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# Bioscience Biotechnology Research Communications

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## Editors Communique

Have we tamed the coronavirus? May be yes,  
as pandemics do not die, they can only be faded !

Science and technology has made it possible, in the shortest span of time, it has shown that with firm determination and international cooperation, we can win over the onslaughts of even the worst of the pandemics. COVID-19 is perhaps fading over now, due to our coordinated efforts worldwide. Though we have lost millions, in the two year period, partly due to the mishandling of the viral attacks and somewhat by our own follies and carelessness. Anyway lessons learnt from the past, always make us more stronger and determined. Let us now not relax and work on a better mode, as all is still not well yet. The almost taming of the virus and its cousins have indicated some of the concealed failures, on which we have to focus now. We have to be more vigilant, and even a bit of laxity can spoil the good work done. On societal and governmental parts, utmost care and caution is required on a long term basis.

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 two years now. We pray for the strength to bear this universal calamity and come up with long lasting fortitude to eradicate it soon.

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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 more to the occasion and will soon provide succor to the already grief stricken humanity.

Sharique A. Ali, PhD  
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# Effectiveness of Indoor Plants Using Home Design Ergonomics in Improving Indoor Air Quality and Human Health: A Systematic Review

Jamil Saeed Asali and Majid Alotaiby\*

Technical and Vocational Training Corporation, Jeddah College of Technology, Jeddah, Saudi Arabia.

## ABSTRACT

Indoor or houseplants are pretty good for health and several studies have shown and confirmed that indoor plants improve the concentration and productivity of the residents. Their presence decreases the stress levels and significantly eliminate common air pollutants. In a limited space, the home garden is an integrated system that produces a variety of foods and agricultural products, including staple crops, vegetables, fruits, medicinal plants and more. The importance of the backyard garden as a source of biological diversity is recognized in this context. When it comes to home gardens, whether in rural or urban regions, the structure and multi-functionality allow for the supply of multiple advantages for both ecosystems and humans. Home gardens conserve a significant amount of genetic variation in plants, both between and within species. Gardening at home is a significant part of social and cultural life, as well as a way for families to earn more money and improve their lives. This review has described the importance of plants in the home and role of home garden prevailing in Saudi Arabia. Biocultural and biological relevance of home gardens are addressed, along with future research challenges and opportunities that could help define and promote the role of house gardens in agricultural biodiversity conservation and cultural legacy preservation.

**KEY WORDS:** INDOOR PLANTS, CARBON DIOXIDE, HOME DESIGN AND SAUDI ARABIA.

## INTRODUCTION

Plants are living organisms, which is present in various colors, sizes, and shapes. Plants requires water, nutrients, air and sunlight for their survival. Planets are majorly covered by plants through every blade of grass, flower, bush, fern and tree. Plants can be found in a variety of sizes and shapes on land and sea, ranging from forests to deserts, and they can be found almost anywhere. Plants are so crucial to human existence that we would not be able to survive without them. In addition to being the primary source of energy for the sun, plants are the building blocks of practically every food chain on the planet. The vast majority of plants are utilized for medical purposes and to treat human diseases. As with animals, the use of therapeutic herbs began instinctively. Because of the lack of information at the time on the causes of illnesses or the plants that could be used to treat them, everything was dependent on personal experience. For a long time, the use of medicinal plants was based solely on empiricism, but this changed throughout time as scientist

began to understand the causes behind the plants' efficacy. Before 16th century iatrochemistry, plants were used to treat and prevent sickness. Although synthetic drug efficacy is falling and there are increasing contraindications to their use, natural medicine is once again becoming a hot topic (Petrovska, 2012).

The concentration of air pollutants can reach lethal levels in current energy-saving airtight structures, posing a major risk to human health. Although the stomatal and non-stomatal processes driving phytoremediation are poorly understood and not yet well characterized, plants can effectively remove contaminants and improve indoor air quality. Most indoor plants are chosen for their aesthetic qualities, rather than their physiological needs, which reflect their ability to eliminate contaminants from the air they breathe. Human health is gravely endangered by indoor pollution, which can be improved by using plants, however this is a cost-effective and environmentally friendly option that is being underutilized. There is, however, a lack of consideration for the physiological processes and mechanisms involved in phytoremediation when selecting plants for indoor use (Brilli et al., 2018). Some researchers have found that rooms with plants have less dust and mold than rooms without

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any foliage. Leaves and other parts of the plants act as natural filters to catch allergens and other airborne particles. Common low-light houseplants like Chinese evergreen or the peace lily can do the job, Der Sarkissian, (2021).

Indoor plants can purify air through a variety of methods: absorption, dilution and precipitation. Indoor plants are regarded to be natural air filters. Photosynthesis is a well-known plant activity that cleans the air by absorbing carbon dioxide (CO<sub>2</sub>) and exchanging it for oxygen. Plants also absorb oxygen and exhale CO<sub>2</sub> during the process of respiration. The stomata, which are the primary plant device for absorbing and removing water and nutrients, are responsible for the movement of air in and out of the plant. There are many ways in which plants might help restore the ecological balance in our atmosphere. Besides removing pollutants like CO<sub>2</sub> and volatile organic compounds, carbonyl, particulate matter, organic compounds, calcium, ozone, and carbonate, plants may also remove these pollutants from the air. As a low-cost alternative to reducing indoor pollution and minimizing human exposure to hazardous substances, indoor plants can be considered (El-Tanbouly, Hassan, & El-Messeiry, 2021).

Indoor plants have been shown to have a positive impact on office workers' productivity and well-being, as per confirmed studies. Psychophysiological stress responses, task performance, emotional states, and room assessments are some of the outcomes that can be measured during the experiment. Limited documented studies on the effects of indoor plants on health and discomfort symptoms associated with sick building syndrome, including attitudes toward plants in the workplace. Only limited studies have observed at how plants in the home can affect people's mental health, and those have all been done in a controlled environment. Using laboratory tests to support statements about causation has significant advantages. Environmental control, self-selection control for varied experimental circumstances, and precision measurement of performance on standardized tasks are a few examples of these methods are in usage. To the point where they can lead to unrepresentative conduct in the workplace because of their artificiality and short duration. The outcomes of laboratory or simulated studies may not be applicable to real-world workplaces (Bringslimark, Hartig, & Patil, 2007).

Plants are frequently used to brighten up a room, whether it be a home or a workplace. Indoor plants have been shown in investigations over the last three decades to greatly lower the majority of urban air pollution. It's also important to look for ways to improve indoor air quality and microclimate conditions without using any additional energy, as these processes utilize a lot of it. 10–20% of ventilation and air conditioning energy costs can be saved by using indoor plant designs. Between 80-90% of humans spend most of their time inside the house. As a result, air quality and comfort within the home are critical considerations.

Sick building syndrome, characterized by headache, eye, nose, and throat irritation, weariness, dizziness, and nausea, can occur if indoor air quality is poor. Sick building syndrome affects an estimated 27 million office workers

in the United States and 30% of new buildings worldwide. People are living in increasingly restricted places due to the energy crisis, which increases the concentration of pollutants in the air. The levels of indoor air pollution are normally two to five times higher than those of outside pollution, but they can reach a 100-fold increase. Ventilation is the simplest way to reduce the amount of air pollution in a structure. In the winter, even a brief period of ventilation results in a significant loss of heat, which leads to insufficient ventilation inside the home. As a result, mechanical ventilation uses a great deal of electricity (Han & Ruan, 2020). A staggering 47% (~2.9 billion people) of humanity resided in cities in the year 2000.

There is a wide range of estimates on how many people would live in cities in the 2030s: between 60-90%. More than two-thirds of the people in European countries live in cities. In addition, those who live in urban areas spend at least 80% of their time indoors. There can be no doubt about the importance of indoor air quality to human health and productivity are directly impacted by a reduction in indoor air quality. CO<sub>2</sub> is one of the gases whose concentration can rapidly fluctuate as a result of human metabolic activity in the home. Inhaled air, which contains 21% oxygen and 0.033% CO<sub>2</sub>, degrades to 16-17% oxygen and 4% CO<sub>2</sub> upon exiting the lungs. CO<sub>2</sub> levels are expected to rise dramatically in places where people congregate, such as public schools, shopping malls, and hospitals. When the concentration of CO<sub>2</sub> in the air rises, it can cause exhaustion, a loss of awareness, and sleepiness inducing a condition called carbon dioxide narcosis, (Drechsler and Morris, 2022).

It's also common for greater CO<sub>2</sub> concentrations to create a number of complaints that result in a decrease in performance that can't be pinpointed. Over 1,000 ppm of CO<sub>2</sub> causes headaches, dizziness, weariness, and a general feeling of drowsiness; 1,500 ppm or more of CO<sub>2</sub> causes throat irritation, nose irritation, nasal discharge, coughing, and discharge from the eyes and eyes, as well as irritation of the respiratory system (Cetin & Sevik, 2016).

Public health is influenced by the quality of the air we breathe. In the same way that poor outdoor air quality can create health issues, poor interior air quality can do the same. It is possible to have symptoms such as rashes and itchy eyes when exposed to indoor air pollution. Chronic obstructive pulmonary disease (COPD) and lung cancer are just two of the most serious long-term health effects that can result from long-term exposure. Most people spend 80–90% of their time indoors, including at home, school, work, the gym, and on public transits, such as buses and trains. Indoor contaminants are 100 times more concentrated than outdoor pollutants, according to the US Environmental Protection Agency (EPA). Indoor air quality must fulfill specified requirements in order to prevent a wide range of diseases, both short and long term, from developing. It is fairly uncommon for countries to have vastly varying criteria for indoor air quality.

World Health Organization (WHO) 2021 has recently released global indoor air quality guideline and recommendations

aimed at preventing dangerous contaminants and reducing exposure to them in space. Guidelines for indoor air sanitation issued by the Ministry of Health of the Republic of Indonesia are the basis for Indonesia's indoor air quality standards. The accumulation of pollutant levels in a room has been studied in a variety of locations and with a variety of different contaminants. According to most studies, the room's pollutant levels are greater than they should be. Household activities such as cooking, cleanliness, candle burning, and cigarette smoking contribute to a higher concentration of particulate matter in the inside environment than outside. PM<sub>2.5</sub> from inside a room was found to be more effective at lowering lung function than PM from outside the room, a study showed Building factors such as the year of construction, the year of restoration, and the heating method utilized all affect pollution levels. Infections of the lungs and airways might be facilitated by high levels of air pollution in the room.

There is an association between the usage of domestic fuels including kerosene, coal, and firewood and the frequency of lung illnesses. Other studies claim that the 96 emits indoor pollutants. The use of coal and firewood to improve indoor air quality may raise the risk of tuberculosis infection. Asthma and COPD have also been linked to higher amounts of pollutants in a room, according to certain studies. 150 preschool-aged children in the US were part of a longitudinal cohort research that found a link between elevated PM levels and an increase in asthma symptoms. As a result of exposure to indoor PM<sub>2.5</sub> particles and the absence of inhaled corticosteroids, school-age children's lung function declines. Indoor NO<sub>2</sub> exposure was found to be linked to an increase in asthma episodes when the concentration of NO<sub>2</sub> in the air went up. After repeated exposure to NO<sub>2</sub>, an asthma patient's asthma reaction to pollen increased. Though a systematic study did not find a substantial link between VOC exposure and asthma, it is possible that it causes inflammation of the airways in those who are exposed to it. According to numerous meta-analyses, exposure to indoor pollutants, particularly those arising from the use of solid fuels such as coal and firewood, can cause COPD and lung cancer (Susanto, Winardi, Hidayat, & Wirawan, 2021).

Plants in the home are a natural component of the environment, but the impact they have on the indoor environment and on people has not been studied. An indirect psychological effect on task performance, health, and stress levels has also been found through the use of plants in the home or work environment. Indoor plants may purify the air in your home, and they have been extensively studied in this regard. Sensory, solar, acoustic, and human health and comfort are just a few of the possible uses for indoor plants. Utilizing all of a plant's properties is beneficial to human health and well-being. Engaging in physical and mental activities in the great outdoors has been shown to have numerous health advantages. This shows that bringing nature indoors can be an effective means of enhancing people's choices connection to the natural world. There has been a long tradition of cultivating plants indoors. Having living things around made people feel more secure and

relaxed, but there is a lack of knowledge about how these psychological impacts work (Deng & Deng, 2018).

Bio-filtration and phytoremediation have been studied in laboratory trials as passive ways for eliminating indoor pollutants from the water, soil, and air. A lack of comprehensive study and meta-analysis of phytoremediation systems' indoor contaminant efficiency, chamber specifications, plant leaf areas, airflow rates, light intensities and duration despite decades of research output in the field. Bio-filtration, botanical filters, and potted plants will be evaluated in this article by comparing the removal efficiency of their air chamber volumes and other building envelope design-related factors (Parhizkar & Elzeyadi, 2020).

Saudi Arabia is a prime region, located in the southwest corner of Asia at the crossroads of Europe, Asia, and Africa. Eastern and western coasts each have the important industrial cities of Jubail and Yanbu. Oil and petroleum-related industries and businesses are encouraged and promoted by the Royal Commission (RC), which administers the two cities. These include the production of organic chemicals and fertilizers, plastics and synthetic materials. Sulfur dioxide, ozone, nitrogen oxides, and hydrogen sulfide are among the characteristics being monitored by air quality monitors and meteorology network stations around Saudi Arabia as a result of the construction of industrial cities like Jubail and Yanbu.

As a result, Saudi Arabia's cities have the region's cleanest air. It was shown that isolating the sulfate reducing-oxidizing and nitrogen fixation bacteria helped the bacterial rhizosphere breakdown nitrogen and sulfate compounds that build up in soil. Sulfur elemental and other higher oxidation states are frequently produced in nature by microbial oxidation of more reduced sulfur forms. There are chemoautotrophs, chemoheterotrophs, and photoautotrophs that can carry out these functions. It is likely that the modest numbers of species involved in nitrification and symbiotic nitrogen fixation make these processes especially vulnerable to pollution disruptions. Bacteria which can break down sulfur and nitrogen compounds were utilized as an indicator of soil pollution by oil refineries and other petrochemical facilities. In comparison to the total bacterial count in the control area, sulfate and nitrogen bacteria were found to be significantly more numerous (Khiyami, 2008).

Customer service representatives in Saudi Arabia's eastern telecommunications providers were the subjects of an Alayis et al study to see how Indoor Environmental Quality affected their level of creativity. On the other hand, there is no statistical evidence that the quality of the air, the sound, the noise, the interior design, or the scenery affects the level of creativity. In addition, while age, experience, and education level are not statistically significant differences, there are statistically significant disparities owing to gender. The findings of this study are critical for business leaders and policymakers, as well as customer service representatives, who are tasked with fostering innovative work cultures (Alayis, Amin, & Abdelmajeed, 2020).

Environmental health issues are typically linked to air pollution, both indoor and outdoor. Biological and non-biological dust particles, poisonous gases, and unpleasant scents pollute the air in slaughterhouses. In contrast, little attention was paid to the quality of the air inside slaughterhouses. In Salama & Berekaa (2016) studies, the levels of particulate matter, hazardous gases, and bio-aerosols in the slaughterhouse indoor environment were assessed. The results of Salama et al studies show that industrial sources and traffic above the slaughtering area have a greater impact on air quality than the regulation allows for. The proximity of the slaughterhouse to Dammam in Saudi Arabia second-largest industrial city, has a considerable impact on the quality of the air both within and outside the building. Bioaerosol production and bacterial contamination should be managed in slaughterhouses by the use of an effective ventilation system and strict adherence to hygiene guidelines (Salama & Berekaa, 2016).

Many studies have been carried out in the Saudi Arabia among indoor plants and home design (Al-Jeelani, 2009; Al-Mazam & Mohamed, 2001; Khiyami, 2008 a). In conclusion, Indoor plants plays a major role in impacts on occupational health. Home gardens' biocultural and biological importance are discussed, as well as prospective research challenges and opportunities that could assist define and promote the role of house gardens in agricultural biodiversity conservation and cultural legacy preservation. This review concludes as indoor plants plays a human impact in the house as well as towards the health and the role of the home garden in Saudi Arabia.

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# A Rare and Unique Case Report of Two-Year-Old Suffering From West Syndrome

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## ABSTRACT

Infantile spasms or West syndrome, may be a serious and severe sort of neurological disease that generally affect infants who are younger than 2 years. West syndrome, or infantile spasms syndrome may be an oft ruinous infantile epileptic neurological disease with a range of aetiologies. We are reporting a 10 months old male child who is a known case of seizure disorder for 6 months and came with the chief complaints of spasm of upper and lower limb, 2-3 episodes per day, which last for 3-4 min for 5 months. The history included that the baby did not cry immediately after birth and was shifted to Neonatal intensive care unit (NICU) and was on ventilator support for 15 days and then discharged. Later at 4 months of age baby started developing convulsions, electroencephalogram (EEG) was done which suggested of epileptic encephalopathy along with hypsarrhythmia. Post EEG baby developed respiratory distress for which he was shifted to Paediatrics intensive care unit (PICU), kept on nil by mouth (NBM) and on oxygen support by nasal prongs, treatment started. Patient was hemodynamically stable and hence being discharged.

**KEY WORDS:** EPILEPTIC ENCEPHALOPATHY, HEMODYNAMIC, INFANTILE SPASM, WEST SYNDROME, REHABILITATION.

## INTRODUCTION

West syndrome or infantile spasms is characterised by abnormal patterns of brain waves referred to as hypsarrhythmia and with intellectual incapacity. The spasms that area unit occurring might vary from violent jack knife or “salaam” movements where the complete body bends in half, or are no over a delicate symptom of the muscles of shoulder or any eye deformity. These varieties of spasms usually begin at intervals the first birth months and will be helped with some medication. The occurrence of this is often usually between three to eight months of birth life. It's characterised by the mixture of salaam spells that is abrupt dropping of the highest and arms flexion, body's biological processes retarded and hypsarrhythmia on encephalogram. Common causes of are hypoxic anaemia disorder, neurocutaneous syndromes, perinatal infections, injury, metabolic disorders, localized structural malformations and disorder (Desnous et

al. 2021; Saravanapandian et al. 2021; Solberg and Riggio 2021).

The spasms occur largely within the cluster kind usually on or whereas waking. Its prognosis for ancient mental development is reasonably poor, adrenocorticotrophin and a few corticosteroids facilitate the period in fact varies from two weeks to twelve weeks, relying upon its response. The Vigabatrin is the drug of selection for the primary approach to the condition. This can jointly occur within the older age patients; they are referred to as “epileptic spasms”. Currently, the ILAE (International League Against encephalopathy) has revised it and presently referred to as epileptic spasms preferentially accustomed comprehend varied age and groups of the onset and their area unit, someday several have varied causes of epileptic spasms and if a specific cause is understood, an identification of symptomatic epileptic spasms is made. If the cause cannot be detected, an identification of cryptogenic epileptic spasms may be created ( Lord 2019; Desnous et al. 2021).

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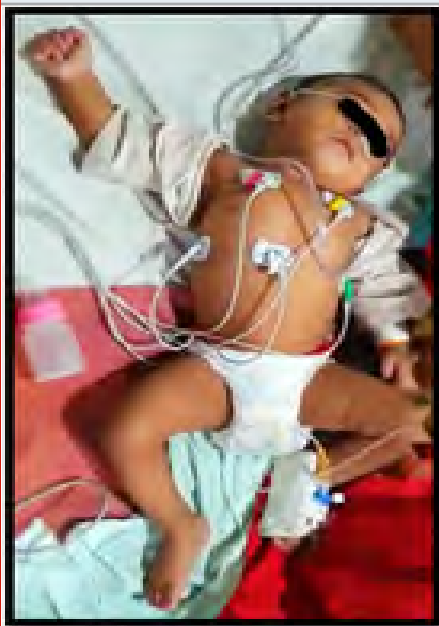
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**Case Description:** 10-month-old male child (Figure 1) who is a known case of seizure disorder for 6 months approached the Paediatrics out patient department (OPD) with the complaint of spasm of upper and lower limbs following 2-3 episodes per day which last for 3-4 min for 5 months. It was associated with drooling of saliva. The history was narrated by the mother of the child. Guidelines of CARE (Consensus Surgical Case Report (SCARE Agha RA et al. 2020 PubMed ID 33181358) for preparing Case Reports involving human subjects has been followed.

**Figure 1: Patient in PICU with nasal prongs on and ECG leads for constant close monitoring of vitals.**



A 2.75 kg male child born to primigravida mother at full term gestational age via lower segment caesarean section (LSCS) with meconium-stained liquor and foetal bradycardia. Baby did not cry immediately after birth and was shifted to neonatal intensive care unit (NICU) following perinatal asphyxia and was then started on IV antibiotics and was on ventilator support for 15 days and then discharged. Later at 4 months of age baby started developing convulsions for which he was taken to nearby hospital where EEG was done which suggested frequent generalised epileptiform discharges along with slow background which was sign of epileptic encephalopathy. He was then referred to AVBRH for further management. Baby did not achieve milestones as per age and has developmental delay. On examination, he was afebrile with heart rate (HR) of 110 beats per min, respiratory rate (RR) of 26 breaths per min. The cardiovascular system examination noted S1 S2 with no murmur. The respiratory system examination revealed no abnormality with equal air entry in bilateral lung fields. The patient was conscious, co-operative and well oriented to time, place and person as per the central nervous system examination. The abdominal examination suggested soft and non-tender viscera on palpation.

Patient was orally allowed and started on syrup Valparin. Dermatology opinion was taken following the hypo pigmented macules over the face to rule out tuberous sclerosis. As suggested calamine lotion was prescribed. EEG was done which was suggesting a sign of hypersarrhythmia. Post EEG baby developed respiratory distress for which he was shifted to Paediatric Intensive Care Unit (PICU). Patient was kept nil by mouth (NBM) and on O2 nasal prongs. He was started on injection Aumentin, nebulization with Levolin and Budecort was given. Tab prednisolone was started at 4mg/kg/day, ophthalmology call was done retinoscopy which was normal in range. Patient was then hemodynamically stable and hence being discharged. During the follow-up, the patient was advised for tapering off Tab. Prednisolone 20mg dissolve on 10ml, given 5ml morning, 4ml evening.

**Review of the case:** Epilepsy (in general) is characterized by recurrent episodic paroxysmal involuntary clinical events which are associated with abnormal electrical activity from the neurons. The patient may present with motor sensory psychomotor phenomena often associated with alteration in sensorium (Vaidyanathan et al. 2016).

An underlying cause for West syndrome is known in or so 70-75% of affected patients. Disorders that may result in the brain injury is associate underlying reason for West syndrome together with trauma, brain malformations like hemimegalencephaly or animal tissue abnormality, infections, body abnormalities like trisomy 21, neurocutaneous disorders like stem induration complicated (TSC), Sturge Weber syndrome, incontinent pigment, completely different metabolic/genetic diseases like aderin deficiency, non-ketotic hyperglycaemia, sirup pee disorder, PKU, mitochondrial encephalopathies and biotinidase deficiency, Otahara's syndrome, associated an abnormality (mutation) within the ARX sequence or CDKL5 sequence situated on the X chromosome (Erdemir et al. 2021). West syndrome may be a rare syndrome that may have an effect on males and on females. The X-linked sort of West syndrome mainly affects males a lot of usually than compared to females (Kankanamage and Gome 2021).

Most kids can have the declension in some adeptness or lag deed skills which need synergism of the muscles and voluntary (willing) movements also referred as psychomotor retardation. Generally, 3rd of the kids affected with West syndrome might results in continual epileptic seizures as those kids ages. This syndrome usually leads to Lennox-Gastaut syndrome along with different forms of seizures, troublesome management along with related to intellectual incapacity (Kohli et al. 2021). West syndrome has been clearly made public as a result of the association between infantile spasms with associate instrument pattern of hypersarrhythmia. The intellectual deficit appears within the majority cases at intervals that infantile spasms are not controlled with medication, this is (often/this can be) often an organic process aspect of the condition and not a manifestation that has got to primarily be gift thus on define the syndrome. The analysis of the interictal and attack encephalogram readings, at the side of the clinical

characteristics of the spasms and additionally the drugs examination of patients, provides some orientation as regards the causations.

Despite the spectrum that the title of this work focuses on, the study does not cover the treatment of early infants with West syndrome. Stress is placed on the completely differential diagnoses of West syndrome with different epileptic syndromes that manifest at intervals the first a pair life, and a load of significantly with a series of abnormal non-epileptic motor phenomena that occur in early infants. Of those last non-epileptic disorders square measure displayed in associate extremely table, but benign spasm of early infancy or Fejerman syndrome is given as a paradigmatic example for the diagnosis. The aboriginal aim is to prevent neurologically healthy early infants from receiving medication and sometimes adrenocorticotropin or some corticoids because of a wrong diagnosing (Kummerfeld et al. 2021; Ayad et al. 2021).

## CONCLUSION

The findings of the present study has shown that by given the chance of weak biological process outcomes is, together with - emergence of different type of seizure disorders and psychological feature and biological process issues, the early recognition and treatment of effected area unit vital the best patient outcomes. The Dissemination of associated access to instructional and validating some resources for the families and those caregivers across the time period of the kid is a pressing want. medical specialty health care suppliers' area unit well positioned to handle these desires.

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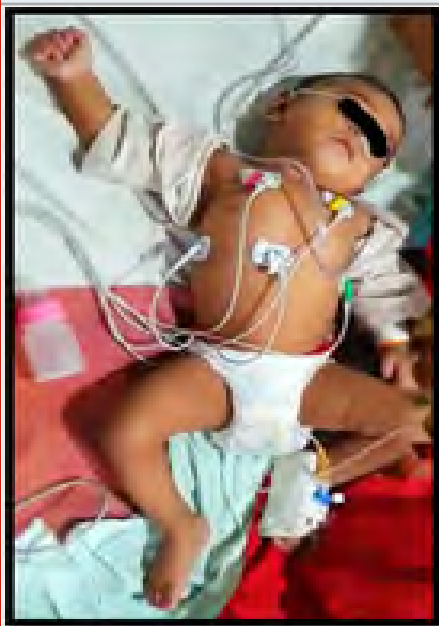
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**Review of the case:** Epilepsy (in general) is characterized by recurrent episodic paroxysmal involuntary clinical events which are associated with abnormal electrical activity from the neurons. The patient may present with motor sensory psychomotor phenomena often associated with alteration in sensorium (Vaidyanathan et al. 2016).

An underlying cause for West syndrome is known in or so 70-75% of affected patients. Disorders that may result in the brain injury is associate underlying reason for West syndrome together with trauma, brain malformations like hemimegalencephaly or animal tissue abnormality, infections, body abnormalities like trisomy 21, neurocutaneous disorders like stem induration complicated (TSC), Sturge Weber syndrome, incontinent pigment, completely different metabolic/genetic diseases like aderin deficiency, non-ketotic hyperglycaemia, sirup pee disorder, PKU, mitochondrial encephalopathies and biotinidase deficiency, Otahara's syndrome, associated an abnormality (mutation) within the ARX sequence or CDKL5 sequence situated on the X chromosome (Erdemir et al. 2021). West syndrome may be a rare syndrome that may have an effect on males and on females. The X-linked sort of West syndrome mainly affects males a lot of usually than compared to females (Kankanamage and Gome 2021).

Most kids can have the declension in some adeptness or lag deed skills which need synergism of the muscles and voluntary (willing) movements also referred as psychomotor retardation. Generally, 3rd of the kids affected with West syndrome might results in continual epileptic seizures as those kids ages. This syndrome usually leads to Lennox-Gastaut syndrome along with different forms of seizures, troublesome management along with related to intellectual incapacity (Kohli et al. 2021). West syndrome has been clearly made public as a result of the association between infantile spasms with associate instrument pattern of hypersarrhythmia. The intellectual deficit appears within the majority cases at intervals that infantile spasms are not controlled with medication, this is (often/this can be) often an organic process aspect of the condition and not a manifestation that has got to primarily be gift thus on define the syndrome. The analysis of the interictal and attack encephalogram readings, at the side of the clinical

characteristics of the spasms and additionally the drugs examination of patients, provides some orientation as regards the causations.

Despite the spectrum that the title of this work focuses on, the study does not cover the treatment of early infants with West syndrome. Stress is placed on the completely differential diagnoses of West syndrome with different epileptic syndromes that manifest at intervals the first a pair life, and a load of significantly with a series of abnormal non-epileptic motor phenomena that occur in early infants. Of those last non-epileptic disorders square measure displayed in associate extremely table, but benign spasm of early infancy or Fejerman syndrome is given as a paradigmatic example for the diagnosis. The aboriginal aim is to prevent neurologically healthy early infants from receiving medication and sometimes adrenocorticotropin or some corticoids because of a wrong diagnosing (Kummerfeld et al. 2021; Ayad et al. 2021).

## CONCLUSION

The findings of the present study has shown that by given the chance of weak biological process outcomes is, together with - emergence of different type of seizure disorders and psychological feature and biological process issues, the early recognition and treatment of effected area unit vital the best patient outcomes. The Dissemination of associated access to instructional and validating some resources for the families and those caregivers across the time period of the kid is a pressing want. medical specialty health care suppliers' area unit well positioned to handle these desires.

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# Screening of Some Local Traditional Plants for their Antitumor and Antibacterial Activities Against the Global Emergence of Multi-Drug Resistant Bacteria

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## ABSTRACT

Saudi Arabia flora has many medicinal plants which are traditionally used in inhibition of many human pathogens. *Echinops macrochaetus* (Camel thistle) and *Xanthium spinosum* (Spiny cocklebur) from the Asteraceae family and Lemongrass (*Oymbopogon citrates*) from the Gramineae family are one of the local plants which are quite popular but less studied scientifically. Hence in the present work, they were analysed for their medicinal efficacies, for which they were collected and extracted using either hot water or organic solvents (n-butanol, diethyl ether and ethyl acetate). The antibacterial activities of the previous plants extracts were detected and the methanolic extract of the three tested plants gave excellent inhibition of the tested bacterial pathogen, *Escherichia coli* while the aqueous extracts recorded the lowest bacterial inhibition. The susceptibility of the tested bacteria to the three methanolic plant extracts and control antibiotic was tested using agar well diffusion method. The methanolic extract of *Echinops* recorded excellent antibacterial activity while *Xanthium* and *Oymbopogon* recorded moderate activities against the Gram negative *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Proteus mirabilis* in addition to the Gram positive *Staphylococcus aureus* and *Enterococcus faecalis*. The mean antibacterial activities (bacterial index) was maximum for the methanol extract of *Echinops* (21.8 mm) followed by *Xanthium* (13.8 mm) and *Oymbopogon* (12.5 mm). Moreover, *In vitro* MTT test and Neutral Red assays were used to detect any antitumor activities of the three tested plant extracts. *Echinops* extract showed excellent antitumor activity against two cell lines, MCF-7 (breast cancer) and HepG2 (hepatocellular carcinoma) with no toxicity (recorded using *Artimia salina* for the assay). Also, using fluorescein diacetate microdilution method, minimal inhibitory concentrations (MICs) and the Fractional Inhibitory Concentrations of the methanolic extract of *Echinops*, ciprofloxacin and their mixture were calculated. FIC index ranged from 0.89-1.15 that means there is an additive effect between *Echinops* extract and ciprofloxacin. In conclusion, the results show that *Echinops* methanolic extract single or mixed with the antibiotic ciprofloxacin demonstrated excellent inhibitory activities for all tested urinary tract pathogenic bacteria.

**KEY WORDS:** ANTIBACTERIAL, ANTIBIOTICS, ECHINOPS, OYMBOPOGON, XANTHIUM.

## INTRODUCTION

The efficacy of the current antibiotics is limited or not effective and pharmacological industries are in constant need of new products to control the harmful effects of dangerous microbes. New active antibiotics from medicinal plants are necessary and urgently needed to cure variety of diseases and contribute in improvement of human health care system. For a long period, medicinal plants are consumed as natural medicine and plants have been considered as the best sources of antibacterial, antifungal, antitumor and antioxidant drugs. Also phyto-materials can be safely used

for stimulation of the immune system which degrades the effects of dangerous pathogens, (Carew and Patterson 1970; Jaleel et al. 2007; Aly et al. 2013; El Sayed and Aly 2014; Chassagne et al. 2021).

According to World Health Organization, herbal plants are used for thousands of years safely as traditional treatments compared to synthetic materials which had unsafe effects on human health. Almost all medical plants had therapeutic importance and human health care due to the presence of many active alkaloids in different plants (Santos et al. 1995; Mordmuang et al. 2019; Wink 2020; Chassagne et al. 2021). Counts of the therapeutic products based plants were enhanced every year because they are easily used, common, wild available, had low prices and suitable to the poor people as antibacterial agents. Many plants extracts recorded

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excellent antibacterial activities against the multidrug resistant bacteria which cause many dangerous diseases compared to commercial antibacterial agents (Ba-Hamdan et al. 2013; NLM 2020; Chassagne et al. 2021).

Asteraceae is a big family of the flowering plant that had more than 1000 genera and about 30000 species, were described in Africa, Mediterranean area and Asia. Most of them are annuals, perennials, shrubs, or small trees. Genus *Echinops* L., was classified in the sub-class Asteridae, order Asterales and family Asteraceae which had about 130 plant species, found mainly in Southern of Europe and Asia in addition to Central and North of Africa. Plants of the genus *Echinops* characterized an erect perennial herb or shrub with long stem which grow up to 1.2 m with massive root, elliptical segmented leaves and white or bright blue corolla. Out of 120 species of the genus *Echinops*, *E. kebericho* Mesfin, *E. buhaitensis* Mesfin, *E. ellenbeckii* O. Hoffm, *E. kebericho* and *E. longisetus* A. Rich were recorded in few localities in Ethiopia. Eighty two species were recorded in few localities in Europe and 15 species were found in Iraq while in Saudi Arabia, ten species were found. In Saudi Arabia, *Echinops* L. is represented by 10 species: *E. abuzinadianus* Chaudhary, *E. erinaceus* Kit-Tan, *E. glaberrimus* DC, *E. hystrichoides* Kit-Tan, *E. macrochaetus* Fresen, *E. mandavillei* Kit-Tan, *E. polyceras* Boiss, *E. sheilae* Kit-Tan, *E. viscosus* DC., and *E. yemenicus* Kit-Tan. Of these species, *E. abuzinadianus*, *E. mandavillei*, and *E. sheilae* are endemic to Saudi Arabia (Singh and Kumar 2001; Shukla 2003; Sharma and Borah 2012; Poulakou et al. 2018; Thielmann et al. 2019; Chassagne et al. 2021).

Species of the genus *Echinops* are useful for treating migraine, many heart and respiratory diseases, hemorrhoid, diabetes, and microbial infections of the urinary tracts in addition to dangerous worm's removal. Moreover, the effect of this plant extracts on pathogenic bacteria was poorly studied whereas *E. giganteus* root extract showed inhibitory activities against *S. saprophyticus* and *E. coli* while the extract of *Echinops ritro* was the most potent extract against three species of the genus *Colletotrichum* which is a famous fungal pathogen of human. Also, three isolated compounds, 5'-(3-Buten-1-ynyl)-2,2'-bithiophen, R-terthienyl and 2-[pent-1,3-diynyl]-5-[4-hydroxybut-1-ynyl]thiophene were active against *Colletotrichum* sp., *Fusarium oxysporum*, *Phomopsis viticola* and *P. obscurans* (Desta 1993; Fokialakis et al. 2006, Parekh et al. 2007; Deyno et al. 2021).

The antimicrobial activities may due to the presence of 83 different compounds like sesquiterpenoids and monoterpenoids which were isolated from *E. grijsii*, *E. ritro*, *E. kebericho* and *E. ellenbeckii*, monoterpenoids limonene and camphor which were isolated from the volatile oils of *E. ellenbeckii*, *E. kebericho* and sesquiterpenoid and sesquiterpenoid compounds which were observed in the oil extract of *E. kebericho* (Hymete et al. 1991; Maurya et al. 2015; Ivana, 2015; Ameya et al. 2016, Deyno et al. 2021).

Previous studies on the *Echinops* genus revealed the presence of diterpenoids, thiophenes, flavonoids, lignans,

steroids, sesquiterpenes, and polyacetylenes but the studies on *E. macrochaetus* are limited (Tene et al. 2004; Hymete et al. 2005b; Yadava and Singh 2006; Abdallah et al. 2013; Ibrahim et al. 2016; Zaynab et al. 2018; Zhao et al. 2019; Wu et al. 2020; Chassagne et al. 2021).

Cancer remains one of the most serious illnesses in the world and finding anticancer treatments is still a priority of studies worldwide. In cancer treatment, chemotherapy is the major option but had many limitations while natural products may be used as anticancer agents with minimal side effects and without ethical approval. About 50-60% of cancer patients in USA use plants secondary metabolites in chemotherapy like curcumin, genistein, polyphenols, resveratrol, sulforaphane, isothiocyanates, silymarin, diallyl sulfide lycopene, rosmarinic acid, apigenin from parsley, and gingerol (Nobiliet al. 2009; Ahmed et al. 2013; Wang et al. 2014; Aro et al. 2019; Belcher et al. 2020; Chassagne et al. 2021).

For cancer treatment, two sesquiterpene glycosides I and II from the aerial parts of *Echinops macrochaetus* extract were studied against MCF-7, HepG2, and HCT-116 tumor cells using sulforhodamine B assay method and the two Compounds showed potent cytotoxic activities towards all tested cell lines with IC<sub>50</sub> of 2.1, 2.9, and 3.6 μM for product I and 1.9, 3.3, and 2.3 μM, for product II, respectively. Also, the dichloromethane extract of *E. macrochaetus* was inhibitor for acetyl cholin esterase. Similarly, *Echinops giganteus* extract showed antitumor activities and can be used to treat cancer cells (Kuetel et al. 2013; Saeed et al. 2015; Zamzami et al. 2019; Chassagne et al. 2021). This study has been aimed to evaluate *in vitro* antibacterial and antitumor activities of some plants, locally collected for evaluation of their synergistic effects along with an antibiotic in urinary tract bacterial inhibition.

## MATERIAL AND METHODS

The tested pathogenic bacteria were *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* (clinical isolate), *Proteus mirabilis* (clinical isolate), *Staphylococcus aureus* (ATCC 29213) and *Enterococcus faecalis* (clinical isolate). All bacteria were obtained from King Abdulaziz University Hospital, Jeddah, Saudi Arabia and used as test organisms. The bacteria were grown and preserved according to standard guidelines (CDC 2019; Chassagne et al. 2021).

The aerial parts (stalk and leaves) of *Echinops macrochaetus*, *Oymbopogon citrates* and *Xanthium spinosum* were collected in May 2020 from various areas of Taif city, Saudi Arabia. The plants were identified by a taxonomist at the Department of Biology, Faculty of Science, KAU, Saudi Arabia, in addition to their morphological features were examined, voucher samples are recorded (Collenette 1999). In sterile plastic bags, the collected plants were preserved at 4°C until extracted with hot water, methanol, n-Butanol, Ethyl acetate and Diethyl ether (10 g/100 ml, w/v) for 10 hrs. The solvent was collected, dried and re-dissolved in 2 ml DMSO to detect the antibacterial activities of the

obtained extract using Agar well diffusion method (Holder and Boyce 1994; El Sayed and Aly 2014).

Ciprofloxacin (Sigma-Aldrich, St. Louis, Missouri, USA) was used as control antibiotic and the minimal inhibitory concentrations (MICs) of the tested plant extracts or the control antibiotic were recorded for some bacterial pathogens in 96 well ELISA trays, using a colorimetric fluorescein diacetate method (Chand et al. 1994, Aly and Gumgumjee 2011). Fractional inhibitory concentration (FIC) was calculated using the formula found below (Petersen et al. 2006; Chassagne et al. 2021).

**FIC index = MIC of extract in combination/MIC of extract alone + MIC of antibiotics in combination/MIC of antibiotics alone.**

The combination defined synergy if  $\sum FIC \leq 0.5$ , additively if  $0.5 < \sum FIC \leq 1$ , indifference if  $1 < \sum FIC \leq 4$  and antagonism as  $\sum FIC > 4$ .

Using brine shrimp lethality test, the cytotoxicity of the tested plant extracts in DMSO was determined at varying concentrations using brine shrimp larvae as test organism. After 8 hrs, the average of survived larvae at each concentration was determined and LD50 was calculated (Meyer et al. 1982; Aly and Gumgumji 2011). The antitumor activity of the three plant extracts against MCF-7 (breast cancer) and Hep G2 (hepatocellular carcinoma), tumor cell lines was studied using *In vitro* MTT and Neutral Red assays. Under sterile conditions and in 96-well plates, the MTT Test on two cell lines was evaluated (Betancur-Galvis et al. 1999).

Cells ( $6 \times 10^3$  cells/well) in 100  $\mu$ l of the culture medium were grown for 24 hrs and treated with different concentrations of the tested plant extracts for 72 hrs. Then, cells were collected and treated with MTT solution (10  $\mu$ l) and the viability of the cell was calculated by the absorption at  $A_{550}$  nm after dissolving the formazan crystals, culture medium plus MTT was used as control. Also, in 96-well plates, Neutral Red Uptake Assay, described by Betancur-Galvis et al. (1999), was used to detect *in vitro* cytotoxicity of three plants extracts on the two selected cell lines (Hep G2 and MCF-7). Cells were grown in 0.1 ml/well of the culture medium with different concentrations of the tested extract for 48 hrs, and then the collected cells were suspended in culture medium (0.1 ml containing  $5 \times 10^{-5}$  g/ml neutral red) and after incubation at 37°C for three hrs, the collected cells were washed, re-suspended in a mixture of acetic acid/ ethanol/ water (1:49:50 v/v/v) and  $A_{540}$  nm was recorded. The plant extract concentration reduced the cell viability by 50% is recorded as  $IC_{50}$  for the tested extract (Chassagne et al. 2021). Three replicates for each experiment were applied, the mean values, standard deviations and analysis of variance using ANOVA were recorded to detect any significant difference at  $P \leq 0.05$ .

## RESULTS AND DISCUSSION

Every minute, the number of the resistant microbes to almost used antibiotic in hospitals was increased causing

dangerous problems to the hospitalized patients (Halwany et al. 2015; Zaman et al. 2015; Shriram et al. 2018; El Sayed et al. 2019). Many vascular plant extracts showed inhibition activity against some bacterial pathogens and the most studied families were Asteraceae, Lamiaceae and Fabaceae in addition to the genera *Cinnamomum*, *Rosmarinus* and *Thymus* while South Africa represented the most sources of the studied plants and methanol was the most used solvents but leaves were the most studied plant part (Chassagne et al. 2021).

*Echinops macrochaetus* (Camel thistle) and *Xanthium spinosum* (Spiny cocklebur) were belonging to Asteraceae family while Lemongrass (*Oymbopogon citrates*) was from Gramineae (Table 1). The aerial parts of the three collected plants were extracted with hot water or organic solvents to detect their antibacterial activities using *E. coli* as tested bacterial pathogen. The methanolic extract of *Echinops macrochaetus* showed the highest inhibition against *E. coli* (inhibition zones diameter about 20 mm while the lowest activity was 11 mm for the aqueous extract (Table 2). Also, the methanol extract of *Oymbopogon* and *Xanthium* had lower antibacterial activities compared to *Echinops macrochaetus* methanolic extract. Additionally, diethyl ether, ethyl acetate and n-butanol extracts exhibited lower antibacterial activities compared to their methanolic extracts. Therefore, the methanolic extract of the three tested plants were selected to determine their antibacterial activities against the six tested bacteria (Table 3).

The highest antibacterial activity was recorded for the methanolic extract of *Echinops* against the two Gram positive *S. aureus* and *E. faecalis* (mean inhibition zone 24 mm) while moderate activities were recorded against *E. coli*, *K. pneumoniae*, *P. aeuroginosa* and *P. mirabilis*, with inhibition zone diameter ranged from 20-22 mm. The extracts of *Oymbopogon* and *Xanthium* recorded the same inhibitory activity against *K. pneumoniae* and *P. mirabilis* with inhibition zone of 14 mm. The antibacterial index was a maximum for *Echinops* methanolic extract (21.8 mm), followed by *Xanthium* extract then finally the *Oymbopogon* extract which considered the less active extract. The susceptibilities of the tested bacteria to differed plant extracts were different according to their cell wall structure, morphology and presence of resistance genes.

Ciprofloxacin was used as a control antibiotic, it showed excellent activities against all the tested bacteria but the activities were more potent against Gram negative bacteria, with inhibition zone diameter ranged from 30-31 mm while lower activities were against *S. aureus* and *E. faecalis* with inhibition zone diameter ranged from 20-24 mm. *Xanthium methanolic* extract was more active against the Gram negative *E. coli* (16 mm) and showed moderate actives against *K. pneumoniae*, *P. mirabilis* and *P. aeuroginosa* (14 mm). The effect of *Oymbopogon* extract was moderate on Gram negative bacteria, especially *E. coli*, *P. aeuroginosam*, *P. mirabilis* and *K. pneumoniae* while showed weak activity against the two Gram positive bacteria, *S. aureus* and *E. faecalis* (Chassagne et al. 2021).

**Table 1. The Scientific names of the collected traditional plants, families and the used parts.**

Used part	Family	Scientific name	Common name
Aerial parts	Asteraceae	<i>Echinops macrochaetus</i>	Camel thistle
Aerial parts	Gramineae	<i>Oymbopogon citrates</i>	Lemon grass
Aerial parts	Asteraceae	<i>Xanthium spinosum</i>	Spiny cocklebur

**Table 2. Diameters of the inhibition zone of the aqueous and organic extracts of the tested plants using *E. coil* as test organism.**

Used plant	Type of the extract				
	Aqueous extract (HW)	Methanol extract	Diethyl ether	Ethyl acetate	n-Butanol
<i>Echinops macrochaetus</i>	11 ±0.04	20 ±1.41cd*	17 ±1.16d*	16 ±0.19d*	14 ±1.01a*
<i>Oymbopogon citrates</i>	08 ±0.70	12 ± 0.20a*	11±0.09a*	11 ±0.06a*	10±1.02ab*
<i>Xanthium spinosum</i>	08 ±0.10	15 ±1.00d*	10 ±0.08a*	11 ±0.05a*	11 ±1.10ab*

\*: significant results compared to aqueous extract at  $p \leq 0.05$ , HW: hot water

**Table 3. The diameter of the inhibition zones (mm) of the methanolic extract of the tested plants using different pathogenic bacteria and compared to the antibiotic, Ciprofloxacin (positive control).**

n-Butanol	Type of the extract				Used plant
	Ethyl acetate	Diethyl ether	Methanol extract	Aqueous extract (HW)	
14 ±1.01a*	16 ±0.19d*	17 ±1.16d*	20 ±1.41cd*	11 ±0.04	<i>Echinops macrochaetus</i>
10±1.02ab*	11 ±0.06a*	11±0.09a*	12 ± 0.20a*	08 ±0.70	<i>Oymbopogon citrates</i>
11 ±1.10ab*	11 ±0.05a*	10 ±0.08a*	15 ±1.00d*	08 ±0.10	<i>Xanthium spinosum</i>

+Activity index was calculated as the mean value of net zones of inhibition (mm) against the pathogenic bacteria, \* Significant difference compared to Ciprofloxacin at  $p \leq 0.05$

Urinary tract infections were caused mainly by Gram-negative bacteria, *E. coli*, *Klebsiella*, *Proteus*, *Enterobacter*, *Pseudomonas* and *Serratia* in addition to some low appearance Gram-positive isolates. Inhibition of these pathogens is difficult in some cases due to low effectiveness, limitation and resistant to the present antibiotics (El Sayed and Aly, 2014). Thus, looking for new plant extracts, safe and potent for bacterial inhibition without any harmful on the host cells describe the needed characters for the therapeutic agent. Thus, screening of local less studied plants is important and *Echinops macrochaetus* is one of these less studied local plants in Saudi Arabia (Chassagne et al. 2021).

The genus *Echinops* L. (Asteraceae) was represented in Saudi Arabia with 10 species and was poorly understood. Moreover, *Echinops macrochaetus* was recorded in Saudi Arabia but studies concerning its morphological characters, chemical compositions and biological activities were very rare and lacking. Morphological variations were affected

by geographical area and environmental conditions and are very useful for taxonomically separating and distinguishing species which need sufficient experience. Root, leaves, inflorescence, corolla, ovary and fruit morphological characters and diameters were applied to distinguish between the different species (Hymete et al. 2005a; Al-Joboury et al. 2021).

In Saudi Arabia folkloric medicine, different plants with therapeutic activities were traditionally applied for treatment of many bacterial pathogens and some important diseases. This study focused on collection and studying genus *Echinops* from local sites and identifies this plant according to plant identification keys. This plant was traditionally used as a fumigant against typhus fever, treatment of migraine, reduction of humans stomach ache, and intestinal diseases and it had strong nematocidal and molluscicidal activities, LD50 = 0.057 mg/ml (Abate and Ayehu 1993; Hymete et al. 2005; Chirayath et al. 2019; Viswanathan et al. 2019; Chassagne et al. 2021).

Aqueous and organic solvents were used in this study to select the strongest plant extract and our results proved that methanol extract is the most effective solvent for the active plant ingredients. Similarly, lemongrass, lantana and olive leaves methanol extracts had good inhibitory activity against some bacterial isolates (Aly et al. 2013). Extraction method, used plant part, type of the plant, the used solvents

and the tested microorganisms, all are factors affected the antimicrobial activities. Growth inhibition of *Enterococcus*, *Escherichia* and *Klebsiella* by some plant extracts owing to the presence of some active metabolites like flavonoid, volatile essential oils; alkaloids; lectins, polypeptides and phenolics compounds were reported before (Chassagne et al. 2021).

**Table 4. Antitumor activity ( $LD_{50}$ ,  $\mu\text{g/ml}$ ) of the methanolic extracts of the three tested methanolic plant extracts against hepatocellular carcinoma (Hep G2) and breast cancer (MCF-7) and their toxicity ( $LD_{50}$ ,  $\mu\text{g/ml}$ ) against *Artemia salina*.**

Tested product Tested cells	MTT Test ( $LD_{50}$ , $\mu\text{g/ml}$ )		Neutral Red Assay ( $LD_{50}$ , $\mu\text{g/ml}$ )		Toxicity ( $LD_{50}$ , $\mu\text{g/ml}$ ) against <i>Artemia salina</i>
	Hep G2	MCF-7	HEp-2	MCF-7	
<i>Echinops macrochaetus</i>	50.0±12.5 *	25.0±12.5 *	75.0±12.5 *	50.0±12.5 *	≥75
<i>Oymbopogon citrates</i>	≥50	≥50	≥50	≥50	≥50
<i>Xanthium spinosum</i>	≥50	≥50	≥50	≥50	50 *
#Bleomycin (control)	0.02±0.001	0.04±0.00	ND	ND	≥ 5

\* Significant result at  $p \leq 0.05$  compared to control (untreated cells), ND: Not detected, #: control antitumor agent

**Table 5. Minimal inhibitory concentrations ( $\mu\text{g/ml}$ ) of *Echinops* methanolic extract, Ciprofloxacin and their combination using Fluorescein diacetate protocol.**

Tested bacteria	Minimal inhibitory concentration ( $\mu\text{g/ml}$ )				
	<i>Echinops</i>	Ciprofloxacin	<i>Echinops</i> + Ciprofloxacin	FIC index	Effect
<i>E. coli</i>	0.50± 0.12	0.12 ± 0.1	0.09 ± 0.0	0.89± 0.2	Additive*
<i>K. pneumoniae</i>	0.50± 0.12	0.12 ± 0.1	0.09 ± 0.0	0.93± 0.5	Additive
<i>P. mirabilis</i>	0.50 ± 0.12	0.14 ± 0.1	0.10 ± 0.0	0.91± 0.1	Additive
<i>P. aeruginosa</i>	0.50 ± 0.25	0.14 ± 0.1	0.10 ± 0.0	0.91± 0.0	Additive
<i>E. faecalis</i>	0.75± 0.9	0.14 ± 0.1	0.12 ± 0.0	1.15± 0.4	Independent
<i>S. aureus</i>	0.62± 0.12	0.17 ± 0.1	0.12± 0.0	0.94± 0.2	Additive

\*Additive : $\sum$ FIC less than 1, Independent : $\sum$ FIC more than 1

The antitumor activity of the three methanolic extracts was determined using two different techniques (MTT and Neutral Red assays) and two cell lines (Hep G2 and MCF-7). *Echinops macrochaetus* extract showed excellent activity against MCF-7 cells and moderate activity against Hep G2 while no activity was recorded for the extracts of *Xanthium* and *Oymbopogon*. No toxicity was found for all the tested extracts except the extract of *Xanthium* which showed moderate toxicity,  $LD_{50} = 50 \mu\text{g/ml}$  (Table 4). A recent study carried by Zamzami et al. (2019) discovered two new rare sesquiterpene glycosides, macrochaetosides A and B; originating from the aerial parts of *E. macrochaetus*. Their structures were discovered using different spectroscopic data while the active phytochemical agents in *E. vitro* were thiophenes, quinoline alkaloids, flavonoids, and sesquiterpenes, as well as fatty acids and alkanes (Biteward

Hymete 2019, Sabo and Knezevic 2019; Chassagne et al. 2021).

The minimal inhibitory concentrations were calculated using Microdilution method using fluorescein diacetate method. Moreover, the minimal inhibitory concentrations (MICs) of the methanolic extract of *Echinops*, Ciprofloxacin and their combination were determined and FIC index was calculated (Table 5). The obtained MICs of the tested methanol extracts were higher than the MIC of the tested antibiotics which was in the range of 0.12-0.14  $\mu\text{g/ml}$  while the MICs of the extract of *Echinops* ranged from 0.5-0.75  $\mu\text{g/ml}$ . The calculated MICs for the mixture of both ciprofloxacin and *Echinops* extract were in the range of 0.09-0.12  $\mu\text{g/ml}$ . The inhibition activity of the combination (Fractional inhibitory concentration index, FIC) was calculated for each tested

bacterial pathogen. They were ranged from 0.89-1.15. The interaction between plant extract and the tested antibiotics was either synergistic, additive where ( $0.5 < \sum FIC \leq 1$ ) or indifference ( $1 < \sum FIC \leq 4$ ) (Chassagne et al. 2021).

Acute and fatal infections of the urinary tract in patients with complicated cystitis are caused mainly by *K. pneumoniae*, *P. mirabilis*, *P. aeruginosa*, *S. saprophyticus* and *E. coli* which were strongly attached to the human bladder epithelium. Unfortunately, they are the most frequent causative agents of many dangerous diseases if they are untreated or not properly treated. *E. coli* (70-90%) followed by *Klebsiella*, *Proteus* and *Pseudomonas* identified as the most important urinary bacterial pathogens while *S. saprophyticus*, *E. faecalis* and *S. agalactiae* were less isolated Gram positive bacteria. Unfortunately, these bacterial infections were generally resistant to more than two classes of already used antibiotics, thus new search for novel agents from plants which playing an important role in improving human health is recommended by many authors (Aly and Gumgumgi 2011; Aly et al. 2013; MNPS 2020; Chassagne et al. 2021).

Furthermore, positive interaction was recorded between plant extract and antibiotic against some bacteria was recorded before. Inhibitory activity of both Ampicillin and garlic extract was excellent against *S. aureus*. Studying and purification of the active plant compounds was carried out and their mode of action is one or more of the suggested protocols, essential inhibition of some important enzyme, degradation of cell wall and cell membranes, prevention of DNA and protein synthesis (Aly et al. 2013; Ali et al. 2015; MNPS 2020; Al-Joboury et al. 2021).

Previous chemical investigation of *Echinops* species indicated the presence many active components including flavonoids compounds which were investigated in these plants (Al-Joboury, 2021). Six flavonoids and phenolic acid components varied in their containment were isolated by high pressure liquid chromatography from the three genera *E. armatus*, *E. nitens* and *E. viscosus* and all these secondary metabolites played a big role in the different recorded biological activities (Algabr et al. 2005, Cvetanovic et al. 2019, Dettweiler et al. 2020; Al-Joboury et al. 2021).

The chemical constituents of the species *E. kebericho* confirmed the presence of sesquiterpene lactones,  $\beta$ -sitosterol, stigmasterol, campesterol,  $\beta$ -amyrene, acetylenic thiophene lupeol and ursolic acid while tricyclic sesquiterpenes, monoterpenoids, sesquiterpenoid and sesquiterpene lactones were reported from *E. grijsii*, *E. ritro*, *E. ellenbeckii* and *E. giganteus*. Also, simple quinoline alkaloids, echinorine, 7-hydroxy-echinazolinone and echinazolinone were identified from over 14 members of the genus which act as either antibacterial or antitumor agents (Tadesse and Abegaz 1990; Afify and Hymete 1997; Weyersthal et al. 1998; Al-Joboury et al. 2021).

## CONCLUSION

The results of this study confirmed the importance of some local plant extracts as a promising treatment for multidrug

resistant bacteria which destroy almost used antibiotics. These plants can be used safely by local people to control some bacteria that cause many diseases. Furthermore, the dried powdered of *Echinops macrochaetus*, extracted with methanol was very effective against more than five different bacterial pathogens and had antitumor activities against two cancer cell lines with no toxicity. Thus, it may be used to develop new antibacterial or antitumor drugs, mainly against resistant bacteria pathogens. Combination of the previous plant extract with some antibiotics enhanced the antibacterial activity.

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# Signs and Symptoms of Dry Eye among Silicone Hydrogel Soft Contact Lens Wearers

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## ABSTRACT

Silicone Hydrogel Contact Lenses (SiH CL) provides better comfort when compared with hydrogel contact lenses. But the signs that show up in wearers with reduced comfort are unclear. Thus, we aimed to study the association between symptoms and clinical signs of dry eye in SiH CL wearers. Forty-eight SiH CL wearers (mean age:  $28.8 \pm 5.0$  years) underwent ocular assessments with and without contact lens, and completed Contact Lens Dry Eye questionnaire-8 (CLDEQ-8). Contact-lens related dryness was reported by 52.1% (n=25) subjects. A significant ( $p < 0.01$ ) Spearman's correlation was found between CLDEQ-8 score and pre-lens tear film non-invasive tear break-up time ( $r = -0.80$ ), pre-ocular tear film non-invasive tear break-up time ( $r = -0.78$ ), invasive tear break-up time ( $-0.87$ ), and Schirmer test ( $r = -0.83$ ). In conclusion, for comfortable SiH CL usage, a healthy tear film is essential, and a thorough clinical evaluation may be beneficial in alleviating discomfort.

**KEY WORDS:** CLDEQ-8, DRY EYE, SIGNS, SILICONE HYDROGEL, SYMPTOMS.

## INTRODUCTION

Dry eye is an ocular surface disorder that is caused by a loss of homeostasis in the tear film. This causes instability in the tear film and inflammation or damage to the ocular surface (Craig et al. 2017). Dry eye disease affects between 5% and 50% of people worldwide (Stapleton et al. 2017). In India, the number of people with dry eyes ranges from 15.4% to 45.4% (Rege et al. 2013; Chatterjee et al. 2021). Age, female gender, Asian ethnicity, contact lens use, environment, drugs, and hormonal imbalances have all been identified as risk factors for dry eye (Wolffsohn et al. 2021). One of the modifiable risk factors for dry eye is contact lens wear. Dryness symptoms are common among contact lens wearers; however, Silicone Hydrogel Contact Lenses (SiH CL) have improved comfort and decreased dryness symptoms as compared to Hydrogel Soft Contact Lenses (Cummings et al. 2020; Wang et al. 2021).

Earlier studies have suggested that contact lens dryness may be caused by various factors, including environmental surroundings, patient-related factors, lens materials and care products, necessitating the use of effective management

strategies (Fernández-Jimenez et al. 2021). Hence, when a SiH CL wearer complains of dryness, it may be due to dry eyes or any of the reasons mentioned above. It is important to figure out what is causing the dryness in the first place. The correlation between signs and symptoms in dry eye patients has been investigated, but not exclusively in SiH CL wearers. The relationship between dry eye symptoms and signs is not linear and varies depending on the type of dry eye disease (Young et al. 2012; Sullivan et al. 2014). However, there is limited understanding about the relationship between the dryness symptoms associated with SiH CL and clinical signs of dry eye. To the best of our knowledge no such study has been done in India. Hence the current study was focused solely on SiH CL to find the association between symptoms and clinical signs of dry eye in SiH CL wearers (Fernández-Jimenez et al. 2021).

## MATERIAL AND METHODS

By convenient sampling, 48 SiH CL wearers (mean  $\pm$  SD age:  $28.8 \pm 5.0$  years, between 18 - 39 years) were recruited from a Hospital in Chennai, India. Subjects were recruited if they were under the age of 40, had been using SiH CL for at least one year on a monthly or biweekly basis, and could understand English. Subjects with any form of contact lens-related complication (except dry eye related complications like conjunctival congestion and staining, filamentary keratitis, mucus clumping, and tear debris); past ocular

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surgery, lid abnormality, Sjogren's syndrome, hormonal imbalance, facial nerve palsy, and under medications like anticholinergic effects, anti-histamines, anti-depressants, anti-psychotics, and diuretics, were excluded from the study. The study followed the protocols recommended by the tenets of the Declaration of Helsinki and the methods were approved by the Institutional Review Board and Institutional Ethical Committee of Sri Jayendra Saraswathi Institute of Optometry, number SJSIO/2016/EC/2015/06/07, and informed consents were obtained.

The subjects completed an interviewer-administered questionnaire regarding their personal details, computer usage, contact lens history, systemic & ocular history. Later the subjects underwent an eye examination including visual acuity, objective and subjective refraction, pupil test, cover test, extraocular motility assessment, slit lamp assessment, and retinal examination. To ensure standardization, all of the following tests were performed in the order mentioned. Pre-Lens Tear Film – Non-Invasive Tear Break-up Time (PLTF-NITBUT), contact lens centration and corneal coverage, contact lens post blink movement, contact lens overall fit, grading of palpebral, limbal & bulbar hyperaemia, meibomian glands, and white spots along with film deposits in contact lenses were all measured while the lens was in place (Fernández-Jimenez et al. 2021).

Pre-Ocular Tear Film - Non-Invasive Tear Break-up Time (POTF-NITBUT), tear prism height, Invasive Tear Break-up Time (ITBUT), corneal fluorescein staining (severity and area), conjunctival lens edge staining, blinks/minute, Schirmer test without topical anesthesia were performed after 15 minutes, which was given to re-distribute tear film, after contact lens removal. The flowchart of the methodology is illustrated in Figure 1. The grading of the contact lens and dry eye, its classification, was done in accordance by Young et al. study guidelines, which can be found in Appendix and Table 1 respectively (Young et al. 2012). The Contact Lens Dry Eye Questionnaire - 8 (CLDEQ-8) was then administered to all the subjects (Chalmers et al. 2012).

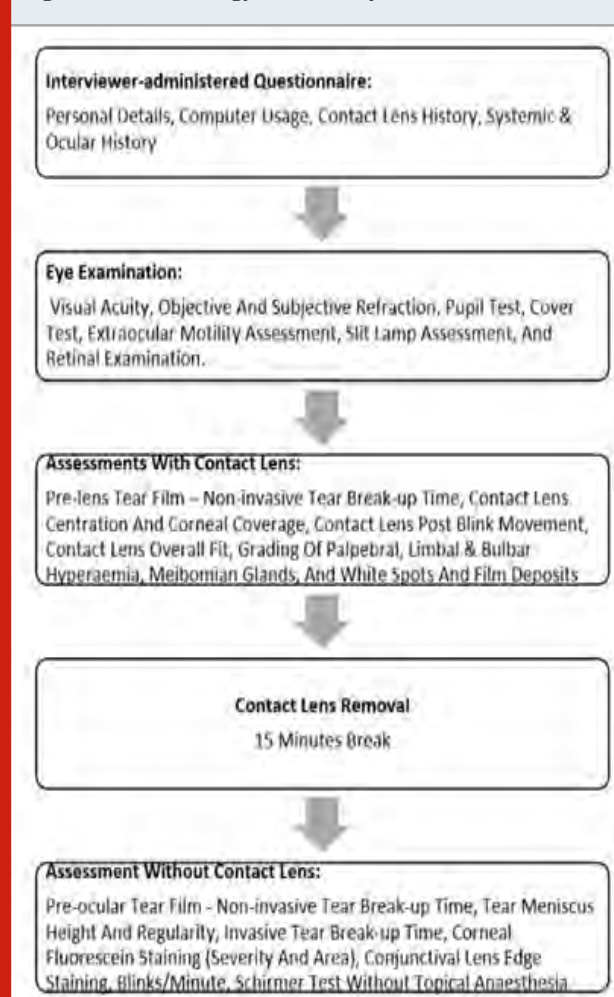
The cumulative CLDEQ-8 score for all subjects was calculated, and if it was less than 12, it was classified as non-contact lens-related dryness, otherwise as contact lens-related dryness. Spearman's Correlation analysis was done to test the correlation between CLDEQ-8 score and variables like computer usage (hours), years of lens wear (n), contact lens (hours/day), comfortable contact lens wear (hours/day), PLTF-NITBUT (sec), POTF-NITBUT (sec), invasive TBUT (sec), Schirmer test (mm). Logistic regression was carried out to investigate the relationship between PLTF-NITBUT (sec), POTF-NITBUT (sec), ITBUT (sec), Schirmer test (mm), corneal staining grade, and symptoms of dryness (Fernández-Jimenez et al. 2021).

## RESULTS AND DISCUSSION

The percentage of female subjects in the study was 65% (n=31). Best corrected monocular visual acuity of 6/6 and 6/9 was noted in 89.6 % (n=43) and 10.4 % (n=5) of subjects, respectively. Of the 48 subjects, 41 (70 %) used

monthly disposable lenses and the remainder used biweekly disposable lenses. There was a moderate association found between contact lens materials and symptoms of dryness (Cramer's V = 0.55, p < 0.01). Table 1 shows the number of subjects in each diagnostic category of dry eye. There were 47.9 % (n=23) and 52.1 % (n=25) of subjects with non-contact lens related dryness and contact lens related dryness, respectively. Table 2 summarises the distribution of the 48 subjects in non-contact lens related dryness and contact lens related dryness according to contact lens wearing time (hours/day), duration of comfortable contact lens wearing time (hours/day), and the clinical findings. Table 3 illustrates the distribution of non-contact lens related and contact lens related dryness symptoms in subjects based on the contact lens materials, and dry eye signs. The Spearman's correlation coefficient (rs) and p value for each factor tested with the CLDEQ-8 Score are shown in Table 4. A strong negative correlation was found between the CLDEQ-8 score and factors like PLTF-NITBUT, POTF-NITBUT, ITBUT, and Schirmer test, which was statistically significant (p < 0.01). A minimal correlation (p < 0.01) was found between contact lens wear (hours/day), and comfortable contact lens wear (hours/day).

**Figure 1: Methodology of the study**



Logistic regression was run to determine the effect of dry eye symptoms on the probability of contact lens wearers reporting symptoms of dry eye. The model had five independent variables (PLTF-NITBUT, POTF-NITBUT, ITBUT, Schirmer Test, and Corneal Staining-Severity). The model containing all predictors was statistically significant,  $\chi^2(5, n=48) = 44.59, p < 0.05$ , indicating that the model was able to discriminate between subjects with and without

dryness. The model showed between 60.5% (Cox and Snell R square) and 80.7% (Nagelkerke R squared) of the variance in dryness symptoms, and correctly identified 93.8% of the subjects. In Table 5, only the PLTF-NITBUT variable had a statistically significant contribution to the model, indicating that for every additional second of PLTF-NITBUT, subjects were 0.29 times less likely to report having complaint of the dryness, controlling for other factors in the model.

**Table 1. Criteria for classification of diagnostic groups of dry eye. Number of subjects in each diagnostic group among contact lens and non-contact lens related dryness.**

Diagnostic Group	Diagnostic criteria	Number of subjects with non-contact lens related dryness	Number of subjects with contact lens related dryness
No dry eye signs	No corneal staining or no reduced Schirmer's. Not considered: if low non-invasive tear-break up time or low tear meniscus height.	20	7
Aqueous tear deficient	Low Schirmer's value or low tear meniscus height with corneal desiccation staining	-	9
Meibomian Gland Dysfunction	Signs of meibomian gland dysfunction with low non-invasive tear-break up time or corneal desiccation staining	-	6
Soft contact lens induced tear instability	Low non-invasive tear-break up time or low invasive tear break-up time (with or without corneal desiccation staining) but no other signs of dry eye.	1	2
Contact lens poor fit or deposits	No signs of DE with poor contact lens fit or lens deposits	2	1

**Table 2. Contact lens wearer details and clinical findings**

	Non-Contact lens related Dryness		Contact lens related dryness		P value
	Median	Min-Max	Median	Min-Max	
Years of lens wear (n)	8	2-13	8	6-13	0.92
Contact Lens wear per day (h)	11	6-16	13	4-15	<0.01
Comfortable Contact Lens wear per day (h)	10	6-16	10	6-15	0.37
Blinks per minute (n)	12	12-15	12	11-14	0.15
Pre-lens tear film non-invasive TBUT (s)	20	17-21	15	12-21	<0.05
Post-lens tear film non-invasive TBUT (s)	20	14-20	14	13-20	<0.05
Invasive TBUT (s)	17	14-20	13	10-17	<0.05
Schirmer's test (mm)	18	13-20	13	8-20	<0.05

Median, minimum and maximum of years of lens wear (number), Contact lens wear per day (hours), comfortable Contact Lens wear per day (hours), blinks per minute (number), Pre-lens Tear Film non-invasive tear break up time (TBUT) (seconds), POTF non-invasive tear break up time (TBUT) (seconds), invasive tear break up time (seconds), Schirmer's test (mm) among contact lens related dryness and non-contact lens related dryness group.

**Table 3. Number (percentage) of subjects with non-contact lens-related and contact lens-related dryness**

	Non-Contact Lens related dryness Number (%)	Contact Lens related dryness Number (%)
<b>Contact Lens Material</b>		
Balafilcon	5 (22)	19 (76)
Lotrafilcon	12 (52)	3 (12)
Senofilcon	5 (22)	2 (8)
Comifilcon	1 (4)	1 (4)
<b>Limbal &amp; Bulbar Hyperemia</b>		
None	16 (70)	7 (28)
Trace	7 (30)	13 (52)
Mild	-	5 (20)
<b>Palpebral Hyperemia</b>		
None	14 (61)	9 (36)
Trace	9 (39)	13 (52)
Mild	-	3 (12)
<b>Corneal Staining – Severity</b>		
None	21 (91)	9 (36)
Micro-punctate	2 (9)	16 (64)
<b>Pre-Lens Tear Film – Non-Invasive TBUT</b>		
Normal ( $\geq 15$ s)	23 (100)	14 (56)
Below normal	-	11 (44)
<b>Pre-Ocular Tear Film – Non Invasive TBUT</b>		
Normal ( $\geq 15$ s)	22 (96)	12 (48)
Below Normal	1 (4)	13 (52)
<b>Invasive TBUT</b>		
Normal ( $\geq 10$ s)	23 (100)	25 (100)
<b>Meibomian Gland Dysfunction</b>		
Normal	22 (96)	12 (48)
Greasy Fluid Expelled	1 (4)	13 (52)
<b>Tear Meniscus Height</b>		
Shallow	-	7 (28)
Acceptable	3 (13)	14 (56)
Optimal	20 (87)	4 (16)

Number (percentage) of subjects with non-contact lens related and contact lens related dryness among types of Contact lens materials, limbal & bulbar hyperemia, palpebral hyperemia, and corneal staining, pre lens tear film non-invasive tear break up time (TBUT), pre ocular tear film non-invasive tear break up time, invasive tear break-up time

**Table 4. Spearman’s correlation coefficient values between CLDEQ-8 scores and factors**

Factors vs CLDEQ-8 Score	r, (p)
Computer usage (h)	0.246 (0.09)
Years of lens wear (n)	0.08 (0.57)
CL wear per day (h)	0.37 (<0.01)
Comfortable CL wear per day (h)	-0.37 (<0.01)
Pre-lens tear film non-invasive tear break up time (s)	-0.80 (<0.01)
Pre-ocular tear film non-invasive tear film break up time (s)	-0.78 (<0.01)
Invasive tear break up time (s)	-0.87 (<0.01)
Schirmer test (mm)	-0.83 (<0.01)
Corneal Staining grade-severity	0.67 (<0.01)

Spearman’s correlation coefficient values between CLDEQ-8 scores and factors like years of lens wear (number), Contact lens wear per day (hours), comfortable Contact Lens wear per day (hours), Pre-lens Tear Film non-invasive tear break up time (seconds), Pre-ocular tear film non-invasive tear break up time (seconds), invasive tear break up time (seconds), Schirmer’s test (mm), Corneal Staining grade-severity

Table 5. Logistic regression analysis

	B	S.E.	Wald	df	Sig.	Odds Ratio	95% C.I. for odds ratio	
							Lower	Upper
Pre-lens tear film non-invasive tear film break up time	-1.23	.55	5.03	1	.02	.29	.09	.86
Pre-ocular tear film non-invasive tear film break up time	1.23	.72	2.96	1	.09	3.43	.84	13.97
Invasive tear film break up time	-.80	.50	3.21	1	.07	.41	.15	1.09
Schirmer's Test	-.87	.63	1.95	1	.16	.42	.12	1.42
Corneal Staining (Severity)	.71	1.84	.15	1	.70	2.04	.06	75.61
Constant	28.58	10.63	7.23	1	.01	2.58		

We observed a significant inverse correlation based on statistical analysis, between the symptoms and clinical signs of dry eye in SiH CL wearers. As the CLDEQ-8 score increased, the values of PLTF-NITBUT, POTF-NITBUT, ITBUT, and Schirmer test decreased. The corneal staining grades showed a positive correlation with the CLDEQ-8 scores. We also found that ITBUT, PLTF-NITBUT, and Schirmer test values are significantly associated with the CLDEQ-8 score. The adjusted R<sup>2</sup> value is 82.7% of the variance in the CLDEQ-8 score can be explained by PLTF-NITBUT, POTF-NITBUT, ITBUT, Schirmer test, and corneal staining grade. Previous research has shown that the PLTF-NIBUT or ITBUT tests are diagnostic in contact lens wearers, but we found no such attributes in the current study (Molina et al. 2020).

In the present study, 44% of the symptomatic subjects had a lower-than-normal PLTF-NIBUT value (less than 10 secs), and none had an abnormal ITBUT value. Even though the latter test value did not fall into the category of "abnormal," the CLDEQ-8 score showed a clear association with it. Subjects with contact lens-related dryness had lesser PLTF-NITBUT, POTF-NITBUT, ITBUT, and Schirmer test values of 5 secs, 6 secs, 4 secs, and 5 mm, respectively, then non-contact lens-related dryness subjects (Molina et al. 2020).

However, from a clinical perspective, the signs of dry eye in those with positive symptoms cannot be specified based on the current findings. Our study results indicate that subjects with better tear stability and tear secretion have fewer dry eye symptoms. Thus, clinically, when a SiH CL patient has dry eye symptoms, it could be assumed that the higher the values of tear stability and tear secretion, less symptoms they will have. According to a systematic review by Bartlett et al. there is a low and inconsistent association between dry eye disease and signs (Bartlett et al. 2015).

While, our study found a significant relation between the signs and symptoms of dry eye, the difference in findings may be attributed to the questionnaire and study participants (Molina et al. 2020).

The reviewed studies employed questionnaires such as the Ocular Surface Index, 25-item National Eye Institute Visual Function Questionnaire, the Symptom Severity of Discomforts scale, McMonnies' questionnaire and not the CLDEQ-8 questionnaire. The CLDEQ-8 questionnaire was used in this study because we wanted to identify symptoms of dryness in SiH CL wearers, and it was constructed to assess dry eye symptoms in contact lens users in particular (Chalmers et al. 2012; Molina et al. 2020; (Wolffsohn et al. 2021).

One of the established non-modifiable risk factors for dry eye is female gender (Wolffsohn et al. 2021). Even though the proportion of females in the sample was higher, the male and female ratios in the dry eye diagnostic groups, except for Meibomian gland dysfunction, were relatively similar. As a result, gender as a risk factor in the correlation analysis may not have played a significant role. All subjects in this study who were diagnosed with Aqueous Tear Deficiency and Meibomian gland dysfunction had symptoms of dry eyes. So, we recommended carefully dispensing contact lenses in subjects with low Schirmer value or tear meniscus height, with corneal staining, and signs of Meibomian gland dysfunction with low NITBUT or corneal desiccation staining. A similar recommendation is also given by the study done in a previous study (Molina et al. 2020; Wolffsohn et al. 2021).

In this study, two out of three subjects with Contact Lens Deposits were asymptomatic, implying that clinicians should counsel patients about the importance of maintaining a proper cleaning routine for a healthy ocular condition. In



our study, 52% of subjects experienced dryness symptoms while wearing SiH CL which could lead to discomfort. Discomfort with lenses is one of the primary causes of contact lens discontinuation (Pucker and Tichenor 2020).

According to Guillon et al. (2015), a change in the lens care system improved wettability and pre-lens tear film consistency, so this regimen can be attempted in patients who are experiencing contact lens-related discomfort symptoms (Guillon et al. 2015). In our subjects' lenses, external surface treatment was used in balafilcon A (plasma oxidation), and lotrafilcon B (dense plasma coating), internal treatment was used in senofilcon A (internal wetting agent in the matrix), and no surface treatment was used in comifilcon A. We found a moderate association between the type of contact lens material and symptoms of dryness. Subjects' wearing balafilcon A had a significant association with dryness symptoms compared to other materials. Hence, for subjects complaining of dryness, it might be advisable to consider changing the material of the lens to alleviate the dryness symptoms. Contact lens wearers can experience dryness as a result of the lens wetting properties, and this may be attributed to deposits on the lens surface and hydrophobicity (Nichols and Sinnott, 2006). The outcome of vision and comfort during lens wear can be influenced by PLTF thinning. In our study, we observed that every increase in PLTF value resulted in a significant reduction in dry eye symptoms (Pucker and Tichenor 2020).

Earlier studies have found that symptoms of ocular dryness and discomfort in contact lens wearers were more pronounced at the end of the day. It is recommended that contact lens wearers be assessed at the end of the day to best identify symptomatic patients (Begley et al. 2000; Miller et al. 2021). In this study, we found no correlation between the number of hours of contact lens wear per day and the CLDEQ-8 score. However, the subjects were not examined at a fixed time of day, which may be one of the study's limitations. Another limitation of the present study is that the subjects who were shifted from hydrogel to silicone hydrogel were not noted, as those subjects could have been more symptomatic than old silicone hydrogel users (Miller et al. 2021).

## CONCLUSION

The findings of the present study have shown an inverse correlation between dry eye symptoms and clinical signs in SiH CL wearers. The symptoms of dryness were associated with signs of dry eye, a healthy tear film is necessary for comfortable contact lens wear. Before considering refitting or recommending an improvement in the lens care system, eye care professionals should conduct a detailed eye examination.

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# Incidence and Antibiotic Susceptibility Pattern of Urinary Tract Infection in Aminu Kano Teaching Hospital (AKTH) Kano, Nigeria

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## ABSTRACT

Urinary Tract Infection (UTI) is a serious health problem that affects people of all ages and genders globally. A study was carried out to determine the incidence of urinary tract infection (UTI) in patients present at AKTH with UTI from January 2019 to January 2020. The study aimed to determine the incidence and identify the infective uropathogens and their antibiotic sensitivity pattern. Urine samples were collected from 128 (Male 63) patients ranging from 0 to 84 years. Culture plates with bacteria count greater than or equal to  $1 \times 10^5$  cfu-ml<sup>-1</sup> were taken as positive for UTI. The bacteria isolates were identified based on colony morphology characteristics, gram stain reaction and biochemical tests. The identified bacteria were then tested in vitro with standard antibiotics disc to determine their antibiotic sensitivity patterns. The result showed that 35 (27.3%) out of 128 patients investigated had UTIs. The urine culture of 65 female patients resulted in 24(36.9%) positive samples, while 11 (17.5%) of the 63 males had significant bacteriuria. *Escherichia coli* constituted the predominant organism and was responsible for 24(68.7%) of the cases of UTI. The other encountered uropathogens were *Klebsiella* sp. 5(14.3 %), and *Staphylococcus saprophyticus* 2(5.5%). One sample (2.9%) each was found to have *Morganella morganii*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Salmonella* sp., respectively. All gram-negative bacteria were highly-sensitive to Meropenem and Gentamicin and were mostly-resistant to Piperacillin. Gram-positive organisms were sensitive to Gentamicin, Chloramphenicol, and Cefoxitin and resistant to Erythromycin. The highest incidence (39.1%) of UTI was found in the age range (10 – 19 years), with a significant gender difference.

**KEY WORDS:** URINARY TRACT INFECTION (UTI), UROPATHOGENS, ANTIBIOTIC SENSITIVITY, KANO.

## INTRODUCTION

Urinary tract infections (UTIs) are a serious health problem that affects millions of people throughout the world every year. The presence and proliferation of microorganisms in the urinary tract cause this infection. It incurs one of the highest total healthcare expenditures among urological cases. One of the most prevalent bacterial illnesses in the community and hospitals is urinary tract infection (UTI) (Oluwafemi et al. 2018). It can cause anything from asymptomatic bacteriuria to serious kidney injury and sepsis, depending on the clinical manifestation (Nguyen 2008; Geerlings 2016). A UTI can be diagnosed based on a

combination of symptoms as well as a positive urine assay or culture (Geerlings 2016). Except in early infancy, UTIs are prevalent in children, and females are at a much higher risk than boys. Urinary tract infections are linked to poor hygiene, malnutrition, and low socioeconomic position, all of which are prevalent in rural areas (Oladeinde et al. 2011; Mattoo et al 2021).

Several studies have been reported on Urinary tract infections from various parts of Nigeria (Oladeinde et al 2011; Iregbu and Nwajiobi-Princewill 2013; Oluwafemi et al. 2018; Okechukwu et al. 2019). *Enterobacteriaceae* such as *Klebsiella* spp., and *E. coli* as well as gram-positive organisms such as *Enterococci*, *Staphylococci*, and *Candida albicans* in patients with underlying physiological debilitation, are the most common causative agents of UTI. Similar etiological agents have been detected for UTIs in other places viz. Enugu, Zaria, Yola, Maiduguri, and Ife in

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Nigeria (Iregbu and Nwajiobi-Princewill 2013; Isa et al. 2013). Other organisms that have been identified include *Staphylococcus aureus* and *Staphylococcus epidermidis*, *Morganella morganii*, and Group B *Streptococcus*. Most UTIs in infants and children is caused by *E. coli*, which is the most commonly isolated pathogen. (Dixon-Umo et al. 2020). Earlier investigations conducted at the AKTH had concluded that the UTI incidence range from 1.3% to 50% in various patient groups (Yakasai et al 2012; Sule et al 2018; Ibrahim et al. 2019). The purpose of the present study was to look into the prevalence and antibiotic susceptibility pattern of urinary tract pathogens among patients coming for treatment at Aminu Kano Teaching Hospital, Kano, Nigeria.

## MATERIAL AND METHODS

A prospective study was conducted in the Department of Microbiology, Aminu Kano Teaching Hospital (AKTH), in northern Nigeria. AKTH is a 700-bed capacity referral hospital, serving a catchment population of 17 million from Kano and neighbouring states (Ado-Kurawa 2006). Prior ethical approval to conduct this research work was obtained from the Research Ethics committee of Aminu Kano Teaching Hospital, Kano, Nigeria (Reference no. AKTH/MAC/SUB/12A/P-3/VI/2858). The selected period of study was from January 2019 to January 2020.

The subjects were patients who presented with urinary tract complaints at the general outpatient department of AKTH. These patients are then referred to the Microbiology department. After getting verbal and written informed consent urine samples were collected from 128 people of different gender and age group. Following enrollment, each subject underwent clinical and physical examinations by the attending physician. The subjects were then given a plastic universal sterile transparent container with a screw cap for urine collection. Clear oral and written instructions on how to collect urine were given to each subject or their parents/guardians. Early morning midstream urine samples were collected. Female subjects were instructed to clean their genitalia before sample collection, while infants were instructed to be held on their laps with their genitals exposed before sample collection. Urine samples were delivered to the laboratory within 1-2 hours of collection.

All urine samples were observed macroscopically to check for turbidity, and colour and using a strip of Combii-10 to check for pH, specific gravity, presence of blood, protein, and other parameters (Wu 2010). The antibiotic susceptibility of the bacterial isolate was tested on Mueller-Hilton Agar using the traditional Kirby-Bauer disc diffusion method, and bacterial isolates were identified based on their culture, gram-stain, and biochemical properties (CLSI 2020). The disk diffusion method was used to evaluate the antibiotic susceptibility of each isolate and control strain (Okechukwu, & Thairu 2019). The following antibiotics were used for the present study Ciprofloxacin, Nitrofurantoin, Chloramphenicol, Gentamicin, Augmentin, Ceftazidime, Ceftriaxone, Meropenem, Piperacillin, and Tigecycline). SPSS version 20.0 was used to analyze the data. The student t-test was used to examine the relationship

between variables. Significant was defined as a p-value of less than 0.05.

## RESULTS AND DISCUSSION

A total of 128 patients with urinary tract complaints were enrolled in this study, the details of the demographic characteristic are given in Table 1. Out of the 128 samples tested 93(72.7%) had no bacterial growth after 24 hours of incubation, while 35(27.3%) were positive for bacterial presence. Among the 35 positive samples, 24(39.6%) were females and 11(17.5%) were males. The female gender was found to be a significant risk factor for acquiring UTIs. The frequency of occurrence of bacteria isolated was as follows: *Escherichia coli* 24(68.75%), followed by *Klebsiella* with 5(14.3%), and *Staphylococcus saprophyticus* with 2(5.5%).

One sample each was found to have *Morganella morganii*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Salmonella* spp., details is presented in Table 2. Among the 35 positive samples, 24(39.6%) were females and 11(17.5%) were males. A significant difference in UTIs was observed between the genders. It was observed that the most number of cases i.e., 39.1 % was from the age group 10-19 years, followed by the age group 0 – 9 years. The detailed breakup is presented in Table 3. In the age group, 0-9 years all the positive samples were from female patients. All the Gram-negative organisms were highly sensitive to Meropenem. Mixed results were seen in the case of Gentamicin, Tigecycline, Ceftriaxone, Ceftazidime, Augmentin, and Ciprofloxacin, respectively. The highest frequency of resistance was observed with Piperacillin, the details are given in Table 4. While five antibiotics were used to determine the susceptibility pattern of the gram-positive isolates. Except for Erythromycin all other antibiotics viz. Gentamicin, Chloramphenicol, Cefoxitin, and Clindamycin, showed positive results (Table 5).

**Table 1. Distribution of the demographical characters**

Demographical	Characters Frequency	Percentage (%)
Age (Years)		
0 - 9	25	19.5
10 – 19	23	18.0
20 – 29	22	17.2
30 – 39	20	15.6
40 – 49	10	7.8
50 – 59	6	4.7
60 – 69	4	3.1
70 – 79	15	11.7
80 – 89	3	2.3
Total	128	100.0
Gender		
Male	63	49.2
Female	65	50.8
Total	128	100.0

**Table 2. Incidence of urinary tract infection**

Suspected Organism	No. of Positive Samples	Incidence (%)
<i>Escherichia coli</i>	24	68.75
<i>Klebsiella pneumonia</i>	5	14.3
<i>Staphylococcus aureus</i>	2	5.5
<i>Morganella morganii</i>	1	2.9
<i>Pseudomonas aeruginosa</i>	1	2.9
<i>Enterococcus faecalis</i>	1	2.9
<i>Salmonella spp</i>	1	2.9
Total	35	100.0

The present investigation revealed the incidence of UTI of 27.3% with a significant gender difference ( $p < 0.1$ ). This result is similar to the earlier investigations conducted by Muhammad et al. (2017) and Muhammad (2015), who reported UTI incidences of 26% and 25%, respectively. However, a low prevalence of UTIs (16%) was reported in malnourished children (Ibrahim et al. 2019). Even lower incidences of UTIs 12-13% have been reported (Aiyegoro et al. 2007; Iregbu et al. 2013). A high incidence of UTI (38%) was reported by Sule et al. (2018); a study done in Onitsha by Nwachukwu et al. (2018) report a prevalence of 46.1%. Even higher prevalence of 56% and 61% UTI was reported by Nwachukwu et al. (2018) and Simon et al. (2019), respectively.

**Table 3. Comparison between Demographical Characters and Isolated Organisms**

Demographical Characters	Isolated Organisms		X <sup>2</sup>	Df	P-value
	No. Examined	Positive (%)			
<b>Age (Years)</b>			M	F	
00 – 09	25	7 (28.0)	-	7	2.883
10 – 19	23	9 (39.1)	3	6	
20 – 29	22	6 (27.3)	3	3	
30 – 39	20	6 (30.0)	3	3	
40 – 49	10	2 (20.0)	-	2	
50 – 59	6	2 (33.3)	-	2	
60 – 69	4	1 (25.0)	-	1	
70 – 79	15	2 (13.3)	2	-	
80 – 89	3	0 (0.0)			
Total	128	35 (27.3)			
<b>Gender</b>					2.828
Male	63	11 (17.5)		1	
Female	65	24 (36.9)			
Total	128	35 (27.3)			

$\chi^2$  = Chi square value, significance level  $p$ -value =  $p < 0.1$ . M = Male; F = Female

**Table 4. Antibiotic susceptibility pattern of Gram negative bacterial organisms**

Antibiotics	MEM		CRO		AMC		CTX		PRL		GN		TGC		CIP	
	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S
A = (24)	0	23	10	14	13	11	13	11	21	3	0	24	6	18	19	5
B = (5)	0	5	4	1	5	0	4	1	4	1	3	2	0	5	2	3
C = (1)	0	1	0	1	0	1	0	1	1	0	0	1	0	1	1	0
D = (1)	0	1	0	1	0	1	0	1	1	0	0	1	0	1	0	1
E = (1)	0	1	0	1	1	0	1	0	0	1	0	1	1	0	1	0

A = *Escherichia coli*; B = *Klebsiella pneumonia*; C = *Morganella morganii*; D = *Pseudomonas aeruginosa*; E = *Salmonella spp*. MEM = Meropenem; CRO = Ceftriaxone; AMC = Augmentin; CTX = Ceftazidime; PRL = Piperacillin; GN = Gentamicin; TGC = Tigecycline; CIP = Ciprofloxacin

The variations in prevalence of UTI in different places of Nigeria might be due to differences in study populations and the criteria used by various centres in sample selecting, mode of screening, and compounding risk factors, like gravidity, type of toilet used, age, drainage system, socioeconomic condition, etc. (Simon-Oke et al 2019;

Ibrahim et al. 2019; Dixon-Umo et al. 2020). The incidence of UTIs varies based on age, sex, and gender. In the present study, the age group with the highest frequency of UTIs was 10-19 years (39.1%). In an earlier study, Aiyegoro et al. (2007) reported 12% of UTIs in the age group of 5-18 years. In resource-rich countries, the prevalence of UTIs is

very less compared to developing countries. Overall, UTIs of children in the United States annually are estimated to be between 1-3% (Moreno 2016). This is to be expected, as infection rates are low due to improved sanitation and easy access to standardized health care (Dixon-Umo et al. 2020). Most of the bacteria isolates in the present investigation are

normal flora of faecal materials. *Escherichia coli* was the most common pathogen encountered in the present study. Much earlier investigation reports also indicated *E. coli* to be the most prominent organism of UTI (Oladeinde et al. 2011; Isa et al. 2013; Simon-Oke et al 2019; Dixon-Umo et al. 2020).

**Table 5. Antibiotic susceptibility of Gram-positive bacterial organisms**

	ERY		GN		C		FOX		DA	
	R	S	R	S	R	S	R	S	R	S
F = (1)	1	0	0	1	0	1	0	1	0	1
G = (2)	2	0	0	2	0	2	1	1	0	2

Key: ERY= Erythromycin, GN = Gentamicin, C = Chloramphenicol, FOX = Cefoxitin, DA= Clindamycin, F= *E. faecalis*, G= *Staphylococcus saprophyticus*

The present study indicated that UTIs are less prevalent in males than females. Different studies have reported female predominance (Aiyegoro et al. 2007; Oladeinde et al., 2011). There is a significant association between sex and positive cases. The higher prevalence rate recorded in females could be due to the proximity of the urethral to the anus, shorter urethra, contraception, pregnancy, and sexual intercourse which introduces bacteria into the female urinary tract (Omoregie 2008) Furthermore, the spread of normal flora in faeces from the anus to the vagina from where it may ascend to the bladder could result from poor anal hygiene. In a contrasting report by (Otajevwo, 2013), a prevalence rate of 30.1% was recorded among males. Even though they stated that the reason for this was not clear, they, however, enumerated the probable causes like lack of circumcision, and receptive anal intercourse (Dixon-Umo et al. 2020).

Among the isolates observed in the present study, gram-negative microorganisms' presence was 96.2%, while Gram-positive organisms accounted for 3.8%. This observation was consistent with an earlier report (Otajevwo 2013). Our investigation also had a marked resemblance with the earlier investigation done by Aiyegoro et al. (2007). *E. coli* was the most predominant isolate causing UTI in this study while *Klebsiella* spp ranked second in prevalence. This is consistent with other studies ( Uwaezuoke et al. 2006; El-Mahmood 2009; Otajevwo 2013; Otajevwo and Eriagbor 2014) and emphasizes the fact that the majority of organisms causing UTIs are found in the lower gastrointestinal tract, it is noteworthy that *E. faecalis* was isolated only in the female population which may be an indication of poor anal hygiene, especially among teenagers. Among the age groups, UTIs occurred highest in the 10–19 years age group with a prevalence of 39.1%. This could be because patients in this age group are sexually active. Infections were observed to be higher in males under 0-9 years of age than in females. Some reports have stated that at advanced ages, males have more complicated UTIs and also more drug-resistant pathogens than females (Alhambra 2004; Dixon-Umo et al. 2020).

The uropathogens isolated in this study showed high sensitivity to Meropenem, gentamicin, and Tigecycline. The sensitivity to Meropenem is in tandem with other reports of 97.6% and above 50% sensitivity (Haruna et al. 2014; Alabi et al. 2014). In a study conducted at Redeemers University, Nigeria Augmentin and Piperacillin were also found to be resistant (Ayoade et al. 2013). This might be due to the abuse of these drugs leading to mutations that may be transferred through bacteriophages or plasmids thereby promoting resistance. The availability of drugs over the counter without the need for a prescription encourages the abuse of drugs. In addition, the use of fake and substandard drugs in Nigeria may also be a contributory factor to the emergence of resistant strains (Mordy et al. 2006). Inappropriate antimicrobial use as a result of a lack of drug knowledge can result in ineffective therapy and contribute to the spread of drug resistance. (Kariuki et al. 2007; Getachew et al. 2012). The strength of this prospective study was that it tested a good number of urine samples for pathogenic bacteria and underlined the presence of susceptible and resistant bacterial strains. This may provide clear-cut scientific information for appropriate UTI treatment, prevention, and control (Dixon-Umo et al. 2020).

## CONCLUSION

The findings of the present study indicated the incidence of UTI to be 27.3%, and most of the infection was noted in children and teenagers. Females showed significantly higher infection rates. As many patients come from rural backgrounds poor hygiene practices and poor sanitation by these patients may be one of the major factors for UTIs. Though all the Gram-negative bacteria were found to be sensitive to Meropenem. Many other antibiotics that were studied indicated mixed results and some like Piperacillin and Erythromycin were found to be resistant to gram-negative and gram-positive bacteria, respectively. However, the sample studied was very small. Antibiotic resistance is a universal problem and can result in a delay in the recovery process and escalation of the treatment cost. It is always

better to have information about the microbe before starting the antibiotic treatment. However, in many situations, if the UTI needs immediate treatment, then it should be started with Meropenem even though it is an expensive drug.

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# Assessment of Avian Road Kill Mortality in the State Highway Passing Through Agricultural Landscape

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## ABSTRACT

Roads are becoming one of the greater threats to avian fauna. In India, very few studies have been carried out to assess the mortality of avian fauna and other vertebrates. The data on the road kill survey was collected from Jan 2015 to Dec 2017. For the survey of a road-killed avian fauna, the road passing through the agricultural landscape (from Amravati to Paratwada) was selected based on geographical location and availability of vegetation diversity. The survey found a total of 694 carcasses of 38 different species belonging to 25 families that were killed on Amravati to Paratwada Highway. The highest mortality of Greater Coucal (*Centropus sinensis*) was recorded on Amravati to Paratwada state highway. While studying the seasonal variations, it was observed that the maximum mortality was found from June to September and the minimum from February to May.

**KEY WORDS:** AVIAN MORTALITY, AGRICULTURAL LANDSCAPE, ROAD VEHICLE COLLISION, STATE HIGHWAY.

## INTRODUCTION

In this era of industrialization and modernization, the activities of humans affect the population of avian fauna. There are approximately 80 million birds are killed due to collisions with road vehicles every year in the United States alone (Erickson et al. 2005). Highways through wildlife reservoirs affect the fauna seriously and the effects range from habitat loss and fragmentation, direct mortality through collision with vehicles (Foster and Humphrey 1995; Das et al. 2007; Row et al. 2007; Baskaran and Boominathan 2010).

For birds and other vertebrate groups, a direct threat of roads is death due to collisions with vehicles (Erritzoe et al. 2003). Increasing traffic speed and volume have been found to also increase mortality and the mortality rates are generally highest during summer and Spring (Case 1978; Loss et al. 2014b). Mortality rates have been found to increase with the increasing width of the road corridor (Oxley et al. 1974; Pintoa et al. 2020). Pintoa et al. (2020) studied the temporal patterns of bird mortality due to road traffic collisions in the Mediterranean region. This study provides evidence that bird mortality due to road vehicle collisions may change

between years and be highest during the breeding season (Pintoa et al. 2020).

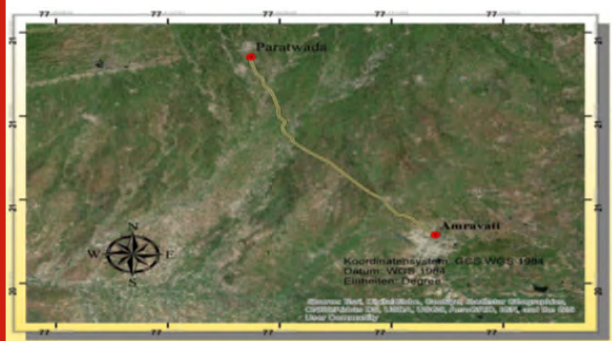
Migratory species travel long distances and they may be even more at risk because they are presumably exposed to more road crossing events than non-migratory species (Harris and Scheck 1991). Higher mortality of adult and juvenile birds having roadside habitats can create sink populations that can only persist through immigration (Mumme et al. 2000; Bishop and Brogan 2013). Betleja et al. (2020) studied how high birds fly above roads, and how they use the road infrastructure such as bridges, lampposts, etc. This study found that species differed significantly in the height at which they crossed over roads, and 30 % of the birds crossing were at heights below 12 m, which is a potential collision height (Betleja et al. 2020). Roads are becoming one of the greater threats to animal and plant populations. In India, the highways are going through many protected areas and cause a severe impact on wildlife and their habitat (Vijayakumar et al. 2001). In India, very few studies have been carried out to assess the mortality of avian fauna and other vertebrates on the roads passing through reserve forests (Chhangani 2004; Das et al. 2007; Baskaran and Boominathan 2010; Selvan et al. 2012; Rawankar and Wagh 2018; Betleja et al. 2020).

These studies provide the data on various casualties due to the vehicular collision in birds and other vertebrate fauna

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in the different parts of India and also suggest the urgent need to undertake such a survey to estimate the magnitude of this threat in different parts of India.

**Figure 1: Map showing Amravati to Paratwada State Highway passing through agricultural land (Satellite image).**



**Figure 2: State Highway from Amravati to Paratwada passing through agricultural land (Actual Photograph)**



## MATERIAL AND METHODS

The Amravati to Paratwada State Highway Number – 6 is passing through the agricultural landscape and large numbers of vehicles are passing through this highway at very high speed every day. Hence this study was carried out to estimate the avian mortality due to road vehicle collisions on this state highway.

**Road Vehicle collision Study:** The data of the roadkill survey was collected from Jan 2015 to Dec 2017. For the survey of avian roadkill, Amravati to Paratwada, 50-kilometer state Highway Number – 6 was selected which is passing through the agricultural landscape. The survey was performed using a motorcycle. The motorcycle was ride at a speed of 10- 20 km/hour on the roads. The survey was performed in the morning hours from 7.00 to 9.00 am. The road, as well as 5 feet, fix area along the side of the road, was screened out for avian carcasses. When the dead bird was encountered, a photograph of the bird was taken. The survey was performed twice a week on this road. This survey method was followed by various researchers and found satisfactory in evaluating the road kills. (Das 2007; Baskaran 2010; Selvan 2012; Betleja et al. 2020).

Once the roadkill was encountered and photographed then it was removed from the road to prevent the double counting. Taxonomic keys were used to identify the road kills. Up to genus and species level identification was done. The road kills reported by locals, birders, and the author's occasional visits and road kills data were also recorded (Grimmat et al. 2009; Rasmussen and Anderton 2012; Wadatkar et al. 2016; Betleja et al. 2020).

The mortality estimation is subject to multiple biases and hence to calculate the annual mortality estimate accurately, the biases such as observer bias (probability of detection) and scavenging activity (persistence time) are considered. To calculate the mortality estimate, the formula provided by Gerow et al. was used (Tajera et al. 2018; Betleja et al. 2020).

$$M = (C / nS) * (I / Tp)$$

Where M is the estimated mortality; C is the number of carcasses found; nS is the number of surveys conducted (nS = 104), I is the number of days between surveys (I = 03); T is the persistence time of each taxon (T= 2.2 days), and p is the probability of detection (p= 67 %). While calculating the mortality per km (with or without bias), an average of three years was taken into consideration. Standard deviation was calculated for each road-killed bird species on Amravati to Paratwada State highway during the year 2015 to 2017 (Betleja et al. 2020).

## RESULTS AND DISCUSSION

The survey found a total of 694 carcasses of 37 different avian species belonging to 25 families that were killed by road vehicles collision on the Amravati to Paratwada Highway. The highest mortality of Greater Coucal (*Centropus sinensis*) was encountered, and followed by Red - vented Bulbul (*Pycnonotus cafer*), Spotted Owlet (*Athene brama*), Common Myna (*Acridotheres tristis*), House Crow (*Acridotheres tristis*), Indian Nightjar (*Caprimulgus asiaticus*), Ashy Prinia (*Prinia socialis*), Indian Roller (*Coracias benghalensis*), Yellow-eyed Babbler (*Chrysomma sinense*), Laughing Dove (*Spilopelia senegalensis*), Jungle Babbler (*Turdoides striata*), Asian Koel (*Eudynamis scolopaceus*), Common Stonechat (*Saxicola torquatus*), Jungle Prinia (*Prinia sylvatica*), Indian Pond Heron (*Ardeola grayii*), Red-wattled Lapwing (*Vanellus indicus*), Barred buttonquail (*Turnix suscitator*), Indian Robin (*Copsychus fulicatus*), Baya Weaver (*Ploceus philippinus*), Indian Thick-knee (*Burhinus indicus*), Common Tailor Bird (*Orthotomus sutorius*), Pied Cuckoo (*Clamator jacobinus*), Rufous Treepie (*Dendrocitta vagabunda*), Coppersmith Barbet (*Psilopogon haemacephalus*), Barn Owl (*Tyto alba*), Large Grey Babbler (*Turdoides malcolmi*), Indian Scops Owl (*Otus bakkamoena*), Yellow Wagtail (*Motacilla flava*), Scaly-breasted Munia (*Lonchura punctulata*), Silver bill Munia (*Euodice malabarica*), Wire - tailed Swallow (*Hirundo smithii*), Red - rumped Swallow (*Hirundo daurica*), Common House Martin (*Delichon urbicum*), Long-Tailed Shrike (*Lanius schach*) and Tawny bellied Babbler (*Dumetia hyperythra*).

**Table 1. Showing the species-wise mortality and its percentage to the total carcasses killed due to a road vehicle collision and standard deviation for each species, on Amravati to Paratwada State highway during the year 2015-2017**

Scientific Name	Common Name	2015	2016	2017	Total (%)	SD
<i>Centropus sinensis</i>	Greater Coucal	29	65	79	173 (24.93)	25.79
<i>Athene brama</i>	Spotted Owlet	16	16	24	56 (8.07)	4.62
<i>Pycnonotus cafer</i>	Red-vented Bulbul	16	18	20	54 (7.78)	2.00
<i>Acridotheres tristis</i>	Common Myna	11	13	14	38(5.48)	1.53
<i>Corvus splendens</i>	House Crow	11	9	8	28(4.03)	1.53
<i>Caprimulgus asiaticus</i>	Indian Nightjar	12	9	6	27 (3.89)	3.00
<i>Coracias benghalensis</i>	Indian Roller	9	3	10	22 (3.17)	3.79
<i>Spilopelia senegalensis</i>	Laughing Dove	8	6	8	22 (3.17)	1.15
<i>Prinia socialis</i>	Ashy Prinia	10	4	7	21 (3.03)	3.00
<i>Chrysomma sinense</i>	Yellow-eyed Babbler	9	7	3	19 (2.74)	3.06
<i>Prinia sylvatica</i>	Jungle Prinia	6	6	4	16 (2.31)	1.15
<i>Turdoides striata</i>	Jungle Babbler	7	2	6	15 (2.16)	2.65
<i>Ardeola grayii</i>	Indian Pond Heron	6	4	5	15 (2.16)	1.00
<i>Burhinus indicus</i>	Indian Thick-knee	6	5	4	15 (2.16)	1.00
<i>Vanellus indicus</i>	Red-wattled Lapwing	6	4	2	12 (1.73)	2.00
<i>Psilopogon haemacephalus</i>	Coppersmith Barbet	5	5	2	12 (1.73)	1.73
<i>Tyto alba</i>	Barn Owl	4	2	6	12 (1.73)	2.00
<i>Eudynamys scolopaceus</i>	Asian Koel	7	2	2	11 (1.59)	2.89
<i>Saxicola torquatus</i>	Common Stonechat	7	4		11 (1.59)	2.12
<i>Orthotomus sutorius</i>	Common Tailor Bird	5	5	1	11 (1.59)	2.31
<i>Turdoides malcolmi</i>	Large Grey Babbler	4	5	1	10 (1.44)	2.08
<i>Copsychus fulvicatus</i>	Indian Robin	6	0	3	9 (1.3)	3.00
<i>Clamator jacobinus</i>	Pied Cuckoo	5	3	1	9 (1.3)	2.00
<i>Otus bakkamoena</i>	Indian Scops Owl	2	5	1	8 (1.15)	2.08
<i>Ploceus philippinus</i>	Baya Weaver	6	0	1	7 (1.01)	3.21
<i>Turnix suscitator</i>	Barred buttonquail	6	0	0	6 (0.86)	3.46
<i>Dendrocitta vagabunda</i>	Rufous Treepie	5	0	0	5 (0.72)	2.89
<i>Bubulcus ibis</i>	Cattle Egret	0	5	0	5 (0.72)	2.89
<i>Lonchura punctulata</i>	Scaly-breasted Munia	0	2	0	2 (0.29)	1.15
<i>Merops orientalis</i>	Green Bee-eater	0	2	0	2 (0.29)	1.15
<i>Euodice malabarica</i>	Silver bill Munia	0	1	0	1 (0.14)	0.58
<i>Motacilla flava</i>	Yellow Wagtail	0	0	1	1 (0.14)	0.58
<i>Hirundo smithii</i>	Wire-tailed Swallow	0	0	1	1 (0.14)	0.58
<i>Hirundo daurica</i>	Red-rumped Swallow	0	0	1	1 (0.14)	0.58
<i>Delichon urbicum</i>	Common House Martin	0	0	1	1 (0.14)	0.58
<i>Lanius schach</i>	Long Tailed Shrike	0	0	1	1 (0.14)	0.58
<i>Dumetia hyperythra</i>	Tawny-bellied Babbler	0	0	1	1 (0.14)	0.58
	Unidentified Birds	7	17	10	34(4.9)	5.13
	Total	231	229	234	694	

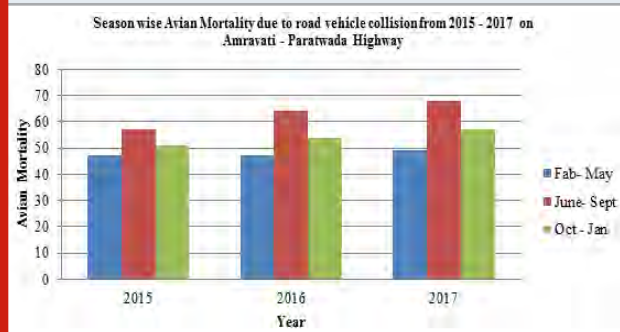
The Family wise mortality of avian species was also calculated. Highest mortality was encountered in the Centropodidae and then followed by Pycnonotidae, Strigidae, Sturnidae, Corvidae, Caprimulgidae, Cisticolidae, Coraciidae, Sylviidae, Columbidae, Leiotrichidae, Cuculidae, Muscicapidae, Ardeidae, Charadriidae, Turnicidae, Ploceidae, Burhinidae, Megalaimidae, Tytonidae, Estrildidae, Motacillidae, Hirundinidae, Laniidae, and Timaliidae. The year wise mortality of the

birds due to road vehicular collisions are shown in Table No. 1 and season wise details in Fig. 3.

Carcasses of Greater Coucal were found more frequently than other reported bird carcasses on the studied highway and perhaps the most dominant species on all the roads of Vidarbha and Maharashtra. This bird species cannot make pace with the speed of the vehicle because of its large size, low-level flight pattern, and ground-dwelling habit

and hence gets killed in collision with the vehicle. Indian Roller and Common Myna are the tree cavity-nesting birds. These birds prefer the holes of the Neem plant (*Azadirachta indica*) and Babul plant (*Acacia nilotica*) along the roadside. The frequency of road vehicle collisions of Indian Roller and Common Myna was observed maximum in summer because these birds' activities get increased near the nest site. This observation correlates with a previously published study (Erritzoe et al. 2003; Betleja et al. 2020).

**Figure 3: Season wise Avian Mortality due to road vehicle collision from 2015 - 2017 on Amravati - Paratwada Highway**



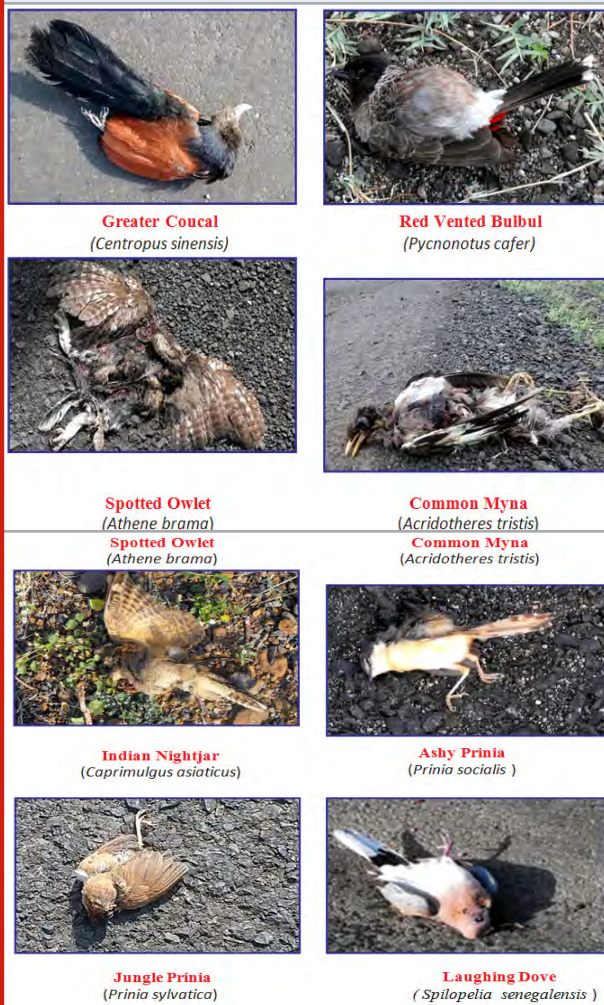
Owls, owlets (Spotted Owlet), and Nightjars are the nocturnal bird species. As Owls and Owlets feed on mice, rats, and other small animals, they used roadside trees for roosting. These birds are also tree cavity-nesting birds and they become active during the night, but due to high-intensity LED flashlights of vehicles they cannot make pace with the speed of the vehicle and get killed. Spotted Owlet and nightjars are the most frequently killed species of birds among the nocturnal birds (Betleja et al. 2020).

While studying the seasonal variations, it is observed that the maximum mortality was found during the months of June to September and the minimum in the months of Feb to May. The majority of the bird species have a breeding season during the rainy months from June to September. During the breeding season, the movements of the bird's increase, and hence the chances of collision also increase. Pintoa et al. (2020) studied the temporal patterns of bird mortality due to road traffic collisions in the Mediterranean region and their study also found that bird mortality due to road vehicle collisions was highest during the breeding season. Betleja et al. (2020) studied how high birds fly above roads, and how they use road infrastructure such as bridges, lampposts, etc. This study found that species differed significantly in the height at which they crossed over roads, and 30 % of the birds crossing were at heights below 12 m, which is a potential collision height (Betleja et al. 2020).

Due to rain, the wings of the birds get wetted, and hence the weight of the wings increases, and birds require more energy to fly. Due to this reason, birds find difficulty in flying due to wet body feathers. Again during monsoon, the frogs, toads, snakes, lizards, and small mammals come on roads

in more number, and the birds which feed on these animals also come on roads frequently to catch these animals. Due to this reason, the mortality of nocturnal raptors (Owls and Owlets) get increased during the monsoon season. On Amravati to Paratwada State Highway, the estimated mean annual mortality was found 4.62 birds/km/year, and when recalculated by considering the biases such as observer bias and scavenging activity it is found 12.44 birds/km/year (Betleja et al. 2020).

**Photo Plate No 1: Photographs of Carcasses of the birds Killed Due to Road vehicle Collision**



Various study results show that the most dominant species found dead due to road vehicle collision belong to the family passerines, Columbidae, nocturnal birds such as owlets and nightjars. On the contrary, the present study recorded the observations that the Greater Coucal was the most dominant species belonging to the family Centropodidae found dead on the roads due to the vehicular collision. This scientific report presents that the Greater Coucal is the most dominant species found dead due to the road vehicle collision on State highways passing through agricultural landscape (Dhindsa et al. 1988; Chhangani 2004; Erickson et al. 2005; Selvan et al. 2012; Betleja et al. 2020).

**Photo Plate No2: Photographs of Carcasses of the birds Killed Due to Road vehicle Collision**



**Jungle Babbler**  
(*Turdoides striata*)



**Plain Prinia**  
(*Prinia inornata*)



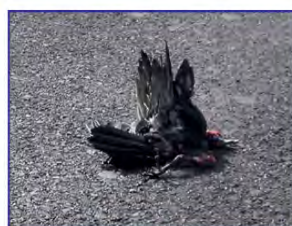
**Indian Roller**  
(*Coracias benghalensis*)



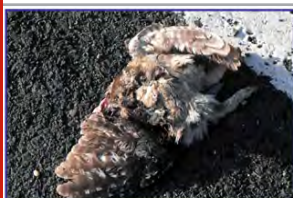
**Yellow-eyed Babbler**  
(*Chrysomma sinense*)



**Asian Koel**  
(*Eudynamis scolopaceus*)



**House Crow**  
(*Corvus splendens*)



**Indian Scops Owl**  
(*Saxicola torquatus*)



**Common Stonechat**  
(*Otus bakkamoena*)

Erritzoe et al. (2003) and Boves and Belthoff (2012) reported that the passerines and Owls are the most dominant among the road-killed species. The present study also recorded the significant mortality of Passerines and Owls due to road vehicle collisions. When the data of road vehicle collision was evaluated based on feeding habitat (Table No. 2), it is found that the maximum number of species were insectivorous (21 species) followed by Omnivorous (06 species), Carnivorous (4 species), Granivorous (3 Species), Frugivorous (2 Species) and Piscivorous (1 species). The insectivorous and omnivorous birds do come on the roads to feed on the small insects and other animals such as amphibians, snakes, crabs, etc., and hence the chance of

their collision with vehicles increases. The granivorous birds come on the roads to feed on the grains dispersed on the roads. Frugivorous birds such as Red Vented Bulbul, Brahmany Starling, and Coppersmith Barbet feed on the fruits of the roadside trees and collide with vehicles when trying to cross the roads. In the Piscivorous bird category, the study found only one species which is the Indian Pond Heron. This bird mainly feeds on fishes, frogs, aquatic insects, etc. The record of these species as roadkill may be a matter of chance only (Betleja et al. 2020).

When a comparison was made between diurnal and nocturnal birds (Table No.2), it is found that the majority of the species which got killed due to road vehicle collisions are diurnal ( 33 species ) and only 4 species were nocturnal. The nocturnal bird species include the Owls (Barn Owl, Scops Owl), Owllet (Spotted Owllet), and Indian Nightjar. The mortality in diurnal birds occurred during the early hours of the day when the diurnal birds are more active and the speed of the vehicle is very high. The mortality in nocturnal birds such as Owls, owllets, and Nightjar occurred during the night-time (Betleja et al. 2020).

Following recommendations will be useful to minimize road vehicle collision and avian species conservation.

1. It is recommended that limiting the speed of the vehicle on the highways passing through the critical areas to 40 to 50 km/hour (Orlowski and Nowak, 2006), further speed breakers and signposts at the roadsides should be introduced in Wildlife conservation policies.
2. Structural elements can encourage birds to fly above traffic or below the road through bridges or culverts. Flight diversion works best for species with direct, rapid flight. Poles that produce an illusion of a solid barrier were effective in reducing bird road kills.
3. Vehicle LED lighting appears to blind Owls and increases the chances of collision with vehicles hence light pollution could be addressed as a part of the strategy.
4. The combined efforts and continuous monitoring of Foresters, Non-Government Organisations (NGO), and Birders are needed for the conservation of the Avian Diversity.

This study provides baseline data on the magnitude of avian mortality due to road vehicle collisions on the Amravati to Paratwada state highway in the Amravati District. Due to such loss of these species, the total avian population becomes severely affected. The number of mortality due to road vehicle collisions may be quite large because the avian carcasses get cleared immediately by scavengers after death and remain unnoticed; sometimes the collided birds have dashed far away from the road and cannot be noticed. Further, this was the first attempt to estimate the mortality of avian fauna due to road vehicle collision on State highway no.6 Amravati to Paratwada (50Km) in the Amravati district. The better estimation and evaluation of avian carcasses need long-term study (Betleja et al. 2020).

**Table 2. Showing feeding habits and diurnal/ Nocturnal habit of birds killed by road vehicle collision Amravati - Paratwada State Highway**

Sr. No	Scientific Name	Insectivorous	Granivores	Frugivorous	Carnivorous	Omnivorous	Piscivores	Diurnal	Nocturnal
1	<i>Prinia socialis</i>	+						+	
2	<i>Eudynamis scolopaceus</i>	+						+	
3	<i>Turnix suscitator</i>					+		+	
4	<i>Ploceus philippinus</i>		+					+	
5	<i>Bubulcus ibis</i>	+						+	
6	<i>Tyto alba</i>				+				+
7	<i>Delichon urbicum</i>	+						+	
8	<i>Acridotheres tristis</i>					+		+	
9	<i>Saxicola torquatus</i>	+						+	
10	<i>Orthotomus sutorius</i>	+						+	
11	<i>Psilopogon haemacephalus</i>			+				+	
12	<i>Centropus sinensis</i>	+						+	
13	<i>Merops orientalis</i>	+						+	
14	<i>Corvus splendens</i>					+		+	
15	<i>Caprimulgus asiaticus</i>	+							+
16	<i>Ardeola grayii</i>						+	+	
17	<i>Copsychus fulicatus</i>	+						+	
18	<i>Coracias benghalensis</i>	+						+	
19	<i>Otus bakkamoena</i>				+				+
20	<i>Burhinus indicus</i>				+			+	
21	<i>Turdoides striata</i>	+						+	
22	<i>Prinia sylvatica</i>	+						+	
23	<i>Turdoides malcolmi</i>	+						+	
24	<i>Spilopelia senegalensis</i>					+		+	
25	<i>Lanius schach</i>					+		+	
26	<i>Clamator jacobinus</i>	+						+	
27	<i>Pycnonotus cafer</i>			+				+	
28	<i>Cecropis daurica</i>	+						+	
29	<i>Vanellus indicus</i>	+						+	
30	<i>Dendrocitta vagabunda</i>					+		+	
31	<i>Lonchura punctulata</i>		+					+	
32	<i>Euodice malabarica</i>		+					+	
33	<i>Athene brama</i>				+				+
34	<i>Dumetia hyperythra</i>	+						+	
35	<i>Hirundo smithii</i>	+						+	
36	<i>Chrysomma sinense</i>	+						+	
37	<i>Motacilla flava</i>	+						+	

+ = Present

## CONCLUSION

The findings of the present study recorded the avian mortality of 37 bird species due to road vehicle collision on Amravati to Paratwada state highway, which is passing through the agricultural landscape. Greater Coucal, Red-vented Bulbul, Spotted Owlet, Common Myna, Nightjars, Indian Roller, Dove, and Babblers were found the most affected agricultural habitats dependent bird species. But Greater Coucal was reported the most dominant species affected due to this road vehicle collision threat. The

maximum mortality of bird species was recorded during the rainy seasons and the minimum during the summer season. This report presents the first-ever detailed quantitative study on avian mortality due to road vehicle collisions on state highways passing through agricultural landscapes.

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**Data availability Statement:** All data / results / information is available with the authors and can be shared on a reasonable request made to the corresponding author when required.

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# Impact of Covid 19 and Its Relevance With Respect to Environmental Functioning

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## ABSTRACT

The world has faced a huge pandemic in the form of Coronavirus 2019 (COVID 19) started the infection from Wuhan city of China to all over the world. COVID 19 is an infectious disease induced by the presence of coronavirus-2 causing severe acute respiratory syndrome (SARS-CoV-2). This infectious disease has changed the entire life of human beings and has started to shut down all the cities in the world. The COVID 19 has shown both positive and negative shades which has impacted on human life. Many publications have been discussed on COVID 19 disease, human health and specifically on human diseases. Only limited studies have been discussed on environmental assessment with reference to different countries. In this review, the main objective is to discuss about the global impact of environment effect with the relation towards COVID 19. The objective of this review is to define the impact of COVID 19 in terms of the role of environmental effects. In spite of the financial impact of the COVID 19 epidemic on the global economy, there was a positive influence for the environment. Though as a result of global warming, the greenhouse gases CO<sub>2</sub>, and NO<sub>2</sub> are increasing in concentration. Ozone layer depletion and changes in the climate have been reported despite low levels of air pollution, water pollution, noise pollution, etc. The findings of the present review provide significant information that COVID 19 had a favorable influence on the environment by enhancing air and water quality.

**KEY WORDS:** COVID 19, SARS-COV2, ENVIRONMENT, AIR QUALITY, WATER QUALITY.

## INTRODUCTION

A novel coronavirus has been identified as the cause of severe acute respiratory syndrome. In December 2019, the coronavirus-2 (SARS-CoV-2) produced a series of acute atypical respiratory infections in Wuhan, Hubei Province, China. The virus-caused disease was termed coronavirus disease 19 (COVID-19). The virus is transmissible to humans and has caused a worldwide pandemic (Yuki et al., 2020). Pandemics, in general, are not only a serious public health issue; they often cause devastating socioeconomic and political crises in the countries that are infected. COVID-19, in addition to becoming the greatest threat to global public health of the century, is viewed as an indicator of inequity and a lack of social advancement (Chakraborty and Maity, 2020). COVID-19 is a type of pneumonia that first occurred on December 31, 2019, in Wuhan, China, and then spread

throughout the world. The spread of the virus, strict isolation measures, and delays in the start of schools, colleges, and institutions across the country are all expected to have an impact on students' mental health. The epidemic has had a psychological impact on the general public, patients, medical personnel, children, and the elderly (Pragholapati, 2020).

Globally, the number of people infected with SARS-CoV2 has increased dramatically (the etiological agent of COVID-19). COVID-19 patients can develop Acute Respiratory Distress Syndrome (ARDS), pneumonia, and organ failure. There is mounting evidence that the immunological patterns of individuals infected with viruses are closely connected with the progression of their disease. A reduction in peripheral T-cell subsets is a distinct feature of SARS patients (Yang et al., 2020).

COVID-19 is not likely to be any different from other pandemics, which have had devastating economic consequences. Worldwide, countries have implemented many public health measures to prevent COVID-19 from

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spreading further, including social distancing. Since the Spanish flu epidemic of 1918 and a flu outbreak in Mexico City in 2009. Mandavilli, (2020) claims that this method saved thousands of lives. Lockdown measures were enforced in many nations as part of the social distancing measures, which included the closure of businesses and schools as well as the prohibition of mass gatherings. Only essential travel was permitted in several countries. For this reason, these initiatives aim to limit the number of new COVID-19 cases each day in order to slow their exponential increase and alleviate the strain on health care providers (Brodeur et al., 2021).

Among the public care techniques are hand washing, face masks, physical distance, and avoiding large crowds and assemblies. Lockdowns and enforced quarantines have been implemented to slow the spread of the disease and prevent it from spreading further (Pokhrel and Chhetri, 2021). The incubation period is the time interval from the moment a virus is ingested and the onset of symptoms. It can take anything from 1-14 days for COVID-19, but it's most normal for it to take about five days. Fever, dry cough, and lethargy are all common clinical signs of COVID-19 infection. Headaches, dizziness, stomach pain, nausea, and vomiting are among the less common side effects.

The five early signs of the disease include loss of smell and taste; nausea and diarrhea; and anorexia and diarrhea, which can occur many days before the fever sets in. However, even while a fever is a crucial indicator, it may not be present in all cases. The spread of the virus has been encouraged not just by the predominance of asymptomatic infections, but also by a global lack of universal testing and personal protective equipment (PPE) for healthcare workers. The enormous surge of COVID-19-infected patients at many institutions warrants an in-depth examination of the clinical, radiographic, and laboratory findings associated with higher disease severity and mortality (Gallo Marin et al., 2021).

Numerous corona viruses are known to cause respiratory infections in humans, ranging from the common cold to more severe diseases such as the Middle East Respiratory Syndrome (MERS) and SARS. To give the virus a crown-like look, the SARS-CoV-2 particle has mushroom-shaped protein spikes projecting from its surface. The virus is able to enter the body because of the spikes' ability to connect to human cells. There is 98% sequence identity between the spike protein of the novel corona virus and the spike protein of bat coronavirus. A CoV-2 SARS-spike protein interacts to angiotensin converting enzyme 2 (ACE2), a cellular receptor that serves as an entry point into human cells. It has a binding affinity 10 to 20 times greater than SARS. Human-to-human transmission is facilitated by a higher binding affinity (Verma and Prakash, 2020).

**Prevalence of COVID-19:** As of April 6, 2022, the total number of infected COVID 19 cases worldwide had surpassed 494 million, with 6.1 million (1.25%) global deaths recorded. With 429 Million, the recovery rate was recorded to be 86.7%. The United States is the most contaminated country, with 81.9 million people infected, followed by India with 43.3 million, Brazil with 30

million, France and Germany with 26.2 and 21.9 million, respectively. Saudi Arabia is ranked 73rd, with 0.75 million cases, 1.08 mortality, and a 98% of successful recovery rate.

**Environmental issues with COVID-19:** Humans have been gradually altering nature from the dawn of civilization for their own gain. Increasing population necessitated industrialization and urbanization, which had a negative impact on the world climate. For their own selfish reasons, human-humans began to harm the natural world in countless ways through human-induced activities without regard for long-term sustainability. As a result, environmental contamination has grown to be a major problem in our time. Infectious diseases that are transmitted by vectors, such as bacterial and viral diseases, will be affected by environmental pollution. For a period spanning from a few weeks to a few months, practically every city and town in the afflicted countries is on partial or entire lockdown owing to the extraordinary outbreak of COVID-19. To prevent the spread of the disease within the community, all local and central government agencies were told to shut down educational institutions and prohibited their inhabitants from traveling outside of their homes or conducting non-essential activities (Verma, 2019).

Many religious, cultural, social, scientific, sporting, and political mass gatherings, such as Hajj and the Olympics, have been canceled. Various enterprises are shut down, and all sorts of travel are canceled. Temporarily, measures to inhibit transmission of the SARS-CoV-2, by reducing the movement have had an amazing environmental effect. Due to the failure of businesses, industrial waste creation has decreased significantly. Vehicles are rarely seen on the roads, resulting in nearly no emissions of greenhouse gases and dangerous tiny suspended particles into the environment. Because of the decreased demand for power in industry, consumption of fossil fuels or conventional energy sources has decreased considerably. Ecosystems are regenerating rapidly.

Many people in major cities are experiencing clear skies for the first time in their lives. The degree of pollution in tourism places such as woodlands, sea beaches, and hill areas is also decreasing significantly. Reviving the ozone layer has been discovered to some degree. Furthermore, environmental issues include air pollution, water pollution, climate change, ozone layer depletion, global warming, ground water depletion, biodiversity and ecosystem change, arsenic contamination, and many others. Global warming is caused by rising levels of greenhouse gases such as CO<sub>2</sub>, CH<sub>4</sub>, N<sub>2</sub>O, and others. Humans began damaging nature in many ways as a result of their desire to drive nature according to their own whims and desires. As a result, pollution in the environment has become a major issue in the modern era (Chakraborty and Maity, 2020).

Poor air quality is a leading cause of respiratory disease and a significant number of deaths around the world. Every year, 4.6 million people die from diseases associated to poor air quality. Emphysema, bronchitis, allergic rhinitis, and other respiratory and cardiovascular disorders all

contribute to the death toll from air pollution. The number of deaths caused by poor air quality outnumbers those caused by car accidents every year. Large amounts of NO<sub>2</sub> and CO<sub>2</sub> are emitted into the atmosphere by numerous manufacturing businesses and automobiles in particular. Pandemic COVID-19 has dramatically improved air quality, particularly in countries and communities that rigidly adhere to lockdown and quarantine restrictions. Near Wuhan, China, large reductions in NO<sub>2</sub> levels were first seen, and their beneficial effects were then studied in other parts of the world, where air quality improved significantly. According to NASA, NO<sub>2</sub> and CO<sub>2</sub> emissions have been lowered by 30% and 25%, respectively, in 2020 (NASA, 2020).

The lockdown has resulted in a number of positive outcomes, including less pollution and lower carbon emissions. An unavoidable positive environmental impact can be seen as industries, institutions, and economic activity come to a halt. The shutdown has improved the air and water quality in the city. In India's biggest cities, air pollution levels dropped significantly. According to the Environment Ministry's Central Pollution Control Board, NO<sub>2</sub> levels had dropped by 71%. Lockdown has had a tremendous impact on water sources like the Yamuna and Ganga rivers, as well. Ganga's water quality has improved and now stands at 27 points, making it safe for swimming and promoting wildlife and fisheries, says the Central Pollution Control Board. It is safe to say that COVID-19 has had a positive impact on the environment. However, the pandemic has resulted in an increase of bio-medical and hazardous waste on the other side of things. Furthermore, as plastics have become more widely used, so has the amount of waste that can be recycled, a trend that may cause problems in the future.

Aside from that, the government has shifted its attention away from economics and the environment and toward people. As a result, money have been allocated to areas such as healthcare and the distribution of basic necessities to the public. If government attention shifts to lowering unemployment and promoting economic activity when the pandemic is ended, the focus on the go green idea, climate change, and environmental development may be overlooked in the near future. Therefore, all countries of the governments or countries documented with high pollutions must devise an adequate plan to ensure that the interests of the people, the economy, and the environment are all properly aligned (Debata et al., 2020).

The COVID-19 epidemic demonstrates that environmental change can be achieved despite the negative impacts of social distancing. The drop in industrial activity and refineries, as well as the reduction in automobile and transportation system use, are all contributing to a decrease in greenhouse gas emissions. A number of cities in Asia, Europe, and the United States have reported lower levels of air pollution, particularly with NO<sub>2</sub>, particulate matter, and black carbon. As a result, there was a decrease in PM10 (-28 to -31.0%) and an increase in O<sub>3</sub> (50%) concentrations. There has been a dramatic reduction in air pollution in major cities throughout the world reported by NASA satellites and the Copernicus Atmosphere Monitoring Service of the European Space Agency. A two-month improvement

in air quality in China alone is expected to save the lives of tens of thousands of children and the elderly. Pollution reductions in the world's most populous cities could have a big impact on health.

The positive environmental effects of COVID 19 were magical and illusory, but there was a counterweight. For large cities, the Covid-19 epidemic will have a long-term detrimental impact on the economy, restricting pollution and the release of greenhouse gasses and particles linked to respiratory sickness (SanJuan-Reyes et al., 2021).

There is a strong association between the number of cases of COVID-19 in Turkey and daily temperature. In other words, the larger the proportion of COVID-19 cases on a given day, the lower the temperature. Temperature average (°C) in DKI Jakarta was also strongly associated with COVID-19. Salé City in Morocco, COVID-19 has resulted in lower levels of PM10, SO<sub>2</sub>, and NO<sub>2</sub>. The economic consequences of the lockdown policy will be significant. The economic impact of the COVID-19 epidemic must be assessed by policymakers in order to make informed decisions. Because of overpopulation, public health issues, and interactions with wild animals in poor countries, diseases like COVID-19 can kill people of any socioeconomic status in any civilization (Caraka et al., 2020). The fact that SARS-CoV-2 appears to exhibit tropism for a variety of organs, including the respiratory system, brain, endothelium, heart, kidney, and liver, highlights the difficulty in forecasting the severity of COVID-19 disease. COVID-19 complication risk factors must be identified in order to improve patient outcomes and allocate precious resources. (Gallo Marin et al., 2021).

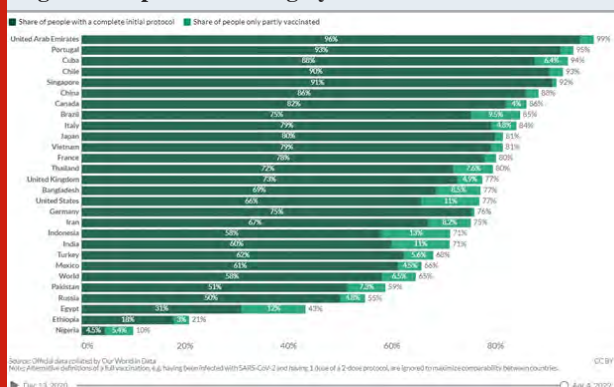
Stay-at-home and preventive measures have had an unsettling influence on waste management, despite the fact that the COVID-19 epidemic has been claimed to reduce air pollution and environmental-related noise and increase biodiversity and tourist sites. Gloves, gowns, masks and other PPE have piled up due to the unusually high amount of garbage being generated by both households and health care facilities. Secondary transmission of COVID-19 might occur if health facilities and households do not properly dispose of their trash. Air quality and health effects could be negatively impacted by the possible rampant dumping, open burning, or incineration (Sarkodie and Owusu, 2021).

**Vaccination:** Vaccination is a key component in the combat against the COVID-19 pandemic. Vaccine candidates with promising findings were quickly authorized by drug authorities to be used in an emergency. Vaccine reluctance has been a hindrance despite the rapid and coordinated vaccination initiatives launched by governments around the world. The World Health Organization (WHO) has ranked vaccine hesitancy as one of the top 10 concerns to world health for 2019. "Delay or refusal to accept or refuse vaccination despite the availability of vaccination services," as defined by the strategic advisory group on immunization, is the result of an intricate interplay between factors such as time of day and location as well as vaccine specificity. To put it another way, vaccines were broadly accepted in non-high-income countries (defined by the World Bank as countries with a 2019 Gross National Income per capita of

US\$12,536 or more) according to the 2018 Welcome Global Monitor poll. When it comes to the COVID-19 vaccination, individuals in low- and middle-income nations and regions like Nepal (97%) are more likely to accept it than those in high-income countries and regions like the United States (6%) (Aw et al., 2021).

Globally, 11.33 billion people were vaccinated, with Asia accounting for 7.77 billion. A total of 4.99 billion people was vaccinated from upper middle-income countries, and 3.82 billion from lower middle-income countries. The United Arab Emirates leads the way with 99% of vaccinations completed successfully, followed by Portugal (95%), Cuba (94%), Chile (94%), Singapore (92%), China (92%), Canada (82%), Brazil (79%), Italy (79%), Japan (78%), Vietnam (78%), France (76%), Thailand (75%), United Kingdom (75%), Bangladesh (73%), United States (68%), Germany (67%), Iran (67%), Indonesia (67%), India (67%), Turkey (65%), Mexico (61%), World (55%), Pakistan (51%), Russia (33%), Egypt (18%), Ethiopia (15%), and Nigeria (10%) (Aw et al., 2021).

**Figure 1: Top 5 listed as highly vaccinated countries**



## CONCLUSION

Based on this review, the conclusion drafted is that there is a huge loss reported with COVID 19 in the form of financial crisis, increase in garbage disposal, biomedical and hazardous waste, but there is a gain in the point of environmental quality such as improved air quality, water quality, lowered noise and water pollution, climate amendments, and ozone layer depletion. Majorly and most importantly, the identification and confirmation of variables that predict COVID-19 disease development is critical to improving health outcomes. Age, comorbidities, immunological response, imaging findings, laboratory markers, and signs of organ failure all have the potential to predict worse outcomes when taken separately or in combination. The fact that SARS-CoV-2 appears to exhibit tropism for a variety of organs, including the respiratory system, brain, endothelium, heart, kidney, and liver, highlights the difficulty in forecasting the severity of COVID-19 disease. COVID-19 complication risk factors must be identified in order to improve patient outcomes and allocate precious resources.

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# Optimizing Balance Using Vestibular Electrical Stimulation to Study its Therapeutic Effect Among Elderly

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## ABSTRACT

The noisy Galvanic vestibular stimulation (nGVS) is established to be a assuring tool to enhance vestibular functioning. Deterioration in vestibular functioning in the geriatric population results in reduced capacity to identify weakened signal which may result into reduction in balance and ultimately fall. Postural sway is produced when nGVS is given over mastoid process. In present study our aim is to find out whether nGVS can be utilized to maximize the outcome of balance training programme among elderly individuals. Community dwelling elderly (N=150, age 65.67±3.4 yrs) were randomly recruited to a control group (Group A, n=50, age 65.54±3.4 yrs), Placebo group (Group B, n=50, age 65.5±3.3 yrs) and a treatment group (Group C, n=50, age 65.98±3.5 yrs). No intervention was provided to Control group while placebo stimulation was given to group B along with balance training exercises and group C was provided with noisy galvanic vestibular stimulation along with balance training exercises. Pre, mid and post data were recorded on Berg Balance Scale (BBS) for balance and Tinetti's fall risk scale for risk of fall and analyzed. Compared to control group there was a significant improvement in balance and reduction in risk of fall in placebo and treatment groups. Significant difference was found in treatment group in comparison with placebo group for both, BBS and Tinetti's fall risk scale. In treatment group early changes in Balance and risk of fall was observed while similar outcomes were not obtained in control and placebo groups. The findings of this study suggests that nGVS can be chosen to optimize the therapeutic efficacy of balance training exercises clinically.

**KEY WORDS:** BALANCE, ELDERLY, GALVANIC VESTIBULAR STIMULATION, REHABILITATION & RISK OF FALL.

## INTRODUCTION

One of the greatest health concern among the people aging above 60 years is fall. Significant increase in number of episode of fall has been recorded with advancing age in both genders among all races (Tinetti and Kumar 2010; Thomas et al. 2019a). Fall and related injuries are among the leading cause of decreased ability to care for oneself, functional decline and greater dependence. Fall and related injuries are also found to be associated with prolonged hospitalization which add extra financial burden (WHO 2021).

Multiple intrinsic factors have been classified as a cause of imbalance, resulting in fall among geriatric population. Elderly peoples are prone to have multiple disease associated with vision, hearing, strength and proprioception (Verghese et al. 2006; Henry and Baudry 2019). Vestibular system and its roll in providing a key sensory inputs to stabilize the posture in variety of static and dynamic positions is very well understood. Specialised nerve endings in the vestibular

canals detects the head position as well as movement and send signals to the area of brain responsible for processing, planning and coordinating with motor area for anticipatory action (Heuninckx et al. 2008; Mouthon et al. 2018; Ko et al. 2020; Anson and Jeka 2016; Coto et al. 2021).

Significant age associated degeneration has been seen in almost all type of vestibular structure including nerve results into the altered sensitivity (Anson and Jeka 2016; Coto et al. 2021). Other than physical therapy, no effective therapeutic techniques for vestibular system dysfunction have been found to yet (Fujimoto et al. 2016). However, Noisy Galvanic Vestibular Stimulation nGVS has lately demonstrated some promise in this area. Noisy Galvanic Vestibular Stimulation (nGVS) is a technique in which a small electrical current is delivered through electrodes put over the mastoids to stimulate the vestibular afferents nerve (Keywan et al. 2020; Coto et al. 2021).

The vestibular organ is stimulated with a mild noise current in this treatment, which has been found to improve vestibular perception and vestibulo-spinal reflex function. nGVS has been demonstrated to increase cognition in healthy people,

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enhance motor responsiveness in patients presenting neurodegenerative illnesses, and improve gait metrics and static balance in patients with vestibular abnormalities in earlier investigations (Keywan et al. 2018; Wuehr et al. 2018). In a study it was observed that bipolar binaural GVS to vestibular afferents (anode to the left and cathode to the right) stimulated network of the right hemisphere only while reversing the polarity resulted into bilateral activation (Coats 1972; Coats 1972; Utz et al. 2010). Sensitivity of the vestibular system to normal vestibular inputs can be increased by adding noise to subthreshold stochastic vestibular stimulation (Chen et al. 2021). This increase in the sensitivity is important for postural stability in more challenging situations. In Humans 0.1-4 mA direct current is used to activate the vestibular afferents resulting in standing subject to lean in different directions depending upon the polarity of the electrodes. If this lean can be utilized upon combining with exercise is not yet studied (Nepveu et al. 2020; Chen et al. 2021).

Multiple evidences are available in support of the activation of otolith system and increase in sympathetic activity after GVS. A binaural application of sinusoidal variant of GVS has shown significant sympathetic nerve activity in lower limb muscles (Hammam and Macefield 2017). The low-frequency changes in vestibular input associated with postural changes, preferentially modify Muscle Sympathetic Nerve Activity (MSNA) (Grewal et al. 2009; Morita et al. 2020). Increase in peripheral vasoconstriction due to MSNA helps to maintain sufficient vascular supply to brain during upright position and this response could be associated with the Otolith system activation (Chen et al. 2021).

Another study suggestive of correlation between vestibular function and sympathetic nerve activity clearly demonstrated the increase in MSNA and calf muscle vascular resistance during head down rotation in prone position (Ray et al. 2002). nGVS can also alters visuomotor activity and motor circuit functioning. This sensorimotor integration and performance might be associated to change in oscillation related to processing of information and error. It has been seen that more erect posture is maintained after mechanical perturbation when appropriate galvanic current is given over the mastoid process (Ap et al. 2001; Lee et al. 2015). If alteration in sympathetic response of vestibular system (achieved thru stimulation) could be integrated along with voluntary control of posture then better postural control can be expected which might reduce the risk of fall. So far none of the study tried to fill this gap of integrating the nGVS to voluntary motor control for better outcome (Mitsutake et al. 2020). The aim of our study was to explore the effect of nGVS among elderly on balance and risk of fall by performing a randomized control trial and to elicit out whether nGVS can be chosen to augment the therapeutic efficacy of balance training exercises clinically among elderly individuals.

## MATERIAL AND METHODS

In this study, a repeated measure design and randomized controlled trial were used. A recruitment of total of 150 subjects were done and each were assigned randomly to

three groups at various Physiotherapy Centers in Noida. Individuals between 60 and 75 years of age, who could walk independently in the community, perform balancing tests without assistance and take part in several balancing exercise sessions. Our study excluded elderly with a history of any type of orthopaedic surgery in lower extremity, cognitive disorders, on psychoactive medications over the past six months, people with progressive neurological conditions that might have a serious effect on balance and gait, orthostatic hypotension, unstable medical conditions, uncontrolled diabetes, hearing loss, history of vertigo/tinnitus/fall in past twelve months, vision-less than 6/6 in either eye (even if not 6/6 in either eye) and patient with high risk of fall (Berg Balance Score <21, Timed up and Go >14 Sec and Tinetti <19).

A total of 546 volunteers were screened for recruitment in this study initially, of which 150 met the inclusion criteria. The first group was a control group of 50 subjects, 48 (2 drop-outs) were retested at 3rd and 6th-week intervals. Placebo Group B was allocated to 50 subjects who received sham stimulation together with balance training and to 50 other subjects in Group C, receiving (nGVS) along with balance training. A structured interview was conducted to gather socio-demographic information, including age and race. After the group allocation all the subjects carried out with pre, mid and post evaluation on Berg Balance Scale (BBS) and Tinetti's Fall Risk scale for stability and risk of fall assessment. BBS is a five pointer scale intend to quantitatively assess the balance in older population. BBS Score less than 41 out of 56 is considered as moderate and less than 21 as severely effected balance. Tinetti's fall risk scale is three pointer scale to assess risk of fall in elderly. Tinetti.s score less than 19 out of 28 indicate high risk of fall. Average assessment time was 45 minute including five minute of rest in between. There were no problem encountered while giving the balancing exercises and nGVS.

A 6-week program of active muscle stretching, endurance Walking, posture control and muscle coordination exercises were given in the group B and C interventions. Exercises began at a low intensity level and progressed slowly. The actual frequency, repetition and resistance of the exercises were modified following the individual ratings of the perceived exercise (equivalent to 11 Borg perceived exercise scale ratings) (Hunter et al. 2020). The follow-up training was conducted at a moderate intensity level (equivalent to 13 Borg perceived exercise scale ratings). These balance exercises were practiced thrice a week for the whole study duration. Borg rating of perceived exertion (RPE) is used to study the perceived stress during any physical activity (pulse, breathing and excessive sweating); based upon maximum exertion of 20 and minimum of 6 points (12-14 considered as moderate intensity) (Paiva et al. 2019; Hunter et al. 2020).

Group C Participants additionally received bipolar binaural (left-cathodic / right-anodic) noisy Galvanic vestibular stimulation (nGVS) of subliminal intensity for 20 minutes during Exercise training session. A wet lint was placed over the mastoid while doing vestibular stimulation. All

statistical analysis of the Berg Balance score and Tinetti's Fall risk score performed with SAS version 9.4 software. The assumptions of normality are based on the Shapiro-Wilk test. All the individual data parameters have been tested for normality, and the test variables follow a normal population distribution of multiple variables (as assumed by the ANOVA Repeated Measures (RMA) as  $n \geq 25$ ). For SAS programming, PROC MIXED was considered to generate results based on residual maximum likelihood (REML) considerations.

The REPEATED statement in PROC MIXED enables the estimation and testing of repeated measurement models with an arbitrary correlation structure for repeated observations. We have 50 subjects in Group-B and Group-C, and we have 48 subjects in Group-A, which has led to unbalanced data and may not turn to symmetric compounds. Since we have only two parameters for each group and visit, this may not lead to an intense computational matrix. Intra-class correlations are generated from the same model between 14 each difference in treatment with-in and between treatment groups to test the reliability of the results.

Summary of demographic statistics presented based on descriptive statistics N, Mean and Standard deviation

Table 1. Demographics Summary					
		Control (n=50)	Placebo (n=50)	Treatment (n=50)	Total (n=150)
Age (Years)	Mean (SD)	65.54 (3.436)	65.50 (3.388)	65.98 (3.485)	65.67 (3.420)
Gender					
Male	n (%)	27 (54.0%)	27 (54.0%)	26 (52.0%)	80 (53.3%)
Female	n (%)	23 (46.0%)	23 (46.0%)	24 (48.0%)	70 (46.7%)
Height (mts)	Mean (SD)	1.65 (0.051)	1.67 (0.049)	1.66 (0.051)	1.66 (0.050)
Weight (kgs)	Mean (SD)	71.95 (3.081)	72.23 (3.303)	71.24 (4.917)	71.81 (3.852)
BMI	Mean (SD)	26.44 (1.685)	26.01 (1.319)	25.99 (1.321)	26.15 (1.457)

The Least squares Mean values for BBS score, test for Treatment and Placebo arms shows a good improvement from Day-1 to Day 21 and to Day-42 in both, which explains an improvement from baseline to the end visit. Like wise with tinetti's assessments subjects from "Moderate Risk of Falls" shifted to "Low Risk of Falls" by Day42. The BBS score improved by 3.1 points from Day 1 to Day 21 and 3.7 points from Day 21 to Day 42 indicating a gain of nearly 7 points in the Treatment group. While in the placebo group a gain of 3.2 points was observed by Day 42, and with no change in the control group.

The treatment contrast based on LS Mean Estimate (Standard Error) is -6.940 (0.185) with 95% CI of [-7.31;-6.57], at Day 42 from Day-1. LS mean is higher in Day 42 with LS Mean Estimate (Standard Error) is 25.160 (0.124) with 95% CI of [24.91;25.41], and thus there is a 3.9 increase in Tinetti's score at Day 42 from Day-1. The test is statistically significant as P-value is less than 0.05 ( $p < 0.0001$ ). For follow-up comparisons between pairs of time points, the

of three quartiles (Q1, Q2 and Q3) with minimum and maximum values. All the groups were presented as Control, Placebo and Treatment for Group A, Group B and Group C. All statistical analysis were performed at 95% confidence interval and alpha at 5% acceptance. This study was accepted at Amity University in Noida, Uttar Pradesh India by the Institutional Ethical Committee. All subjects were fully informed about the nature of the research and signed informed consent. The interests of all subjects were secured.

## RESULTS AND DISCUSSION

In this study, we assessed the effect of vestibular stimulation on Balance and risk of fall among elderly subjects, randomized into experimental, control and placebo groups. We measured change in balance with Berg Balance scale and change in risk of fall with Tinetti's fall risk scale. All subjects of both gender included in this study were between 60 to 74 years. The demographics of the subject are summarized in Table 1: age, gender, height, weight and BMI. The age and gender match was done among all the groups.

Tukey's-Kramer method is considered for the adjustment and it also shows statistical significance ( $p < 0.001$ ). The treatment and placebo effect show statistical significance ( $P < 0.05$ ) and thus we reject the null hypothesis and can say there is a significant difference for both Treatment and placebo groups within the visits (Chen et al. 2021).

A table 2 and table 3 shows the evaluation of BBS and Tinetti's fall risk score respectively for the three groups. The Pre-Post difference is high in Placebo group with respect to control group. The finding of this study is like those of many researchers who have identified lower limb strength and balance training as effective ways to clinically reduce the risk of falling. Almost all research that look at the risk of falling among the elderly concluded that physical activity, including leisure exercises, are efficient and productive ways to restore balance and prevent falls (Thomas et al. 2019b). Results obtained in the treatment group indicate the added improvement of balance and reducing risk of fall up on combining galvanic stimulation to the existing

balance training program. Since the balance is directly associated postural sway which is a function of vestibular system (Chen et al. 2021).

This finding is in support of those studies who reported improvement in vestibular function after giving nGVS. As patients with vestibular problems have longer postural sway route lengths and mean velocities, the data suggest that increased vestibular afferent function may have contributed to the reduced postural sway seen in this investigation (Talebi et al. 2016). The reduction of postural sway during nGVS may be due to the activation of cortical areas involved

in multimodal input, including vestibular information (Piccolo et al. 2020; Chen et al. 2021). Interestingly we found increasing improvement over the whole duration of treatment nearing to the clinically significant levels in contrast to the study who found no improvement in balance after administering nGVS. The clinical improvement of balance in our study can be understood as we have used subliminal intensity induces imperceptible vestibular stimulation along with voluntary motor task in contrast to the higher intensity of unpleasant perception delivered for short duration before the functional exercises (Hassan et al. 2021).

**Table 2. Pre, Mid and Post by shift difference - within Group – Berg Balance Score**

	Estimate	SE	95% CI	P-value
Treatment With Group Difference				
Pre-Mid vs. Mid-Post Difference	0.580	0.177	[ 0.23; 0.93]	0.0015
Mid-Post vs. Pre-Post Difference	3.760	0.192	[ 3.38; 4.14]	<0.0001
Pre-Mid vs. Pre-Post Difference	3.180	0.177	[ 2.83; 3.53]	<0.0001
Placebo With Group Difference				
Pre-Mid vs. Mid-Post Difference	2.160	0.100	[ 1.96; 2.36]	<0.0001
Mid-Post vs. Pre-Post Difference	2.680	0.131	[ 2.42; 2.94]	<0.0001
Pre-Mid vs. Pre-Post Difference	0.520	0.100	[ 0.32; 0.72]	<0.0001
Control With Group Difference				
Pre-Mid vs. Mid-Post Difference	-0.021	0.047	[-0.11; 0.07]	0.6588
Mid-Post vs. Pre-Post Difference	-0.083	0.056	[-0.19; 0.03]	0.1370
Pre-Mid vs. Pre-Post Difference	-0.063	0.047	[-0.16; 0.03]	0.1872

**Table 3. Pre, Mid and Post by shift difference - within Group – Tinetti's Fall Risk Scale**

	Estimate	SE	95% CI	P-value
Treatment With Group Difference				
Pre-Mid vs. Mid-Post Difference	-0.220	0.127	[-0.47; 0.03]	0.0867
Mid-Post vs. Pre-Post Difference	1.840	0.138	[ 1.57; 2.11]	<0.0001
Pre-Mid vs. Pre-Post Difference	2.060	0.127	[ 1.81; 2.31]	<0.0001
Placebo With Group Difference				
Pre-Mid vs. Mid-Post Difference	1.900	0.084	[ 1.73; 2.07]	<0.0001
Mid-Post vs. Pre-Post Difference	2.060	0.105	[ 1.85; 2.27]	<0.0001
Pre-Mid vs. Pre-Post Difference	0.160	0.084	[-0.01; 0.33]	0.0600
Control With Group Difference				
Pre-Mid vs. Mid-Post Difference	-0.042	0.033	[-0.11; 0.02]	0.2052
Mid-Post vs. Pre-Post Difference	-0.063	0.040	[-0.14; 0.02]	0.1218
Pre-Mid vs. Pre-Post Difference	-0.021	0.033	[-0.09; 0.04]	0.5251

Furthermore, GVS-induced more afferent vestibular excitement can activate brain areas related to multisensory input (areas 2, 3a/b, and 7, as well as the parieto-insula vestibular cortex) via delivering direct current through the vestibular nuclei in the brainstem and vestibular thalamus (Inukai et al. 2018). GVS with an alternating current can

also activate parts of the brain that interpret vestibular information for head and body positioning in space (i.e. the supramarginal gyrus, posterolateral thalamus, cerebellar vermis, posterior insula and hippocampus). Stimulating brain areas along with peripheral voluntary contraction during exercise has been offered as a possible explanation



(Ferreira et al. 2019; Helmchen et al. 2020; Chen et al. 2021).

Findings (Table 4 and 5) of this study suggest the consistent and added improvement during two halves of the study period in the treatment group indicate no adaptation and carry over effect as we have used small, repeated session considering neural adaptation as in previous studies relatively negligible difference in effects were found after vestibular stimulation for longer duration of three hours compared to thirty minutes (Fujimoto et al. 2016; McLaren et al. 2021). Appropriateness of nGVS for repetitive treatment sessions for bringing change in balance can be understood due to long lasting effects, non-invasion and absence of adverse effects. Though this study does not

determine that small duration of stimulation can bring the optimal therapeutic effects.

But we advocate the further exploration of factors which helps to identify appropriate duration to bring lasting effects among various subject population and with various vestibular disorders. No difference in the first half of study duration among control and placebo group indicating balance training exercise alone is insufficient to bring early detectable change in balance. This could be explained as lack of cortical excitability during balance training exercise may delay the changes to reflect clinically. Cortical excitability achieved in treatment group resulted in enhancement in motor evoked potential targeting the lower limb muscles elicited resting motor threshold could be the possible explanation (Fleming et al. 2018; Kudo et al. 2022).

**Table 4: Between the groups shift difference – Treatment estimates – Berg Balance Score**

	Estimate	SE	95% CI	P-value
Between Treatment group effects				
Treatment Pre-Mid vs Placebo Pre-Mid	-2.660	0.158	[-2.97;-2.35]	<0.0001
Treatment Pre-Mid vs Control Pre-Mid	-3.239	0.162	[-3.56;-2.92]	<0.0001
Placebo Pre-Mid vs Control Pre-Mid	-0.579	0.160	[-0.89;-0.26]	0.0003
Treatment Mid-Post vs Placebo Mid-Post	-1.080	0.158	[-1.39;-0.77]	<0.0001
Treatment Mid-Post vs Control Mid-Post	-3.842	0.162	[-4.16;-3.52]	<0.0001
Placebo Mid-Post vs Control Mid-Post	-2.762	0.160	[-3.08;-2.45]	<0.0001
Treatment Pre-Post vs Placebo Pre-Post	-3.740	0.158	[-4.05;-3.43]	<0.0001
Treatment Pre-Post vs Control Pre-Post	-7.085	0.162	[-7.40;-6.77]	<0.0001
Placebo Pre-Post vs Control Pre-Post	-3.345	0.160	[-3.66;-3.03]	<0.0001

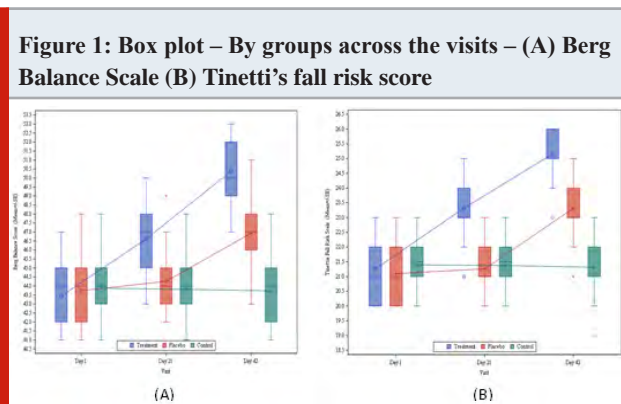
**Table 5. Between the groups shift difference – Treatment estimates – Tinetti's Fall Risk Scale**

	Estimate	SE	95% CI	P-value
Between Group Difference				
Treatment Pre-Mid vs Placebo Pre-Mid	-1.900	0.112	[-2.12;-1.68]	<0.0001
Treatment Pre-Mid vs Control Pre-Mid	-2.089	0.114	[-2.31;-1.86]	<0.0001
Placebo Pre-Mid vs Control Pre-Mid	-0.189	0.113	[-0.41; 0.03]	0.0959
Treatment Mid-Post vs Placebo Mid-Post	0.220	0.112	[-0.00; 0.44]	0.0503
Treatment Mid-Post vs Control Mid-Post	-1.905	0.114	[-2.13;-1.68]	<0.0001
Placebo Mid-Post vs Control Mid-Post	-2.125	0.113	[-2.35;-1.90]	<0.0001
Treatment Pre-Post vs Placebo Pre-Post	-1.680	0.112	[-1.90;-1.46]	<0.0001
Treatment Pre-Post vs Control Pre-Post	-3.984	0.114	[-4.21;-3.76]	<0.0001
Placebo Pre-Post vs Control Pre-Post	-2.304	0.113	[-2.53;-2.08]	<0.0001

We observed (Fig. 2) early improvement upon adding noise to the stimulation which is further supported by the findings of other studies who reported Stochastic resonance, a process in which a signal that is too weak to exceed a specific threshold is amplified by adding noise, is thought to be the reason for these ameliorating effects of nGVS (M et al. 2017). The sensory system's information processing

appears to be aided by stochastic resonance. Processing of subthreshold signals is augmented by lowering the vestibular detection threshold upon adding noise to GVS (Wuehr et al. 2018). Identification and processing of the subthreshold signal which helps to modulate MSNA can result into the motor firing in the muscles of the lower limb (Fleming et al. 2018; Kudo et al. 2022).

If this motor recruitment is integrated with exercises including static and dynamic voluntary control of different posture and repeated regularly, it could have resulted into the extensive neuroplasticity in the vestibular system. Stimulating Vestibular end organs excite the ipsilateral extensor motor neuron and inhibit the reciprocal flexor motor neuron thru vestibular nuclei via lateral vestibospinal tract (Puyal et al. 2003). The signal from vestibular nuclei then passes to the vestibular thalamus resulting in recruitment of brain areas associated with multisensory input (Utz et al. 2010). Activation of brain areas associated with vestibulospinal relay and sensory vestibular input might have resulted into the improvement in the balance during various task involved in balance assessment. A study on animal model demonstrate that the stimulating vestibular neuron can induce long term potentiation (LTP) and long term depression (LTD) of vestibular nuclei field potential (Grassi and Pettorossi, 2001; Smith et al. 2020; Kudo et al. 2022).



In general there are multiple evidences available which establish the benefits of nGVS for improving balance and reducing risk of fall, but exact mechanism is still lacking a strong evidence. Due to the complexity of the functioning of the vestibular organ, sometime it is also thought to be the involvement of multiple mechanism. Evidence for modulation of vestibular hair cell activity is available but further what frequency and intensity is appropriate is still yet to know.

Exercises included in the groups were advised based upon the rate of perceived exertion which was variable for individuals considering age and no prior involvement in any exercise regime, but it is important to explore further in future about the effects of nGVS keeping exercises regime constant for all or selecting the subjects with similar level of physical activity at the stage of inclusion. Although various methods of delivering galvanic stimulation is used by different researchers, we considered transcranial delivery of current to be more appropriate due to non invasive and no side effects, but consensus is lacking which method is most effective. No episode of fall had been reported during the study period (Steinhardt and Fridman 2021; Kudo et al. 2022).

## CONCLUSION

The findings of this study suggest that vestibular electrical

stimulation can significantly improve balance and reduce risk of fall among elderly individual. We can also conclude from the findings of this study that vestibular electrical stimulation also brings the early improvement in balance and can be used as therapeutic tool among elderly with impaired balance. Findings suggest that the improvement in the balance and reduction in risk of fall is not a placebo effect of vestibular stimulation. The finding of this study may be useful in further exploring what frequency, intensity and duration is appropriate to have optimal benefit.

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

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**Data Availability Statement:** The database generated and/or analysed during the current study are not publicly available due to privacy, but are available from the corresponding author on reasonable request.

**Author Ethical Statement:** This study was approved at Amity University in Noida, Uttar Pradesh by the University Ethics Committee on 25 July 2016. All subjects were fully informed about the nature of the research and signed informed consent. The interests of all subjects were secured.

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# Defluoridation of Groundwater with the Help of *Azadirachta indica* leaves as Bioadsorbent in Korba, Chhattisgarh, India

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## ABSTRACT

In this paper, we have used a removal technique of fluoride from groundwater in Korba district, Chhattisgarh, using thermally activated neem (*Azadirachta indica*) leaves as adsorbents. For this purpose, we collected the groundwater sample in January–March 2021. The Ion-Selective Electrode (ISE) technique was used to assess the fluoride concentration in groundwater samples. Neem leaves were efficient at removing fluoride in this study. Fluoride has a split personality in the human system, having a damaging impact when fluoride concentration is more than 1.5 mg/L, causing dental and skeletal fluorosis, and a positive effect when concentration is less than 1.0 mg/L, causing caries preclusion, and health promotion. This small project provides the outcomes of a study on neem leaf powder for water defluoridation. The analysis here discusses the applicability of inexpensive leaf adsorbents for successfully remediating fluoride contaminated water: contact time, pH, and adsorbent concentration all influence fluoride ion sorption effectiveness. The effects of treated leaf powder on pH, adsorbent dose, and contact time with aqueous solutions containing 2.28–10.04 mg/L fluoride ions were investigated. Fluoride adsorption is most substantial at pH 2. Fluoride removal diminishes dramatically when the pH exceeds 2. At adsorbent doses of 10 g/L, the necessary time for fluoride ion adsorption equilibrium is 120 minutes, and the highest removal efficiency attained was 85%, during that amount of adsorbent was 12 g/L. This research also discusses fluoride's adsorption isotherm and kinetics by activated neem leaf powder.

**KEY WORDS:** ADSORPTION, AZADIRACHTA INDICA, BIOADSORBENT, DEFLUORIDATION, REMOVAL EFFICIENCY.

## INTRODUCTION

Fluoride ions are negatively charged and are found in various minerals and may be in water and soil (Tolkou et al. 2021). Fluoride in ordinary water has developed into a severe anxiety in many parts of the world for humankind, and it should be within 0.6 – 1.5 mg/l (Patil et al. 2015; Prasad et al. 2021). India is one of the countries with many people affected by fluorosis due to drinking groundwater. Dental and skeletal fluorosis, neurotoxicity, endocrine impacts, and attention deficit hyperactivity disorder are among the side consequences of excessive fluoride exposure (Akafu et al. 2019; Kashyap and Ghosh 2021).

Because the difference between the intended and unwanted fluoride dose is so tiny, it's critical to monitor and consider the quality of drinking water and, when required, eliminate

excess fluoride from water to safeguard human health (Premathilaka and Liyanagedera 2019). The National Research Council of the United States evaluated EPA fluoride standards in (2006), They concluded that fluoride has direct and indirect effects on the brain, that excessive fluoride levels in water is also neurotoxic, for which a lot of analysis is required (Philippe Grandjean 2019). Although groundwater provides protected potable water for billions of people worldwide, there are rare instances where pollution levels are too high, and groundwater cannot be utilized for potable purposes without treatment. Fluoride poisoning of drinking water is a significant environmental hazard that affects much of the world's populace (Gronwall and Danert 2020; Kashyap and Ghosh 2021).

Fluoride concentrations in the water are so high in nations like India, Ghana, China, Pakistan, Bangladesh, and Tanzania (Yadav et al. 2019; Nde-Tchoupe 2019) that hundreds of thousands of people suffer from fluoride-related diseases. Fluoride concentrations in the water are so high that thousands of individuals are affected by fluoride-related

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conditions. Effective fluoride exclusion in these areas is a complex and fascinating undertaking due to chemical hurdles preventing fluoride from being successfully sorbed by most traditional adsorbents utilized in the drinking water industry (Rasool et al. 2018; Yadav et al. 2019). In addition, a lack of required infrastructure and technological know-how plays a crucial role. On the other hand, China and India are putting up significant effort to resolve the issue, and the situation is slowly improving (Wang et al. 2019; Kisku and Sahu 2019; Kashyap and Ghosh 2021).

Consequently, fluoride exclusion from drinking water has attracted a lot of interest in recent years, with many methods being explored in labs or used in the field (Yadav et al. 2018; Tolkou et al. 2019). Bio-remediation is the most common technology used to eradicate fluoride from drinking water. Adsorption methods are becoming more popular due to their ease of use and extensive selection of adsorbents. With varying degrees of success, research has been conducted on various types of low-cost adsorbents (Kanaujia et al. 2018; Kumar 2019; Yadav and Bhattacharya 2020). Plant-based bioremediation to enhance water quality has become a hot topic of research. For defluoridation, several plant materials such as coconut shells, tamarind seeds, neem leaves, and neem bark have been utilized as adsorbents. In the treatment of water, activated carbon is frequently utilized. For the cleaning of polluted water, bioremediation is widely regarded as a cost-effective and environmentally beneficial solution. This research aims to scrutinize the usage of neem tree leaf powder for groundwater Defluoridation (Mihavo et al. 2021).

**Figure 1: Location of Korba district in Chhattisgarh ([www.korbadistmapimage.com](http://www.korbadistmapimage.com))**



## MATERIAL AND METHODS

The Korba district in Chhattisgarh's coal-based thermal power center from 22°02'50" to 23°01'20" N and from 82°07'20" to 83°07'50" E. The study area in different areas is full of natural possessions (Singha et al. 2019). Coal is the largest resource in the field of research. Talchir, Karaharbhari, Barakar, and Kamthi formations are characteristic of the Gondwana rock supergroup. The construction of the Talchir consists mainly of shale, sandstone, and a boulder bed. The formation of Karaharbhari is made up of subgraywacke, sandstone, and gallstone (Singha and Pasupuleti 2020). The appearance of Barakar includes arcose sandstone, shale, and carbon seam. Kamthi formation consists of coarse ferruginous carbonated sandstone with extremely fine coal

seams, whereas the CGC consists of undifferentiated granite and metamorphic litho units (Singha et al. 2019; Singha and Pasupuleti 2020).

**Table 1. Fluoride Concentration in selected groundwater samples of Korba district in mg/L**

SN	Sample spot and Sample code	Fluoride Concentration in groundwater samples in mg/L
1	Tanera (S1)	2.28
2	Fulsar (S2)	4.52
3	Aamatikra (S3)	6.0
4	Aamatikra basti (S4)	7.46
5	Pondikala (S5)	8.18
6	Pondikala basti (S6)	10.04

**Table 2. The significance of the initial adsorbate concentration, Operating order for the consequence of initial adsorbate concentration, Adsorbent dose - 10 g/L, Temperature was adjusted 27°C ± 0.5°C, Time of contact - 120 Minutes, Volume of sample - 100 ml, and pH 2.**

SN	Ci (mg/L)	Ce (mg/L)	Elimination % of F-
1	2.28	0.04	98
2	4.52	0.36	92
3	6.0	0.90	85
4	7.46	1.49	80
5	8.18	1.75	80
6	10.04	2.20	78

For the sampling of Groundwater and materials, borewells and hand pumps gathered field samples in various places around the Korba district. At least 1000 ml of each sample was acquired in cleaned plastic cans or bottles and kept at room temperature in the lab for analysis. Six field samples were obtained. For this study, groundwater samples were collected from the highly fluoridated zone of the Korba district. This study was conducted from January to March 2021. This device has the ISE and meter (Thermo Scientific, Orion Star A214), TISAB-III, and a fluoride standard of 100 mg/L. In the fluoride analysis, plastic goods were employed.

For the preparation of adsorbent, the leaves of the neem tree (*Azadirachta indica*) were exploited as a low-cost natural adsorbent. These were found in Naya Raipur, India, behind the Kalinga University Campus. The neem leaves were dried, crushed, and thoroughly washed with double distilled water. They were dried for 24 hours in a hot air oven at 60–90 °C, following which the dried material was crushed in a jaw crusher and screened at ASTM 50 m mesh. For the alkali treatment, a leaf biomass fine particle sample (40 gm) and 400 ml of 0.5 N NaOH was mixed in a 1000

ml round bottom conical flask. The liquid was progressively heated on the burner for 20-25 minutes after it began to boil. The treated biomass was rinsed with double distilled water until the color was gone entirely, and very clear water was generated.

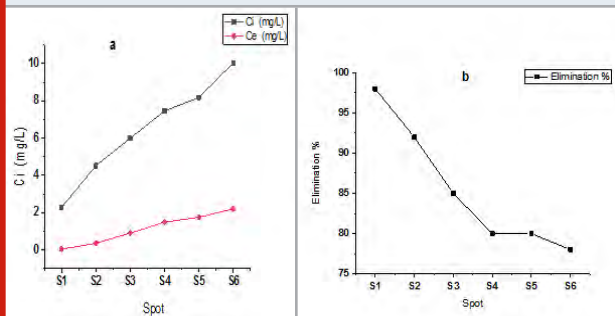
## RESULTS AND DISCUSSION

For this investigation, high fluoridated six groundwater samples were selected from Korba district regions for fluoride analysis of samples used the ISE method. The fluoride content in this region varied from 2.28 to 10.04 mg/L shown table 1.

The successful use of the adsorption process necessitates developed low-cost, non-toxic, conveniently accessible, and locally available materials. Bioadsorbents meet these requirements. If the ideal circumstances were known, a better design and modelling process would be ushered in. From a kinetic standpoint, various essential factors, such as pH, contact duration, starting adsorbate concentration, and the adsorbent dose, were studied. Experiments like batch adsorption investigations were carried out (Birhanu et al. 2020). Experiments were conducted by agitating 100 ml of obtained groundwater samples containing F<sup>-</sup> at a speed of 200 strokes per minute while shaking 10 g/L of adsorbent. To maintain samples containing F<sup>-</sup> at the proper pH, 0.5 N HNO<sub>3</sub> was added. At room temperature (27°C ± 0.5°C), all of the measurements were taken (Birhanu et al. 2020).

**Effect of Initial Adsorbate Concentration:** During the optimal time of contact, the rate of a purely adsorptive reaction varies in lockstep with the concentration of adsorbate. As the initial fluoride ion concentration grows, the capacity of the adsorbent materials is rapidly exhausted. The adsorption capability of treated biosorbents was studied methodically by increasing the starting concentration of F<sup>-</sup> from 2.28 to 10.04 mg/L.

**Figure 2: a. Effect of initial adsorbate concentration, b. Removal efficiency of F<sup>-</sup> from groundwater**



The percent of elimination of fluoride ions varies through starting concentration at various initial pH levels. When the starting concentration of F<sup>-</sup> was raised from 2.28 to 10.04 mg/L with a constant sorbent dosage of 10 g/l at a pH of 2, treated bioadsorbents were shown to be reasonably active in decreasing fluoride ions from 98 to 78% shown in table 2 and figure 2 a and b (Philip et al. 2021).

**Adsorption Isotherm model:** To fit the investigational adsorption equilibrium data (Yuan et al. 2020; Philip et al. 2021) of fluoride on a bioadsorbent (neem leaves), the isotherm models of Langmuir, Freundlich, and Temkin were applied (Ali and Ismail 2021). These models are mathematically expressed as follows:

**Langmuir isotherm:**

$$\frac{C_e}{q_e} = \frac{1}{q_m} C_e + \frac{1}{K_a q_m}$$

Where  $C_e$  is the equilibrium concentration of adsorbate (mg/L),  $q_e$  denotes the quantity of adsorbed per gram of the adsorbent at equilibrium (mg/g),  $K_a$  represents the Langmuir isotherm constant (L/mg) and  $q_m$  is the highest adsorption capacity (mg/g) of the adsorbent. As shown in Table 3 and figure 3 a.

**Freundlich isotherm:**

$$\log q_e = \log K_f + \frac{1}{n} \log C_e$$

Where  $K_f$  is the Freundlich isotherm constant  $\text{mg}^{1-(1/n)} \text{L}^{1/n} \text{g}^{-1}$ ,  $n$  indicates the adsorption intensity, (Priyantha and Kotabewatta 2019; Yuan et al. 2020). As shown in Table 3 and figure 3 b.

**Temkin isotherm:**

$$q_e = \frac{RT}{b} \ln K_T + \frac{RT}{b} \ln C_e$$

Where  $b$  is Temkin constant which is related to heat of sorption ( $\text{J mol}^{-1}$ ), (Altun et al. 2021) and  $K_T$  is Temkin isotherm constant ( $\text{Lg}^{-1}$ ). As shown in Table 3 and figure 3 c.

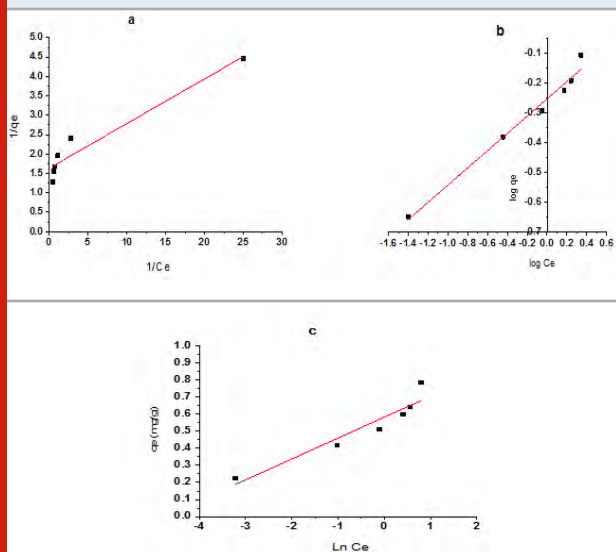
**Effect of contact time:** The elimination of F<sup>-</sup> is shown to increase with increasing contact time duration to some extent. Increased contact time does not improve absorption due to fluoride ions on the accessible adsorption sites on the adsorbent material. According to early studies, fluoride ions appear to absorb quickly on the adsorbent material at their ideal pH values. Within the first hour of interaction with fluoride ions at a concentration of 10.04 mg/L and an adsorbent dose of 10.04 mg/L for treated bioadsorbents, 62% of the adsorption occurs. After this rapid adsorption, a more gradual approach to equilibrium occurs, with saturation happening in 1.5 to 3 hours. This contact time was used as the equilibrium period for further optimization of other parameters (Ali and Ismail 2021).

**Adsorption kinetic studies:** Adsorption kinetics is a critical parameter that indicates adsorption efficiency. Figure 4 depicts the fluoride adsorption rate on the surface of adsorbents as a function of time (Siva kumar et al. 2019; Akinyeye et al. 2020). The pseudo-first-order rate (Lagergren 1898), equation is represented by follows:

$$\log (q_e - q_t) = \log q_e - \frac{K_1 t}{2.303}$$

Where  $q_t$  and  $q_e$  are the total of fluoride adsorbed (mg/g) at contact time  $t$  and at equilibrium respectively and  $k_1$  is the pseudo-first-order rate constant ( $\text{min}^{-1}$ ). Pseudo-first-order rate constant  $k_1$  and the equilibrium adsorption capacity  $q_e$  were determined from the slope and intercept of the plots of  $\log(q_e - q_t)$  against time are shown in figure 4a and table 5 along with the correlation coefficient ( $R^2$ ) (Ali and Ismail 2021).

**Figure 3: a. Langmuir isotherm studies plot not fitted for activated neem (*Azadirachta indica*) leaves as bioadsorbents for used defluoridation of groundwater. b. Freundlich isotherm studies plot fitted for activated neem (*Azadirachta indica*) leaves as bioadsorbents for used defluoridation of groundwater. c. Temkin isotherm studies plot not fitted for activated neem (*Azadirachta indica*) leaves as bioadsorbents for used defluoridation of groundwater (Ali and Ismail 2021).**



**Table 3. In the case of bioadsorbent, a comparison of isotherm parameters from different isotherm models.**

Langmuir isotherm		Freundlich isotherm		Temkin isotherm	
$Q_m$ (mg/g)	0.611139	$K_f$ ( $\text{mg}^{-1} \cdot (\text{ln}) \text{L} \cdot \text{g}^{-1}$ )	0.55856	$B$ ( $\text{J mol}^{-1}$ )	0.1222
$K_s$ (L/mg)	14.22985	$1/n$	0.28973	$K_T$ ( $\text{Lg}^{-1}$ )	117.0022
$R^2$	0.9196	$R^2$	0.97551	$R^2$	0.87558

The rate-determining step is the pseudo-second-order kinetic model, which may be represented as:

$$\frac{t}{q_t} = \frac{1}{k_2 q_e^2} + \frac{1}{q_e} t$$

Where  $k_2$  denotes the adsorption rate,  $q_t$  is the total amount of fluoride adsorbed at any given time, and  $q_e$  denotes the equilibrium adsorption capacity. The slope and intercept of the plot of  $t/q_t$  against  $t$  (Wolowicz and Wawrzkiwicz, 2021) displayed in figure 4 b, as well as the kinetic data in table 5.

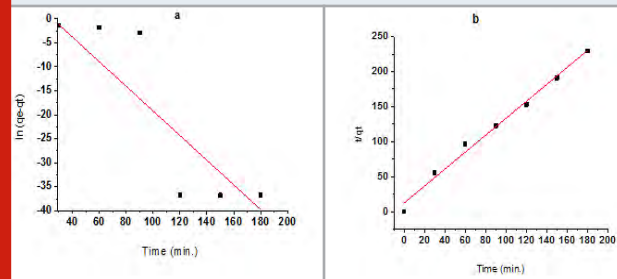
**Table 4. Effect of contact time, Operating order for the consequence of contact time, Adsorbent dose - 10 g/L, Temperature was adjusted  $27^\circ\text{C} \pm 0.5^\circ\text{C}$ ,  $C_i = 10.04$  mg/L, Volume of sample - 100 ml, and pH 2.**

Time (min)	$C_i$ (mg/L)	$C_e$ (mg/L)	Elimination % of F-
30	10.04	4.61	54
60	10.04	3.81	62
90	10.04	2.71	73
120	10.04	2.20	78
150	10.04	2.20	78
180	10.04	2.20	78

**Table 5. Virtual study of kinetic parameters in case of used bioadsorbent for defluoridation of groundwater.**

Pseudo-first-order		Pseudo-second-order	
$q_e$ (mg/g)	653.43953	$q_e$ (mg/g)	0.82540
$K_1$ ( $\text{min}^{-1}$ )	-0.00142	$K_2$ ( $\text{g/mg} \cdot \text{min.}$ )	0.056069
$R^2$	0.73822	$R^2$	0.98862

**Figure 4: a. Pseudo-first-order kinetic studies for activated neem (*Azadirachta indica*) leaves as bioadsorbents for used defluoridation of groundwater b. Pseudo-second-order kinetic studies fitted for activated neem (*Azadirachta indica*) leaves as bioadsorbents for used defluoridation of groundwater.**



**Effect of pH:** The adsorption process is influenced by the pH of the aqueous solution (Mia and Jha, 2020). The effect of  $\text{H}^+$  concentration was studied at pH values 2, 4, 6, and 8. This was attained by mixing 0.5N  $\text{HNO}_3$  for 120 minutes with 100 ml of a standard fluoride solution containing 10.04 mg/L fluoride and a dose of 10 g/L treated bioadsorbent.



With increasing the pH of the resolution, we noticed a reduction in the amount of fluoride ion removal. At pH 2, this was found to be 78%, while at pH 4, it was found to be 75%. As a result, more research was done at these pH levels. In treated adsorbents, the entitlement of adsorption varies almost linearly between 2.0 and 8.0, achieving a maximum clearance at pH 2.0 after 120 minutes of contact time (Ali and Ismail 2021).

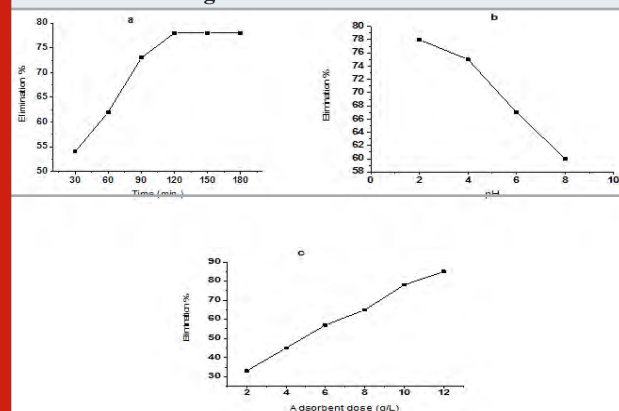
**Table 6. Table for effect of pH, Operating order for the consequence of pH, Adsorbent dose - 10 g/L, Temperature was adjusted  $27^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ , Time of contact - 120 Minutes, and Volume of sample - 100 ml,**

pH %	Ci (mg/L)	Ce (mg/L)	Elimination of F-
2	10.04	2.20	78
4	10.04	2.51	75
6	10.04	3.31	67
8	10.04	4.01	60

**Table 7. Table for effect of adsorbent dosage, Operating order for consequence of adsorbent measure, Effect of contact time, Operating order for the consequence of contact time, Temperature was adjusted  $27^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ , Ci = 10.04 mg/L, Volume of sample - 100 ml, and pH 2.**

Adsorbent dose (g/L)	Ci (mg/L)	Ce (mg/L)	Elimination % of F-
2	10.04	6.62	33
4	10.04	5.52	45
6	10.04	4.31	57
8	10.04	3.51	65
10	10.04	2.20	78
12	10.04	1.50	85

**Figure 5: a. The effect of contact time relations on F-subtraction, b. The effect of pH on F- subtraction, c. Effect of adsorbent dosage on the subtraction of F-.**



Higher hydrogen ion concentrations at lower pH values may have neutralized negative charges on the surface of the treated bioadsorbents in this example. This makes it simpler for negatively charged fluoride ions to diffuse onto the increased active surface of the modified bioadsorbent.

**Effect of adsorbent dosage:** The elimination of fluoride ions appears to augment when the quantity of adsorbent is increased. The starting fluoride ion concentration in all of these experiments was set at 10.04 mg/L. The adsorbent dosage in an aqueous solution was adjusted between 2 and 12 g/l at their optimum pH levels. Table 7 and figure 5c. demonstrate that treated bioadsorbent was efficient for 33 – 85% removal of fluoride ions, with maximum exclusion of 85 % recorded at 12 g/L, at a room temperature of  $27^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$  (Ali and Ismail 2021).

Contact duration, pH, and adsorbent concentration all affect the performance of neem leaf adsorbents in successfully remediating tainted fluoride water (Patel and Gupta, 2020; Pandey et al. 2020).

## CONCLUSION

The findings of the present study used Azadirachta indica leaves as bioadsorbents to eliminate fluoride from groundwater samples from Korba district of Chhattisgarh. Fluoride in this region varied from 2.28 to 10.04 mg/L. As per the result analysis, we saw that contact duration, pH, and adsorbent concentration influence the effectiveness of low-cost leaf adsorbents in successfully remediating fluoride-polluted water. Maximum fluoride elimination was 78% at pH 2 and up to 85% at 12 g of the adsorbent dose. In the future, this technique of removing fluoride from highly fluoridated water will give positive results and be cheap and available everywhere.

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

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**Data Availability Statement:** The database generated and /or analysed during the current study are not publicly available due to privacy, but are available from the corresponding author on reasonable request.

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# Assessment of Risk Factors for Severe Coronavirus Disease -19 in Taif Province, Saudi Arabia

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## ABSTRACT

Coronavirus 2019 (COVID 19) is one of the pandemics registered for 2020, with the entire world affected. COVID 19 disease is diagnosed based on symptoms and risk factors, and both swab and RT-PCR tests are available to confirm the disease. The aim of this study was to the underlying health issues which might be connected to development of severe COVID19. A retrospective study was carried out in a tertiary hospital (Corona center) in Saudi Arabia from August 2020 to December 2020 to identify the risk factors of mortality in ICU admitted COVID-19 patients and to assess the underlining sociodemographic and medical conditions that may lead to the development of severe COVID-19. Data was collected from patients' medical records. In this study, 174 subjects were recruited and 71.3% of the population is male and 28.7% as female. Survival was 52%, whereas non-survivors was 48%. The average age was 57.7±16.0 years old. The most common clinical manifestations were shortness of breath (75.9%), cough (67.8%), and fever (64.9%). T2DM was one of the highest co-morbidities was documented. Binary logistic regression analysis confirmed both the age and fever showed the positive association ( $p<0.05$ ) and the serum parameters such as CRP, d dimer, neutrophils and lymphocytes showed the elevated levels and positive association ( $p<0.05$ ). In conclusion, the current study results confirmed HTN, CKD, and heart disease comorbidities all played a substantial impact in the development of COVID-19, and that older age (>60 years) also played a role in COVID 19 patients. Saudi Arabia is one of the nations that has successfully managed the COVID 19.

**KEY WORDS:** COVID 19, COMORBIDITIES, SERUM PARAMETERS, SURVIVORS AND NON-SURVIVORS.

## INTRODUCTION

The story of the coronavirus begins at the end of 2019 in China, in a well-known location known as the Wuhan market (Farasani, 2021). Because of the threat to the entire human population, the World Health Organization (WHO) has declared coronavirus 2019 (COVID19) a pandemic (Daniel, 2020). Severe acute respiratory syndrome Coronavirus 2 (SARSCoV-2), the seventh human coronavirus, has been discovered. As a result of this, the

virus has spread throughout the entire world (Ciotti et al., 2020). Coronaviruses (also known as Coronavirinae/CoV) are enclosed, single-stranded, positive-sense RNA viruses. It's likely that the CoVs genome, which ranges from 26 to 32 kilobases in length, is the biggest viral RNA yet discovered. CoVs have historically been recognized to cause disease in humans, and there are currently six identified CoVs of which two falls into the low pathogenic and two falls into the highly pathogenic category.

Between 10-30% of all upper respiratory tract infections are caused by CoVs that have a low pathogenicity, including 229E, HKU1, OC43, and NL63. In contrast, CoVs with high pathogenicity, such SARS and MERS, primarily infect the

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lower respiratory system and produce deadly pneumonia (Han et al., 2020). SARS-CoV's has a diameter of ~50–200 nm, ranging from 26-32 kb. SARS-CoV-2 is a  $\beta$ -coronavirus that contains a single positive RNA strand. Its 60-140 nm diameter envelope provides it with a square, elliptical form. In its genome, there are special parts derived from 9 Wuhan patients that consist of a close-knit 29,903 basis pair of single stranded RNA pairs (88%) of  $\beta$ -coronavirus isolated bats. The untranslated area (UTR) of the SARS-CoV-2 genome is composed of 50 leader sequence, 1a/b free reading frame genes, spike protein, the protein envelope, protein membrane/ matrix and auxiliary protein, nucleoprotein and sequenced 3'UTR (Farasani, 2020).

The most typical symptoms of COVID 19 are fever, cough, and weariness. One of the first signs is a loss of taste or scent. Shortness of breath, muscle aches, headache, runny nose, nausea, vomiting, diarrhea, and rashes are the remaining symptoms. These factors, such crowding, literacy levels, and health care employees, are all involved in increasing the likelihood of infection and infection is often a problem in the elderly, men, and those with preexisting conditions (Rashedi et al., 2020). Because of community transmission, the risk of COVID 19 in the general population is currently very high. COVID-19 harm to the population with established risk factors for severe illness outcome and COVID-19 can cause serious disease in people of any age who have specific underlying medical issues. COVID-19 is a newly discovered disease. COVID-19 can affect anyone, and the disease can cause mild to severe symptoms. Some persons may be more likely than others to suffer from severe disease because they have qualities or medical conditions that put them at risk.

There is currently little evidence and information available about the impact of underlying medical disorders and if they raise the risk of severe disease from COVID-19. Based on what we know at this time, it is extremely vital to recognize the persons at high risk of serious disease from COVID-19, as well as those who live with them, in order to protect yourself from obtaining COVID-19. If you have COVID-19, you are more likely to be admitted to the hospital or ICU if you have certain risk factors. Knowing the risk factors connected with COVID-19 is important because it can help people who are at high risk take further precautions. Almost 98% of COVID-19 cases from Saudi Arabia has recovered and 1% of the mortality cases was documented and only <1% of active cases were listed. Limited studies in the Saudi Arabia has performed with the risk of developing with severe form. Therefore, the current study was aimed to investigate the underlying health issues which might be connected to development of severe COVID-19.

## METHODOLOGY

This is a retrospective and hospital-based study that was conducted at King Faisal Medical Complex hospital in Taif Province from August to December 2020. Following authorization from the Ministry of Health, clinical details, personal and family history data were gathered from medical records of patients admitted to Intensive Care Unit. This rationale study will address one of the most

important questions to address the currently unfolding pandemic: "what are the risk factors for severe illness or death and will allow us to understand the risk factors for disease severity providing a comprehensive picture of the characteristics of COVID-19 patients." The inclusion criteria of the cases were patients diagnosed with COVID 19 in any nationality and the patients without COVID-19 infection can be considered as exclusion criteria of the enrolled subjects. In this study, we have selected 174 cases in which 50 females and 124 males were involved. In this study, 77 non-Saudi and 97 Saudi subjects were involved. Both the symptoms and comorbidities details were recorded in all the enrolled patients.

**Table 1. Clinical and sociodemographic characteristics of COVID 19 patients hospitalized to the ICU**

Sociodemographic			
Gender			
		Frequency	Percent
	Female	50	28.7
	Male	124	71.3
Age (Mean $\pm$ SD)	57.7 $\pm$ 16		
Age group	< 35	15	8.6
	36 - 59 Y	76	43.7
	$\geq$ 60	83	47.7
Nationality	Non-Saudi	77	44.3
	Saudi	97	55.7
Symptoms			
SOB	No	42	24.1
	Yes	132	75.9
Cough	No	56	32.2
	Yes	118	67.8
Fever	No	61	35.1
	Yes	113	64.9
Diarrhea	No	151	86.8
	Yes	23	13.2
Myalgia or fatigue	No	159	91.4
	Yes	15	8.6
Headache	No	160	92
	Yes	14	8
Comorbidity			
Type 2 DM	No	91	52.3
	Yes	83	47.7
Hypertension	No	105	60.3
	Yes	69	39.7
Heart disease	No	147	84.5
	Yes	27	15.5
Chronic kidney disease	No	153	87.9
	Yes	21	12.1
Asthma, Bronchial Asthma (BA)	No	159	91.4
	Yes	15	8.6

**Biochemical parameters:** In this study, serum blood was collected from all the patients who have involved and used for c-reactive protein (CRP), lymphocytes, neutrophils and d dimer tests.

**Statistical analysis:** Statistical analysis was performed with SPSS software and we have analyzed both the numerical and categorical variables with frequency and percentages. Binary logistic regression analysis was performed with the obtained data. P values less than 0.05 is considered as statistically significant ( $p < 0.05$ ) (Khan et al., 2019).

**Table 2. Shows the survival and non-survival rates of COVID 19 patients hospitalized to the ICU.**

Variable	Survivor	Non- Survivor	Total	P-value
<b>Sociodemographic</b>				
Gender N (%)				
Female	21(23.3%)	29(34.5%)	50 (28.7%)	0.131
Male	69(76.7%)	55(65.5%)	124(71.3%)	
Age N (MIN - MAX)	90(14 - 82)	84(23 - 92)	174(14 - 92)	
Age (mean $\pm$ SD)	51 $\pm$ 14	65 $\pm$ 14	57.7 $\pm$ 16	
Age group				
$\leq$ 35	13(14.4%)	2(2.4%)	15(8.6%)	
36 - 59 Y	51(56.7%)	25(29.8%)	76(43.7%)	0.00
$\geq$ 60	26(28.9%)	57(67.9%)	83(47.7%)	
<b>Symptom</b>				
Dyspnea SOB	61(67.8%)	71(84.5%)	132(75.9%)	0.013
Cough	54(60%)	64(76.2%)	118(67.8%)	0.02
Fever	49(54.4%)	64(76.2%)	113(64.9%)	0.004
Diarrhea	14(15.6%)	9(10.7%)	23(13.2%)	0.37
Myalgia or fatigue	6(6.7%)	9(10.7%)	15(8.6%)	0.4
Headache	8(8.9%)	6(7.1%)	14(8%)	0.7
<b>Comorbidities</b>				
Type 2 DM	38(42.2%)	45(53.6%)	83(47.7%)	0.17
Hypertension	26(28.9%)	43(51.2%)	69(39.7%)	0.003
Bronchial Asthma, (BA)	9(10%)	6(7.1%)	15(8.6%)	0.5
Chronic kidney disease	5(5.6%)	16(19%)	21(12.1%)	0.006
Heart disease	7(7.8%)	20(23.8%)	27(15.5%)	0.003

## RESULTS

In this hospital-based study, we have opted 28.7% of women and 71.3% of men. The mean age of the 174 participants were found to be 57.7 $\pm$ 16.0. The age was categorized into <35 (8.6%), between 36-59 (43.7%) and >60 (47.7%) years of age. Saudi subjects (44.3%) were found to be higher than non-Saudi participants in this study (55.7%). Table 1 presents information on sociodemographic, symptoms, and comorbidities. The general COVID 19 symptoms such as high in SOB (75.9%), cough (67.8%), fever (64.9%), diarrhea (13.2%), fatigue (8.6%) and headache (8%). Type 2 diabetes mellitus (T2DM; 47.7%), hypertension (HTN; 39.7%), heart disease (15.5%), chronic kidney disease (CKD, 12.1%) and bronchial asthma (BA, 8.6%) were among the comorbidities revealed in this studied participant. According to Figure 1, 48% of hospitalized patients were determined to be alive, while the remaining 52% were confirmed to be dead. Table 2 shows the survival and non-survival rates of participants infected with COVID-19 who were admitted to the Taif hospital in the ICU.

Table-2 shows the list of COVID-19 survivors and non-survivors admitted to the ICU. Males were found to be higher than females in both categories among survivors and non-survivors ( $p=0.13$ ). The survivors' minimum and maximum ages were determined to be 14-82 years old, whereas the non-survivors' minimum and maximum ages were found to be 23-92 years old. The mean ages of survivors and non-survivors were reported to be 51 $\pm$ 14 and 65 $\pm$ 14, respectively, with a non-significant association ( $p>0.05$ ). Among survivors, the majority of the age groups was found to be high in between 36-59 years of age with 56.7%, followed by 28.9% in 60 and above years of age and 14.4% in below 30 years of age, while in non-survivors, 67.9% was found to be high in below 60 years of age, followed by 29.8% in between 36-59 years of age, and only 2.4% in above 35 years of age with the statistical association ( $p<0.05$ ).

Among the symptoms, dyspnea SOB, cough, and fever were found to be more prevalent in non-survivors when compared to survivors and showed a significant association

( $p < 0.05$ ), whereas in survivors, both diarrhea and headache were found to be more prevalent when compared to non-survivors with a non-significant association ( $p > 0.05$ ), and fatigue prevalence was found to be more prevalent in non-survivors when compared to survivors ( $p = 0.7$ ). T2DM and BA were shown to have a non-significant association when compared between survivors and non-survivors ( $p > 0.05$ ) in the comorbidities. Other comorbidities, such as HTN, CKD, and heart disease, were found to be substantially associated when comparing survivors to non-survivors ( $p < 0.05$ ).

Binary logistic regression analysis among the survival subjects of independent factors was defined in Table-3. In this study, numerous variables such as age, gender, fever, cough, fatigue, dyspnea, diarrhea, headache, T2DM, HTN, BA, CKD and heart disease were involved in the survivor subjects and only the age (OR-5.48 [95%CI:2.54-11.81];  $p = 0.001$ ) and fever (OR-3.15 [95%CI:1.10-8.97];  $p = 0.03$ ) variables was associated. The other variables were found to be non-significant when performed the logistic regression analysis ( $p > 0.05$ ).

**Table 3. Binary logistic regression survival status and independent factors**

Variables	OR	95% C. I		P-value
		Lower	Upper	
Age	5.48	2.541	11.815	0
Gender	0.675	0.3	1.517	0.341
Fever	3.15	1.105	8.979	0.032
Cough	1.22	0.418	3.562	0.717
Myalgia or fatigue	3.676	0.888	15.212	0.072
Dyspnea	1.426	0.534	3.809	0.479
Diarrhea	0.569	0.194	1.669	0.304
Headache	0.506	0.13	1.973	0.326
Type2DM	0.504	0.212	1.194	0.119
Hypertension	1.654	0.701	3.905	0.251
Bronchial Asthma (BA)	1.195	0.3	4.759	0.8
Chronic kidney disease	3.195	0.865	11.799	0.081
Heart disease	1.977	0.676	5.781	0.213

**Table 4. Lab abnormalities of COVID 19 patients admitted to ICU**

Variables	Survivor	Non- Survivor	Total*	P-value
C Reactive Protein				
Elevated	60(83.3%)	70(98.6%)	130(90.9%)	0.001
Normal	12(16.7%)	1(1.4%)	13(9.1%)	
Lymphocyte				
Lymphopenia	35(58.3%)	45(73.8%)	80(66.1%)	0.05
Normal	25(41.7%)	16(26.2%)	41(33.9%)	
Neutrophils				
Elevated	19(31.7%)	35(57.4%)	54(44.6%)	0.004
Normal	41(68.3%)	26(42.6%)	67(55.4%)	
D dimer				
Elevated	57(80.3%)	55(93.2%)	112(86.2%)	0.001
Normal	14(19.7%)	4(6.8%)	18(13.8%)	

\*Total is differing for each test according to number of patients tested

Table 4 shows the biochemical parameters such as CRP, lymphocytes, neutrophils, and d dimer in COVID-19 survivors admitted to the ICU. When compared to survivors, the elevated levels were found to be higher in non-survivors, indicating a positive association. CRP levels were found to be increased in non-survivors at 98.6% and survivors

at 83.3% ( $p = 0.001$ ). The aberrant lymphocyte counts were 73.8% and 58.3% in non-survivors and survivors, respectively ( $p = 0.05$ ). The number of increased neutrophils was 44.6% in non-survivors and 57.4% in survivors ( $p = 0.004$ ). The enlarged levels of d dimer in non-survivors were 93.2% and 80.3% in survivors ( $p = 0.001$ ).

**Figure 1: Status of COVID 19 patients admitted into ICU**

## DISCUSSION

The purpose of this study was to investigate at the underlying health issues associated with demographic information, symptoms, and comorbidities among hospitalized patients in the ICU with COVID-19 in the Taif city of Saudi Arabia. The current study results confirmed the significant association with age group, documented symptoms such as Dyspnea SOB, cough, fever and other comorbidities such as HTN, CKD and heart disease ( $p < 0.05$ ). Binary logistic regression analysis revealed the positive association with age and fever ( $p < 0.05$ ) and all the biochemical parameters such as CRP, lymphocytes, neutrophils and d dimer showed the statistical association ( $p < 0.05$ ).

There have been no studies conducted in Saudi Arabia among COVID-19 patients admitted to the ICU, and this is the first research study was carried out in the Saudi population from Taif city. T2DM, HTN, BA, CKD, and heart disease were among the comorbidities studied in our study.

Unfortunately, due to the pandemic crisis, we did not record body mass index values to categorize obesity levels, despite the fact that obesity is one of the known risk factors for COVID-19. One of the meta-analysis and systematic reviews discovered that HTN, DM, and cardiovascular disease (CVD), along with age and being male, are all highly connected to severe COVID-19 and can provide a better estimate of risk for severe COVID-19 (Matsushita et al., 2020). Another systematic reviews and meta-analysis studies found that those over the age of 65, men, HTN, CVD, diabetes, and cancer were all more likely to die from COVID-19 infection. Patients with a poor prognosis could be identified based on these data (Parohan et al., 2020).

The immune system varies with age in a similar manner to the differences in immunological response related to sex. It is thought that as people age, their immune systems become chronically inflamed, which makes them more vulnerable to infection and tissue damage. Aging is related with comorbidities and a lower reserve capacity in essential organs, resulting in greater frailty and increased risk of mortality and infection with COVID-19 when infected. In this study, age was divided into three categories: (a) <35 years, (b) 36-59 years, and (c) >60 years, with 47.7% being >60 years, 43.7% being 36-59 years, and 8.6% being <35

years of age. A positive association was observed among the three age groups compared between the survivors and non-survivors ( $p = 0.001$ ). Our study was found to be in association with the previous studied results (Pijls et al., 2021). Similar studies have been conducted in different regions of Saudi Arabia based on our current study.

From the beginning of the pandemic crisis to current scenario, WHO has strongly recommended to follow three rules to avoid the COVID-19 infections: social distance, sanitization and wearing a mask. All countries have rigorously enforced and extra lockdown was performed to stop the infection rate, and Saudi Arabia is one of the countries that has successfully controlled the COVID-19. Until September 5th, 2021, the overall number of infected cases was 545,243, the number of recovered cases was 534062, and the total mortality was 8579, with 2602 active cases. Apart from Saudi Arabia, there are other countries which have been successfully managed the infection. Ministry of Health from Saudi Arabia has played a pivotal role in controlling the infections and proper measures have been taken, as well as residents of the kingdom received a couple of doses of free vaccines.

Aside from RT-PCR, biochemical markers were important in COVID-19 patients. A recent review supported the importance of CRP and d dimer levels in identifying COVID-19 patients (Farasani, 2021). In our study, additionally with CRP and d dimer, we have screened lymphocytes and neutrophils levels in the COVID-19 patients and unfortunately, all the elevated levels were confirmed in the non-survivors and all the parameters showed the significant association ( $p < 0.05$ ). Strong evidence suggests that neutrophils play a crucial role in pathogenesis, particularly in COVID 19 patients (Reusch et al., 2021).

One of the limitations of this study was not incorporating the RT-PCR details. Additionally, clinical details were not incorporated. The other limitations of our study were to skip the severe forms of diagnosis which is mandatory, but due to COVID-19, we couldn't document the data. The strength of this study was to involve the 174 subjects who were infected with COVID-19. Although the sample size was small but we have managed to enroll the limited subjects during the pandemic situations.

## CONCLUSION

The current study shows that HTN, CKD, and heart disease comorbidities all played a substantial impact in the development of COVID-19, and that older age (>60 years) also played a role in COVID-19 patients. Developing a unique multi-item scale method to predict COVID-19 patients is indicated as a future proposed study.

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**Abbreviations:** ICU, Intensive Care Unit, DM, Diabetes Mellitus, T2DM, Type 2 Diabetes Mellitus, CRP, C Reactive Protein, CVD, Cardiovascular disease, HTN, Hypertension, CKD, chronic kidney disease, RT-PCR, Real Time polymerase Chain Reaction, RNA, Ribonucleic Acid, SOB, Shortness of Breath, BA, Bronchial Asthma, SPSS, Statistical Package for the Social Sciences.

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# *In vitro* Callus Induction from Immature Fruits of *Elaeocarpus ganitrus*: A Tissue Culture Approach

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## ABSTRACT

In tissue culture investigations for tree species, the growth medium with a perfect concentration of growth regulators and other components has a crucial role in the *in vitro* callus initiation from explants material. The present study has highlighted the effects of various concentrations of plant growth regulators on callus initiation from immature fruits of *Elaeocarpus ganitrus* in Murashige & Skoog (MS) medium. The immature fruits were collected as explants source during fruiting season and cultured on 1/2, 3/4 and full-strength MS medium. In this study, 1.8mgL<sup>-1</sup> of 2, 4-D, and full-strength MS medium was observed best for callus initiation which further supported for the multiplication from immature fruits of *E. ganitrus*. The callus initiation from immature fruits of *E. ganitrus* was the crucial step towards the conservation study of this tree species. This study can be helpful for tissue culture investigations concerning callus development from immature fruit explants material of several other species of *Elaeocarpus* and even for the other endangered tree species.

**KEY WORDS:** CALLUS, E. GANITRUS, IMMATURE FRUITS, MS.

## INTRODUCTION

The genus *Elaeocarpus* consists of a large number of species. Among these many, Rudraksha are popularly known. Rudraksha has tremendous importance in rituals and prayers. Several investigations were carried out by researchers in the past on different species of *Elaeocarpus*. *Elaeocarpus sphaericus* is well known for its antioxidant properties and through several literature reviews, it was inferred that it owns notable biomedical capability. Through GC-MS *E. sphaericus* phytochemicals have been analyzed in the previously published studies (Mahajanakatti et al. 2022). *E. sphaericus* is rich in antimicrobial and anti-inflammatory characteristics. Along with phytochemicals, *E. sphaericus* is a very important and useful source of antibiotics and antioxidants. Findings on *Elaeocarpus ganitrus* fruit set and dispersal may have high implications for species regeneration (Koirala et al. 2022; Khan et al. 2005).

The blue colour is most prevalent in *Elaeocarpus* spp. in its entire distribution. Regeneration failure from seeds of *E. venustus* leads to an approach toward other techniques. Further vegetative propagation was first reported successfully (Lee 1998; Saravanan et al. 2011). The callus initiation using leaf stalk as explants material of *E. grandiflorus* was reported on MS medium (Habibah et al. 2019). Using small and immature soft leaves of *E. ganitrus*, callus was developed (Rishi et al. 2021). A procedure for *in-vitro* propagation was developed for *E. serratus* fruit tree. For the investigation, mature nodal explant material was collected. In the study 1/2, 3/4 strength MS medium was used during the study. Other media like White's, B5 Gamborg and WPM (Woody Plant Medium) were also taken during the investigation (Raji and Siril 2021). For several other trees of different genus, tissue culture has shown its importance and necessity. For Baramasi mango, a variety of micropropagation protocols were developed by nucellar embryogenesis. B5 Gamborg MS minor and major salts along with several other additives were used for basal media. 2,4-D is very essential for callus induction and pro-embryonic calli generation in nucellar embryogenesis (Al-Busaidi 2016). Young soft leaves of *Litchi chinensis* Sonn. were taken as explants for the

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development of callus to get *in vitro* plantlets (Puchooa 2004). Somatic embryogenesis of 'Feizixiao litchi' was first reported. The lactalbumin hydrolysate helped in getting a high frequency of callus initiation when taken in combination with plant growth regulators in MS medium (Wang et al. 2016).

The somatic embryogenesis was achieved in callus cultures of *C. wightii*, a medicinally important woody tree species (Kumar et al. 2003). The MS medium was found suitable for callus initiation from leaf explants of *Ailanthus excelsa* Roxb., and plant growth regulator kinetin was found better than TDZ (Dhaval and Nataraj 2018). For the tropical woody plant, *Parasponia andersonii* Planch the callus initiation was obtained with 0.1-0.2mg L<sup>-1</sup> TDZ with 0.05 mgL<sup>-1</sup>NAA (Knyazev 2018). For direct and indirect shoot organogenesis in *Ficus religiosa* using hypocotyls as explants, two valuable protocols were developed (Hesami and Daneshvar 2018). In zygotic embryos of *Cedrus deodara* formation of adventitious bud was examined. Four different tissue culture growth media variously fortified with and without plant growth regulators were used (Tamta and Palni 2004). In the present study growth regulators and antioxidants were used in MS (Murashige and Skoog 1962) medium for *in vitro* callus initiation from immature fruits of *E. ganitrus*. It is difficult to do tissue culture work with fruits of *E. ganitrus*. Therefore, immature fruits of *E. ganitrus* (Roxb.) were taken into consideration as explants source to get new insights.

**Figure 1:** [A] Tree of *E. ganitrus* [B] Inflorescence on *E. ganitrus* [C] Growing *E. ganitrus* fruit.



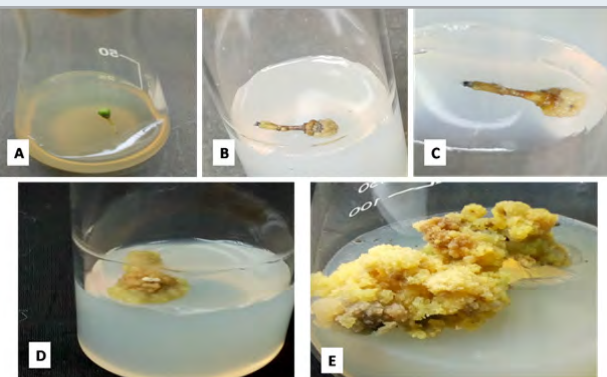
## MATERIAL AND METHODS

The samples (Fig. 2A) were obtained from 6-7 years old tree located inside SIET, Meerut, Uttar Pradesh, India (Fig.1A) which were brought into the laboratory and further washed gently using distilled water. The samples were dipped in distilled water to treat under bavistin (1%) (w/v) for 35mins (Rishi et al. 2021). Under the laminar air hood the surface sterilization of explants was performed using 70% ethanol for time period of 30 seconds. The explants samples were treated with HgCl<sub>2</sub> 0.1% (w/v) for 4 mins (Rishi et al. 2021). The explants were rinsed for 6 times each for 4 min. with sterile double distilled water within the laminar hood. The immature fruits after surface sterilization were cultured on the respected medium of various strength. The temperature of 25±2°C was set for maintaining the *in vitro* cultures with

60-70% humidity. The light and dark conditions of 16h/8h were provided to the respective cultures.

For the investigation MS medium with 1/2, 3/4 and full strength was used and fortified with 2, 4-D (2, 4-Dichlorophenoxyacetic acid) and TDZ which were taken in the concentration range of 0.2 mgL<sup>-1</sup> to 2.0 mgL<sup>-1</sup>. The antioxidants like ascorbic acid (165 mgL<sup>-1</sup>), citric acid (5 mgL<sup>-1</sup>) polyvinyl pyrrolidone (PVP,165 mgL<sup>-1</sup>) were used. The pH of 5.8±0.5 was maintained for the medium and 0.8% agar was added. The medium was autoclaved for 15-20 min. For statistics, analysis of variance, one way ANOVA was carried out for significant difference p≤ 0.05 (n=10).

**Figure 2:** Different stages of callus development from immature fruit explants on full strength MS medium. [A] Immature fruit; [B]&[C] Callus initiation; [D] Entire immature fruit of *E. ganitrus* converted into callus; [E] Callus multiplication.



## RESULTS AND DISCUSSION

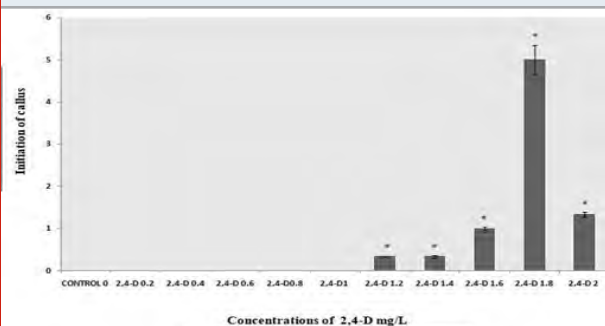
The callus induction was started after 5 weeks (Fig.2B) on full strength MS medium. It was observed that the entire explants material converted into callus after 9-10 weeks (Fig.2D). The initial callus appeared to be whitish to light green in colour, but after some time, when it started getting older, the callus started to appear light yellow and green in colour (Fig.2E). From immature fruits of *E. ganitrus* initiation of callus was observed maximum at 1.8mgL<sup>-1</sup> of 2,4-D as compared to 1.2mgL<sup>-1</sup>, 1.4mgL<sup>-1</sup>, 1.6mgL<sup>-1</sup> and 2.0mgL<sup>-1</sup> of 2,4-D when considered alone (Fig.3).

On the other hand, with the concentrations of TDZ 0.6mgL<sup>-1</sup> analyzed better in comparison with 0.4mgL<sup>-1</sup> and 0.8mgL<sup>-1</sup> (Fig.4). With combination of both the growth regulators i.e., 2,4-D 1.8mgL<sup>-1</sup> and TDZ 0.4mgL<sup>-1</sup> concentration, callus initiation was observed (Fig.5).

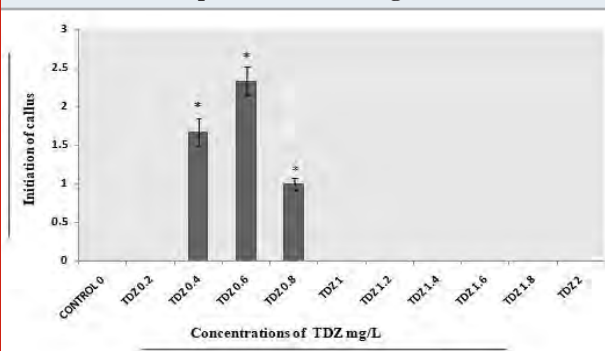
In this study, it was observed that low concentrations of TDZ responded much better in contrast to higher concentrations. The organogenic callus of *E. robustus* was observed using the MS medium consisting of 2,4-D (Rahman 2004). Callus initiation was reported in *E. tuberculatus* when MS medium was fortified with 2, 4-D (Arshad and Kumar 2006). Callus initiations for *E. sphaericus* were best observed in MS

medium fortified with  $1 \text{ mgL}^{-1}$  BAP in combination with  $0.5 \text{ mgL}^{-1}$  2, 4-D (Dubey and Das 2011). The compact callus induction and basal callus formation were observed in the *in vitro* cultures of *E. blascoi* in WPM using TDZ (Siva et al. 2015). Callus formation was successfully reported in *E. grandiflorus* when 2,4-D was used (Habibah et al. 2019). The sterilization component i.e.,  $0.1\%$   $\text{HgCl}_2$  was found best for surface sterilization of immature fruit explants.  $\text{HgCl}_2$  with  $0.1\%$  concentration was analysed as best for surface sterilization of leaf explants of *E. ganitrus* (Rishi et al. 2021).

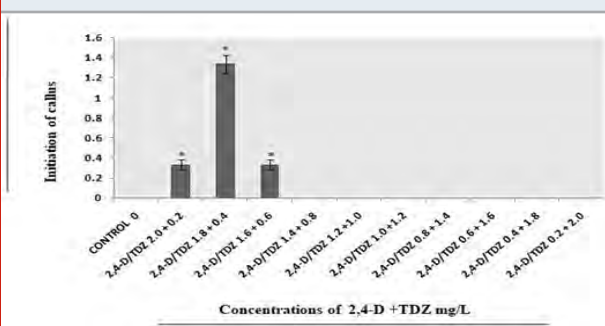
**Figure 3: Different concentrations of 2, 4-D used for callus initiations from explants on full strength MS medium.**



**Figure 4: Different concentrations of TDZ used for callus initiations from explants on full strength MS medium**



**Figure 5: Different concentrations of 2, 4-D+TDZ mg/L used for callus initiations from explants on full strength MS medium**



The previously published studies on tissue culture for different tree species including *E. ganitrus*, were observed quite promising based on plant conservation aspects.

The results obtained from such reported studies can be utilized to develop deep insights into the tissue culture of *Elaeocarpus* due to its high importance in several research disciplines. Therefore, the investigation of immature fruits of *E. ganitrus* (Rudraksha) was taken for research purposes which further may open the various ways of holding other studies. The study can become very important in the coming future for the conservation of endangered tree species of not only *Elaeocarpus* but for other species belonging to different genera through plant tissue culture.

## CONCLUSION

The findings of the present study have shown that the initiation and multiplication of callus observed best in full strength MS medium with 2, 4-D at the concentration of  $1.8 \text{ mgL}^{-1}$ . The study indicated that 2,4-D plays a crucial role during callus induction, growth and development to influence the entire explant material. Hence, the procedure may be useful for callus studies of other *Elaeocarpus* species, especially the ones which are known for different types of Rudraksha beads.

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

**Data Availability Statement:** The database generated and/or analysed during the current study are not publicly available due to privacy.

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# Elephant Foot Yam Peels as Substrate for the Production of Alkaline Xylanase from *Aspergillus terreus* Using Solid State Fermentation

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## ABSTRACT

Xylanase is a significant enzyme which contributes to the breakdown of xylan and is utilized in an enormous range of biotechnological applications. In the present study, alkaline xylanase was produced using Elephant Foot Yam peels (EFY) in Solid State Fermentation (SSF) by *Aspergillus terreus* Thom isolated from elephant dung, which was procured from Indore Zoo. The optimization of xylanase production using One Factor at a Time (OFAT) approach exhibited  $121 \pm 2.5$  U/ml of the highest xylanase activity and obtained at 60°C, pH 8.0 in 96 h culture with inoculum size of  $1 \times 10^6$  spores/ml, 90% moisture and 2 mm particle size in SSF. Further, the BBD (Box-Behnken design) based on Statistical software analysis i.e., Response Surface Methodology (RSM) was employed for optimizing xylanase production which predicted 4.2% increase in value which was in concurrence with the investigational design model.

**KEY WORDS:** ASPERGILLUS TERREUS THOM, ELEPHANT FOOT YAM PEEL, SOLID STATE FERMENTATION, STATISTICAL OPTIMIZATION, XYLANASE.

## INTRODUCTION

Xylan represents 30-35% of the total dehydrated mass of plants and is the next most abundant carbohydrate after cellulose in plant cell walls Izidoro and Knob 2014; Marimuthu et al. 2019; Champreda et al. 2019; Xue et al. 2022). The comprehensive disintegration of xylan is embraced by exploitation of several enzymes that comprises endo  $\beta$ -1,4 xylanase,  $\beta$ -xylosidase,  $\alpha$ -arabinofuranosidase, and acetyl xylan esterase, all of which release xylo-oligosaccharides and D-xylose as the main products Chukwuma et al. 2020; Najjarzadeh et al. 2020; Wu et al. 2020). Xylanases are extracellular enzymes produced by various bacteria like *Bacillus circulans*, *B. subtilis*, fungi like *Aspergillus*, *Trichoderma* species, yeasts and actinomycetes like *Thermomonospora fusca* (Hrmova et al. 1984; Ball et al. 1989; Sunna and Antranikian 1997; Liu et al. 1998, 1999; Shah and Madamwar 2005; Thomas et al. 2016; Rodrigues et al. 2017; Alves et al. 2020; Romero Victorica et al. 2020; Joshi et al. 2022).

Fungi are more efficient for enzyme production in SSF as compared to bacteria owing to their mycelial nature and

requirement of lesser amount of water (Walia et al. 2017; Bhardwaj et al. 2019). Xylanase has been largely employed in animal feed, biorefinery, food, pulp and paper, and textile industries and can be rational for manufacturing numerous useful economical products such as sugar syrups, single cell proteins (SCPs), liquid and gaseous fuels. (Techapun et al. 2003; Dhiman et al. 2008; Xiao et al. 2019; Yepes-Bentacur et al. 2021; Ayubi et al. 2021). Enzyme production using SSF provides many financial benefits over Submerged Fermentation, like lower capital and operational costs, higher product yield and an effective fermentation medium (Krishna 2005; Soccol et al. 2017; Aita et al. 2019).

Several researchers effectively reported on xylanase production using an experimental design model based on Response Surface Methodology for enhancing biotechnological processes (Ezeilo et al. 2019; Azzouz et al. 2020; Prabhu et al. 2022). Many different fruit and vegetable peels viz. lemon peel, orange peel, lemon pomace, apple pomace, cassava peel, mausambi peel, pomegranate peel, banana peel have been used as solid substrates for xylanase production (Seyis and Aksoz 2005; Olanbiwoninu and Odunfa 2016; Kaur et al. 2017; Atalla and El Gamal 2020; Zehra et al. 2020). Elephant foot yam (EFY), *Amorphophallus paeoniifolius* (Araceae), commonly known as Suran, is widely grown in India as a profit crop

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due to its high-level manufacturing potential and lucrative return value (Jogi and Lahre 2020). It supplies a satisfactory amount of protein and simple carbohydrates, and is often used as a vegetable (Singh et al. 2007). EFY has also been used for medicinal purposes such as blood purifier, piles treatment, abdominal disorders, tumours, asthma and rheumatoid arthritis (Kirtikar and Basu 1989; Mishra et al. 2001a; 2001b). The peel of suran which is nearly 20 to 25% of the corm weight and 1 to 4 cm in thickness is used as a supplement in animal feed or a waste disposed into the environment causing biological litter (Ravi et al. 2011; Kumar 2020).

EFY peels were used in present study as solid substrate for production of xylanase from *Aspergillus terreus* Thom isolated from elephant dung. The production was optimized using the OFAT approach and further statistically optimized using BBD based on RSM. EFY peels are an underutilized but potential bio waste that can be used as substrate for production of useful enzymes and other valuable industrial products like Bioethanol and Xylitol. However it has few anti-nutritional factors such as – it may cause itchiness due to the presence of oxalates. Hence, more investigations are needed to utilize the agro-residue elephant foot yam peels for production of useful commercial compounds.

## MATERIAL AND METHODS

Birchwood xylan, xylose and xylo-oligosaccharides were procured from Sigma-Aldrich, USA and other analytical grade chemicals used were procured locally. Agricultural residues purchased from local market were Sun-dried and used. Samples were collected from different natural habitats like agricultural soil, dumping sites, garden area and agro-waste material from nearby locations of Indore and Mhow regions. Excreta of different animals were also collected from Indore Zoo. Samples from soil were collected after removing the upper layer of the soil upto 5 cm depth, using a spatula. The collected samples were aseptically stored in Zip lock poly bags at 4°C till further use.

**Figure 1: Morphological appearance of fungal isolate EF1 identified as *Aspergillus terreus* on PDA plate.**

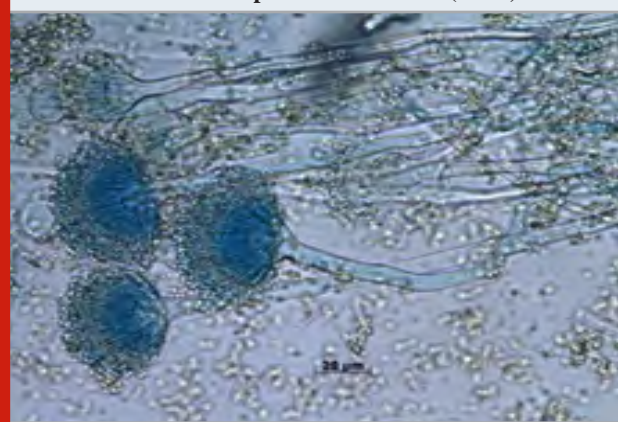


Xylanase producing fungi were screened on xylan potato dextrose agar (XPDA) medium consisted consisting of (g/l) dextrose 20, potato 200, xylan 10, agar 25 and pH 5±0.5 (Sakthiselvan et al. 2014). The XPDA plates were streaked

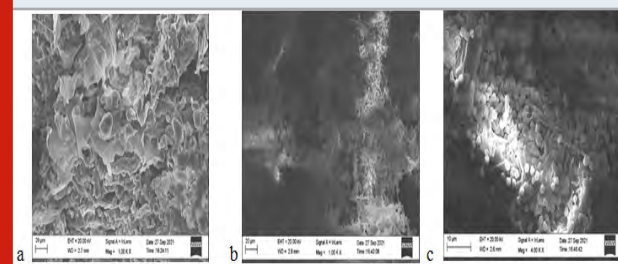
with a loop full of 10<sup>3</sup> diluted soil samples and incubated at 30 ± 2°C for 120 h. A clear xylanolytic zone was observed around the fungal growth on XPDA plates using Congo red staining dye as per the method of Teather and Wood (1982). The pure cultures were maintained at 4°C on PDA slants. Primarily, the isolate was identified by determining the physical appearance on the PDA plate followed by microscopic observations. The structural morphology and molecular characterization were employed for detection of the maximum xylanase producing fungi *Aspergillus terreus* Thom (Bhardwaj et al. 2019).

ITS (Internal Transcribed Spacer) region – partial sequencing was performed at the National Fungal Culture Collection of India, NCCCI, Pune. Sequence kit ABI-Big-dye Terminator v3.1 Cycle was used for PCR sequencing. DNA sequencer entitled automated ABI 3100 was employed for the attainment of raw sequence which was edited for checking irregularity on the routine basis. Publicly accessible sequences were used for the alignment of the sequence data and analysis was done to check its identity (<http://www.ncbi.nlm.nih.gov/>). The construction of the Phylogenetic tree was performed by employing the Maximum Likelihood tree method (Tamura et al. 2011) using MEGHA X software. The isolated fungus from elephant dung was recognized as *Aspergillus terreus* Thom and was applied for production of xylanases.

**Figure 2: Microscopic image of *Aspergillus terreus* NCCCI 4986 stained with lactophenol cotton blue (100X).**



**Figure 3: SEM images a. EFY Peel without fungal spores b. EFY Peel with fungal spores c. Growth of fungal spores over substrate (EFY peel).**



A 5-days old fungal culture was used for the inoculum development as described by Nenava et al. (2021).

Neubauer's counting chamber was used to count the fungal spores. A spore suspension of  $1 \times 10^6$  spores/ml which is used for SSF inoculating the Bio-wastes wheat bran, elephant foot yam peel, orange peel, papaya peel, garadu peel, sugarcane bagasse, pea pods and apple peels were selected as solid substrates for SSF. The Sun-dried peels were pulverised using a grinder and sieved through a 0.5-3 mm mesh. Sun-dried substrate (2 mm particle size) 10 g was moistened with 70% (v/w) sterile distilled water and autoclaved at  $121^\circ\text{C}$  (15 lbs pressure) for 20 min. 1 ml of inoculum ( $1 \times 10^6$  spores/ml) was inoculated in the flasks and incubated for 96 h at  $30^\circ\text{C}$ . Xylanase enzyme extraction was carried out as described by Nenava et al. (2021) in which using 50 mM citrate buffer (pH  $5 \pm 0.5$ ) was used and .The filtrate was centrifuged at  $1500 \times g$  for 20 min at  $0$  to  $-4^\circ\text{C}$  temperature and the supernatant was used for xylanase assay.

Birchwood xylan was used as a substrate for xylanase enzyme assay. The method employed for xylanase enzyme assay and protein estimation was previously mentioned described by Nenava et al. (2021). The reducing sugar released was determined by employing 3' 5'- dinitrosalicylic acid (DNS) method (Miller 1956; Beiley et al. 1993). One unit of endo-1, 4-  $\beta$ -xylanase has been well-defined as the quantity of the enzyme required to release 1  $\mu\text{mole}$  of wood sugar xylose equivalent in one min under the conditions of enzyme assay (Bhalla et al. 2015). Lowry et al. (1951) method using BSA (Bovine Serum Albumin) as a standard protein was used for the estimation of protein present in the reaction mixture. 10 gm each of different sun dried agricultural residues (apple peel, banana peel, orange peel, pea pod, mausambi peel, corn husk, EFY peel, garadu peel, wheat bran, sugar cane bagasse) were pulverised and used for solid state fermentation. EFY peel gave the maximum production of xylanase when fermented by *Aspergillus terreus* Thom.

To determine the effect of fermentation period, 1 ml of *Aspergillus terreus* spores ( $1 \times 10^6$  spores/ml) exposed were inoculated to 10 g of pulverized suran (EFY) peel, and kept at  $28 \pm 2^\circ\text{C}$  for 10 days. Fermented substrate (0.5 g) was withdrawn after every 24 h for enzyme extraction and assay. In order to determine the effect of moisture content 10g of EFY peel was moistened with sterile distilled water. The moisture content ranges was varied from 40% to 110% (v/w) with an interval of 10%. The flasks were autoclaved and inoculated with spore suspension of  $1 \times 10^6$  spores of *Aspergillus terreus* Thom and incubated for 96h.

The flasks were incubated at  $10^\circ\text{C}$ ,  $20^\circ\text{C}$ ,  $30^\circ\text{C}$ ,  $40^\circ\text{C}$  and  $50^\circ\text{C}$  for optimization of fermentation temperature. The washed and dried EFY peel was milled and sieved to get 0.5, 1.0, 1.5, 2.0, 2.5- and 3-mm particle sizes. To study the effect of particle size, 10 g of different particle sized EFY peels were transferred into different 250 ml capacity Erlenmeyer flasks, and each was incubated with 1ml of *Aspergillus terreus* having  $1 \times 10^6$  spores/ml. The final moisture content of 90% was maintained by addition of distilled water as a moistening agent. The incubation was done at  $28 \pm 2^\circ\text{C}$  for 96 h.

Spore count of *Aspergillus terreus* was varied from  $1 \times 10^3$  to  $1 \times 10^9$  spores/ml for optimization of inoculum size. Spore suspension of 1ml was inoculated in autoclaved 10 g EFY peel moistened with 9 ml of distilled water and incubated for 4 days at  $28 \pm 2^\circ\text{C}$ . The optimization of agitation frequency was exhibited by pounding the pre-inoculated flask onto the palm surface of the hand for a minute for about one to five times a day till the end of fermentation process. For the agitation study, 10g of EFY peel with optimized conditions were utilized.

SEM analysis was performed at SEM Facility Unit, IISER, Bhopal. SEM Instrumentation used was Zeiss, Ultra Plus model, Germany. Samples were dehydrated and then, uninoculated EFY peel (without spores) and inoculated EFY peel (with spores) were discretely placed on sample stub. Thereafter, the sample was placed into a gold sputtering system. Using a mini-gold sputter, gold was sputtered for 30 s at  $\sim 70$  m Torr pressure. Next, a sample stub containing a sample was inserted and the SEM chamber was allowed to reach the desired vacuum pressure by turning pumps on and operating under voltage range 1-30 kV for better image analysis. A BBD experimental model based on RSM was executed for optimizing production of xylanase. For optimization studies, elephant foot yam peels were used as solid substrate. In all the experimental arrays assays, the pH was maintained at 8.0. In the present statistical design model, four important process parameters such as fermentation time (A), moisture content (B), particle size (C) and inoculum size (D) were finalized as self-regulating (independent) variables and xylanase activity (Y) as dependent variable response. Above mentioned self-regulating variables were deliberated at different levels, -1 (low), 0 (middle), +1 (high), separately with the help of the BBD model. The trial runs (N) mandatory for the advancement of statistical model BBD is represented by the subsequent calculation:

$$N = 2k(k-1) + C_0 \quad (1)$$

k denotes the Number of Variables and  $C_0$  denotes Number of Central Points. Using the above equation mathematical correlation was developed amongst the four important variables (A-D) in consideration for xylanase enzyme production. A set of 29 experimental run was performed using 5 replicates at a central point and executed in the polynomial model implementing the succeeding equation:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (2)$$

The pH and temperature of the medium were kept persistent at  $8.0 \pm 1$  and  $28 \pm 2^\circ\text{C}$ , respectively, for all the experimental runs. Experiments designed in the BBD are subjected for analysis of xylanase activity at particular time intervals. One flask was prepared as control in order to examine the activity of the enzyme, if it exists before the initiation of the SSF process. Statistical analysis of obtained experimental values was accomplished using the software Design Expert® 12. The optimum interpretations of chosen parameters of fermentation process and its cooperative outcomes were analysed exploiting the same software.



## RESULTS AND DISCUSSION

### Screening and isolation of fungal species for xylanase production:

During primary screening, a total of 62 isolates were evaluated for xylanase activity. Thirteen isolates screened from 28 samples, exhibited a clear zone on xylan agar plate at pH 5.5 on staining with Congo red dye (0.2%). Amongst the four isolates giving a clear zone > 5mm, isolate EF1 (*A. terreus*) isolated from Elephant dung, Indore Zoo gave maximum endo-1,4- $\beta$  xylanase production of  $94.6 \pm 2.6$  units/ml. All the experimental studies were conducted using *A. terreus*. Its occurrence and morphological observations (Fig. 1) showed a brown colour (cinnamon); velvety appearance, and aerial hyphae with brown spots. Staining was performed using lactophenol cotton blue showed that, the conidial head was columnar while the conidial globose was slightly ellipsoid as observed through 100 X magnification (Fig. 2). Scanning electron microscopy (SEM) images of EFY peel without fungal spores and with fungal spores are represented in Fig. 3 (Intasit et al. 2022).

### OFAT approach for optimization of xylanase production:

**Solid substrate selection:** EFY peel was projected to be an effective solid substrate for xylanase production displaying 83.10 U/ml activity followed by garadu peel 64.85 U/ml, and wheat bran 53.24 U/ml; while other solid wastes showed moderate to low xylanase activity (Fig. 4). In order to minimize the cost of xylanase production, use of low-cost substrate in the production media is recommended for which also adds to the sustainability of environment (Sadaf and Khare, 2014; Gupta et al. 2015; Walia et al. 2015; Zhang and Sang, 2015; Behnam et al. 2016; Chilakamarry et al. 2022; Prabhu et al. 2022). The fermentation process is also governed by the configuration of substrate and different process parameters implemented. Different substrate combinations were also used to enhance the xylanase production potential of the medium (Fig. 5). The combination SP +OP (Suran (EFY) peel + Orange peel) (1:1) and SP+SB (Suran (EFY) peel + Sugarcane bagasse) (1:1) showed maximum activity of 71.59 U/ml 61.91 U/ml, respectively. But the combinations used in this research investigation did not represent any prominent increase in xylanase activity, hence EFY peels were solely used as a substrate for xylanase production. Some researchers have effectively employed the combined strategy WB, WS and RB ratios in 1:1 for the production of xylanase (Rana et al. 2021; Intasit et al. 2021; Intasit et al. 2022).

In 2013, Singh et al. reported that elephant foot yam/suran is a fine source of starch, sugars, proteins and minerals. Normal nutritive profile comprises of starch (11-28%), sugar (0.7-1.7%), protein (0.8- 2.60%), fat (0.07-0.40%) and mean energy value of 236 to 566.70 KJ/100g. According to the author's information, this is the first report on EFY peel, a biowaste which is has been employed for the production of xylanase enzyme (De Oliveira et al. 2022). Fig. 4 Different agro-residues used as solid substrate showing xylanase enzyme activity

Figure 4: Different agro-residues used as solid substrate showing xylanase enzyme activity.

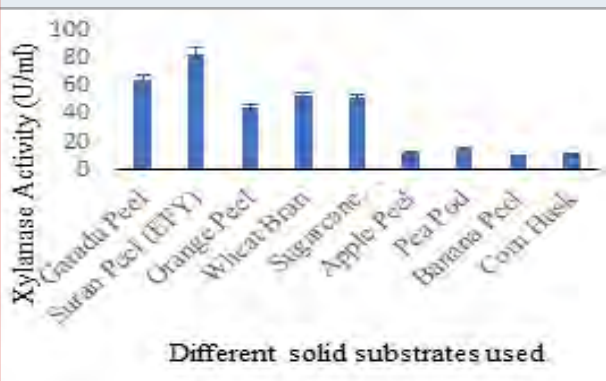


Figure 5: Different substrate combinations employed for production of xylanase showing xylanase activity.

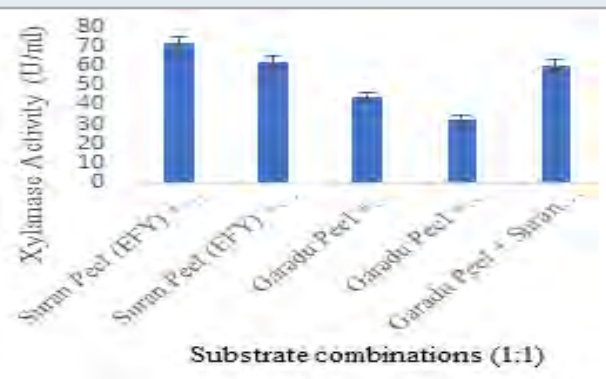
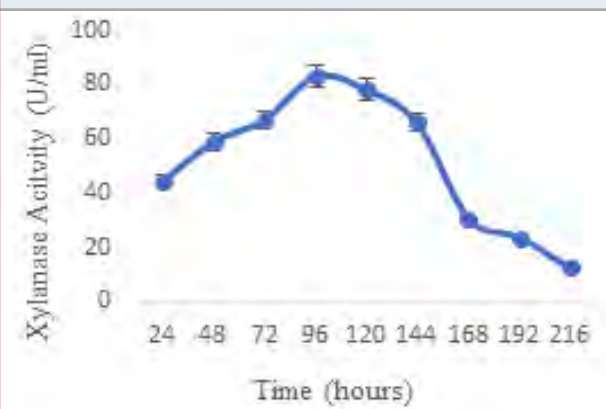


Figure 6: Effect of fermentation period on the production of xylanase by *Aspergillus terreus*.



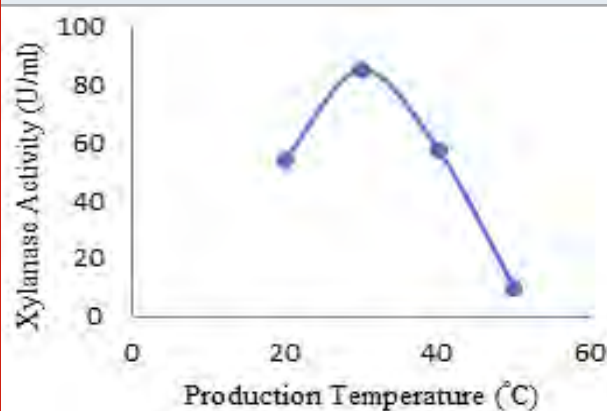
**Effect of fermentation period:** Xylanase production by *A. terreus* was studied by incubating the SSF medium for 9 days. A proportionate increase in enzyme units was observed for 96 h reaching a maximum of 83.04 U/ml. On further incubation, a regular reduction was observed in the activity of xylanase (Fig. 6). The decline in activity over the incubation period could be because of non-specific proteases in the medium. Decker (1983) emphasized the effect of nature of substrate, organism and other fermentation parameters on the optimum fermentation period. Optimum xylanase production was achieved in 4

days using *Aspergillus foetidus* MTCC and in 7 days using *Aspergillus* sp. S9 (Shah and Madamwar 2005; Sharma et al. 2015). The present results also correspond with the previous studies. Similarly, the maximum xylanase activity was reported in 7 days of SSF conducted by De Oliveira et al. (2022).

**Figure 7: Effect of Moisture Content on xylanase enzyme production.**



**Figure 8: Effect of temperature on the secretion of xylanase enzyme.**

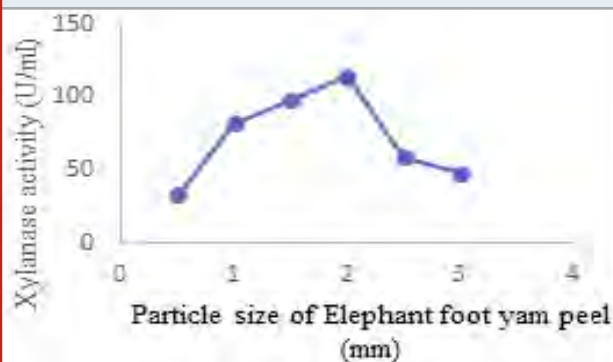


**Effect of moisture content:** The optimal moisture content was found to be 90% for the highest production of xylanase enzyme (Fig. 7). Amongst the different substrates, the moisture level at which free water is available varies considerably depending on their water bonding characteristics. However, Bakri, et al. (2008) reported that for the solid-state fermentation process preliminary moisture content of 30–80% was critical factor because it has a substantial impact on growth, manufacture and release of different metabolites and products. Additional increase in moisture levels beyond 90%, did not elevate xylanase production. Razali et al. (2021) concluded that the moisture content along with the inoculum size triggers varied changes in the production of xylanase. On the contrary, maximum xylanase activity was reported with 60% moisture content under SSF using *Lentinus strigosus* from Amazon (De Oliveira et al. 2022).

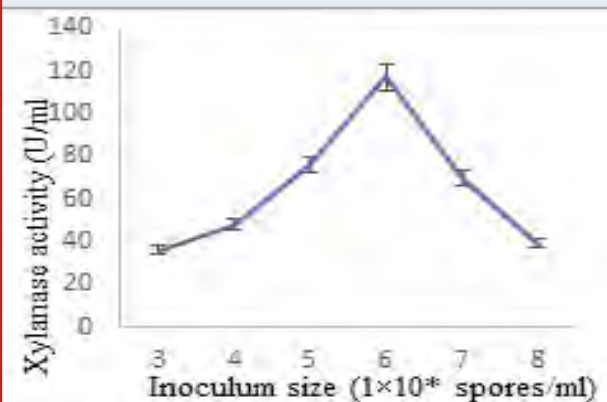
**Effect of temperature:** The enzyme production of  $85.69 \pm 2.5$  U/ml was obtained at a temperature of 30°C with EFY peel. Kuhad et al. (1998) also reported optimum

temperature 30°C for *Fusarium* sp. for xylanase production. Temperature has an important role in the advancement of biological processes as it stimulates the denaturation of protein, enzyme inhibition, and cell progress. In the present study, the enzyme activity decreased with increase of temperature up to 50°C (Fig. 8). Optimum production of endo-1,4-β xylanase was also reported to be 30 °C by Kanimozhi and Nagalakshmi (2014) and Subbulakshmi and Iyer (2014). Temperature specific xylanases (extremozymes) and their metagenomic studies were revealed by (Verma and Satyanarayana 2019; Verma and Satyanarayana 2020). Zarafeta (2020) reported thermostable xylanase belonging to GH-10 family.

**Figure 9: Effect of particle size of substrate (EFY peel) on xylanase production by *A. terreus***



**Figure 10: Effect of inoculum size on xylanase secretion by *Aspergillus terreus*.**

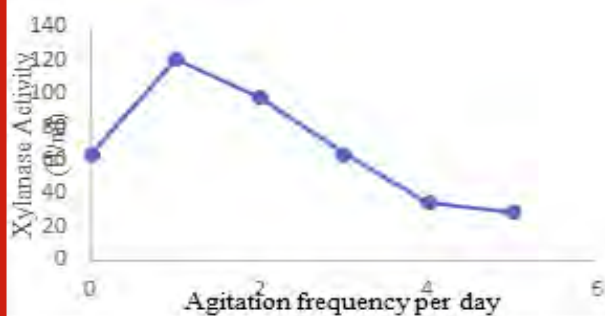


**Effect of substrate particle size:** The maximum xylanase production ( $114.6 \pm 1.5$  U/ml) was obtained at particle size of 2 mm which further decreased on changing the particle size either to higher or lower values (Fig. 9). In SSF, appropriate particle size of solid substrate plays a vital role in improving enzyme activity. Previous studies have reported that the smaller particles have a reduced amount of permeability which encourages lowering of gas diffusion, while the larger particles absorb lesser moisture and swell slightly smaller, thereby drying speedily resulting in a sub-optimal growth of the fungi (Zadrazil and Puniya, 1995). The different particle sizes of the same substrate significantly contribute to the specific degradation of substrate along with the fungal strain used for fermentation and composition of substrate

Therefore, it is significant to detect the optimal particle size for xylanase production. (Lakshmi et al. 2011; Nawawi et al. 2022; Prabhu et al. 2022).

**Effect of inoculum size:** Highest Maximum production of xylanase (117.23± 4.42 U/ml) was obtained with using *Aspergillus terreus* Thom inoculum of 1×10<sup>6</sup> spores/ml (Fig. 10). An adequate concentration of inoculum must be employed to confirm good fungal growth and substrate colonization (Simotilde and Tauk-Tornisielo 2006). An inoculum size extending from 10<sup>5</sup> to 10<sup>7</sup> spores/g of substrate is mostly used for enzyme production (Silva et al. 2005, Sood et al. 2011).

**Figure 11: Effect of agitation frequency on xylanase secretion by *Aspergillus terreus***



**Table 1. Coded levels and Actual response generated by *Aspergillus terreus* in SSF**

Run	A Fermentation time	B Moisture Content	C Particle size	D Inoculum size	Response R1	Predicted R2
1	0	0	0	0	124.3	123.1
2	0	-1	0	-1	58.6	56.8
3	-1	1	0	0	69.1	72.1
4	0	0	0	0	118.7	120.8
5	0	0	-1	1	71.04	69.8
6	1	0	0	1	71.5	72.98
7	1	0	1	0	76.06	75.9
8	1	0	-1	0	59.1	61.18
9	0	0	0	0	121.4	124.5
10	-1	0	1	0	70.4	66.4
11	0	0	0	0	126.4	128.1
12	-1	0	0	1	69.1	67.57
13	0	-1	1	0	60.9	61.49
14	0	1	0	1	63.67	63.62
15	0	1	-1	0	64.85	65.2
16	1	-1	0	0	65.4	64.7
17	-1	0	0	-1	75.7	73.6
18	0	0	-1	-1	66.8	68.7
19	1	0	0	-1	58.47	59.4
20	-1	-1	0	0	64.5	68.09
21	0	0	1	-1	63.4	67.0
22	0	-1	0	1	75.8	76.53
23	-1	0	-1	0	76.06	76.81
24	0	0	1	1	72.87	74.02
25	0	-1	-1	0	76.5	73.38
26	0	1	0	-1	78.4	74.02
27	1	1	0	0	67.9	75.8
28	0	0	0	0	118.6	121.9
29	0	1	1	0	76.1	77.9

The higher inoculum size resulted in reduced enzyme titre because of the primary competition amongst the microbial cell population for nutrients. (Bayoumi et al. 2008; Razali et al. 2021; Prabhu et al. 2022).

**Effect of agitation frequency:** In SSF, agitation study is important and plays a crucial role in the enhancement of production of target enzymes. Inoculated flasks when

tapped gently over the palm of the hand one time a day, gave maximum xylanase production of 121.3±1.5 U/ml (Fig. 11). The enzyme activity declined on increasing agitation frequency. Ibrahim et al. (2012) presented that, dissimilarities in optimum level of agitation rate may be correlated to the category of microorganism used, the level at which evolution of heat occurs, the dissipation of the quantity of carbon dioxide and other volatile metabolites, the

thickness of substrate bed height, and also the dimensions of pore space in the substrate. Syarifah et al. (2014) noticed that mixing was an essential phenomenon for few substrates used in SSF where it can induce the fungal progression and yield of enzymes. Ooijkas et al. (2000) conveyed that the activity of microorganism is adversely affected by mixing on some solid supports whereas in others no adverse effect is observed. However, agitation is not recommended in static reactors in several aerobic SSF processes like tray fermenters (Lonsane et al.1992; Viesturs et al.1987; Wang et al. 2021; Prabhu et al. 2022).

**3.2.8 RSM for Statistical Optimization:** BBD was employed for the evaluation of xylanase activity with an optimum level of each parameter varied along with their interactions. In Table 2, several amalgamations of process parameters such as fermentation period (A), moisture content (B), particle size (C) and inoculum size (D) are represented. The tentative results of response generated were tailored using the second order polynomial equation (1) (Mitri et al. 2022). Thus, the second order polynomial equation can be represented as follows:

$$R^2 \text{ (Xylanase units)}$$

**Table 2. Analysis of Variance of Xylanase**

Source	Sum of Squares	df	Mean Square	F-value	p-value	Level
Model	12196.63	14	871.19	94.17	< 0.0001	Significant
A-A	58.21	1	58.21	6.29	0.0251	
B-B	27.97	1	27.97	3.02	0.1040	
C-C	2.41	1	2.41	0.2607	0.6176	
D-D	42.60	1	42.60	4.61	0.0499	
AB	1.10	1	1.10	0.1192	0.7351	
AC	127.92	1	127.92	13.83	0.0023	
AD	96.33	1	96.33	10.41	0.0061	
BC	180.23	1	180.23	19.48	0.0006	
BD	254.88	1	254.88	27.55	0.0001	
CD	6.84	1	6.84	0.7392	0.4044	
A <sup>2</sup>	4583.11	1	4583.11	495.43	< 0.0001	
B <sup>2</sup>	4651.03	1	4651.03	502.77	< 0.0001	
C <sup>2</sup>	4129.81	1	4129.81	446.43	< 0.0001	
D <sup>2</sup>	4494.74	1	4494.74	485.88	< 0.0001	
Residual	129.51	14	9.25			
Lack of Fit	101.61	10	10.16	1.46	0.3825	not significant
Pure Error	27.90	4	6.98			
Cor Total	12326.14	28				

Std. Dev.- 3.13, C.V.% - 4.0, R- Squared – 0.9895, Adj. R-Squared- 0.9790, Pred. R- Squared- 0.9508

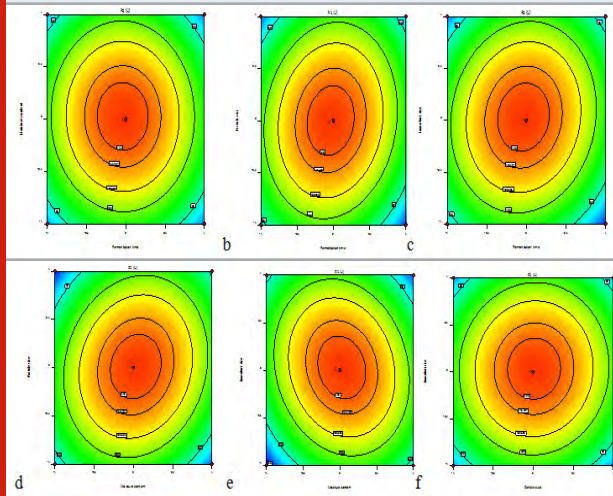
$$= 121.30 - 2.20A + 1.53B + 0.448C + 1.88D - 0.525AB + 5.66AC + 4.91AD + 6.71BC - 7.98BD + 1.31CD - 26.58A^2 - 26.78B^2 - 25.23C^2 - 26.32D^2 \quad (\text{Eq. 3}).$$

Detailed range of all the chosen variables executed in the optimization process are given in Table 1 along with the design of coded and uncoded levels. The statistical implication of the second order polynomial equation (Eq. 3) was confirmed by Fisher distribution (F- test) and the outcomes are revealed in Table 2. The statistical model BBD represents the F-value of 94.06. The remarkable model terms are A, D, AC, AD, BC, BD, A<sup>2</sup>, B<sup>2</sup>, C<sup>2</sup>, D<sup>2</sup> which show a P-value less than 0.05. Values greater than 0.1 indicate that model terms are not important. The lack of Fit F-value of 1.16 indicates that it is possibly due to error with 48.29% chance that could have occurred owing to noise. Significance of model is exhibited by the coefficient of determination (R<sup>2</sup>) of 0.98 value.

The Predicted R<sup>2</sup> of 0.950 is in rational arrangement with the Adjusted R<sup>2</sup> of 0.979; i.e., the difference is less than 0.2. The motion to noise ratio is measured by adequate precision and comparability of the range of the predicted values at the design points to the average prediction error. A ratio of more than 4 is desirable. In the existing study, the motion to noise ratio was 29.366 which specified that the system contained adequate signal. Navigation of the design space can be implemented by this statistical model. Additionally, the coefficient of variation (CV= 4.0 %) was low, indicative of an improved accuracy and evenness of the experimental runs (Mitri et al. 2022).

The response fitted for the above model was strategized for the duo grouping of the utmost collaborative variables (Fig. 12 A-F). The maximum xylanase production was acquired at 96 h of fermentation period, 90% of Moisture content, inoculum size of  $1 \times 10^6$  spores/ml and particle size

**Figure 12: Contour plots showing the effect of (a) Fermentation period and moisture content (b) Fermentation Period and Particle size (c) Fermentation period and Inoculum Size (d) Moisture content and Particle size (e) Moisture content and Inoculum size (f) Particle size and Inoculum size**



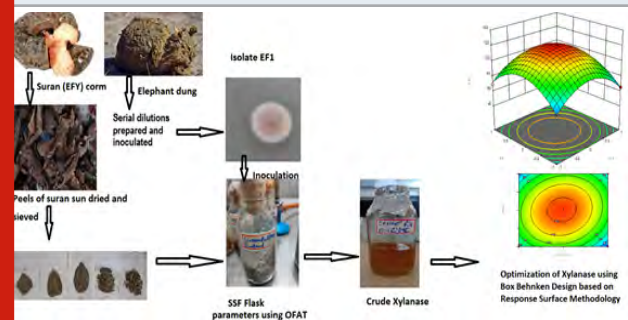
of 2 mm. Highest probable response was intended to be 126.4 U/ml for production of xylanase enzyme. The Fig. 12 (A, B) indicated production of xylanase enzyme upto 89% of moisture content, higher or lower values resulted in reduction of yield of enzyme yield. The xylanase production deteriorated decreased with higher moisture level, probably because of the reduction in the exchange of gases and air, degradation of the substrate structure, inhibition of fungal growth due to increase in adhesiveness (Shah and Dutta 2005; Azzouz et al. 2020).

On adjusting the fermentation time to superior (120 h) or inferior (72 h) values the yield of xylanase production decreased (Fig. 12 A-C). The maximum xylanase enzyme was produced using fermentation time of 96 h. The xylanase production reduced below and above the optimized fermentation time may be because of minimized fungal growth and accumulation of secondary metabolites promoting inhibition of fungal growth followed by enzyme liberation. (Ajijolakewu et al. 2017; Azzouz et al. 2020; Mitri et al. 2022). The Fig. 12 (D-F) showed that xylanase production is increased due to the particle size variable. The maximum production of xylanase occurred at 2 mm particle size. By employing suitable particle size of the solid substrate, the flow of air and mycelial growth was distributed in a space of the medium used (Manan and Webb 2017; Manan and Webb 2020).

Xylanase production was influenced by high and low values of inoculum size. An elevated xylanase secretion was predicted at  $1 \times 10^6$  spores/ml. Inoculum density confirms to be a substantial factor in production of enzymes (Krishna 2005). A low concentration of inoculum may cause underprovided biomass followed by less product formation whereas high concentration of inoculum may cause excessive supply to biomass which triggers reduction in the availability of essential nutrients to the substrate for

product development (Kavya and Padmavathi 2009; Naidu et al. 2020). Recently, Razali et al. (2021) inferred that the temperature, moisture content and inoculum size affect the production of xylanase. Temperature and moisture content also affected the enzyme activity (Razali et al. 2021). Thus, the investigative plan may conclude that the statistical model employed for analysis of variance was satisfactory. Also, it reflects the accuracy and suitability of RSM in optimization of the process parameters employed for xylanase production (Ajijolakewu et al. 2017; Razali et al. 2021).

**Figure 13: Graphical Abstract**



**Validation of RSM model:** During analysis the result obtained after selecting important parameters such as fermentation time, moisture content, inoculum size and particle size for augmented medium model. The response surface model was also validated in triplicate. Experiments designed in BBD are subjected for analysis of xylanase activity at optimum time interval to determine the precision of the model and to authenticate the optimized results. Under optimized conditions,  $128.1 \pm 1.2$  U/ml of xylanase production was attained. This value matches precisely to the predicted value of 126.4 U/ml, by the model which is 4.2% more than the OFAT optimized value 121.3 U/ml. Azzouz et al. (2020) reported that xylanase activity increased phenomenally from 8475.87 to 14766.28 U/mL from *Trichoderma afroharzianum* isolate AZ 12 representing that RSM experimental design has established itself to be a successful tool in governing the optimum parameters for production of enzyme. Ezeilo et al. (2019) inferred xylanase production from a novel *Trichoderma asperellum* UC1 along with other enzymes with maximum xylanase activity of 255.5 U/g. However, the production of xylanase-pectinase cocktail using RSM based on CCD was reported to be as an effective tool used for enhancing production of enzymes using *Bacillus amyloliquefaciens* AD12 conveyed by Nawawi et al. (2022). A brief pictorial representation of present work in shown in graphical abstract (Fig.13) (Nawawi et al. 2022).

## CONCLUSION

The findings of the present study show that the elephant dung isolated *Aspergillus terreus* Thom produces xylanase maximally in statistically optimised SSF on EFY peels. Alkali and thermo-tolerant Xylanase produced can be of effective use in industries such as pulp and paper industry, recycling by deinking in paper industry, bioethanol

production, bakery, fruit juice clarification and many more. The utilization of biowaste for production of industrially useful enzymes can be an effective strategy for Bio sustainability.

**Abbreviations:** SSF- Solid State fermentation, SEM- Scanning Electron Microscopy, EFY- Elephant foot yam, OFAT- one factor at a time, RSM- Response surface methodology, BBD- Box Behenken Design, XPDA- Xylan potato dextrose agar. DNS (3,4- dinitrosalicylic acid) method, BSA- Bovine serum albumin,

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# Antimicrobial Activity of Apple Cider Vinegar Treated Selected Vegetables Against Common Food Borne Bacterial Pathogens

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## ABSTRACT

Vinegar-treated eatables are widely used to improve digestion and are also known for their antimicrobial activity. The evaluated antimicrobial activity of apple cider vinegar (ACV) treated and untreated eatables- ginger (*Zingiber officinale*), garlic (*Allium sativum*), onion (*Allium cepa*), raw papaya (*Carica papaya*), white radish (*Raphanus sativus*) and green chilli (*Capsicum annum*) were analysed against selected common food borne pathogens named *Escherichia coli* (ATCC8739), *Bacillus subtilis*, *Staphylococcus aureus* (ATCC 6539), *Shigella flexneri* (ATCC 12022), *Salmonella typhi* (ATCC 14028), *Cronobacter sakazakii* (ATCC 29544), *Vibrio parahaemolyticus* (ATCC 17802) and *V. cholera* (ATCC 3906) using agar well diffusion technique. Different methods for extraction of phytochemicals have been compared. The eatables were soaked in water for 24 hours, then followed by centrifugation which yielded highest number of phytochemicals. All untreated eatables showed high to moderate antimicrobial activities against all test pathogens, while ACV-treated showed higher antimicrobial activities.

**KEY WORDS:** ANTIMICROBIAL ACTIVITY, APPLE CIDER VINEGAR, EATABLES, EXTRACTION METHODS, PHYTOCHEMICALS.

## INTRODUCTION

Eatables like ginger (*Zingiber officinale*), garlic (*Allium sativum*), onion (*Allium cepa*), white radish (*Raphanus sativus*), raw- papaya (*Carica papaya*), and green chili (*Capsium annum*) are commonly used in different household preparations with and without vinegars, and also in various food products within the industry. Apple cider vinegar (ACV) is known to possess a wide range of biological activities that include antimicrobial, antioxidant, anti-diabetic, anti-inflammatory, anti-hypertensive, immunostimulatory, anticancer and others (Kalaba et al. 2019; Benedek et al. 2022). Ginger (*Zingiber officinale*) which belongs to the family-Zingiberaceae, is cultivated for its medicinal and spices/condiment purposes. This spice is also useful in the treatment of common cold, headache, muscular and rheumatic disorders. Ginger has a distinct spicy flavour and a pleasant aroma. The specific fragrance is attributed to its essential oil which is approximately up to 3% (Hara et al. 1998; Unuofin et al. 2021).

Gingerol is the main bioactive compound in ginger. This spice also contains zingerone, shogaol, gingerol, paradol,  $\beta$ -phellandrene, curcumene, cineol, geranyl acetate, terpineol, terpene, borneol, geranyl, limonene, zingiberol, linalool,  $\alpha$ -zingiberene,  $\beta$ -sesquiphellandrene,  $\beta$ -bisabolene, zingiberenoland  $\alpha$ -farnesene (Manuhara et al. 2018; Yan et al. 2021; Benedek et al. 2022). Ginger has high amounts of antioxidants including phenolic compounds, alanine, and vitamin C. Due to this, ginger is often used by boiling in water to be consumed as a beverage. These antioxidant compounds have an important role in maintaining human health. Besides this, the antioxidants are also widely used as food additives to prevent food damage and add value to the food (Tsai et al. 2005; Habinshuti et al. 2018; Priya et al. 2019; Yan et al. 2021). White Radish (*Raphanus sativus*) belongs to the family- Brassicaceae. It helps in weight loss and increases the metabolism for improved bodily processes (Gamba et al. 2021).

Similarly, white radish helps in treatment of colon, kidney, intestinal, stomach and oral cancers, it also improves the immunity, removes mucous from throat and fight cold and cough. Onion (*Allium cepa*) belongs to the family- Liliaceae. Onion has recorded 6000 years BC old roots and is widely used for its several minerals and vitamins. Onion is also

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used as a medicine. Available research has shown that it is better to use raw onion because after boiling it loses its medicinal properties (Elisabetsky 1991; Sami et al. 2021; Benedek et al. 2022).

Papaya (*Carica papaya*) belongs to the family Caricaceae. It is a rich source of antioxidant and vitamin (A, B, C and E) and some minerals including magnesium and potassium. Papaya has medicinal properties that makes it effective against dyspepsia, hyperacidity, dysentery and constipation. It is also useful in digestion of proteins because it has high proteolytic enzymes (Dawson and Emma 1997; Parin 2020). Chilli (*Capsicum annuum*) belongs to "Solanaceae" family and is rich in vitamins, especially vitamin C and is known for its characteristic non-pungent and pungent taste (Brito-Argaez et al. 2009; Batiha et al. 2020).

Various enteric pathogens are known to adversely affect human health and cause several intestinal diseases. However, these are constituent part of the normal gut microflora of small intestine and act as opportunistic pathogens that are responsible for wide range of infections. *E. coli* is a Gram's-ve, facultative anaerobe, rod-shaped, coliform bacterium, commonly found in lower intestine of healthy people and animals. Few strains of *E. coli* (O157:H7) produces a powerful toxin that damages the lining of the small intestine, which can cause bloody diarrhoea (Mead et al. 1999; Braz et al. 2020; Haindongo et al. 2022).

*Salmonella typhi* is also a Gram-ve, rod-shaped, flagellated bacterium which causes systemic infection and typhoid fever in humans. It is transmitted through food and water. Shigella is another Gram's -ve, facultative anaerobe, non-spore forming, non-motile, rod shaped and a leading bacterial pathogen cause of diarrhoea. The most common symptoms are fever, nausea, vomiting, stomach cramps and flatulence. It is also commonly known to cause large and painful bowel movements. Many commercial antibiotics like Azithromycin (Zithromax), Penicillin, Amoxicillin, Trimethoprim, Ciprofloxacin etc. are being used to fight against such enteric pathogens, but use of such antibiotics is not a good choice due to their high cost, multi-drug resistance and side effects e.g., amikacin and gentamycin used against *E. coli* leads to hearing loss, vertigo and kidney damage (Lewis et al. 2012; Haindongo et al. 2022).

The aim of this study was to determine the antimicrobial activity of untreated and apple cider vinegar treated ginger (*Zingiber officinale*), garlic (*Allium sativum*), onion (*Allium cepa*), white radish (*Raphanus sativus*), raw-papaya (*Carica papaya*) and green chilli (*Capsicum annuum*) extracts on different pathogenic bacteria to find out their beneficial uses and to carry out their phytochemical screening of the extracts so as to evaluate the impact of vinegar treatment on their antimicrobial activities.

## MATERIAL AND METHODS

All eatables were collected from the local market of Meerut (Uttar Pradesh) India and washed well to clean the soil particles, peeled off and were further washed thoroughly again with clean water. After washing, these were dried

under sunlight for 2-3 days and 5 g of each material was soaked in 25 mL of distilled water and 25 mL of apple cider vinegar (ACV) separately for one week. After soaking, these were crushed. Phytochemical analysis and antimicrobial activities were evaluated.

For the physical evaluation of eatables, Evaporable Moisture Content and Total Ash Content were calculated. Evaporable Moisture Content was determined by subtracting the fresh weight from sun dried mass. For Total Ash Content, weighed number of dried eatables was heated at 550°C temperature for 6 h in muffle furnace and the ash content was determined using following formulae:

$$\text{Ash content \%} = \frac{\text{wt of ash}}{\text{wt of sample}} \times 100$$

Different methods were used for extraction of phytochemicals from ginger and finally the best was followed for rest eatables. For Soxhlet Method, 5 g dry powder of ginger was dissolved/suspended in 500 mL of distilled water and kept at 70°C for 6 to 7 h to evaporate the water, the pellet was dissolved in sterile distilled water to make final volume of 25 mL.

For Heating Method with Filtration, 5g powder of ginger was dissolved/suspended in 25mL of distilled water and heated till boiled, mixed properly and filtered through normal filter paper, final volume was adjusted to 25mL. For Heat without Filtration: 5g powder of ginger was dissolved/suspended in 25 mL of distilled water and heated till boiled, kept for 2 h and just decanted, final volume of extract was adjusted to 25 mL. During Simple Soaking followed by Centrifugation, 5g ginger powder was soaked in 15 mL of distilled water over night and was simply centrifuged at 2000rpm for 15 min. The extraction procedure was repeated 3 times, each time with 3 mL water and finally all extracts were mixed and final volume of 25 mL was made. Besides this, the antimicrobial activity of different eatables with or without apple cider vinegar (ACV) was evaluated using agar well diffusion method on Müller Hinton agar. The plates were incubated in an upright position at 37±1°C for 24 to 48 h. and the diameter of zone of inhibition (in mm) was measured against test human pathogens. Lastly, Qualitative standard chemical tests were carried out for phytochemical screening of untreated and ACV- treated eatables using AOAS protocol as described (Trease and Evans 1978; Rawat and Garg 2021).

## RESULTS AND DISCUSSION

Moisture evaporated by sundry method revealed that sun dry weight of garlic was highest followed by onion, green chili and ginger (Fig 1) while the ash content of ginger was highest followed by garlic, white radish and onion. It shows that ginger, garlic and white radish contain high amounts of inorganic minerals. Chili had lowest ash content (1.2% only) which suggests that it has lesser mineral contents but its other contents contributes to medicinal and digestive value (Batiha et al. 2020). White radish had highest evaporable moisture content showing its greater and easy availability

during digestion (Gamba et al. 2021). On comparison of phytochemical composition of ginger extracted by Soxhlet method, heating with and without filtration, and simple soaking followed by centrifugation, it was found that latter method yielded highest amount of all phytochemicals followed by heating without filtration and Soxhlet method showed poor results (Table 1) (Gamba et al. 2021).

It may be due to the loss of vital phytochemicals by evaporation during Soxhlet procedure. Overnight soaking followed by centrifugation avoided the heat treatment which allowed greater release/leaching of phytochemicals. It, was therefore, suggested that overnight soaking followed by centrifugation should be used for phytochemical analysis of biomaterials. Each phytochemical was analyzed using two different tests to ensure their presence or absence in untreated and ACV- treated eatables and the results (Table 2, Fig. 2) revealed that ACV- treated ginger showed tannins; onion and raw papaya possessed terpenoids and green chili gave positive test for saponins while these phytochemicals were absent in the untreated vegetables. It was also found that no phytochemical was lost due to apple cider vinegar treatment and ACV treatment improved the nutritive value of the vegetables (Benedek et al. 2022).

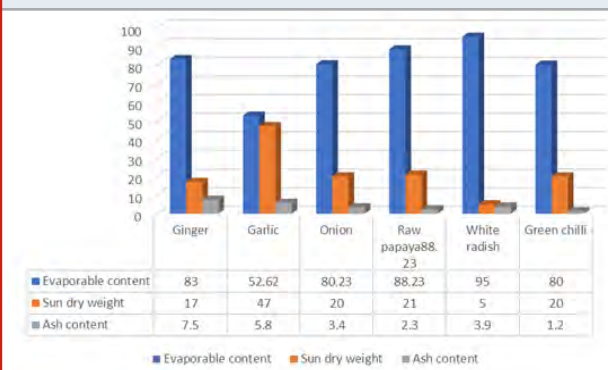
To assess the antimicrobial activity of untreated and ACV-treated eatables, agar well diffusion assay was used against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Shigella flexneri*, *Salmonella typhi*, *Cronobacter sakazakii*, *Vibrio parahaemolyticus*, *Bacillus subtilis* and *Vibrio cholera* (Table 3, Fig. 3, 4) and comparison of their antimicrobial activity showed that ACV-treated ginger should not be used if suffering from typhoid fever as its treatment reduced antimicrobial activity of ginger against *Salmonella typhi* (Table 3). ACV-treated green chili either reduced or showed little effects on antimicrobial activity of almost all vegetables. It suggests that green chilies should not be eaten after treatment with apple cider vinegar. ACV-treated white radish and raw papaya generally enhanced antimicrobial activity against all test species except *Pseudomonas aeruginosa* where it showed little or no effects. ACV-treated onion showed reduced antimicrobial activity against *E. coli* which suggests that apple cider vinegar-treated onion should not be eaten by a person suffering from colitis.

The presence of medicinally active constituents like flavonoids, alkaloids, saponin, tannins, anthraquinones, terpenoids and glycosides in untreated and ACV-treated eatables determines their nutritive value and antimicrobial activity (Manuhara et al. 2018; Benedek et al. 2022). The present study shows that the extracts of eatables possess antimicrobial compounds and are beneficial for health. Vinegars are commonly used as food condiment and preservatives. Apple cider vinegar is also used in the Ayurvedic pharmaceutical industry because of its medicinal properties. Ginger is well known for its antimicrobial activity and is widely used in Ayurveda for various treatments and also in food industry for flavor and aroma (Rajsekhar 2012; Kalhor et al. 2022). Antibacterial activity of some leafy vegetables has been reported in previous studies against *S. aureus*, *S. pyogenes*, *B. subtilis*, *E. coli* and

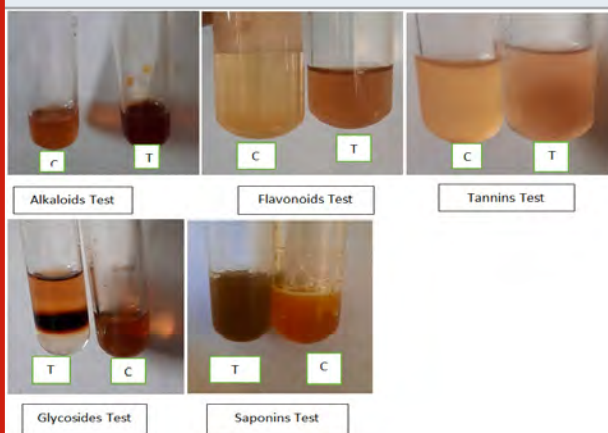
*P. aeruginosa* (Bhat and Al-Daihan 2014; Mahendranathan and Abhayarathne 2021).

Fruits and vegetable are also known for their antimicrobial activity and papaya, potato, cucumber, beet root and ginger have been found to inhibit *E. coli*, *S. aureus*, *Lactobacillus*, and *Proteus vulgaris* (Narinder et al. 2017; Kumar et al. 2021). Antimicrobial activity of fermented vegetable byproducts of some vegetable has been evaluated in a previous study which showed that fermented extracts of tomato, melon and carrot possessed higher antimicrobial activity than commercial preservatives against *Salmonella* spp., *Listeria monocytogenes* and *B. cereus* (Ricci et al. 2021).

**Figure 1: Percent moisture, sun dry and ash contents of eatables used.**



**Figure 2: Qualitative phytochemical tests for analysis of various groups from eatables**



Hossaini et al. (2020) have evaluated antimicrobial effects of medically relevant green leafy vegetables and have found that ethanolic and methanolic extracts of *Azadiracta indica*, *Coccinia grandis*, *Ipomoea aquatica* and *Paederia foetida* leaves extracts possess antimicrobial activity against *Staphylococcus* spp., *Klebsiella* spp., and *Pseudomonas* spp., while crude and hot water extract showed almost no effect on bacterial growth (Hossaini et al. 2020; Ricci et al. 2021). Antimicrobial activity of vinegar is attributed to the phenolics, organic acids, microbial metabolites of the fermenter organism as well as its high acid component and has been found effective against *E. coli*, *P. aeruginosa*, *S.*

*aureus* using agar well diffusion technique (Yagnik et al. 2018; Ousaaid et al. 2021; Benedek et al. 2022).

Antimicrobial activity of ginger extract against *P. aeruginosa*, *S. aureus*, *Proteus mirabilis*, *E. coli*, *B. subtilis* and *S. typhi* has been demonstrated in previous studies (Akintobi et al. 2013; Unuofin et al. 2021). In view of the increasing demand of natural products with health

promoting attributes, the antimicrobial activity of grape vinegars against *S. aureus*, *E. coli* and *Candida albicans* is reported in the previous study (Antoniewicz et al. 2021). Our results reveal that apple cider vinegar -treated white radish and raw papaya should be used while ACV-treated ginger should be avoided when suffering from typhoid fever and similarly ACV-treated onion should not be consumed in colitis. Green chili should not be treated with ACV at all and should be used raw (Benedek et al. 2022).

**Table 1. Comparison of different extraction methods for estimation of various phytochemical groups using ginger as a model.**

Extraction methods	Phytochemical tests						
	Flavonoid	Alkaloid	Tannins	Saponins	Anthraquinone	Terpenoid	Glycoside
Soxhlet method	+	+	-	++	-	+	++
Heat filtration method	++	+	-	+	-	++	+++
Heat without filtration method	+++	++	-	+++	-	++	+
Maceration method	++	+	-	++	-	+	++
Centrifuge method	++++	+++++	-	+++++	-	++++	+++++

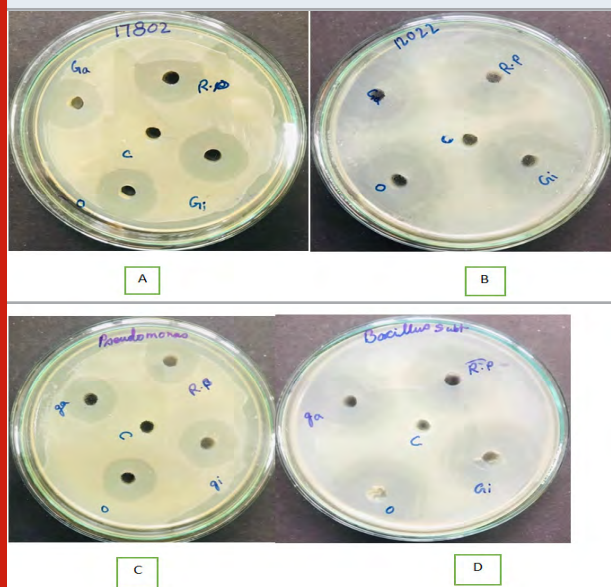
**Table 2: Phytochemical analysis of various biomolecules from apple cider vinegar (ACV) treated and untreated eatable in water extract (WE) using centrifugation method.**

Phytochemicals	Eatable's extract											
	Ginger		Garlic		Onion		Raw papaya		Green chilli		White radish	
	WE	ACV	WE	ACV	WE	ACV	WE	ACV	WE	ACV	WE	ACV
Alkaloids	+	+	+	+	+	+	+	+	+	+	+	+
Saponins	+	+	+	+	+	+	+	+	-	+	+	+
Tannins	-	+	+	+	+	+	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+	+	+	+	+	+	+
Anthraquinones	-	-	+	+	+	+	-	-	+	+	+	+
Terpenoids	+	+	+	+	-	+	-	+	+	+	+	+
Glycosides	+	+	+	+	+	+	+	+	+	+	+	+

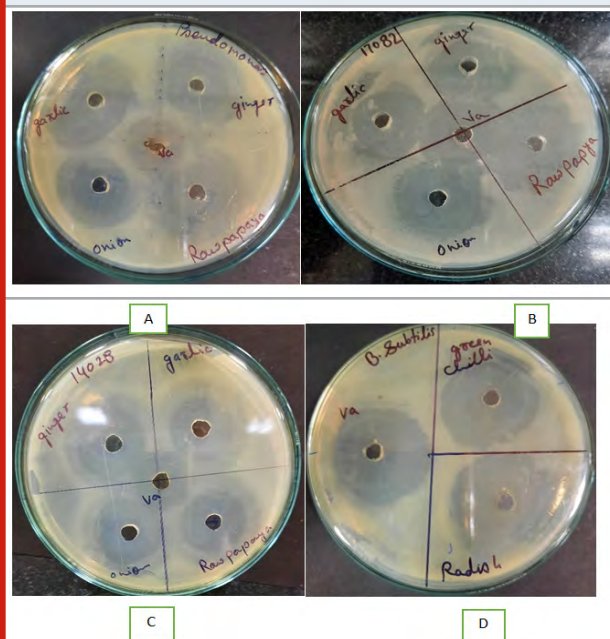
**Table 3. Zone of inhibition (mm in diam.) exhibited by water extracts (WE) of and apple cider vinegar (ACV) treated eatable's extracts.**

Test pathogen	Garlic		Ginger		Onion		White radish		Green chilli		Raw papaya	
	WE	ACE	WE	ACE	WE	ACE	WE	ACE	WE	ACE	WE	ACE
<i>Escherichia coli</i>	9.5	10	7.5	9	7.7	8	10	13.5	9	10.5	9	10.5
<i>Pseudomonas aeruginosa</i>	7.0	7.5	7.75	10	9.5	11.25	8.25	7	6.0	7.25	9	10.25
<i>Staphylococcus aureus</i>	6.5	7	8.25	10.75	7.72	12.25	9.0	11.25	8	8.5	7	7
<i>Shigella flexneri</i>	9.0	11.25	9.25	12	10.25	12	7.0	12.25	7.94	9.25	8.5	11.75
<i>Salmonella typhi</i>	9.5	12	7.57	9.75	7.0	11.25	8	8.25	7.68	9.75	8.5	8
<i>Cronobacter sakazakii</i>	10.25	10.75	7.5	7.75	8.75	7.75	10.65	12.25	7	7.75	9	9.75
<i>Vibrio parahaemolyticus</i>	8.5	11.75	9	10.75	7.5	12	8.8	8.5	8.23	8.25	9	12
<i>Bacillus subtilis</i>	8.5	9	9	9	8.25	8.25	7.89	9.5	7.5	9.25	9	9.25
<i>Vibrio cholera</i>	7	8.75	6.5	9.5	6	10.5	8.25	7.75	6	8.25	8	9.25

**Figure 3: Zone of inhibition of water extract of different eatables: ginger (Gi), garlic (Ga), raw papaya (Rp) and onion (O) against *Vibrio parahaemolyticus* plate A; *Shigella flexneri* plate B; *Pseudomonas aeruginosa* plate C and *Bacillus subtilis* plate D.**



**Figure 4: Zone of inhibition of Apple cider vinegar extract of different eatables: ginger, garlic, raw papaya and onion against *Pseudomonas aeruginosa* plate A, *Vibrio parahaemolyticus* plate B, *Salmonella typhi* plate C and *Bacillus subtilis* plate D and Va denotes apple cider vinegar used as control.**



## CONCLUSION

The findings of the present study have concluded that overnight soaking followed by centrifugation yields

better quality of phytochemicals. Ginger, garlic, onion, raw papaya, green chili and white radish possess high antimicrobial activities against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Shigella flexneri*, *Salmonella typhi*, *Cronobacter sakazakii*, *Vibrio parahaemolyticus*, *Bacillus subtilis* and *Vibrio cholera* and their treatments with apple cider vinegar improves their antimicrobial activity. ACV- treated white radish and raw papaya should be used while ACV-treated ginger should be avoided in typhoid fever. ACV-treated onion should not be consumed in colitis. Green chili should not be treated with ACV and should be used raw.

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## ***In vivo* Immunotoxicity Assessment of Atrazine in two Economically-Important Marine Pelecypoda Species**

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### **ABSTRACT**

Two commercially significant marine Pelecypoda species named *Perna viridis* (green mussel) and *Paphia malabarica* (short neck yellow clam) were exposed to different concentrations of Atrataf (commercial brand of atrazine available in India) in an acute toxicity test. The 96 h LC50 values of Atrataf to *P. viridis* and *P. malabarica* were 6.10 mg L<sup>-1</sup>, and 4.90 mg L<sup>-1</sup> respectively. This study showed that there is a significant increase in mortality in both species as the dose and duration of Atrataf exposure are increased further, exposure to sublethal concentrations of the Atrataf. Moreover, following 14 days of exposure to sublethal doses of Atrataf, the immunotoxic potential of atrazine was examined by measuring viable haemocytes using the Trypan Blue Exclusion Assay. After 14 days of exposure to the highest sublethal doses of Atrataf, the percentage of viable hemocytes decreased to 74.51 (*Perna viridis*) and 78.39 (*Paphia malabarica*), relative to the control. Since Haemocytes are the most critical cells in the immune system of Pelecypoda, any decrease in the hemocyte count will have a detrimental impact on the immune system activities. This study is first of its kind to investigate and report atrazine as a potential compound, which can induce immunotoxicity in Pelecypoda.

**KEY WORDS:** ATRAZINE, PELECYPODA, IMMUNOTOXICITY, IN VIVO, TRYPHAN BLUE EXCLUSION ASSAY

### **INTRODUCTION**

Agricultural chemicals such as pesticides and weedicides contaminate surface water locally but have a tremendous potential to impact at global scale by either entering into the food chain or by bioaccumulation in the soft tissues (Shomar, Muller and Yahya 2006). Pesticide and herbicide toxicity to non-target organisms, particularly aquatic species, has been widely documented in scientific studies (LeBlanc, Bain and Wilson 1997; María et al. 2007; Palma et al. 2008; Flynn and Spellman 2009; Nwani et al. 2010; Tongo and

Ezemonye 2015; Ullah, Hasan and Dhama 2015; Westlund et al. 2018; Klementova et al. 2019). Pesticides have long been the focus of toxicological research, while weedicides have gotten less attention. Weeds drastically reduce agriculture produce by tapping available resources, thus deprive crops of indispensable nutrients and vital minerals. Farmers use variety of weedicides to manage this menace but Atrazine (ATZ) is a favourite choice due to its efficacy and ease of application (Opote et al. 2021).

Atrazine is a generic weedicide, which controls weeds by interfering with the protein biomolecules involved in the photosynthetic machinery of the plants (Trebst 2008). Atrazine is widely used in paddy fields, other cereal crops,

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Sugarcane and most of the horticulture-based plants since from almost sixty years (Muller 2008). Regrettably, this weedicide is always used in excess quantity that leads to the advent of weedicide tolerant species. Efficacy of the atrazine increases if used in right quantities (Muller 2008; Heap 2014). International survey of herbicide-resistance weed data base has reported development of ATZ related weed-resistance in more than sixty species (Short and Colborn 1999). ATZ is predominant contaminant of surface runoff due to its extensive use, and its ability to linger in the milieu for a considerably longer duration (Koskinen and Clay 1997; Opute et al. 2021). Toxicological studies carried out in vertebrate species establish it as toxic to the reproductive, endocrine, and immunomodulation systems (Cummings, Rhodes and Cooper 2000; Stoker et al. 2002; Laws et al. 2003; Whale et al. 2003; Filipov et al. 2005; Opute et al. 2021). Established facts from erstwhile studies, paves way for further studies in other important animal models to understand the hazardous effects of the ATZ. Given the widespread usage of ATZ, more research is particularly essential to corroborate its risks to susceptible nontarget organisms (Opute et al. 2021).

Rampant irrational use of this weedicide has the potential to result in the adverse and precarious effects on almost all ecosystems in general, aquatic ecosystems in particular (Flynn and Spellman 2009). Toxic effects of ATZ have already been reported on several species of fishes, amphibians, and reptiles (Solomon et al. 1996; Glen et al. 2014). Bulk of these studies have focused on physiological and biochemical aspects (Ralston et al. 2009; Nwani et al. 2010; Ullah, Hasan and Dhama 2015). Some studies have reported the influence of ATZ on the aggregation behaviour in freshwater mussels (Flynn and Spellman 2009). Nonetheless marine species are more susceptible to the harmful manifestation of ATZ, which necessitates the need for further research on these species. A special attention is needed on the pelagic Pelecypoda due to their vulnerable habitat that exposes to the high levels of environmental contaminants (Opute et al. 2021).

Given the widespread consumption of mussels, oysters, and clams as food, it is imperative that safety standards be established. Baring seldom reports on the genotoxicity of heavy metals, the risk assessment of environmental contaminants in Pelecypoda is not as exhaustive as it should have been (Prakash and Rao 1995; Sokolova 2004). Other vital aspects such as physiology, particularly those related to immunity, have never been studied. In the present investigation we have evaluated the immunotoxic manifestations of ATZ on two commercially important edible marine pelecypods (Opute et al. 2021). Pelecypoda like any other invertebrate species, solely rely upon innate immunity to defend themselves from pathogens. Haemocytes play a critical role to confer immunity against pathogens to these marine species. (Canesi and Pruzzo 2016; Burgos-Aceves and Faggio 2017; Destoumieux-Garzon et al. 2020; Opute et al. 2021).

Erstwhile studies have provided comprehensive information on the molluscan hemocyte morphology and classification (Cheng 1984). Apart from conferring innate immunity,

Haemocytes are responsible for wide range of functions (Cheng 1977; Opute et al. 2021). In this study, we are evaluating the toxic effects of ATZ on the Haemocytes of two commercially important pelecypods viz. *Perna viridis* (Order: Mytilidae) and *Paphia malabarica* (Order: Venerida) by Exclusion Assay (Strober 2015). This procedure is simple yet tremendously precise and unswerving to determine the viable hemocyte count. Viable / nonviable Haemocytes can be identified simply by observing clear/blue cytoplasm.

## MATERIAL AND METHODS

Atrazine (ATZ) sold under the trade name TATA-Atrataf, was procured from the local dealer of pesticides and weedicides. It was a white amorphous powder, which was soluble in water (7 mg mL<sup>-1</sup>).

*Perna viridis* (Order: Mytilidae) were collected from the Kali River estuary and carefully transported them to the laboratory in a container filled with marine water. Green mussels were acclimatized for five days by feeding ad libitum with phytoplankton, *Chaetoceros* sp. (Iqbal, Khan and Goswami 2008). *Paphia malabarica* (Order: Veneroida) were excavated through sediments at Kali River estuary and carefully transported to laboratory and acclimatized as described for green mussels.

The ninety-six (96) hrs LC<sub>50</sub> calculations were done along with the preparation of sub-lethal doses of *Perna viridis* and *Paphia malabarica*. LC<sub>50</sub> of TATA-Atrataf (Commercial brand of ATZ) for green mussel was determined according to established procedures (Islam et al. 2019). *Perna viridis* were treated with 0 mg/L (Control), 2 mg/L, 4 mg/L, 6 mg/L, 8 mg/L, 10 mg/L, and 12 mg/L of ATZ each in 3 replicates. The treated water was replaced with fresh ones after every 24 hours. Inspection of the experimental setup was done after every six hours to remove the dead animals from experimental containers. Mortality was carefully recorded at 24 hrs., 48 hrs., 72 hrs. and 96 hrs. The data was statistically analyzed by using ANOVA (one way) to determine the differences in mortality between control and experimental groups. Furthermore, the data was also analyzed by the Dunnett T test to compare the control and experimental groups. Based on the mortality, LC50 with 95 % confidence Interval was determined by PROBIT regression analysis. All statistical tests were performed by using IBM SPSS version 24.0.

Total of 50 (Fifty) *Perna viridis* were treated with sublethal doses of 0.00 mg/L (Control), 0.25 mg/L (1/25th of LC<sub>50</sub>), 0.50 mg/L (1/12th), 0.75 mg/L (1/7th), 1.00 mg/L (1/6th) and 1.25 mg/L (1/5th) atrazine in 25 liters of estuarine water, each with three replicates. The experiment was carried out for fourteen (14) days with regular change of water containing sublethal doses after every 24 hours. One hundred short neck yellow clams were exposed to sub lethal doses of 0.00 mg/L (Control), 0.20 mg/L, 0.40 mg/L, 0.60 mg/L, 0.80 mg/L and 1.00 mg/L atrazine in 30 liters of estuarine water, each experimental setup was carried out in three replicates. The experiment was carried out for fourteen (14) days with regular change of water containing sublethal doses after every 24 hours.

Extraction of the hemolymph of the green mussels and clams was done with the help of 5 cc syringe (25-gauge needle) from posterior adductor muscles on 2, 4, 6, 8, 10, 12 and 14 day from control (0.00 mg/L) and five experimental groups (0.25 mg/L, 0.50 mg/L, 0.75 mg/L, 1.00 mg/L and 1.25 mg/L). Exclusion Assay (Trypan Blue) was done as per the instruction given by the manufacturers instruction (Sigma). Viable Haemocytes with clear cytoplasm and nonviable Haemocytes with blue colored cytoplasm were recorded and tabulated separately. More than two thousand each of viable and nonviable Haemocytes were screened on the 2nd day of the experiment from each of the control and five experimental groups of *Perna viridis* and *Paphia malabarica*. Methodology was repeated on 4th, 6th, 8th, 10th, 12th and 14th day of the experiment.

Following calculations were done by using the formulas provided by the manufacturer (Sigma).

1. Haemocytes  $\text{mL}^{-1}$  = Avg count per square  $\times 5 \times 10000$
2. Total count = Haemocytes/mL  $\times 10 \text{ mL}$
3. % VHC=Unstained Haemocytes with clear cytoplasm / Total count $\times 100$

4. % NVHC= Haemocytes with blue colored cytoplasm / Total cells  $\times 100$

Percentage of viable and nonviable haemocytes were analyzed statistically to determine the  $\text{LC}_{50}$  with 95 % CI by using regression test by using IBM SPSS version 24.0.

## RESULTS AND DISCUSSION

***Perna viridis*: Acute toxicity test:** The ability of ATZ to induce cytotoxicity or immunotoxicity was never been investigated on any pelecypoda. This reports for the first time the acute toxicity of ATZ in two commercially as well as ecologically important edible species of pelecypoda. In 5 experimental groups and a control group, mortality of the mussels was recorded at 24 hrs., 48 hrs., 72 hrs., and 96 hrs. The results of the one-way ANOVA revealed a significant difference in mortality rate between the experimental and control groups, with  $F=79.64$  ( $P<0.0001$ ), and further analysis of multiple comparisons by using the Dunnett-T test revealed a significant difference ( $P<0.0001$ ) between the experimental and control groups. The Lowest Observed Effective Concentration (LOEC) was recorded as 2.00 mg/L ( $P<0.009$ ) with 95 % CI (1.18, 8.81). The results of the one-way ANOVA and the Dunnett-T test are presented in Table 1.

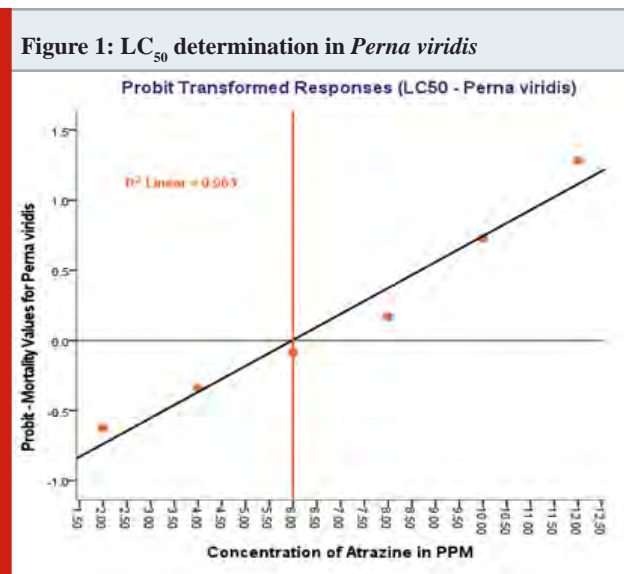
**Table 1. ANOVA (One Way) and Dunnett-T test for Acute toxicity in *Perna viridis***

Dose in mg/L	Number of Animals	Mortality	SE	95 % CI for mean				Dunnett-T Test				
				LB	UB	F - Value	Sig.	SE	Sig.	95 % CI		
										LB	UB	
0	30	3	0.57	0.51	5.48							
2	30	8	1.73	0.55	15.45	79.64	0.000*	1.3	0.009*	1.18	8.81	
4	30	11.33	0.33	9.89	12.77				0.000*	4.52	12.14	
6	30	14	1.15	9.03	18.96				0.000*	7.18	14.81	
8	30	16.66	0.33	15.23	18.1				0.000*	9.85	17.48	
10	30	23	0.57	20.51	25.48				0.000*	16.19	23.81	
12	30	26.66	0.88	22.87	30.46				0.000*	19.85	27.48	

**Table 2. Parameter estimates of analysis in *Perna viridis***

Parameter Estimates							
	Parameter	Estimate	SE	Z	Sig.	95% CI	
						LB	UB
a	Concentration	.214	.028	7.733	.000	.160	.269
	Intercept	-1.372	.198	-6.928	.000	-1.570	-1.174
a. model: (p) = Intercept + BX							

The results of the regression determined LC<sub>50</sub> as 6.10 mg/L. Parameter estimates of analysis presented in Table 2. Dose-response curve (R<sup>2</sup> linear = 0.963) shows that, as the dose and duration of the experimental groups increases, there is a concomitant increase in the rate of mortality (Figure 1).



**Viable Haemocyte Count in *Perna viridis*:** Acute toxicity test was done over 14 days to evaluate the % of viable haemocytes and nonviable haemocytes. Viability and non-viability were determined by simply observing the staining of the cytoplasm, viable haemocytes have clear cytoplasm without any staining whereas nonviable haemocytes have cytoplasm, which is stained as blue. The average count of viable haemocytes (VHC), nonviable haemocytes (NVHC), total haemocytes (TC) and %viability is presented in Table 3. The counting of VHC and NVHC were done on 2<sup>nd</sup>, 4<sup>th</sup>, 6<sup>th</sup>, 8<sup>th</sup>, 10<sup>th</sup>, 12<sup>th</sup> and 14<sup>th</sup> day for the experimental and control groups, %of viability was calculated and presented in Figure 3A and 3B.

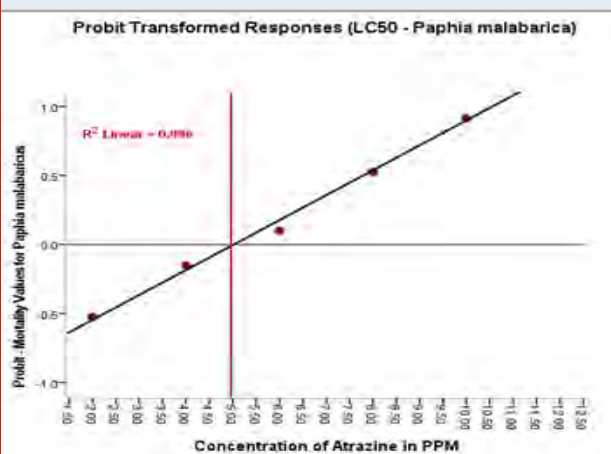
The % of viable haemocytes in the control group was found to be between 94.34 (2.034/mL×10<sup>7</sup>) on the 2<sup>nd</sup> day to 92.34 (1.952/mL×10<sup>7</sup>) on the 14<sup>th</sup> day. No significant difference in the rate of mortality was evident within the control group with no ATZ treatment. In experimental groups of lowest observed effective concentration (LOEC) is found to be 0.25 mg/L. The number of viable haemocytes decreased steadily from 90.5 (1.96/mL×10<sup>7</sup>) on the 2<sup>nd</sup> day to 85.31 (1.85/mL×10<sup>7</sup>) on the 14<sup>th</sup> day. In the second experimental group with 0.50 mg/L of atrazine, the percentage of viable cells decreased from 87.6 (1.92/mL×10<sup>7</sup>) to 84.47 (1.72/mL×10<sup>7</sup>) on day 2 and day 14 respectively.

**Table 3. Haemocytes count and Percentage of viability in *Perna viridis* (× 10<sup>7</sup> Haemocytes/mL)**

			Duration of the Treatment						
			2 days	4 days	6 days	8 days	10 days	12 days	14 days
Atrazine treatment	1.25 mg/L	VHC	1.68	1.63	1.59	1.57	1.54	1.51	1.42
		NVHC	0.40	0.40	0.40	0.40	0.42	0.44	0.49
		THC	2.08	2.04	1.99	1.97	1.96	1.95	1.91
		%	80.86	80.16	79.82	79.8	78.75	77.3	74.51
	1.00 mg/L	VHC	1.81	1.78	1.73	1.67	1.65	1.60	1.58
		NVHC	0.31	0.33	0.34	0.35	0.38	0.39	0.40
		THC	2.12	2.10	2.07	2.02	2.03	2.00	2.00
		%	85.55	84.41	83.4	82.51	81.1	80.27	79.76
	0.75 mg/L	VHC	1.94	1.93	1.85	1.76	1.72	1.68	1.72
		NVHC	0.28	0.29	0.32	0.35	0.36	0.37	0.40
		THC	2.22	2.22	2.18	2.10	2.08	2.06	2.12
		%	86.76	87.48	85.2	83.41	82.66	81.82	81.1
	0.50 mg/L	VHC	1.92	1.94	1.89	1.84	1.79	1.77	1.77
		NVHC	0.27	0.23	0.28	0.30	0.32	0.33	0.33
		THC	2.19	2.17	2.16	2.14	2.11	2.10	2.10
		%	87.6	89.39	87.22	86.17	85.01	84.35	84.47
	0.25 mg/L	VHC	1.96	2.05	2.02	1.99	1.94	1.91	1.85
		NVHC	0.20	0.19	0.20	0.22	0.24	0.28	0.32
		THC	2.17	2.24	2.22	2.22	2.19	2.20	2.17
		%	90.5	91.53	90.89	89.9	89.13	87.07	85.31
0.00 mg/L (Control)	VHC	2.03	1.98	1.98	1.96	1.94	1.96	1.95	
	NVHC	0.12	0.13	0.14	0.13	0.14	0.16	0.16	
	THC	2.16	2.11	2.12	2.09	2.08	2.12	2.11	
	%	94.34	93.66	93.21	93.69	93.07	92.47	92.34	

The trend of decrease in % viable haemocytes has continued with the concomitant increase in the doses of 0.75 and 1.00 mg/L. The experimental group with the highest treatment of 1.25 mg/L, % viable haemocytes was recorded as 80.86 (1.68/mL $\times$ 10<sup>7</sup>) on the 2nd day and further decreased to reach a minimum of 74.51 (1.42 $\times$ 10<sup>7</sup> ml<sup>-1</sup>) on 14th day.

**Figure 2: LC<sub>50</sub> determination in *Paphia malabarica***



#### Nonviable Haemocyte count (NVHC) in *Perna viridis*:

In order to overcome the errors associated with this assay technique, we have also examined the % nonviable haemocytes. The result of this counting of the dark blue-coloured cells clearly shows a drastic increase in the % nonviable haemocytes in *Perna viridis*. In the control group percentage of nonviable haemocytes on the 2nd day of the experiment was found to be 5.781 (0.122/mL $\times$ 10<sup>7</sup>) and on the last day of the experiment i.e., on the 14th day 7.66 (0.160/mL $\times$ 10<sup>7</sup>). The result of this experiment clearly shows no significant change in the % nonviable hemocytes. However, in the case of experimental groups steady increase in the % nonviable haemocytes was clearly evident (Figure 4A and 4B).

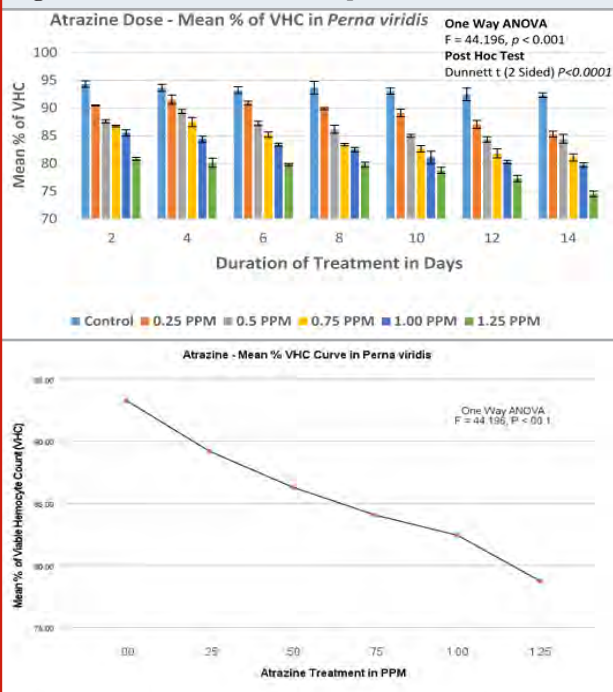
In the case of the lowest observed effective concentration (LOEC) of 0.25 mg/L on the 2nd day, the % nonviable haemocytes were 9.18 (0.206/mL $\times$ 10<sup>7</sup>) and on the 14th day % went up to 14.68 (0.318  $\times$  10<sup>7</sup>). Similarly, there was an incremental increase in % nonviability in experimental groups of 0.50mg/L, 0.75 mg/L, and 1.00 mg/L. Finally experimental group with highest treatment of 1.25mg/L % nonviability recorded as 18.91 (0.398/ml $\times$ 10<sup>7</sup>) on 2nd day itself and 24.47 (0.486/ml $\times$ 10<sup>7</sup>). ANOVA (One Way) test was repeated by using IBM SPSS version 24.0, which showed a significant difference between the groups with P<0.001 (F = 47.118). Further, in the multiple comparisons by using the Dunnett-T test, the mean difference is significant for all the experimental groups when individually compared with the control group (P < 0.001) with 95 % CI.

***Paphia malabarica*: Acute toxicity studies:** In this paper, we reported the LC<sub>50</sub> of atrazine and the lowest observed concentration (LOEC) by conducting 96 hrs. of exposure. All experiments were carried out in triplicates. The mortality rate was recorded after 24 hrs., 48 hrs., 72 hrs., and 96

ANOVA (One Way) was applied to determine the significance level between the viable haemocyte Count in the Control group and in the experimental groups. This test confirms the significant difference in the viability of haemocytes with P<0.001 (F=28.566). Furthermore, multiple comparisons were performed by the Dunnett-T test, which was found to be significant with P<0.0003 between the groups with 95 % CI.

**Figure 3A. % Viable Haemocytes in *Perna viridis***

**Figure 3B: Atrazine dose – VHC response curve in *Perna viridis***



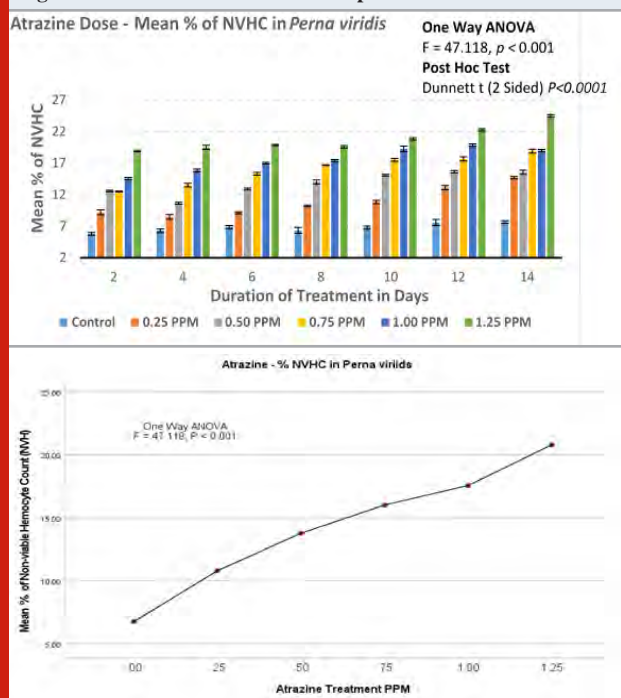
hrs. Results of ANOVA (one way) showed a significant difference in the rate of mortality between control and experimental groups with P < 0.0001 (F = 138.6), and Dunnett-T test results of multiple comparisons also show a significant difference between the experimental groups and control group with P<0.0001. The LOEC in this species was also found to be 2.00mg/L (P< 0.004) with 95 % CI (2.24, 11.76). The results are written in Table. 4.

The regression test LC<sub>50</sub> as 4.90mg/L. Parameter estimates for analysis and Dose- response curve (R<sup>2</sup> linear=0.996) is depicted in Table 5 and Figure 2.

Viable Haemocyte Count (VHC) in *Paphia malabarica* The average number of viable haemocytes, nonviable haemocytes, total haemocytes and % viability is shown in Table 6.

Based on the number of viable haemocytes, the % viability was calculated and depicted in Figure 5B. The mean % viability in the control group without ATZ was found to be 95.29 (1.54/mL $\times$ 10<sup>7</sup>) on the 2nd day whereas, on the last day of the experiment that is on the 14th day, the % viable haemocytes were calculated to be 94.19 (1.59/mL $\times$ 10<sup>7</sup>). No significant difference was found in this group over 14 days. Among the experimental groups, LOEC was found

**Figure 4A: % Nonviable Haemocytes in *Perna viridis*,  
Figure 4B: Atrazine dose– NVHC response curve in *Perna viridis***



to be 0.20mg/L. From this experiment, it is evident that the total number of viable haemocytes has steadily decreased from 90.56 (1.52/mL×10<sup>7</sup>) on the 2nd day to 84.69 (1.36/mL×10<sup>7</sup>) on the last day (14th day) of the experiment. The trend of decrease continued with the concomitant increase in the doses (0.40mg/L, 0.60 mg/L and 0.80mg/L). Finally, at peak treatment of 1.00mg/L, % viable haemocytes were calculated as 85.69 (1.35/mL×10<sup>7</sup>) on the 2nd day and reached a minimum of 78.39 (1.23/mL×10<sup>7</sup>) on the 14th day (Figure 5A and 5B).

ANOVA (One-Way) test showed significant difference in % viability between the groups with P<0.001 (F = 32.238) but no significant difference within the groups.

**Nonviable haemocyte count (NVHC) in *Paphia malabarica*:** In the control group without ATZ, % nonviable haemocytes on the 2nd day of the experiment were calculated as 5.781 (0.122/mL×10<sup>7</sup>), and on the last day of the experiment (14th day) there is a marginal increase in the percentage of haemocytes to about 7.66 (0.160/mL×10<sup>7</sup>). In experimental groups, on the 2nd day of the experiment at 0.20mg/L, % of the nonviable haemocytes was found to be 9.43 (0.16/mL×10<sup>7</sup>) and on the 14th day of the experiment, % of nonviable haemocytes increased to 14.55 (0.23/mL×10<sup>7</sup>).

**Table 4. ANOVA (One Way) and Dunnett-T test for Acute toxicity in *Paphia malabarica***

Dose in MG/L	Number of Animals	Mean Mortality	SE	95 % CI for mean				Dunnett T Test					
				LB	UB	F - Value	Sig.	SE	Sig.	95 % CI			
										LB	UB		
0	30	8.66	0.33	7.23	10.1								
2	30	15.66	0.66	12.8	18.53	138.6	0.000*	1.6	0.004*	2.24	11.76		
4	30	21.66	0.33	20.23	23.1				0.000*	8.24	17.76		
6	30	27	0.57	25.51	30.48				0.000*	14.58	24.08		
8	30	34.66	2.6	23.46	45.87				0.000*	21.24	30.76		
10	30	41.66	0.33	40.23	43.1				0.000*	28.24	37.76		
12	30	45.66	1.2	40.49	50.83				0.000*	32.24	41.76		

**Table 5. Parameter estimates of analysis in *Paphia malabarica***

Parameter Estimates						
Parameter	Estimate	Std. Error	Z	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Concentration	.180	.024	7.337	.000	.132	.228
Intercept	-.904	.177	-5.095	.000	-1.081	-.726

a. model: (p) = Intercept + BX

As the dose and the duration of treatment increased, there was a progressive increase in % nonviability in experimental groups of 0.40 mg/L, 0.60 mg/L, and 0.80 mg/L. In the

experimental group with the highest exposure of 1.00 mg/L, % nonviability was recorded as high as 14.30 (0.23/mL×10<sup>7</sup>) on the 2nd day itself and a remarkable increase of 21.6 % (0.34mL×10<sup>7</sup>).

ANOVA (One Way) test was applied by using IBM SPSS version 24.0, the results of this test clearly show a significant difference between the groups with P<0.001 (F=34.08). The results are represented in Figure 6A and 6B. Further, results of the Dunnett-T test also show significant increase in the % nonviable haemocytes with P<0.001 with 95 % CI.

Humanity is facing several challenges and one of the most pressing issues of the present time is environmental contaminants. This is not only adversely affecting human

health but also deteriorating the quality of each and every ecosystem. Thousands of chemicals are being synthesized on daily basis, which at one point in time gain access to the environment. Most of these chemicals enter the environment without being evaluated for their risk. Some of them are subjected to toxicity related studies, others are ignored or insufficiently studied. Perhaps there is no match between new pesticides and weedicides being introduced into the environment and the assessment of their risk. Scientific reports published focused on the toxicological studies done

on laboratory animals such as mice, rats, rabbits and fishes. However, from the perspective of the method of application of some of these chemicals specifically, pesticides and weedicides were taken into consideration aquatic animals are much more vulnerable. Slower degradation of these chemicals leads to long term persistence of these chemicals in surface water, which poses greater challenges to the aquatic animals. Amongst aquatic animals, Pelecypoda species are more likely to be affected by pollutants and environmental contaminants (Opote et al. 2021).

**Table 6. Haemocytes count and Percentage of viability in *Paphia malabarica* ( $\times 10^7$  Haemocytes/mL)**

			Duration of the Treatment						
			2 Days	4 Days	6 Days	8 Days	10 Days	12 Days	14 Days
Atrazine treatment	1.0 mg/L	VHC	1.35	1.34	1.31	1.31	1.29	1.26	1.23
		NVHC	0.23	0.25	0.27	0.29	0.31	0.31	0.34
		THC	1.58	1.58	1.59	1.6	1.59	1.58	1.56
		%	85.68	84.46	82.79	82.11	80.82	80.08	78.39
	0.8 mg/L	VHC	1.31	1.32	1.31	1.30	1.30	1.31	1.29
		NVHC	0.22	0.24	0.26	0.27	0.28	0.28	0.31
		THC	1.54	1.56	1.56	1.57	1.59	1.59	1.59
		%	85.43	84.86	83.50	82.89	82.23	82.26	80.67
	0.6 mg/L	VHC	1.40	1.37	1.34	1.35	1.31	1.30	1.26
		NVHC	0.19	0.20	0.23	0.26	0.27	0.29	0.30
		THC	1.59	1.58	1.57	1.61	1.57	1.59	1.56
		%	88.03	87.05	85.09	84.07	83.11	81.85	80.94
	0.4 mg/L	VHC	1.43	1.41	1.36	1.35	1.34	1.33	1.33
		NVHC	0.17	0.19	0.22	0.24	0.25	0.25	0.26
		THC	1.60	1.60	1.58	1.59	1.54	1.58	1.58
		%	89.49	87.97	85.94	84.78	87.27	84.07	83.71
	0.2 mg/L	VHC	1.52	1.46	1.45	1.42	1.38	1.37	1.36
		NVHC	0.16	0.17	0.19	0.19	0.21	0.23	0.23
		THC	1.67	1.62	1.64	1.61	1.59	1.60	1.60
		%	90.57	89.77	88.50	88.19	87.01	85.76	85.45
0.00 mg/L (Control)	VHC	1.54	1.55	1.53	1.5	1.56	1.59	1.59	
	NVHC	0.08	0.08	0.09	0.10	0.11	0.10	0.10	
	THC	1.61	1.63	1.63	1.65	1.67	1.69	1.68	
	%	95.29	94.85	94.22	93.83	93.31	94.07	94.20	

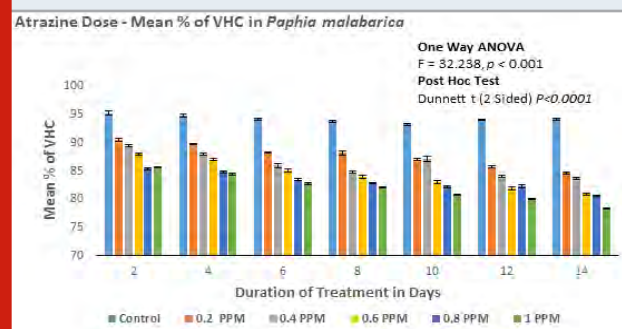
ATZ induced toxicity is reported in several studies already published on fishes, amphibians and mammals. The majority of the studies have reported deleterious effects of ATZ on the aspects like reproduction, endocrinology, developmental biology and the immune system. Some of the toxic manifestations reported on mice models are reproductive organ anomalies, failure of implantations, termination of gestations and delayed onset of puberty (Cummings, Rhodes and Cooper 2000; Stoker et al. 2002; Laws et al. 2003). Some reports implicate ATZ with Immunotoxicity in mice and other mammals. The ATZ is also known to significantly decrease the hematopoietic progenitor cells, reticulocytes and CD4+ lymphocytes in mice and rats (Pistl et al. 2002; Laws et al. 2003; Filipov et al. 2005). Few reports suggest the immunomodulatory functions by significantly

increasing the natural killer cells, T cell proliferation, IgM secreting plasma cells, macrophage-mediated phagocytic and cytolytic functions (Filipov et al. 2005; Rowe et al. 2006; Opote et al. 2021).

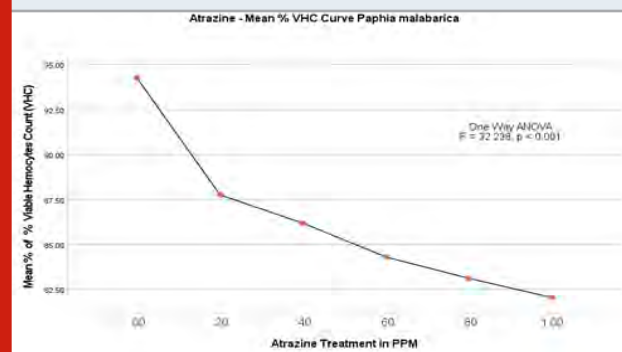
In fishes, ATZ is reported to have toxic effects on the secondary lymphoid organs in salmonid fishes like Coho salmon (*Oncorhynchus kisutch*) and lake trout (*Salvelinus namaycush*) (Zeeman and Brindley 1981). Some reports even suggest ATZ is a potential chemical, which can trigger leucopenia and disintegration of macrophages in fishes (Zeeman and Brindley 1981). Besides immunotoxicity, the ATZ is also known to have endocrine toxicity in catfishes (Opote et al. 2021). In studies done on the amphibians, the ATZ is reported to have an ability to decrease the number of

spleenocytes and leucocytes in *R. pipines*, *R. sylvatica* and *X. laevis* (Kiesecker 2002; Christin et al. 2013; Opute et al. 2021). Apart from the aforementioned literature, no other studies have been done to assess the immunotoxic potential of ATZ in invertebrates. Nevertheless, immunotoxicity of other environmental contaminants such as pesticides, heavy metals, and PCBs have been investigated in a few invertebrate species (Ellis et al. 2011; Renault 2015; Destoumieux et al. 2020; Teresa et al. 2021).

**Figure 5A: % Viable Haemocytes in *Paphia malabarica***



**Figure 5B: Atrazine dose – VHC response curve in *Paphia malabarica***

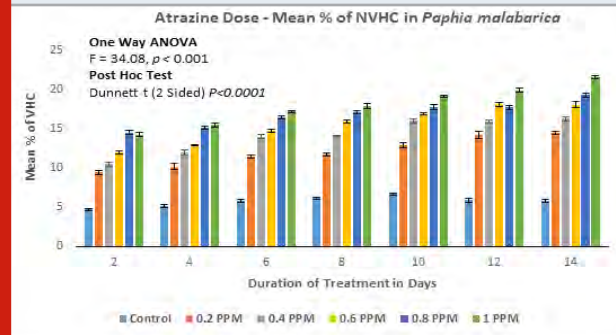


Even molecular mechanisms involved have also been discussed in a few species (Gerdol and Venier 2015; Bachere et al. 2015; Burgos-Aceves and Faggio 2017; Teresa et al. 2021). In the present study, we have evaluated the acute toxicity of ATZ on *Perna viridis* (Order: Mytilida) and *Paphia malabarica* (Order: Venerida), and we have also examined the effect of sub-lethal doses of ATZ on the viability of haemocytes. The results of this study indicate that the ATZ significantly decreases the number of haemocytes in Pelecypoda. This study relies on an exclusion assay - a simple, economical and reliable assay to identify viable and nonviable haemocytes (Piccinini et al. 2017). This assay requires minimum time and is very convenient for the assessment of a large number of samples in a short period. Analysis by one way ANOVA showed a significant decrease in the viable cells in experimental groups when compared to the control group (P < 0.0001) in both Pelecypoda species studied (Teresa et al. 2021).

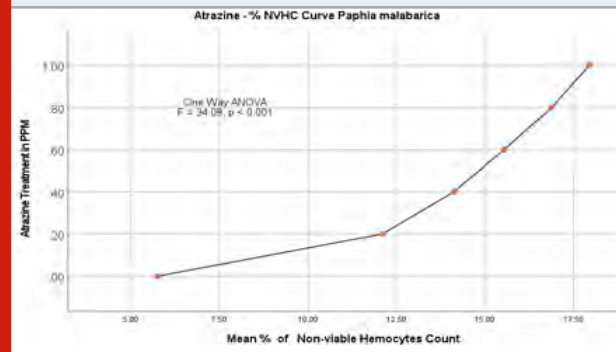
In *P. viridis*, in the control group, the % viability was found to be 94.34 on the 2nd day and 92.34 on the 14th day. In the case of the experimental group at the highest concentration

(1.25mg/L) the % viability was recorded as 80.86 on 2nd day and further decreased to 74.51 by 14th day of ATZ treatment. Correspondingly there was an increase in the number of nonviable haemocytes. In the control group the % of nonviability was recorded to be 5.78 on the 2nd day which marginally increased to 7.66 on the 14th day but in the experimental group with the highest concentration of 1.25mg/L, % nonviability was recorded to be 18.91 on day 2 but drastically increased to 24.47 on 14th day. Similarly, in *P. malabarica*, similar results have been recorded.

**Figure 6A: % Nonviable Haemocytes in *Paphia malabarica***



**Figure 6B: Atrazine dose – NVHC response curve in *Paphia malabarica***



## CONCLUSION

The findings of the present investigation show the immunotoxic potential of ATZ by decreasing the number of viable haemocytes. Analysis by one way ANOVA showed a significant decrease in the viable cells in experimental groups when compared to the control group (P < 0.0001) in both Pelecypoda species studied. In *P. viridis*, in the control group, the % viability was found to be 94.34 on the 2nd day and 92.34 on the 14th day. In the case of the experimental group at the highest concentration (1.25mg/L), the % viability was recorded as 80.86 on the 2nd day and further decreased to 74.51 by the 14th day of ATZ treatment. Correspondingly there is an increase in the number of nonviable haemocytes. In the control group the % of nonviability was recorded to be 5.78 on the 2nd day which marginally increased to 7.66 on the 14th day but in the experimental group with the highest concentration of 1.25mg/L, % nonviability was recorded to be 18.91 on day



2 but drastically increased to 24.47 on 14th day. Similarly, in *P. malabarica*, similar results have been recorded.

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

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# Biodegradation Potential of Phenol and Toluene by Marine *Staphylococcus pasteurii* Isolated from the Red sea, Saudi Arabia

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## ABSTRACT

Hydrocarbons including phenol and toluene are considered as the major source of energy and raw material of different industrial products. Although toluene and phenol have various beneficial applications, many studies reported the massive negative impacts of these contaminants on the environment and on human health. This study aims to provide an assessment of biodegradation potential of *Staphylococcus pasteurii* isolated from the industrial area at the coast of Red Sea, Jeddah, Saudi Arabia. From 29 isolates, a strain, that exhibits a notable growth on mineral salt medium supplemented with phenol and toluene as a sole carbon source, was chosen for further investigation. Different optimization conditions have been examined for optimal degradation; two concentrations of phenol and toluene (0.5% & 1.0%) and different incubation temperatures. Growth assessments was measured by optical density (OD) of phenol and toluene using spectrophotometer. Maximum OD for phenol and toluene: ( $OD_{max} = 0.787$ ) and ( $OD_{max} = 0.969$ ) compared to the abiotic control of ( $OD_{max} = 0.152$ ) and ( $OD_{max} = 0.182$ ) respectively. Degradation of phenol and toluene was also measured using high performance liquid chromatography (HPLC). In addition, molecular identification of the isolates was carried out using 16S rRNA analysis highlighted the isolated strain is *Staphylococcus pasteurii* (strain ATCC 51129) with the accession number (NR114435). This promising strain ATCC 51129 can be used in further biotechnological applications including oil biodegradation processes.

**KEY WORDS:** BIODEGRADATION; HPLC; HYDROCARBON CONTAMINANTS; STAPHYLOCOCCUS.

## INTRODUCTION

Phenol and toluene are highly toxic compounds compared to other types of hydrocarbons. Major problems associated with these compounds are their slow degradation rate which cause massive negative impacts on the environment. In addition, small concentrations of aromatic hydrocarbons such as benzene and toluene will cause vomiting, nausea, skin irritation, and inflammation. Constant exposure to these toxic components will cause long-last effects. In addition, these compounds can induce cancerous cells as a result of genetic mutations. All these contaminants and their processes cause harmful and lethal effects on the aquatic and marine organisms (Abdel-Shafy et al. 2016, Bianco et al. 2020, Ossai et al. 2020).

Various methods have been previously applied in an effort to overcome hydrocarbon contaminants such as adsorption

or membrane filtration. Nevertheless, these methods are proven to be costly and inefficient especially when applied on large scales. Therefore, studies are shifting towards more cost-efficient and eco-friendly methods, one of which is biodegradation. Biodegradation is a principle where microorganisms are responsible for the partial or complete degradation or detoxification of contaminants. Many studies in recent years have focused on the degradation potential benefits of contaminants by some microorganisms i.e., bacteria, fungi, and algae (Basak et al. 2020, Giovanella et al. 2020, Khalel et al. 2021).

Importantly, some microorganisms can consume hydrocarbon contaminants and use them as an organic carbon source such as *Aspergillus*, *Penicillium*, and *Bacillus* (Varjani 2017, Hazim et al. 2019, Younis et al. 2020). However, application of bioremediation of hydrocarbons and petroleum at the contaminated sites is still not achieved on a large scale. This might be due to the poor biodiversity of microorganisms capable of degrading aliphatic and aromatic hydrocarbon compounds. Therefore, this study aims to isolate and identify a bacterium with capability of degrading phenol

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and toluene as hydrocarbon contaminants from aquatic contaminated sites in the Red Sea, Jeddah. In addition to the biochemical and molecular identification of the most efficient isolates with the optimal conditions affecting the process of biodegradation.

## MATERIAL AND METHODS

The aromatic hydrocarbons: phenol and toluene were purchased from Sigma, with 98-99% purity. In addition, all other chemicals and reagents used in the study were obtained from Scharlau and Himedia.

Samples were collected from the industrial area in Red Sea, Jeddah at 21.4934694, 39.17148778. 7 °. Samples were collected from aquatic regions and obtained at 10 – 30 cm depth. Five aquatic samples were placed into sterilized test cups in an aseptic condition. The collected samples were transported to the King Fahd Medical Research Centre (KFMRC), Microbiology laboratory using ice box and kept at refrigerator temperature 4°C until processed. Standard procedure of serial dilution was performed on all aquatic samples according to (Farsi et al. 2021). Diluted samples were mixed in the vortex for 10 minutes then inoculated into nutrient agar and incubated at 37°C for 2-5 days. After the incubation period, each colony of mixed bacterial isolates were picked and sub-cultured three times on a new agar plate resulted in 29 pure isolates. One strain was chosen for further investigations.

The mineral salt medium (MSM) used for enrichment is composed of the following (g/l):  $\text{KH}_2\text{PO}_4$ , 2.25;  $\text{K}_2\text{HPO}_4$ , 2.25;  $(\text{NH}_4)_2\text{SO}_4$ , 1.00;  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.20;  $\text{NaCl}$ , 4.00;  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , 0.02 and  $\text{CaCl}_2$ , 0.01 (pH 7.0). Bacterial isolates were grown on nutrient broth for 24 hrs. at 37°C in rotating shaker. Then, samples were centrifuged at 14000 rpm for 10 minutes. After centrifuging, normal saline solution was added to isolates to dissolve completely. MSM was added as final step before inoculation. Isolate was inoculated in 50 ml of sterilized mineral salt medium (MSM) in Erlenmeyer flask which supplemented with different concentrations of 0.5% - and 1.0% -mM Phenol and Toluene as the only source of carbon for two weeks. Broth cultures were changed from Transparent white color to dark grey color. Bacterial growth was measured by spectrophotometer to evaluate optical density (OD) at 600 nm. Both negative control (abiotic MSM) and positive control (identified bacterium) were used in the study (Oberoi et al. 2015, Rocuzzo et al. 2020).

Estimation of biodegradation potential of phenol and toluene was carried out using Spectrophotometer and High-performance liquid chromatography (HPLC). For HPLC, 1 ml of each MSM samples was transmitted into spectrophotometer cuvette. Blank was the negative control. Growth measurements was estimated from inoculation (0 Time) to the 10th day. Absorbance of bacterial OD was measured at 600 nm.

Phenol and toluene degradation rate was estimated by HPLC (Shimadzu LCsolution Method). C18 column (4.6  $\mu\text{m}$ , 5-micron x150mm, Zorbax Eclipse plus) was utilized in stationary phase with wavelength 256 nm to detect

phenol and toluene throughout biodegradation process. The degradation concentration was calculated based on the retention time and peak area (Oberoi et al. 2015, Xu et al. 2021). Different temperature degrees were applied during cultivation period to estimate the optimum temperature for the isolate. Cultivation the isolated bacteria were applied in 50 ml of sterilized MSM at two different temperatures (25 and 37 °C) into shaking incubator for two weeks.

Two different concentrations (0.5 and 1.0 Mm) were supplemented in 50 ml of MSM, then incubated at 37 °C for two weeks. Evaluation the bacterial growth was obtained in different time intervals during the cultivation (Wongbunmak et al. 2021). Biochemical tests, genomic DNA extraction of the bacterial isolate was done as according to the protocol by (Atashpaz et al. 2010). PCR amplification and 16 S rRNA gene sequencing was done using universal primers. The Forward primer 27F AGAGTTTGATCMTGGCTCAG and Reverse primer 1492 R TACGGYTACCTTGTTACGACTT) were used in the sequencing. Analysis of sequences was done by Blast search9 to find and compare between the similarities that found in NCBI GenBank. In addition, phylogenetic analysis was carried out for the blast results.

## RESULTS AND DISCUSSION

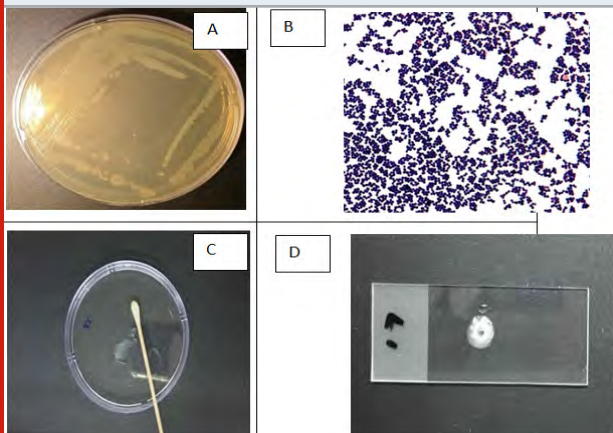
This study was carried out to isolate bacteria capable of biodegrading phenol and toluene from the Red Sea coast, Jeddah, Saudi Arabia. Samples were collected from industrial aquatic regions. A total number of pure 29 isolates were found. The most active isolate, which grow well using phenol and toluene, was selected for identification. After characterization of bacteria, *Staphylococcus pasteurii* was chosen for further identification and estimating its potential for phenol and toluene biodegradation. Strain *Staphylococcus pasteurii* was Gram positive, cocci, colonies appearance was smooth and creamy with white color (Fig. 1). In addition, *Staphylococcus pasteurii* was oxidase negative and gelatinase negative and catalase positive. In addition, antibiotic sensitivity test was done to evaluate resistance of the strain to some antibiotics (Table 1, Fig 2). Furthermore, change in color was noticed in MSM flasks during cultivation period from transparent white to dark grey (Hentati et al. 2021). Recent study for (Chaoui et al. 2022), referred that; the World Health Organization (WHO) has classified the strain of *Staphylococcus maltophilia* as a multidrug- resistant bacteria (MDR). Since the bacterium found to be related to a large range of infections.

The molecular analysis of the strain exhibited a sequencing similarity with 99.85% to *Staphylococcus pasteurii* (strain ATCC 51129). The 16S rRNA gene sequencing (strain ATCC 51129) composed of 1419 nucleotides with accession number of NR114435 according to NCBI GenBank. Phylogenetic trait position of *Staphylococcus pasteurii* (strain ATCC 51129) is illustrated by a neighbor-joining dendrogram (Fig. 3).

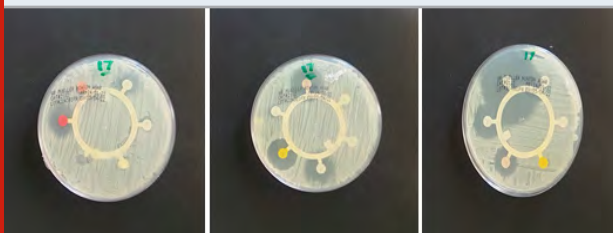
Biodegradation of different concentrations of phenol and toluene was studied by *Staphylococcus pasteurii*. *Staphylococcus pasteurii* was cultivated in MSM medium

supplemented with two different concentrations of phenol and toluene (0.5% and 1.0% mM). The best degradation rate was observed at 0.5 mM comparing to 1.0 mM. Measuring the growth of the strain illustrated by monitoring the OD at 600 nm for 10 days. The isolate showed a significant growth on phenol and toluene as ( $OD_{max} = 0.787$ ) and ( $OD_{max} = 0.969$ ) compared to the abiotic control of ( $OD_{max} = 0.152$ ) and ( $OD_{max} = 0.182$ ) respectively. Moreover, HPLC analyses were achieved to evaluate the degradation rate of phenol and toluene at different interval times (0 time, 1 day, 4 days, 8 days and 10 days). Results showed a prominent capability of the strain to break down phenol and toluene as its sole carbon source. Figure 4 shows degradation percentages of phenol and toluene according to HPLC. The degradation rate of the isolate with phenol was as follows; 0% at 0 time, 12% at 1 day, 20% at 4 days, 16% at 8 days and 14.4% at 10 days. The degradation rate for toluene was 0% at 0 time, 20% at 1 day, 20.8% at 4 days, 12.4% at 8 days and 12% at 10 days.

**Figure 1. Characteristics and biochemical analyses of isolated bacteria from Red Sea, Jeddah, Saudi Arabia. (A) Colony characteristics of isolated strain on nutrient agar plate. (B) Gram staining results of isolated bacteria showing violet cocci bacteria. (C) Negative result for oxidase production test. (D) Positive result for catalase production test.**



**Figure 2: Antibiotic sensitivity test with different inhibition zone for the isolated Strain**



The maximum degradation rate was observed at 4 days equivalents to 96 hours in both phenol and toluene. On the other hand, the least degradation rate was noticed after 10 days of monitoring. This might be cause of consuming most of the hydrocarbon compounds or due to the lethal effect of phenol and toluene on the isolate. Another study carried

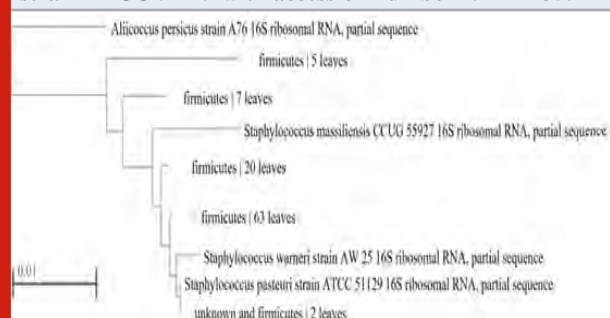
out using a bacterium isolated from soil contaminated areas (*Staphylococcus pasteurii*) has identified this bacterium as a degrader of poly-cyclic aromatic hydrocarbons in addition to n-alkane (Kiamarsi et al. 2019). In addition, Imron et al. (2020) reported that; *Staphylococcus* sp. and *Pseudomonas aeruginosa* have proved a strong ability to perform biodegradation for diesel.

**Table 1. Antibiotic sensitivity test for marine isolated strain**

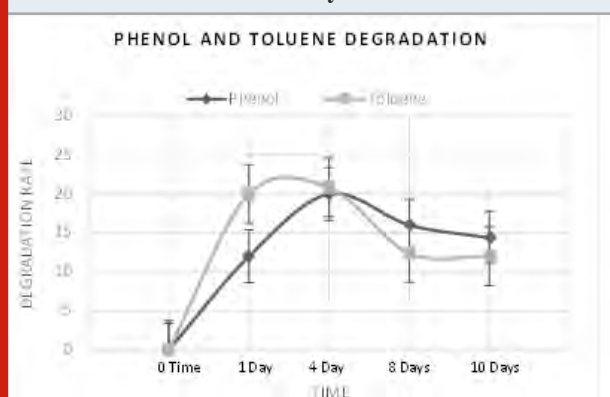
Antibiotic	Results (mm)	Sensitivity
Ciprofloxacin	22	S
Amikacin	16	S
Imipenem	46	S
Ampicillin	-	R
Augmentin	-	R

S: sensitive, I: intermediate, R: resistance.

**Figure 3: Phylogenetic tree analyses based on 16S rRNA gene sequencing showing the position of *Staphylococcus pasteurii* strain ATCC 51129 with accession number NR114435.**



**Figure 4: Degradation rates of phenol and toluene by the strain ATCC 51129 obtained by HPLC**

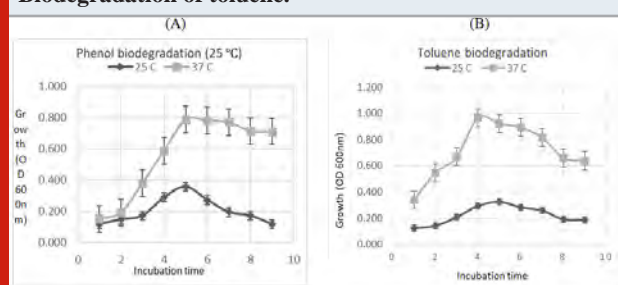


These microorganisms were isolated from different environmental sites contaminated with diesel and considered them as hydrocarbons degrader. In addition, *Staphylococcus pasteurii* strain CO10 is another strain that considered as hydrocarbon degrader. Previous investigation on marine bacterium, *Staphylococcus* sp. CO100 strain has

provide a great ability to biodegrade aliphatic hydrocarbons includes in crude oil with more than 70%. Growth of strain CO100 utilizing crude oil was evaluated by measuring OD using spectrophotometer. The maximum optical density was 0.65 compared to the optical density of control = 0.17, (Hentati et al. 2021).

Investigation of different temperature degrees was carried out in the study. Estimation of biodegradation potential of phenol and toluene was done at 0.5mM with two different temperature (25 and 37 °C) for ten days. At 37°C temperatures, the maximum observation of OD was noticed after two days of incubation (OD= 0.787 and 0.969) for phenol and toluene respectively. On the other hand, OD at 25 °C was (0.357 and 0.325) for phenol and toluene (Fig. 5). The obtained result was in agreement with (Hentati et al. 2021) study on *Staphylococcus* sp. as a hydrocarbon degrader, which reported 37 °C as an optimum growth temperature. The previous study investigated *Staphylococcus* sp. ability for biodegradation on a large temperature scale (15 to 55°C). It should be noted, enzymatic activities also are one of the most important biological factors. The enzymatic activity showed significant inhibition with the increase of hydrocarbon contamination.

**Figure 5: Effect of different incubation temperatures (25 and 37°C) on phenol and toluene biodegradation by the tested bacterium, (A): Biodegradation of phenol (B) Biodegradation of toluene.**



Moreover, species of microorganisms is another important factor that will affect the success of biodegradation process. *Staphylococcus* sp., *Bacillus* sp., *Pseudomonas* sp., and *Acinetobacter* sp. are well known to have high efficiency in degrading aliphatic and aromatic hydrocarbon compounds. Furthermore, temperature variations affect hydrocarbon viscosity directly. High temperatures cause reducing to the viscosity and subsequently leads to better hydrocarbons diffusion as well as a better biodegradation process. Because the well-diffused contaminant is much easier to be degraded by the microorganisms. Generally, hydrocarbon's viscosity decreases with low temperatures. *Staphylococcus* sp. is considered a promising bioagent and there is less research on *Staphylococcus* genus compared to the genera of *Pseudomonas* and *Bacillus* (Eddouaouda et al. 2012, Alrumman et al. 2015, Imron et al. 2020, Xu et al. 2021).

## CONCLUSION

Since the industrial revolution, hydrocarbons are becoming an integral part in daily life. Hence, massive amount of

hydrocarbon waste is produced daily. In this study, several of bacterial strains isolated from aquatic contaminated sites. Among of other bacterial isolates evaluated for their biodegradation potential, *Staphylococcus pasteurii* was selected for further analyses as it exhibits biodegradation potential. Biochemical and molecular identification indicate that the isolate is *Staphylococcus pasteurii* with accession number (NR114435). Also, impact of different parameters was illustrated in the study such as temperatures and different phenol and toluene concentrations. The findings of the present study provided a handful information about the strain and its capability of phenol and toluene degradation which can be an excellent way of finding an alternative solution to achieve high biodegradation rates on a large scale.

**Conflict of Interests:** The author declares no conflicts of interests to disclose.

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