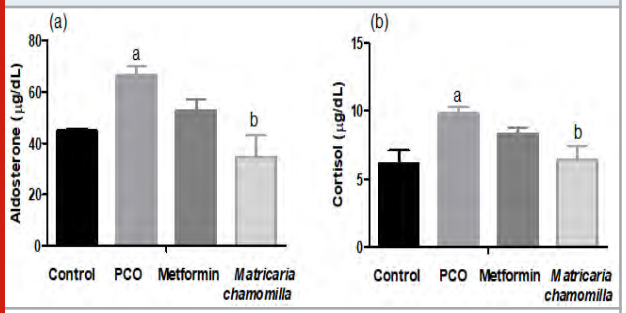
less than 0.05 or at $p < 0.05$, the results were considered statistically significant.

RESULTS AND DISCUSSION

Effect of *Matricaria chamomilla* flowers extract on serum ACTH and estrogen levels measured in estradiol valerate-induced PCO in rats: PCO-induced rats showed significantly increased serum ACTH and estrogen levels compared to the control group. Treatment of PCO-induced rats with *Matricaria chamomilla* and metformin significantly decreased serum ACTH and estrogen levels compared to the PCO group. *Matricaria chamomilla* significantly decreased serum ACTH level compared to the metformin group (Table 1).

Effect of *Matricaria chamomilla* flowers extract on serum aldosterone and cortisol levels measured in estradiol valerate-induced PCO in rats: PCO-induced rats showed significantly increased serum aldosterone and cortisol levels compared to the control group. Treatment of PCO-induced rats with *Matricaria chamomilla* significantly decreased serum aldosterone and cortisol levels compared to the PCO group. There was no difference between the metformin group and PCO group concerning serum aldosterone and cortisol levels (Figure 1).

Figure 1. Effect of *Matricaria chamomilla* flowers extract on serum aldosterone and cortisol levels measured in estradiol valerate-induced PCO in rats.

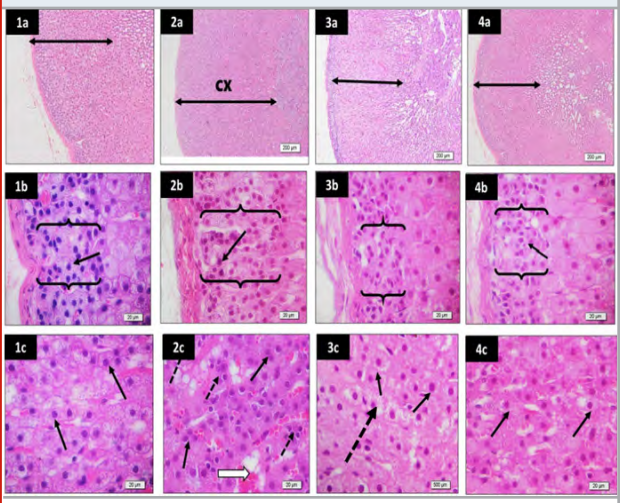


Results are expressed as mean ± SE (n=6). [a]significant difference compared to the control group. [b]significant difference compared to the PCO group.

Effect of *Matricaria chamomilla* flowers extract on adrenal glands histopathological changes: The examination of H & E-stained adrenal glands sections of PCO-induced rats revealed a potential increase in cortical zone thickness, an increase in thickness of zona glomerulosa, increase in cellularity of both zona glomerulosa and zona fasciculata. Treatment of PCO group with either metformin or *Matricaria chamomilla* decreased cortical zone and zona glomerulosa thickness, besides normal cellular features of zona fasciculata (Figure 2).

Effect of *Matricaria chamomilla* flowers extract on adrenal glands zona glomerulosa PCNA immune expression: Sections of PCO-induced rats were showed relative increase in zona glomerulosa thickness, besides, increased number of nuclei expressing PCNA. A mild or moderate decrease in nuclei expressing PCNA is observed in the metformin and *Matricaria chamomilla* treated rats (Figure 3).

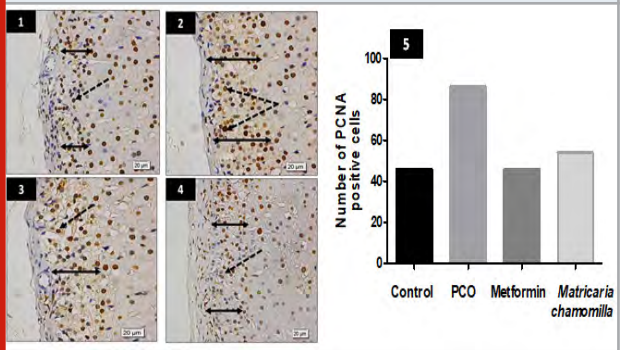
Figure 2. Effect of *Matricaria chamomilla* flowers extract on adrenal glands histopathological changes.



Sections from rat adrenal glands (low power x40) and higher magnification (x400) of zona glomerulosa and zona fasciculata stained by H&E to show a potential increase in cortical zone (cx) thickness in PCO group (2a) compared to control (1a). Besides, an increase in thickness of zona glomerulosa of PCO group (brackets) (2b) compared to control (1b) is observed. The cellularity of both zona glomerulosa (2b) and zona fasciculata (2c) of the PCO group is increased compared to control (1b, and 1c). Mild dilation of sinusoids (white arrow) and presence of lymphocytes (dotted arrows) are also evident in the zona fasciculata of the PCO group (2c). The metformin group showed decreased cortical zone (3a) and zona glomerulosa (3b) thickness compared to the PCO (2a and 2b) and the control groups (1a and 1b). Besides, moderate return to normal cellular features (black arrows) of zona fasciculata is observed in the metformin group (3c), although frequent cells showed unstained cytoplasm (dotted arrow). The Matricaria chamomilla group show relatively normal cortical zone thickness (4a), zona glomerulosa thickness (4b), and cellular features of zona fasciculata (4c) compared to the PCO (2a and 2b) and the control groups (1a and 1b).

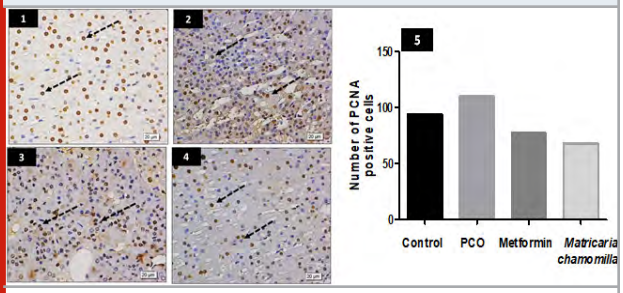
Effect of *Matricaria chamomilla* flowers extract on adrenal glands zona fasciculata PCNA immune expression: Sections of PCO-induced rats show relative increase in zona fasciculata, the number of nuclei expressing PCNA. A mild or moderate decrease in nuclei expressing PCNA is observed in the metformin and *Matricaria chamomilla* treated rats (Figure 4). The current study results showed a significant increase in the level of estrogen in the PCO model developed by exogenous estrogen injection. There was also a significant increase in the level of the ACTH in the PCO rats. It was also clear that the adrenal gland cortisol and aldosterone concentrations were increased in the PCO group. The histological examination (both H&E and PCNA staining) of adrenal sections confirmed these findings. Similar to this study findings, previous research showed an overproduction of various adrenal gland hormones, including cortisol, in PCO women (Azziz et al., 1998).

Figure 3: Effect of *Matricaria chamomilla* flowers extract on adrenal glands zona glomerulosa PCNA immune expression.



Sections from rat adrenal gland zona glomerulosa stained for PCNA show relative increase in zona thickness (double head arrows) in the PCO group (2) compared to the control group (1). Besides, the number of nuclei expressing PCNA looked to be more numerous (dotted arrows) (2). A mild or moderate decrease in nuclei expressing PCNA is observed in the metformin and Matricaria chamomilla groups (dotted arrows) (3 and 4). (5) A bar chart shows the number of PCNA positive cells in all groups.

Figure 4: Effect of *Matricaria chamomilla* flowers extract on adrenal glands zona fasciculata PCNA immune expression.



Sections from rat adrenal gland zona fasciculata stained for PCNA show increasing nuclei expressing the marker in the PCO group (2) compared to the control group (1) (dotted arrows). PCNA immuno-expression is still high in the metformin group (3) (dotted arrows). PCNA immuno-expression is nearly normal in Matricaria chamomilla group (4). (5) A bar chart shows the number of PCNA positive cells in all groups.

The researchers attributed this to the increased sensitivity of adrenal glands to the ACTH, 7α-hydroxylase, and increased cytochrome P450c17α. According to previous research, ladies with PCO were observed to have enhanced peripheral cortisol metabolism, which was linked to raised ACTH production in order to maintain optimal cortisol levels. The possible causes for elevated adrenal hormones levels in PCO ladies incorporate changes in adrenocortical biosynthesis, increased responsiveness of the adrenal to ACTH stimulation, or abnormalities in adrenal product metabolism (Stewart et al., 1990; Bhathena, 2005; Yildiz and Azziz, 2007; Baskind and Balen, 2016; Alahmadi et al., 2020). Numerous probable theories can describe how ACTH and cortisol levels were raised after estradiol valerate therapy. One probability is that estrogen treatment directly or indirectly impacted ACTH production from the pituitary gland, which encouraged cortisol release from the adrenal gland. In pregnant ladies, it is affirmed that the hypothalamic-pituitary-adrenal axis is activated, and hypercortisolism is recognized. Different probability is that high estradiol suppressed transcription of 11-β hydroxysteroid dehydrogenase (11-β HSD), which is identified to decrease cortisol synthesis. It is likely that if local cortisol concentration is reduced in the pituitary and/or hypothalamus, ACTH concentration is raised by the low cortisol concentration (Carr et al., 1981; Ho et al., 2007; Chapman et al., 2013; Anno et al., 2019; Sotiropoulou et al., 2020).

Previously reported data indicated that estrogen impacts 11B-HSD, however, it persists questionable how ACTH and cortisol concentrations were raised following estradiol valerate therapy. This study results showed that *Matricaria chamomilla* flowers extract modified all the hormonal changes associated with this PCO model including, estrogen, ACTH, cortisol, and aldosterone. Similarly, metformin significantly decreased estrogen and ACTH in PCO rats. On the other hand, metformin did not affect the hormonal changes associated with this model concerning the hormones of the adrenal glands (cortisol and aldosterone) (Low et al., 1993; Pepe et al.,2001; Anno et al., 2019; Sotiropoulou et al., 2020).

The histological examination (both H&E and PCNA staining) of adrenal sections confirmed these findings. The active phytoestrogen ingredients of *Matricaria chamomilla* extract are coumarins, which may inhibit estrogen production. Moreover, phytoestrogens interfere with the action of cytochrome P450 enzymes, preventing cholesterol from being converted to pregnenolone and reducing estrogen synthesis. The chamomile flower, *Matricaria chamomilla*, has traditionally been used to relieve tension (Brueggemeier et al., 2001; Löfgren et al., 2004; Ronis, 2016; Witchel et al., 2019; Delgado and Lopez- Ojeda, 2021).

Furthermore, apigenin, the main ingredient in chamomile, lowers cortisol levels in the blood. An earlier study found that breathing chamomile oil vapor alleviated restriction stress in ovariectomized rodents by lowering plasma ACTH levels. In bovines, *Matricaria chamomilla* CH12 reduced stress by inhibiting cortisol secretion. According to the current study's findings, *Matricaria chamomilla* extract may be responsible for the modification of adrenal gland hormones by lowering the pituitary hormone ACTH by restoring estrogen levels to their normal levels. However, it appears that there is a mechanism behind the impact of *Matricaria chamomilla* other than regulating estrogen and ACTH, as metformin changes estrogen and ACTH but does not reduce cortisol and aldosterone levels, implying another mechanism that has to be investigated further (Yamada et al., 1996; Reis et al., 2006; Sotiropoulou et al., 2020; Leon et al., 2021).

CONCLUSION

The PCO model produced by estradiol valerate was linked to higher serum ACTH and estrogen levels. The PCO model also showed that cortisol and aldosterone levels of the adrenal glands were also increased. The hormonal alterations associated with this PCO model were altered by *Matricaria chamomilla* flowers extract, including estrogen, ACTH, cortisol, and aldosterone. These findings were corroborated by histological investigation of adrenal sections (both H&E and PCNA staining).

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Environmental Communication

Environmental Effects of Covid-19 in India: A Qualitative Analysis

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ABSTRACT

The Corona Virus Disease 2019 (COVID-19) is an acute virus creating respiratory disease and gastro intestine disease in humans. The outbreak of novel corona virus (COVID-19) has brought serious impact on all counties around the world. Spread of COVID-19 was controlled by countries through restricted movement, self-hygiene practices and social distancing. Despite all the efforts made by the governments, this pandemic brought serious effect on economy and environment. The impacts of COVID-19 on air, water and waste management were assessed and were observed that air and water quality has improved due to lockdown but the management of waste is a serious issue. This article describes the results of study performed on the environmental effects particularly in air and water by assessing the environmental conditions before and after the outbreak of pandemic COVID-19. The study results yields that the purity of air and water has been improved during the pandemic period when compared with the period before the outbreak of COVID-19 virus. Waste generated from self-quarantine houses, hospitals and self-hygiene practices followed by people has posed an enormous effect on waste management sector. Disposal of infectious waste along with municipal solid waste has created threat to people handling the waste and the environment. Based on the environmental analysis performed on air, water and waste management, solid guidelines has been provided in treating the waste management effectively. This article recommends the need for improving the waste treatment methodology and the significances of policy framework to face pandemic situation in future. This study improves the hope that, implementation of proposed guidelines will improve the purity level of environment and management of biomedical wastes effectively.

KEY WORDS: COVID-19, ENVIRONMENTAL ANALYSIS, POLLUTION ASSESSMENT, WASTE MANAGEMENT, MUNICIPAL SOLID WASTE.

INTRODUCTION

The outbreak of corona virus (COVID – 19) as a pandemic has brought a serious impact on environmental health and economy. Corona virus was found to be spread from a seafood market in Wuhan, China. Then its effect was seen across countries due to human-to human transmission in short period (Sarkodie and Owusu 2020; WHO 2020c). In January 2020, WHO (World Health Organization) found that transmission of COVID-19 is through respiratory droplets (WHO, 2020). In February, rise of corona cases started in Iran, Italy and different nations around the world. Due to the short period of transmission, WHO declared COVID-19 as pandemic. By the end of March, half of the human population was under different forms of lockdown (Tosepu et al. 2020). This transmissible disease spread across 114 countries by 118,000 cases and 4291 deaths (Sarkodie and Owusu 2020; WHO 2020e).

As of October 25, 2020, there were 34,804,348 reported cases about the globe of which 1,030, 738 deaths were recorded alongside 7,733,778 active cases and 27,665,198 recovered cases. Now, the USA has the greater number of cases (7,256,234) and deaths (207,366) among 235 countries, followed by India (6,549,373 confirmed cases and 101,782 deaths), Brazil (4,880,523 confirmed cases and 145,388 deaths), Russia (1,215,001 confirmed cases and 21,358 deaths), Colombia (841,531 confirmed cases and 26,397 deaths) and Peru (821,534 confirmed cases and 32,609 deaths). Total number of deaths in top ten countries due to novel corona virus has been represented in figure 1. Corona virus (COVID-19) has brought worldwide discussion and uncertainty compared to SARS (2002–2003), Avian flu (2003–2009), Swine flu (2009–2010) and Ebola

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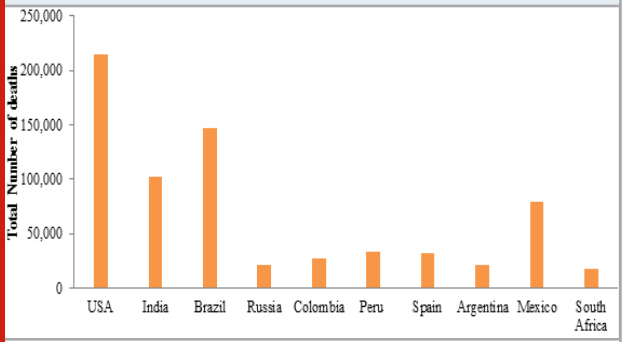
(2014–2016) since 1996 (Ahir et al. 2018). Even the health care systems of world's most developed countries are on the verge of collapse (Armocida 2020).

Source: ECDC, BBC- data to 4th October 2020.
Figure 1: Total Number of deaths in Top 10 countries



Time between catching of virus and showing up the symptoms of disease is known as incubation period. It was found that the symptoms are seen in people within 14 days, nearly around 5 days (Wang et al. 2020). Symptoms of COVID-19 are similar to that of previous outbreak such as MERS and SARS, but the death rate is high compared to them (Wang et al. 2020). Transmission of corona virus is due to the droplets formed due to coughing, sneezing, speaking and accidently inhaling the droplets in a crowded proximity of an affected person. Common signs of COVID-19 are tiredness, fever, sore throat and dry cough. Some patients also have body pain, nasal blockage or diarrhea (Verma 2020). Most of the people recover without special treatment.

Approximately 1 out of each 6 affected persons develops serious breathing conditions, thus needing exceptional care. Aged people with existing medical problems such as hypertension, diabetes or heart issues are found to develop serious illness. Communication of corona virus is also due to contact of the surface which is contaminated by the infected individual. Contaminated surfaces include entryway chimes, lift catches, and steps, vegetables, organic products and so on which may interact with people often times. Certainly, even fecal matter of infected individual is discovered to be the spreading source henceforth it can reach through fecal-oral transmission (Verma 2020).

Therefore, only way of preventing the transmission of corona virus is by restricting the gathering. Compared to other diseases and their effects, COVID-19 has brought greater human agony and serious public health concern. This pandemic situation has triggered political and financial emergency in the world. As immunization for COVID-19 is not available, it has spread quickly and posed enormous health, economic, ecological and social difficulties to the whole human populace. Thus, even large and small cities of affected nations were brought under part of total lockdown. This paper discusses about the effects of COVID-19 in air, water and waste management. This pandemic situation has brought the need of change in policy framework of waste management and hence it becomes necessary to incorporate certain rules and regulations to be followed during future pandemic situations (WHO 2020).

Remedial Measures Taken By Government: The significances of performing remedial measures to bounce back from the pandemic situation are considered to be more vital. The government of all countries are framing security policies and imposing these policies towards the public to follow, such that to avoid the spreading of COVID-19 virus. The government has implemented certain measures like lockdown, quarantine of COVID infected patients, restricted access to market resources, vaccinations, wearing face mask, usage of hand sanitizers and cloves in public places, spraying disinfectants to all public places and containment zones etc (Asumadu 2020). As the vaccination for COVID-19 is not available till date, the government and health care experts have proposed to impose lockdown to contain the spread of corona virus. Government has also forced some rules and regulations such as social distancing, wearing of masks, etc (Somani, 2020).

Control measures such as isolation, restriction on travel to various countries, limit of people in social gathering and occasions, closure of religious places and restriction on public transport were followed during lockdown. Lockdown posed in India, applicable for the entire population started on 25th march, 2020. Till 18th may-2020, lockdown was restored based on the prevailing conditions across the country. Based on that India had various phases of lockdown such as 21days, 14 days, 30 days and so on (Sharma 2020). During the first phase of lockdown, there were severe restrictions such as suspension of various modes of transportation in and across the nation. This was relaxed by the government based on the number of cases prevailing in the particular area. Based on the number of cases, government categorized the entire nation into three different zones such as Red for disease hotspots, Orange for places with limited cases and green for places with nil cases (Sharma 2020). After separating the entire nation based on zones, government announced relaxations based on them from 8th June 2020. Relaxations were given on various phases from June, 2020 and they were termed as "Unlock 1.0" to "Unlock 5.0" till 31st October, 2020 (Mandal 2020).

Effect On Air: Air pollution has attributed to more than 7 million deaths across the world and around 1.4 million in India. It has been reduced considerably due to the lockdown. Restriction on travel resulted in non-movement of aircrafts and vehicles throughout the world. This resulted in decrease in harmful emissions in the atmosphere (Polk 2019; Verisk 2020). NASA satellite pictures showed a decline in the levels of nitrogen oxide by 70% because of reduction in the utilization of non-renewable energy sources during the lockdown (UCAR 2020). Carbon monoxide and aerosol concentration were also reduced (Gautam and Trivedi 2020; Holthaus 2020).

Based on the observation by the contaminant levels in the air was seen reduced in Delhi during lockdown. Rather than other contaminants, PM10 and PM2.5 was found to be reduced by around 50 % [Machado and Samina, 2020].

Due to the fall of PM2.5 concentration in the atmosphere, premature mortality rate was reduced (Venter et al. 2020). Air Quality Index (AQI) was seen throughout the nation during lockdown, it represented a reduction of about 30 % when compared to earlier year data (Sharma et al. 2020). Figure 2 and 3 represents the changes in AQI previously (September 2019 to February 2020) and after lockdown (March to October 2020) in Delhi, Mumbai and Chennai (CPCB AQI Bulletin; Mahato and Pal 2020).

Figure 2: AQI from September 2019 to February 2020for Delhi, Mumbai and Chennai

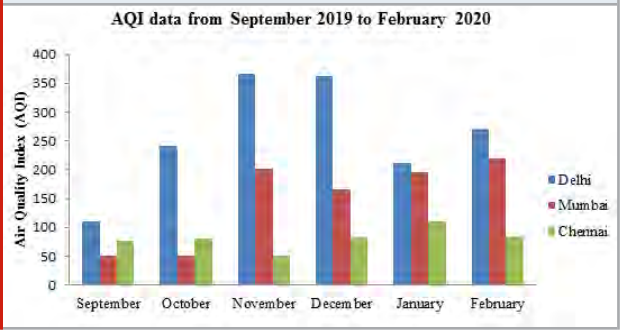
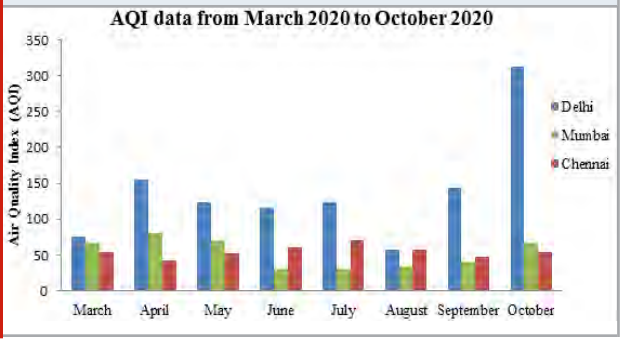


Figure 3: AQI from March to October 2020 for Delhi, Mumbai and Chennai



Effect On Water: Lockdown has brought a reset for nature and human population. Articles from various dailies and magazines reported that there is an improvement in the quality of streams like Ganga, Cauvery, Yamuna and so on (Lokhandwala and Gautam 2020; Pathak and Mishra 2020). This improvement was seen due to the closure of industries which let their untreated effluent into the rivers. River quality parameters such as DO and BOD were considerably reduced (Pathak and Mishra 2020). CPCB inspected the quality of various rivers in India during March and April. The river water quality was assessed on parameters pH, Dissolved Oxygen (DO), Biochemical Oxygen Demand (BOD) and Fecal Coliform (FC). The results were compared with the Primary Water Quality Criteria for Outdoor Bathing notified under Environment (Protection) Rules, 1986. As per their report submitted to National Green Tribunal (NGT) on September 16, 2020, water quality of major rivers in India was not improved significantly. As the lockdown was relaxed from June, the water quality parameters were seen to be increased due to human activities (Lokhandwala and Gautam 2020; Pathak and Mishra 2020).

Effect On Waste Generation: Though COVID-19 has brought a considerable change in air and water quality, it posed a serious effect on waste management. Increase in the quantity of waste generation was due to the isolation and stay at home practices. Not only the municipal solid waste but also the amount of biomedical waste was increased due to hygiene practices followed to prevent the spread of COVID-19. As the developing nations do not have proper waste disposal and management facilities it resulted in the improper disposal of biomedical waste. Thus proper disposal of waste with appropriate care is needed to prevent the spread of corona virus through disposed waste (Ferronato and Torretta 2019; ISWA 2020).

Figure 4: Solid waste management practices during Pandemic

Inadequate maintenance and carelessness in safety measures has brought the need of the hour to safeguard biomedical waste. Proper functioning of waste management needs continuous function of workers and safety measures to be followed by them during collection to avoid spread of corona virus during collection and disposal. Appropriate handling of biomedical waste from health care facilities and households should be followed by public and health care workers. Figure 4 represents the necessary practices to be followed during pandemic situations (ISWA 2020).

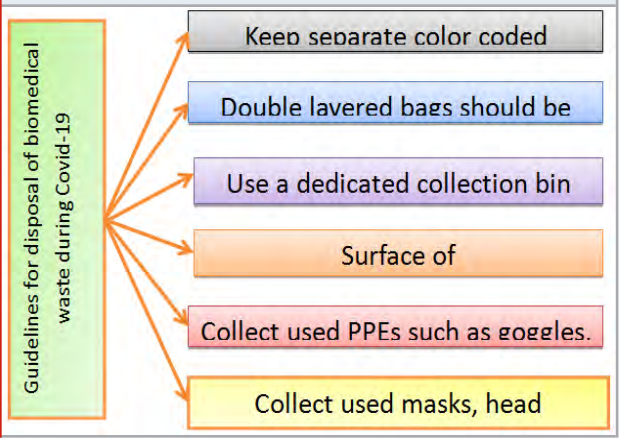
Effect On Improper Disposal Of Bio Medical Waste: During pandemic situations like COVID-19, the amount of biomedical waste generated will be high. It is due to the disposal of contaminated masks and PPE in large quantities. Improper use and disposal of masks and gloves has resulted in risk of human and aquatic life. It was also seen that face masks were disposed without proper care along the streets thus resulting in pressure on waste management system (Emily 2020). Biomedical waste management system needs proper collection, storage, transportation and disposal with proper safety measures and training (UNEP 2020). Till now, India has only limited facilities to handle and dispose the biomedical waste. It has been reported that there are only 198 common bio-medical waste treatment facilities (CBMWTFs) and 225 captive incinerators are operational in the country. Due to the sudden outbreak of COVID-19 and unexpected rise in the quantity of biomedical waste

generation has brought a serious issue in handling and disposing them (Datta et al. 2018; UNEP 2020).

Present And Future of Waste Management: Measures to be taken on treatment and disposal of biomedical waste produced during treatment, determination and isolation of COVID-19 patients was proposed by the Central Pollution Control Board, New Delhi, and Government of India on March 18, 2020. It recommended that that isolation wards should maintain separate coded bins for disposal of waste. Based on their endorsements, workers dealing with disposal of biomedical waste should be given adequate training on handling and sorting them (Ramteke et al. 2020).

Figure 5: Improper disposal of face masks (Source: Independent News)

Figure 5a: Guidelines for disposal of Biomedical waste by CPCB



Though rules and regulations were proposed on handling and disposal of biomedical waste by the government, it's in the hands of an individual to follow and prevent the spread of COVID-19. Hence to avoid sudden enforcement of rules and regulations, waste management during emergency situations should be made as a part of disaster planning and management. Thus, the health care workers all around the world should be trained to handle the infectious wastes. Also, proper classification of waste based on their type and nature may help in preventing unnecessary waste generation (Ramteke et al. 2020).

Corona virus has not only affected human life but also indirectly affected air, water and land. Lockdown has brought positive impact on air quality as many industries were closed and movement of vehicles were restricted. Concentrations of greenhouse gases were found to be reduced in most of the cities around the world. Illegal disposal of effluent without treatment in rivers was reduced due to lockdown, thus enabling improvement in water quality. Most of the rivers in India are revived back to its best condition. Though COVID-19 has brought positive effects on air and water quality, its short termed and it's the responsibility of citizens to preserve the nature which has revived itself. As to prevent the spread of corona virus people are advised to wear mask, gloves, PPE kits etc (Ramteke et al. 2020).

CONCLUSION

The findings of the present study confirms that COVID-19 pandemic has made all the developed and developing countries face severe economic and social instability. Although several measures were taken by them, the spread of COVID-19 is becoming unstoppable. Governments around the world are trying to prevent the spread of virus through lockdown, social distancing and self-hygiene practices. Improper disposal of all those has brought a serious threat to waste management. Health care workers involved in waste management are susceptible to exposure of COVID-19. Biomedical waste quantity has increased drastically, but the disposal options are very less. Incineration facilities are used to dispose biomedical waste but it may release toxic gases in environment. Hence Government should frame necessary policies to handle pandemic situations and waste management options should be modified to adapt increased waste quantities.

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

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Biomedical Communication

Preferences of Indian Women on COVID-19 Medical Solutions

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ABSTRACT

Since the dawn of modern human civilization, the daily lifestyle of humans has been evolved so many times because of either cultural, political, social effects or calamities and epidemics. Whenever any community faces an epidemic, it makes everyone learn a lot many new things for a better life and this time it was not a single commonality but the whole world which was facing a common pandemic COVID-19 which almost stopped the whole world for a time. The phrase normal life has now been converted to a new normal life where people became used to certain precautions to prevent themselves from COVID-19. The use of masks and sanitizers has become a part of day-to-day life but besides that there are many houses routine precautions that are being followed by us to save our families from the pandemic. In India, women are doing so many things for the safety of themselves and their families. In this paper, one online survey has been conducted for Indian women to find out the actual scenario of their preventive actions against COVID-19. This online survey was conducted on women belonging to different professions to check if the profession affects the perception and preferences of women for COVID-19 safety measures or not. The statistical analysis was then applied to the survey data collected. To analyze the results, a factor analysis, reliability test followed by ANOVA test was applied through SPSS tool. At last, the relation between Indian women's approach and their profession has been evaluated and it was found that there was no significant relationship between women's profession and her COVID-19 safety preferences.

KEY WORDS: COVID-19, MEDICAL SOLUTION, PREFERENCES, STATISTICAL ANALYSIS, SURVEY.

INTRODUCTION

With the rise in the World's population, health issues are indeed increasing. In the past decades, the World's unsettling level has risen as a result of life-treating infectious diseases caused by microorganisms. An emerging disease is an infectious disease that has arisen or is spreading rapidly in the population or geographical area recently (Hatfill et al. 2014; Mishra et al. 2017; Mishra et al. 2018). At the end of (2019), China began to see the initial epidemic situation and later in a few months that became a pandemic. India is also one of the very first countries to face a global coronavirus-induced COVID-19 pandemic. On 4 September 2020, India has seen more than 4,020,239 cases and more than 69,635 deaths because of COVID-19 (Mishra et al. 2021).

India has a significant number of COVID patients amid repeated lockdowns. Government plays a major role across every nation in developing and applying appropriate rules and regulations and issuing various advisories. But only Government cannot do everything, all people are expected to do their part, by respecting all advisories and provisions, to protect themselves and others from the COVID-19 virus. Covid-19 has affected India and the world on global scale. Following the SARS-CoV-2 epidemic, various countries like India introduced travel restrictions and visa suspensions. To monitor the tragedy, it is necessary to take ordinary care of the houses and emergency clinics (Hatfill et al. 2014; Mishra et al. 2021).

The normal proposal to limit the disease is-cleaning off your zone. The most critical thing is not to sneeze and cough on the public spot. It is necessary to clean hands with soap and sanitizer, wear the mouth and nose mask while sneezing and coughing. Careful food washing can aid in this respect before cooking. The outbreak can be managed periodically by surface cleaning by disinfectants. Interactions with others having signs including sneezing, coughing, respiratory problems, etc should always be avoided (Hatfill et al.

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2014). On December 30, 2019, the primary example of the novel coronavirus was represented in Wuhan city, 2 Hubei districts, P.R. China (Qamar et al. 2020; Wang et al. 2020). The Center for Disease Control and Prevention (CDC), the Chinese authorities, and experts took swift steps. It was soon identified as a novel coronavirus (2019-nCoV) by the World Health Organization (WHO) (Cui et al. 2019; Lai et al. 2020; Mishra et al. 2021).

As of May 01, 2021, a total of 19,164,969 affirmed cases, 15,684,406 recouped cases, and 211,853 passing were represented in India (Barman et al. 2020; Wang et al. 2020). According to information accessible on different sites concerning COVID-19 diseases around the World, the cases are expanding exponentially. On May 01, 2021, in World, there were 152,038,419 announced cases, which included 3,194,337 deaths and 129,328,584 recovered cases. On 15 August 2020 i.e., Independence Day of India, the total number of cases confirmed was more than 2.53 M; the death toll was about 50,040 (Ramteke et al. 2020). In this paper, one survey study has been conducted by the authors to study the preventive measures taken by Indian women to prevent their families from pandemic from COVID-19. It has also been evaluated if there was any relationship between the professional of Indian women and preventive measures taken by them against COVID-19.

MATERIAL AND METHODS

Since the goal was to perform a study explicitly on the viewpoint of women during the lockdown process, the best approach was to perform an outline survey to reach the maximum population. Google's form, a feature Google used to create a questionnaire on the survey form. For the pilot analysis, the questionnaire was sent to experts and then this questionnaire was circulated over emails, social media channels etc. In this research work, only some of the questions and answers were used as per the necessity according to the hypothesis taken. A total of 225 responses have been received but only 222 answers have been taken as a sample for this report. All the respondents were females belonging to the various Indian States. All the respondents belonged to at least one category from the list having three options as working in any organization, running their own business, and smart house maker.

The survey form was sent through online modes so that the data was also collected online. The data was stored in Google Form Excel sheets created automatically so that all data was protected and not editable. The parameters taken in the Likert scale question were the use of sanitizers, washing and cleaning, social distancing, washing the hands properly, healthy food, and exercise. The respondents were asked to select one Likert scale category according to their preference for the given parameter.

RESULTS AND DISCUSSION

Figure 1 and 2 shows the responses given by Indian women about their preference for various preventive measures against COVID 19. The graph was plotted on a scale of 25, 50, 75, 100, and so on.

Figure 1: Indian Women's Response for various preventive measures against COVID-19

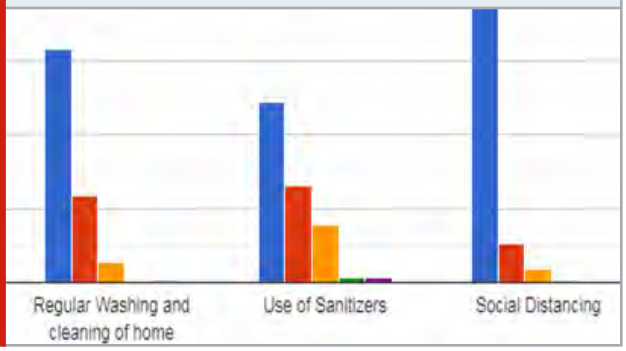
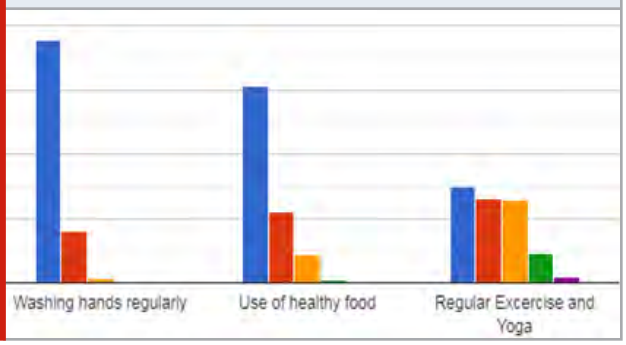


Figure 2: Indian Women's Response for various other preventive measures



1. Null Hypothesis 1: There was "no significant relationship" between the profession of Indian women and their choice of using different parameters to save their home from COVID-19.
2. Alternative Hypothesis 1: There was a "significant relationship" between the profession of Indian women and their choice of using different parameters to save their home from COVID-19.

Table 1. Reliability Analysis

Reliability Statistics	
Cronbach's Alpha	**No. of Items**
0.667	6

Various studies have been reported related to impact of COVID-19 on various aspects of woman upon searching on various electronic databases (Kapoor et al. 2019; Laxminarayan et al. 2020; Kumar et al. 2020; Han et al. 2020; Kelley et al. 2020; Akseer et al. 2020; Roberton et al. 2020). Overall, the primary symptoms of COVID-19 were less likely to be recognized by women due to problems in accessing information or less accurate COVID-19 information. Women were also less likely to practice the most effective preventive activities and to report depressive symptoms (Pinchoff et al. 2020). In 2020, the broad data was published on the outbreak of COVID 19 in India and in reference to that study, an analysis on the precautions taken by Indian women against COVID 19 has been analyzed in

this manuscript (Khandelwal et al. 2020). Among Indian women, around 225 responses were collected, and finally, 222 responses were taken for the analysis. Some of the responses were removed because of some missing values and blank fields.

Table 2. Various parameters with principal component analysis

	Communalities	
	Initial	Extraction
Wash Clean	1.000	0.613
Sanitizers	1.000	0.612
Social Distancing	1.000	0.542
Wash hands	1.000	0.390
Healthy Food	1.000	0.657
Exercise	1.000	0.635

*Extraction Method: Principal Component Analysis

For the analysis of both hypotheses, the authors had first performed the reliability test on SPSS statistical tool. Table 1 shows the reliability analysis performed on the parameters taken with a question in the survey questionnaire. Firstly, we performed reliability analysis to check if all the factors taken were properly interrelated as Cronbach alpha (0.667) was greater than 0.5. It showed a good co-relation. After that we perform factor analysis wherein, we got a combined value for all these factors, and then we did regression wherein the dependent variable was the combined value of factor and the independent variable was the profession. Therein we found that the significance value was 0.545 and hence null hypothesis was accepted. Table 2 shows various parameters considered for the hypothesis. Further, the extraction values calculated with principal component analysis for each parameter are also shown in Table 2.

Table 4 and Table 5 are generated after performing the ANOVA test on SPSS. The significant value generated was 0.545 which showed that the null hypothesis cannot be rejected.

Table 3. Total Variance Analysis

Total Variance explained						
	Initial Eigen Values			Extraction Sums of Squared Loadings		
Component	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %
1	2.408	40.137	40.137	2.408	40.137	40.137
2	1.041	17.358	57.495	1.041	17.358	57.495
3	0.872	14.541	72.036			
4	0.658	10.96	82.996			
5	0.58	9.661	92.657			
6	0.441	7.343	100			

* Extraction Method: Principal Component Analysis

Table 4. ANOVA Test Analysis

Model	R	R square	Adjusted R square	Std. error of the estimate
1	0.41a	0.002	-0.003	1.00143225

* Predicators (Constant), Profession

Table 5. ANOVA Test variable Analysis

Model	Sum of Square	df[a]	Mean Square	F	Sig.
Regression	0.369	1	0.369	0.368	0.545
Residual	220.631	220	1.003		
Total	221	221			

a Degree of Freedom
* Dependent Variable: REGR factor score 1 for analysis 1
* Predicators (Constant), Profession

CONCLUSION

The findings of the present study reflected that Indian woman is very careful about the risk of COVID-19 and takes all the required precautions. Every woman had been taking proper preventive measures required for the mitigation of COVID-19 but as the null hypothesis was accepted, it showed there was no association between the Indian women's preferences for safety precautions taken by them and their profession. On 30 January 2020, in Kerala, one of India's most literate states, the very first case of the novel Coronavirus (COVID-19) was detected, since then a lot of precautions were taken by Indian citizens. Pandemic is still not under global control, and the possibility of continuous infections continues.

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