Chemical Composition and Pesticidal activity of *Cinnamomum malabatrum* Leaf Essential Oil against *Sitophilus oryzae*

Dissertation report submitted in partial requirement for M.Sc. degree course in Zoology with Environmental Biology as special subject

In

University of Calicut

By

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KOZHIKODE -08

2021-2023

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EXAMINER 1:

EXAMINER 2:

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Report of the project (Students project / field project / internship) submitted to St. Joseph's College (Autonomous) Devagiri

Affiliated to the University of Calicut

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Program: M.Sc. Zoology

Semester: FOURTH

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This is to certify that the report entitled **"Chemical Composition and Pesticidal activity of *Cinnamomum malabatrum* Leaf Essential Oil against *Sitophilus oryzae*"** is an authentic record of studies and research work done by **Ms. Akshaya K M.** (Reg No: **DVAVMZL001**) under the guidance and supervision of **Dr. Arunaksharan N.** Assistant Professor, Department of Zoology, St. Joseph's College, Devagiri, Calicut. The work has been carried out for the partial fulfilment of the requirements for the award of the degree of Masters of Science in Zoology.

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CERTIFICATE

This is to certify that the dissertation entitled **"Chemical Composition and Pesticidal activity of *Cinnamomum malabatrum* Leaf Essential Oil against *Sitophilus oryzae*"** is an authentic record of studies and research work done by **Ms. Akshaya K M** (Reg No: **DVAVMZL001**) under my guidance and supervision and that, to the best of my knowledge and belief, this work has not been presented earlier for any degree or fellowship.

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DECLARATION

I hereby declare that the dissertation entitled **"Chemical Composition and Pesticidal activity of *Cinnamomum malabatrum* Leaf Essential Oil against *Sitophilus oryzae*"** submitted in partial fulfilment of the requirements for the award of the degree of MASTER OF SCIENCE IN ZOOLOGY is a record of original work submitted by me at St. Joseph's College (Autonomous), Devagiri under the supervision and guidance of **Dr. Arunaksharan N.** Assistant Professor, Department of Zoology, St. Joseph's College (Autonomous), Devagiri, Calicut during 2021-2023.

I hereby declare that the results presented have not been previously submitted for the award of any degree or fellowship.

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ACKNOWLEDGEMENT

It is with a deep sense of pleasure that I express my sincere gratitude to **Dr. Arunaksharan N.** M.Sc, Ph.D., Assistant Professor, P.G and Research Department of Zoology, St. J oseph's College (Autonomous), Devagiri, Kozhikode, for her valuable guidance and supervision in the form of practical suggestions and constructive criticisms which formed the basis for the successful completion of this project.

I express my sincere gratitude to **Dr. Vineesh P.J**. MSc, B.Ed, PhD, Assistant Professor and Head, PG and Research Department of Zoology for his support and sincere encouragement.

I thank **Visakh N U** (PhD Scholar) and Berin Pathrose (Associate Professor, Department of Agricultural Entomology, Kerala Agricultural University, Thrissur) for providing essential oil samples and supporting the chemical characterization by GC-MS analysis.

I extend my deep gratitude to **Ms. Aswathi m k**, M.Sc, Department of Zoology, for his wholehearted support and advice that helped me throughout the project I had undertaken.

I would not forget to remember **Mr. Joice Tom J**, M.Sc. M.Phil., Assistant Professor, Department of Zoology, for his kind support and advice that helped me a lot during the project I had undertaken.

I am greatly indebted to **Ms. Najiya v k, Ms. Nitha k m, Ms. Nivya, Ms. Shadiya**, and **Ms**. **Shana Jebin M** the co-researchers for helping me to complete the project and providing their valuable time in editing and proofing my thesis.

I extend my heartfelt thanks to the non-teaching staff of the department for their valuable

Cooperation.

Finally, I express my deep gratitude to my family and friends for their sincere support

Throughout the project. Above all, I thank God Almighty for the blessings for the successful Completion of the project.

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INTRODUCTION

Stored-product pests are a major threat to the agriculture sector and cause high economic losses and contamination of foods, which in turn affect public health (Stejskal *et al.,* 2003). Numerous stored grain bugs prefer devouring grain embryos, which diminishes the protein content of feed grain and the percentage of seeds that germinate as a result. Lesser grain borer, rust red flour beetle, and rice weevil are a few significant stored grain pests.

The *Sitophilus oryzae,* also named rice weevil, comes under the family Curculionidae and sub-family Dryophthorinae. It is a commonly stored product pest that feeds on several crops including wheat, rice, and maize. Morphologically an adult grows up to a size of 3-4 mm long with a long snout. Black/brown coloured rice weevil may exhibit orange/ red spots arranged across the wing covers. Maize weevil is always a layer that of the rice weevil, the difference is the external genital structure and key identification of both species.

Different preventive measures will be used starting in the early stages depending on the level of infestation. These include handling and sanitizing grains, cleaning stores, and using physical controls like the application of low and high temperatures, mixing inert dust, activated clay, irradiation, and controlled atmosphere, as well as several mechanical controls, plant products, fumigation, polyphyletic treatment of grains, and chemical pesticides. One of the basic notions of integrated pest management (IPM) and management techniques for pest pesticide resistance is the selective administration of insecticides. Chemical pesticides are frequently used as remedies to stored product pests. A pesticide should Ideally, be fatal to the targeted pests, but not to off-target species, embracing humans. Miserably, this is not the case, so dissension concerning the use and misuse of pesticides has surfaced. The rampant use of these chemicals, under the adage, “if little is good, a lot more will be better” has wreaked havoc on both humans and other living things (Aktar *et al.*, 2009). Additionally, the administration of pesticides causes genetic defects in organisms, environmental pollution also adversely affects pollination, etc.

Administration of plant essential oil, a potent biological source alternates with this and can meet the drawbacks of chemical pesticides. Essential oils are any volatile, aromatic, lipophilic organic extracts derived from plants, that give a plant its distinctive scent and are reputed to possess medicinal benefits. *Cinnamomum*

malabatrum, commonly known as wild cinnamon, country cinnamon, and malabatrum, is an indigenous species of India's Western Ghats and a member of the Lauraceae family. It can become as tall as 15 meters (49 feet). It contains fragrant leaves that are used in cooking and medicine. In classical and medieval eras, it is thought to have served as a prominent origin of the medicinal plant leaves recognized as malabatrum. (Or malabatrum).

The term "Malabar" has historically been used to describe the coastal region of southwestern India, which includes the current state of Kerala and surrounding regions(Duguta, 2021). Based on the phytochemical constitution of the essential oil of *Cinnamomum malabatrum* contains cinnamaldehyde, cinnamyl acetate, caryophyllene, trans-α-bergumontene, caryophyllene oxide etc. which exhibit the properties of antimicrobial, antifungal, pesticidal and various disease prevention. So it is used as a significant bioactive substance against numerous pests. Traditional medical systems all around the world recommend essential oils for a range of health issues. Numerous biological and pharmacological actions are attributed to them, including antibacterial, anticancer, antifungal, antimutagenic, antiviral, antidiabetic, antiprotozoal, and anti-inflammatory effects. The characterisation and identification of key EO components, which are of broad relevance, notably to the cosmetic and pharmaceutical industries, have been made possible by extensive phytochemical investigation(Raut & Karuppayil, 2014). Plant essential oils have been utilized for centuries as natural solutions for various ailments and as insecticides to control pests. The pesticidal activity of essential oils has gained attention due to their effectiveness, low toxicity, and environmental friendliness compared to synthetic pesticides. Essential oils contain a complex mixture of compounds, including terpenes, phenolics, and aldehydes, which contribute to their pesticidal activity. The study also shed light on the mechanisms underlying the pesticidal activity of essential oils, which involve disruption of insect cuticles and membranes, interference with neurotransmitter receptors, and inhibition of enzyme activity. These discoveries indicate that essential oils could be employed in IPM programs to reduce the use of synthetic pesticides and minimize their impact on the ecosystem and human well-being.

This project extends to investigate the pesticidal activity of plant essential oil of *Cinnamomum malabatrum* and their potential use as a substitute for synthetic pesticides. And also it extends the larvicidal property against mosquito larvae, explores the mechanism underlying the pesticidal activity and evaluates their potential

for use in integrated pest management (IPM) programs. This study also sheds light on the effect of essential oils on non-target organisms. Future research should explore more pests in various fields.

OBJECTIVES

- To evaluate the pesticidal activity of the essential oil from *Cinnamomum malabatrum* against *Sitophilus oryzae*.
- Analyze the phytochemical composition of the essential oil derived from *Cinnamomum malabatrum*.
- Assess the mechanism behind the pesticidal activity of essential oil extracted from *Cinnamomum malabatrum* against *Sitophilus oryzae*.

REVIEW OF LITERATURE

Spices are a category of plant-derived compounds that are utilized for enhancing the flavour, fragrance, colour, and appearance of food. In ancient and medieval civilizations, spices held a position of immense worth as coveted commodities in trade. Traditional healers have utilized plants for medicine (Vasanthi & Parameswari, 2010). A spice is a dehydrated seed, root, fruit, bark, or vegetative material that is added to meals as a flavouring agent but has minimal nutritional value. An intensely flavoured or fragrant material of vegetable origin, produced from plants in the tropics, and frequently used as a condiment is what is referred to as a spice(Rathore and Shekhawat, 2008). India contributes 12 per cent of the total world trade in spices and is one of the significant producers and exporters of spices worldwide (Pruthi, 1993). And 80 types of spices are present all over the world with which 50 types of them produced by India alone(Rathore and Shekhawat, 2008). Owing to their powerful anti-inflammatory and antioxidant characteristics, spices and herbs are utilized in numerous traditional remedies, particularly in nations like India, for the treatment of a variety of chronic ailments(Kunnumakkara *et al.*, 2021). Spices are used for a variety of things, including seasoning food, making cosmetics, and even medicine(Senthilkumaran and Amudhavalli, 2007). Spices are also utilized for their therapeutic benefits. For example, ginger prevents dyspepsia, garlic lowers cholesterol and blood pressure, fenugreek helps develop resistance, pepper frequently acts as an antihistamine, and turmeric helps stomach ulcers and promotes skin radiance. (Singh *et al.*, 2012). Assafoetida (*Ferula assafoetida L.*), Bay leaves (*Cinnamomum tamala L.*), Turmeric (*Curcuma longa L.*), Bishop's weed (*Trachyspermum copticum L.*), Cardamom (*Elettaria cardamomum L.*), Cassia (*Cinnamomum cassia L.*), Cumin (*Cuminum cyminum L.*), Pepper (*Piper nigrum L.*), Nutmeg (*Myristica fragrans Houtt.*), Fennel (*Foeniculum vulgare Mill*), Fenugreek (*Trigonella foenum-graecum L.*) are some common Indian spices. Most of the spices are characterized by the presence of potent phytochemicals like carotenoids, curcumins, catechins, and lignin respectively which have significant roles in the field of cancer treatments and for the treatment of numerous diseases. This made enthusiastic researchers explore the properties of antimicrobial, antihistamine, larvicidal, and insecticidal activity of spices(Lai and Roy, 2004).

Clove (*Syzygium aromaticum*) ranks among the most well-known and extensively used spices and has been proven to have excellent dietary worth and antibacterial properties(Dahiru and Paliwal, 2021). Cloves are used commercially to make clove oil, which has numerous pharmacological properties including antioxidant, anti-inflammatory, and antiviral properties. Clove is utilized in a variety of products besides medicine, such as insect repellents, biofuels, and the perfume industry(Sharada and Lalitha). Based on the analysis of the gas chromatography-mass spectrometer reveal the presence of eugenol (83.58%), acetate (12.07%), caryophyllene (4.35%), and phenol, 2-methoxy-4-(2-propenyl)-, were detected and responsible for the antimicrobial property, which is applicable in the field of herbal toothpaste formulations (Oluwasina *et al.*, 2019).Anti-inflammatory studies from(Esmaeili *et al.*, 2016).

Ginger is a member of the family Zingiberaceae and is thought to have its origins in south-eastern Asia. It is cultivated using rhizomes, and it thrives in tropical and subtropical climates.(Rashmi and Tiwari, 2016). *Zingiber officinale Roscoe, Zingiberacae* (ginger) is a spice that is commonly utilized in food all over the world. It has long been used as a key component in Chinese, Ayurvedic, and Tibb-Unani herbal remedies for the therapeutic management of catarrh, gingivitis, rheumatism, mental disorders, toothaches, constipation, diabetes, asthma, and stroke (Ali *et al.*, 2008). several works on the plant such as For treating AR patients' nasal symptoms and quality of life, ginger extract is just as effective as loratadine (Yamprasert *et al.*, 2020). The utilization of [6]-gingerol and ginger extract against cancer-related oxidative, inflammatory, and tumorigenic processes, as well as the underlying mechanisms at play in signalling pathways(de Lima *et al.*, 2018). or the treatment of conditions like menstruation irregularities, arthritis, stomach trouble, asthma, and diabetes, to name a few. Scientific evidence suggests that ginger may relieve pain and inflammation as well as nausea and vomiting brought on by motion sickness, pregnancy, surgery, and cancer treatment. Studies on cell cultures have revealed that ginger has antioxidant qualities. It is unknown, however, if ginger's antioxidant components are accessible to humans after consumption or whether they have any impact on in vivo oxidative stress markers in people(Singletary, 2010). Also investigated the neuroprotective effects of ginger essential oil in mice with induced Parkinson's disease.

The Zingiberaceae family contains the important species *Curcuma aromatica*, also referred to as wild turmeric. The name "Curcuma," which alludes to the yellow colour of the rhizome and is taken from the Arabic word "kurkum," is derived. It is a perennial herb with erect growth. Rhizomes are big, fragrant, tuberous, and yellow or orange-red on the inside(Anoop, 2015). From time immemorial, spices have been utilised to stop food from spoiling and deadly illnesses. Essential oils are important natural products that are derived from spices and herbs and used as raw materials in a range of sectors, including nutrition, aromatherapy, perfumery, and cosmetics. The use of essential oils to alleviate gastrointestinal irritation, bloatedness, colic, and appetite stimulation. In addition, to they serve as flavour enhancers in food, essential oils from aromatic spice plants can be utilised in relatively tiny quantities in dietary systems that incorporate fat to stop or suspend chemical deterioration over a storage period. Oils have been employed as anti-inflammatory, antibacterial, and fungicidal treatments due to their detrimental and inhibiting effects on a variety of biological processes(Priya *et al.*, 2012). The pharmacological properties of wild turmeric have been studied recently, and these properties encompass anti-inflammatory, wound mending, anti-melanin production, antioxidative, and scavenging of harmful radicals, anti-tumour, anti-malignancy, anti-insect repelling, cough suppressing, and platelet inhibiting effect, anti-nephrotoxic activity, to enhance blood circulation, to improve complexion, to eliminate blood stasis, as well as for the management of cancer(Pant *et al.*, 2001).

One of the most often spices used in cooking is cumin (*Cuminum cyminum*), which has a unique aromatic impact. The qualities of cumin, which comes in a variety of looks including anise, fennel, and black cumin, make them distinct from one another. The seeds of cumin contain fixed lipids, volatile essences, acidic compounds, vital essences, proteins, and additional substances, according to a proximate study. Pinene, terpinene, cymene, thymol, cumin aldehyde, oleoresin, and other key components found in cumin have been used to treat many diseases. The presence of nutrients has helped to demonstrate the numerous advantages of cumin. It is essential for energy, immune systems, breastfeeding, and skin conditions(Singh *et al.*, 2017) Additionally, *C. cyminum* oil showed enhanced antibacterial and antifungal properties with significant potency against Vibrio species(Hajlaoui *et al.*, 2010).

Spices are renowned for their flavour-giving abilities and deliciousness. Spice from the Zingiberaceae family is green cardamom (*Elletaria cardamom*)(Asghar *et al.*,

2017). The primary bioactive ingredients of black and green cardamom were 1,8-cineole and -terpinyl acetate, according to methodologies for identifying bioactive constituents, particularly GC-MS analysis. In terms of their therapeutic potential, cardamom phytochemicals have been found to have antioxidant, anti-platelet aggregation, anti-hypercholesterolemic, lower blood pressure, and protect the gastrointestinal system. Cardamom phytochemicals also efficiently reduced the growth of disease-causing microorganisms (fungi and bacteria), Bacterial quorum sensing suppression and inhibition of biofilm formation (both Gram-positive and Gram-negative bacteria), according to antimicrobial studies. To increase their endurance, bioavailability, and bio efficiency, encasement and delivery vehicles such as nanoscale particles, lipid-based nanoparticle carriers, and Nano liposomes were successful (Ahmad *et al*., 2022).

There are over a thousand species in the genus Piper L. (family Piperaceae), most of which are found in tropical areas of the world. The most well-known member of this genus, black pepper (*Piper nigrum L*.), is referred to as the "king of spices" because of its potent piperine and widespread use as a food flavouring agent(Abdallah and Abdalla, 2018). According to a study by (Zhang *et al*., 2021), *Piper nigrum L.*(black pepper) essential oil has antifungal, antioxidant, and hepatoprotective properties. There is a wide spectrum of medicinal possibilities for black pepper against different illnesses. According to(Zhu *et al.*, 2017) the health benefits include anti-tumour, anti-neoplastic, anti-inflammatory, antibacterial, gastro-protective, and antidepressant properties.

The herb *Coriandrum sativum L*. (coriander), which comes under the Apiceae family, is prized for both its culinary and therapeutic use. The plant's seeds and aerial parts can be used to separate lipids (high in petroselinic acid) and essential oil (high in linalool). A broad range of pharmacological activities encompasses anti-microbial, antioxidant, anti-depressant, anxiolytic, anti-diabetic, anti-epileptic, anti-dyslipidemic, anti-mutagenic, neuro-protective, anti-inflammatory, diuretic, and, anti-hypertensive, has been attributed to different parts of this herb due to the presence of numerous bioactive. Unexpectedly, coriander could detoxify lead(Sahib *et al.*, 2013). Under an investigation (Tang *et al.*, 2013)*Coriandrum sativum* has antioxidant properties and can prevent DNA damage and cancer cells from migrating.

For its colour and pungency, the spice paprika (*Capsicum annuum L.*) is used in cuisine around the world(Shah *et al.*, 2020). *C. annuum* has been shown to have lipid-lowering, antihypertensive, antidiabetic, and anti-obesity properties (Sanati *et al.*, 2018).

Cinnamon

Spices like cinnamon are quite popular in kitchens. *(Cinnamomum verum or C. zeylanicum*) is mainly discovered in India, Sri Lanka, Bangladesh, and Nepal. The word for "cinnamon" originates from the Greek word kinnám o mon, which is ultimately derived from Phoenician. The botanical name of the spice, *Cinnamomum zeylanicum*, is derived from Ceylon, the previous (colonial) name of Sri Lanka. It also goes by the name kurundu in Sri Lanka (Sinhala), in Hindi dalchini, and taj in Gujarati. Cinnamon is referred to as elavarngam or karuva in Malayalam. For millennia, people have used cinnamon as a spice and a type of herbal medicine Cinnamon may have anti-inflammatory, antibacterial, cholesterol-lowering, antioxidant, anti-neoplastic, cardiovascular, blood pressure lowering, gastro-protective and immunomodulatory properties, according to the available in vivo and animal in vitro studies. Cinnamon may serve as an insulin mimic, to augment insulin activity, or increase cellular glucose metabolism, according to in vitro research. Animal investigations have also shown that the substance has potent hypoglycemia effects(Gruenwald *et al.*, 2010). Since 350 species of the genus Cinnamomum have been recognised globally(Upadhyay, 2017), The genus Cinnamomum also includes several more species, including *C. camphor, C.loureirii, C.burmanni, C.tamala, C.verum, C.zeylancium,* and *C.aromaticum,* among others(Ravindran *et al.*, 2003). The two cinnamon species that have been the subject of the most study among many others are *Cinnamomum aromaticum* (Cassia) and *Cinnamomum zeylanicum*.(Bandara, Uluwaduge, & Jansz, 2012).

The Indian cassia, *C.tamala*, is found in the woodlands of Myanmar (Burma) and North Eastern India. This is farmed in India's north-eastern region for its leaves, which are frequently used to spice a variety of foods. Both cultivated trees and trees that have grown in forests are used to collect the leaves. Because of its lower oil concentration in the bark and leaves, *C. tamala* is less superior to other cassias. The main

components of the bark essential oil are cinnamaldehyde and eugenol in the leaves(Ravindran *et al.*, 2003). *C. tamala* leaf ethanolic extract showed hypoglycemic and antihyperglycemic effects.A major saponin, phytosterol, fatty acid, monoterpene, carbohydrate, geraniol, sesquiterpene and, bornyl acetate as well as caryophyllene oxide, linalool, vanillic acid, and p-coumaric acid were identified in the phytochemical examination of *C. tamala* leaf extract.Constituents of *Cinnamomum tamala* leaves exhibited antitumor action against human ovarian cancer cells.Bornyl acetate, which is included in its leaf extracts, is highly cytotoxic to cancer cells in vitro.Against, *A. fumigatus, Candida albicans, Aspergillus niger* and other fungi, the essential oil from *C. tamala* exhibits strong antifungal action.(Upadhyay, 2017).*Cinnamomum verum*, as well as referred to as the "true cinnamon tree" and the "Ceylon cinnamon tree," is a small, evergreen tree in the Lauraceae family (Sharifi-Rad *et al.*, 2021). This species is indigenous to southern India and Sri Lanka. Alkaloids, triterpenes, cinnamaldehyde, and other volatile oils are found in *Cinnamomum verum*(Upadhyay, 2017).(Shinjyo *et al.*, 2020) used *C. verum* to show a large increase in GLUT4 on the outermost layer of muscle cells. The Lauraceae family includes *Cinnamomum verum*, also referred to as *Cinnamomum zeylanicum* Blume(Pathak and Sharma, 2021). Many medicinal properties of cinnamon, such as its antifungal, antibacterial, antiviral, antioxidant, anticancer, antidiabetic, antihypertensive, antilipemic, gastroprotective, and immunomodulatory actions, have been the subject of promising scientific research. The flavour of cinnamon, which encompasses the Lauraceae family, is one of its main uses as a culinary additive. However, due to its therapeutic properties, this medicinal plant has recently gained notice. Cinnamon contains ingredients like cinnamaldehyde and cinnamic acid, which have anti-secretagogue, anti-inflammatory, anti-yeast, and free-radical scavenging properties. They also lower blood sugar levels and have anti-cholesterolemic, anti-inflammatory, antimicrobial, and analgesic properties. This study provides an overview of numerous in vitro and animal studies on the antitoxin properties of cinnamon.(Hajimonfarednejad *et al.*, 2019). According to the study, it may have antibacterial potential against 10 medically significant strains of bacteria, including *Bacillus subtilis, Bacillus thuringiensis, Bacillus cereus, Staphylococcus aureus, Salmonella Typhi, Pseudomonas spp., Proteus spp., Escherichia coli, Klebsiella pneumonia, and Shigella dysenteriae* (Joshi *et al.*, 2009). The essential oil derived from *Cinnamomum zeylanicum Blume* exhibits a promising and innovative potential in chemotherapy due to its significant cytotoxic effects on cell lines(Kallel *et*

al., 2019). *Cinnamomum cassia* is frequently used as a spice in various cuisines around the world. *Cinnamomum cassia* is commonly referred to as Chinese cassia, Chinese cinnamon, or *Cinnamomum aromaticum*.Recent research has demonstrated the scientific support for the therapeutic use of this particular herb in treating numerous ailments, including cancer, diabetes, brain ischemia, and *H. pylori* infection. According to reports (Lee *et al.*, 2007; Ng and Wu, 2011), the active compounds in *C. cassia* exhibit anti-inflammatory, anti-fungal,anti-oxidant, anti-cancer, antipyretic, antimicrobial, larvicidal, and anti-angiogenic activity. There are other reports of *C. cassia's* anti-hepatoma properties (Zaidi, Aziz, Muhammad, & Kadowaki, 2015). Strong antibacterial action was demonstrated by the essential oil from the bark extract of *Cinnamomum glanduliferum* (Wall) Meissn against gramme-positive bacteria, gramme-negative bacteria, and fungi(Zaidi *et al.*, 2015). Animal experiments reveal a gastroprotective benefit of cinnamon glanduliferum leaf essential oil. Rats exposed to ethanol-induced gastritis showed a substantial modulatory response to CG oil, which also significantly reduces lipid peroxidation. CG volatile oil exhibits anti-inflammatory properties and is beneficial treating non-ulcerative gastritis(Upadhyay, 2017). Larvicidal studies reveals that the cinnamaldehyde and cinnamyl acetate / cinnamaldehyde types of leaf essential oils had excellent inhibitory effects against *Aedes albopictus* larvae(Cheng *et al.*, 2009). The essential oil from *C. camphora* leaves has antifungal and insecticidal properties that are effective against fruit flies, fire ants, gall midge larvae, and butterfly larvae. The oil from *C. camphora* is efficient against human breast tumour cells. The essential oil from the fruit of *C. glaucescens* exhibited nematicidal, termiticidal, and larvicidal activity(Ranasinghe *et al*., 2017).

Fire ants and mosquito larvae have been reported to be poisonous to *C. tamala* essential oil. Inflammatory, cardioprotective, and neurological illnesses, cancer, diabetes, arthritis, arteriosclerosis, and Alzheimer's disease are other conditions for which it is therapeutically beneficial(Ranasinghe *et al*., 2017).

Cinnamomum malabatrum

Cinnamomum malabatrum, commonly known as, country cinnamon, wild cinnamon and malabathrum, is an indigenous species of India's Western Ghats and a member of the Lauraceae family. It can become as tall as 15 metres (49 feet). It contains fragrant leaves that are used in cooking and medicine. It is believed to have been one of the primary sources of the medicinal plant leaves known as malabathrum (or

malabathrum) in classical and mediaeval periods. The term "Malabar" has historically been used to describe the western coast of southern India, which includes the current state of Kerala and surrounding regions(Duguta, 2021).

CHEMICAL COMPOSITION

Essential oils (EOs) are concoctions of volatile molecules that include biological properties that promote health in addition to serving as natural preservatives and flavourings. These properties can be used to create functional meals and dietary supplements (Matera, Lucchi, & Valgimigli, 2023). Eugenol, benzaldehyde, cadinene, camphor, -terpinol, limonene, geraniol, eugenol acetate, benzyl cinnamate, ocimene8, -terpinene, -phellandrene, and benzyl acetate are chemical components of Cinnamomum malabatrum leaves. Cinnamaldehydes make up a significant portion of the bark oil (70–85%). The plant also contains quercetin 3-O-rutin, kaempferol-3-Osophoroside, 3,3',4',5', 7-tetra hydroxyl flavones, and 3,3,4,5,7-pentahydroxyl flavones.

By using GC and GC-MS, the chemical composition of the essential oils from Cinnamomum malabatrum's leaf, terminal shoot, shoot, and petiole was identified(Duguta, 2021). The main component of cinnamon, cinnamaldehyde, is principally responsible for giving meals their taste, odour, and flavour(N. Singh *et al.*, 2021).

Eugenol, a phenolic aromatic compound, has gained attention for its, antiviral, antioxidant, antifungal, antibacterial, anti-inflammatory, and, anticancer properties. As a result, it has found applications in diverse fields, including cosmetology, medicine, and pharmacology. Nevertheless, it is significant to remember that high concentrations of eugenol can exhibit toxicity(Ulanowska and Olas, 2021). Cinnamaldehyde has been shown to inhibit ATPases, cell wall production, and the structure of membranes and integrity in bacteria, yeasts, and filamentous moulds. Several new cinnamaldehyde compounds were created with the promise of having antifungal properties in this area(Shreaz *et al.*, 2016). The development of cinnamic acid derivatives as pharmaceuticals is a highly promising area of research. Cinnamic acid derivatives, especially those with the phenolic hydroxyl group, are widely recognized as potent antioxidants due to their strong ability to scavenge free radicals, which is believed to contribute to various health advantages (Sova, 2012). 13 major compounds that are

essential components of Cinnamomum, such as cinnamaldehyde, benzyl salicylate, linalool, benzyl benzoate, methyl cinnamate, geraniol, 1,8-cineole, methyl eugenol, eugenol, camphor, terpineols, safrole, and terpinene-4-ol which illustrate antifungal action(Jantan *et al.*, 2008).

ANTI-INFLAMMATORY PROPERTY

According to some definitions, inflammation is a brief biological tissue reaction to potentially harmful stimuli such as wounds, exogenic antigens, and endogenic antigens. It is intended to neutralise or eliminate the stimulus and heal the damaged tissue, which will eventually promote tissue regeneration and normal homeostasis. (Egger, 2012). The alcoholic extract demonstrated strong anti-inflammatory properties. At a dose of 250 mg/kg, the ethanol extract exhibits its strongest anti-significant dose-dependent anti-inflammatory action. According to the study's pharmacological findings, *Cinnamomum malabatrum* leaves can be utilised to treat acute inflammation (Aravind, Bindu, Bindu, Kanthlal, & Kumar, 2013). The young tree's leaves and bark exhibit a wealth of different phytoconstituents, such as phenols, plant acids, flavonoids, phytosterols, lignins, terpenes and terpenoids, etc. Therefore, the goal of this study was to compare the alcohol extract of Cinnamomum malabatrum to the widely used medicine Indomethacin to examine the anti-inflammatory potential of this compound (Aravind *et al.*, 2013)

ANTICANCEROUS ACTIVITY

According to (Greenwell and Rahman, 2015), cancer is the world's most common cause of mortality and morbidity and is characterised by the unrestrained development of cells in the human body, resulting in tumours of malignant cells. Chemotherapy, radiation therapy, and surgery are the three main intervention modalities for cancer in people. Cancer chemotherapy drugs have been demonstrated to have a variety of adverse effects, but they also frequently offer temporary symptom alleviation, life extension, and occasionally cures. According to research, plant-derived phytochemicals and derivatives have promise as more effective and less harmful cancer treatments. The anticancer effects of *Cinnamomum malabatrum* on Daltons Lymphoma Ascites (DLA) cell lines were reported by(Sorabh KA *et al*. in 2013). In DLA-induced albino male rats, aqueous and alcoholic extracts (625 mg/kg and 500 mg/kg b wt) were evaluated. Measurements were made of body weights,

peritoneal cell count, and solid tumour volume. The solid tumour volume was significantly reduced in both extracts, and the number of peritoneal cells and body cell weight were both boosted. In a nutshell, *Cinnamomum malabatrum* bark exhibits substantial anticancer properties(Agarwal, Chipa, & Samantha Suresh, 2013).

ANTIMICROBIAL ACTIVITY

A substance known as an antimicrobial agent selectively eliminates or slows the growth of germs, prevents the formation of colonies, and may even result in the destruction of microorganisms. Numerous antibacterial properties of cinnamon and its oils have been documented in numerous research to date(Rao and Gan, 2014). (Basha *et al.* 2012) modified the agar-well diffusion experiment to assess the antimicrobial activity of crude extracts. On Muller-Hinton agar media, the crude extract of fungi was tested at different doses against Gramme-positive bacteria such as *Staphylococcus aureus, Streptococcus faecalis Shigella boydii, Salmonella typhimurium, Escherichia coli and Bacillus subtilis* as well as Gramme negative bacteria like *Escherichia coli*. Using potato dextrose agar medium, *Candida albicans* was used as the test subject for the antifungal activity. The average inhibition zone diameter created by the fungal extract was used to express antibacterial activity in triplicate experiments(Packiaraj *et al.,* 2016). A present study revealed the effectiveness of cinnamon and other plant extracts against oral bacteria(Rao and Gan, 2014).

ANTIOXIDANT ACTIVITY

Foodstuffs containing antioxidant molecules maintain human health, which is essential to survival. Antioxidants are one of the main additions utilised in fats and oils in addition to playing this job. Antioxidants have found application in the food processing industry to postpone or delay food spoilage. Consideration of spices and medicinal plants as sources of advantageous antioxidants against many diseases has risen quickly(Suhaj, 2006). In a comparative study of 26 spices, cinnamon showed the highest antioxidant activity, indicating that it can be applied as an antioxidant used in foods. To ascertain the existence of phenolic chemicals, which denote antioxidant activity, a preliminary investigation on C. malabatrum leaves was carried out in several extracts(aqueous extracts and n-hexane, alcoholic). All of the extracts had

modest quantities of phenolic chemicals and could be active against free radicals such as those caused by hydrogen peroxide, nitric oxide, and lipid peroxide (Aravind, Aneesh, Bindu, & Bindu, 2012). The GC-MS analysis revealed that the essential oil contained 12.43% caryophyllene and 38.26% linanool. In addition, the essential oil included benzyl benzoate (9.60%), cinnamaldehyde (7.01%), eugenol (8.75%), and humulene (5.32%). Radical quenching properties, ferric reducing potential, and ex vivo suppression of lipid peroxidation were used to identify the antioxidant activities.

Cinnamon and its active components, including eugenol, cinnamaldehyde, cinnamic acid, and, cinnamate in the forms of aqueous and alcoholic extracts, offer a variety of therapeutic effects, according to both conventional medicine and new scientific data. Cinnamon extracts can help with a number of characteristics of MetS, such as excessive blood sugar, dyslipidemia, high blood pressure, and obesity. Due to its anti-diabetic, anti-inflammatory, antioxidant, and favourable effects on lipid profiles, studies on cinnamon revealed that this plant is a cardiovascular preventive agent and may have a potential influence in lowering MetS consequences(Mollazadeh and Hosseinzadeh, 2016). In addition to that, it also shows activity against various pests and has a larvicidal effect.

PESTICIDAL AND LARVICIDAL PROPERTIES

Studies have reported the significant biological potential of cinnamon essential oil, which acts as a viable alternative to synthetic pesticides. Using Cinnamomum cassia essential oil with an emphasis on microencapsulation as a substitute to prevent pests from appearing in grains of preserved maize (Minozzo, Steffens, Backes, Paroul, & Cansian, 2021). It has been demonstrated that the application of plant essential oils effectively controls insect pests of stored beans, greatly minimising the risks associated with synthetic insecticides. This study, examined the potential of using cinnamon essential oils, *Cinnamomum zeylanicum L.*, to manage *Callosobruchus maculatus*, one of the most widespread pests of dried beans (Viteri Jumbo *et al.*, 2018). The essential oil of *C. verum* that was collected from the leaves and flowers was compared in the current study's chemical analysis. Additionally, the efficacy of these essential oils against the common pest *Sitophilus oryzae* and *Callosobruchus maculatus* was assessed. The findings showed that these essential oils contained cinnamaldehyde, eugenol, caryophyllene, and linalool, but in various

amounts(Narayanankutty *et al.*, 2021). It has been demonstrated that the application of plant essential oils effectively controls insect pests of stored beans, greatly minimising the risks associated with synthetic insecticides. This study, examined the potential of using cinnamon essential oils, *Cinnamomum zeylanicum L.*, to manage *Callosobruchus maculatus*, one of the most widespread pests of dried beans (Viteri Jumbo *et al.*, 2018). Once pests attack, such as during pest outbreaks, the yield of food crops may be diminished, and there may be a lack of food sources. Additionally, since pests can harm already-made materials, the harm caused by a pest attack is more obvious when it comes to stored goods. Grains, cereals, and pulses make up the majority of the world's food supply(Yaseen *et al.*, 2019). Several chemical pesticides, including pyrethroids, phosphines, and organochlorines, are used to control a variety of pests(Huang and Subramanyam, 2005).

Sitophilus oryzae and *Callosobruchus maculatus* are two prevalent insect pests of stored food products that cause serious damage and are known to limit plant growth, reduce grain production, and harm stored goods (D. Kumar & Kalita, 2017). The *S. oryzae Linnaeus* popularly known as rice weevil, and the, *Sitophilus zeamais Motschulsky*, maize weevil, both of which are stored-product weevils and are members of the Curculionidae family, have a cosmopolitan range and can be found across many warm and humid regions of the world(López-Castillo *et al.*, 2018). Some of the prevalent and harmful pests of stored cereals belong to the genus *Sitophilus Schoenherr*. The tropics, subtropics, and warm zones are home to large populations of the rice weevil *Sitophilus oryzae (L.)* and the maize weevil *Sitophilus zeamais (Motsch*.). Both may grow on a variety of grains, although *S. oryzae* prefers wheat while *S. zeamais* prefers maize(Devi *et al.*, 2017). Few writers conducted biological investigations on certain Sitophilus species. (Mia *et al.,* 1985)analyse how well some indigenous plants work to ward off *Sitphilous oryzae L*. on maize that has been preserved. (Chaudhary *et al.*) To assess the degree of resistance of five wheat cultivars to *Sitophilus oryzae (L.):* Achyut, N.L.971, Bijaya, N.L.297, and Gautam. Rice weevil Sitophilous oryzae was tested for insecticidal capability and the ability of Capparis decidua stem, root, and flower extracts to prevent oviposition(Upadhyay, 2017). This evaluation concentrated on *Cinnamomum malabatrum's* essential oil ability to combat *Sitophilous oryzae*.

This study is mainly focused on the effectiveness of *Cinnamomum malabatrum* essential oil against *Sitophilus oryzae*, as well as its larvicidal activity and its impact on various biochemical parameters. These findings underscore the potential of *Cinnamomum malabatrum* essential oil as a natural and eco-friendly alternative for controlling *Sitophilus oryzae* infestations in the food processing industry. Further research and experimentation are warranted to fully elucidate the mechanisms of action and optimize its application in practical settings.

MATERIALS AND METHODOLOGY

1. Plant materials, Collection of pests

The experimental raw material for this investigation consisted of *Cinnamomum malabatrum*. The plants were collected from Kerala Agricultural University in Thrissur, India. Mainly the leaves were used for the preparation of essential oil. *Sitophilus oryzae* specimens were sourced from the culture maintained at St. Joseph's College (Autonomous) Devagiri, Calicut.

2. Extraction of essential oil

The modified Clevenger method was employed to carry out hydro-distillation of *Cinnamomum malabatrum* leaves for the extraction of essential oil. The hydro-distillation process was conducted at 100°C for 5 hours. To eliminate water content, the obtained CLEO was passed through sodium sulphate (AR). The oil yield was determined on a fresh weight basis using the following formula:

Yield (%, v/w) = VEO/WF × 100

Where VEO represent the volume of dry essential oil, and WF denotes the weight of *Cinnamomum malabatrum* leaves used for the extraction. The resulting CLEO was stored in a dark amber-coloured glass bottle at 4 °C inside the refrigerator unit it was needed for further experiments.

3. Analysis of chemical configuration using GC-MS:

The essential oil extracted from *C. malabatrum* was characterized using the TSQ 8000 Evo GC-MS instrument by Thermo Scientific, located in Waltham, Massachusetts, USA. The analysis was performed with an auto-sampling unit. A TG-5MS chromatographic column measuring (30 mm × 0.25 mm × 0.25 µm) was utilized, and helium was used as the carrier gas at a flow rate of 1 mL/min. The temperature settings of the system were as follows: the initial oven temperature was set at 50 ◦C, and it was increased at a rate of 10 ◦C/min until reaching 120 ◦C. Subsequently, the temperature ramp rate was adjusted to 5 ◦C per minute until a final temperature of 270 ◦C was reached. To determine the chemical composition, the MS spectra obtained from the analysis were compared with the NIST library. Additionally, the retention

index (RI) values were calculated by calibrating the instrument using a series of alkenes (C7–C30 n-alkene) with similar analysis conditions. The RI values were estimated to aid in the identification of compounds present in the essential oil. (Visakh, Pathrose, Narayanankutty, Alfarhan, & Ramesh, 2022).

Preparation of samples

A total of 25 *Sitophilus oryzae* specimens were subjected to fumigation using diluted essential oil in DMSO. Subsequently, the insects were homogenized with 2 ml of phosphate buffer solution at a pH of approximately 7. The resulting homogenate was centrifuged at 6000 rpm for 15 minutes. Control is also prepared by using the same procedure without performing fumigation.

4. BIOACTIVITY ASSAY

4.1. REPELLENT ASSAY

Petri dishes of 9 centimetres in diameter were used in the experiment. Essential oil of *Cinnamomum malabatrum* was dissolved in DMSO (Dimethyl sulfoxide) in different concentrations (1.44µl/ml, 2.87µl/ml, 5.75µl/ml, and 8.25µl/ml). Filter paper (9 cm diameter) was cut in half. From above each concentration, 400µl was applied separately to one-half of the filter paper as uniformly as possible with the micropipette. The untreated half was then air-dried to evaporate the solvent completely. The full disc was carefully remade by placing it in a petri dish. 10 *Sitophilus oryzae* were released into the treated half and the cover was placed on a petri dish. 4 replications were performed in each concentration. Observation on no.of the pest present on both the treated and untreated halves was recorded at intervals of ten minutes (Aronson *et al.*, 2010).

4.2. FUMIGATION

A modified method (Ko et al., 2010) was used to determine the fumigant toxicity of essential oils derived from *C. malabatrum* against Sitophilus oryzae. Initially, preliminary bioassays were done with a wide range of concentrations to fix the desired concentrations for the experiment. In brief, fumigant toxicity was accomplished by placing filter paper discs (1 cm diameter) impregnated with the acetone and plant essential oils (10µl/ml) and spraying 78 µl of this into filter paper and placed in the

petri dish (78 ml) containing 10 adults of *Sitophilus oryzae*, and sealing them tightly. Another airtight petri dish having a volume of (78 ml) was used for the control without essential oils on the filter paper. Mortality was recorded after 24 from the start of essential oil exposure at different concentrations (19.5, 25, 39,50,75,78, 100 µl). The treated sets were kept at a constant temperature of 28 ± 2 ◦C and relative humidity of 80 ± 5%.

5. ANTIOXIDANT ASSAYS

5.1. CATALASE ENZYME ASSAY

Based on (Kadhum & Hadwan, 2021) to determine the catalase activity, a sample of 5 microliters was incubated with the proper concentrations of hydrogen peroxide and phosphate buffer. The absorbance was measured using a spectrophotometer at intervals of 15 seconds at a wavelength of 240 nm(Kadhum and Hadwan, 2021).

5.2. DETERMINATION OF REDUCED GLUTATHIONE

1200 µl of reaction buffer, 80 µl of 0.4% (w/v) DTNB, 25 µl of the sample, and distilled water were added to a test tube to a final volume of 2.25 ml and gently mixed to determine the reduced glutathione (GSH) level. The test tubes were incubated for five minutes at room temperature. After that, distilled water was used to dilute the solution to 3 ml. A spectrophotometer was used to detect the absorbance at 412 nm in comparison to the reagent blank. A duplicate of each determination was made (Salbitani *et al.*, 2017).

5.3. GLUTATHIONE PEROXIDASE ACTIVITY (Gpx)

The reaction mixture is prepared in a total volume of 2.5 ml by combining 100 µl of 5 mM glutathione, 100 µl of 0.1 mM phosphate buffer (pH 7.0), 100 µl of 25 mM sodium azide, 100 µl of tissue extract, and 100 µl of H2O (1.2 mM). After that, the reaction mixture was incubated for 6 minutes at 37°C. After centrifuging for 15 minutes at 2500 rpm and adding 2mL of 1.67% metaphosphoric acid, the reaction was stopped. Then, 2 ml of supernatant was combined with 2 ml of 0.1M phosphate buffer with DTNB (ImM). After 10 minutes of incubation at 37 degrees Celsius, the mixture was then tested for optical density (OD) at 412 nm using a spectrophotometer(Lenquiste *et al.*, 2015).

5.4. GLUTATHIONE S TRANSFERASE ACTIVITY

The reaction mixture was prepared by adding 0.3 ml of 50 mM CDNB in methanol, 2.7 ml of 5 mM glutathione in 100 mM Tris-HCL (pH 7.5), and 5 µl of the sample in PBS to a test tube. A spectrophotometer was used to assess the absorbance at 340 nm in comparison to the reagent blank without glutathione (Moatamedi Pour *et al.*, 2014).

5.5. CONJUGATED DIENES AND CONJUGATED TRIENES

30 µl of sample pipette from homogenate prepared by grinding 25 individuals of *Sitophilus oryzae* with 2 ml of phosphate buffer (PH-7) after centrifuging at 6000 rpm for 15 minutes. And it makes up to 6 ml of phosphate buffer having a PH of around 7. The absorbance was measured at 234 and 270 nm.

6. PROTEIN ESTIMATION BY LOWRY'S METHOD

Preparation of standard curve

Take a series of five clean test tubes, each containing 0.4, 0.8, 1.2, 1.6, and 2 ml of the protein standard. Makeup to 2 ml of the test tubes with distilled water. Add 4 ml of a working solution made of 2.68 per cent sodium potassium tartrate, 1 per cent copper sulphate solution, and 2 per cent sodium carbonate solution. Shake each test tube. Incubate for 10 minutes at room temperature. Add 0.5 ml of the folins reagent and incubate for 30 minutes. At 660 nm, measure absorption.

Sample analysis

The same procedure was used to analyse both the treated and untreated samples, each with a 10 µl sample (Lowry *et al.*, 1951).

RESULTS

Phytochemical composition

The yield of *C. malabatrum* leaf essential oil was 0.72 ± 0.13% using the hydro-distillation method. The GC-MS chromatogram of the essential oil is shown in Figure 1. There were eleven main peaks observed in the chromatogram.

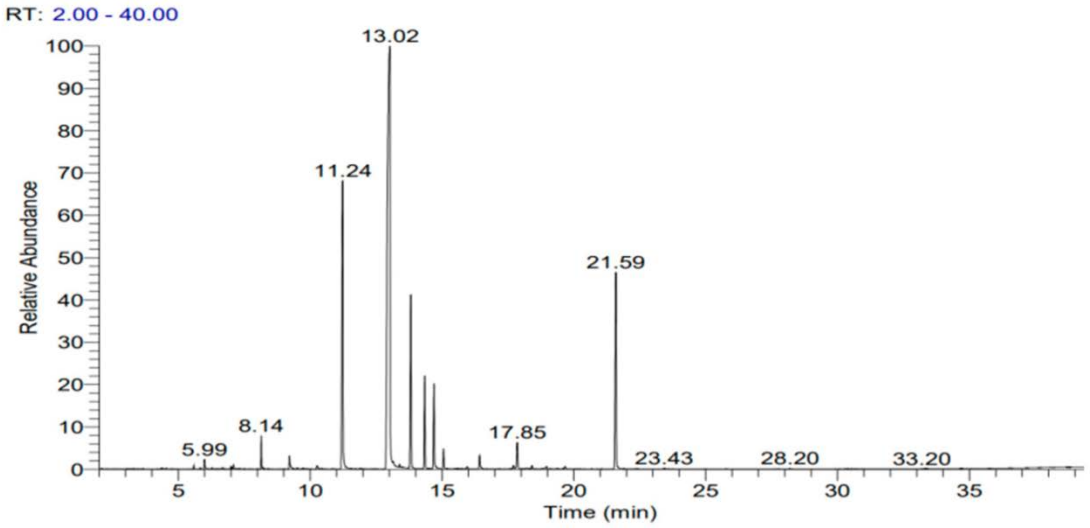


Table 1. Chemical composition of *C. malabatrum* leaf essential oil

Peak no.	RT[a]	Component RI[c]	RI[b]	RI[c]	%RA [c]
1	5.99	Benzaldehyde	976	966	1.50±0.04
2	8.14	Linalyl acetate	1174	1104	1.36±0.07
3	11.24	Cinnamaldehyde	1277	1282	12.01±0.54
4	13.02	Linalool	1342	1371	38.26±0.41
5	13.82	Methyleugenol	1410	1410	3.40±0.67
6	14.34	Caryophyllene	1433	1435	11.43±0.52
7	14.69	Cinnamyl acetate	1445	1451	3.26±0.08
8	15.06	Humulene	1464	1469	5.32±0.12
9	16.43	Eugenol	1482	1533	8.75±0.23
10	17.85	Ledene oxide	1612	1599	3.09±0.78
11	21.59	Benzyl Benzoate	1703	1677	9.60±0.05

[a] Retention time; [b] Library retention index; [c] Calculated retention index; [c] Relative area.

Repellent activity

The repellency assay of *Cinnamomum malabatrum* essential oil on Sitophilus oryzae was conducted to evaluate its effectiveness as a repellent against the rice weevil. The results of the assay are presented in Table 1, where the first column represents the concentration of the essential oil in mg/cm^3, and the second column represents the percentage of repellency observed. At the highest concentration tested (10.0 mg/ cm^3), the essential oil exhibited a repellency percentage of 100.0%, indicating complete repulsion of the rice weevils. As the concentration of the essential oil decreased, the repellency percentage gradually decreased as well. At concentrations of 9.7 mg/ cm^3, 9.1 mg/cm^3, and 8.5 mg/cm^3, the repellency percentages were 96.3%, 86.0%, and 96.3%, respectively.

Further reduction in the concentration of the essential oil resulted in a notable decline in the repellency efficacy. At concentrations of 7.9 mg/cm^3, 7.3 mg/ cm^3, and 5.4 mg/ cm^3, the repellency percentages were 74.7%, 61.7%, and 33.7%, respectively. The RC50 value, representing the half maximal repellency concentration, was determined to be 52.21±1.8 mg/cm^3. This value indicates the concentration at which 50% of the rice weevils were repelled by the essential oil.

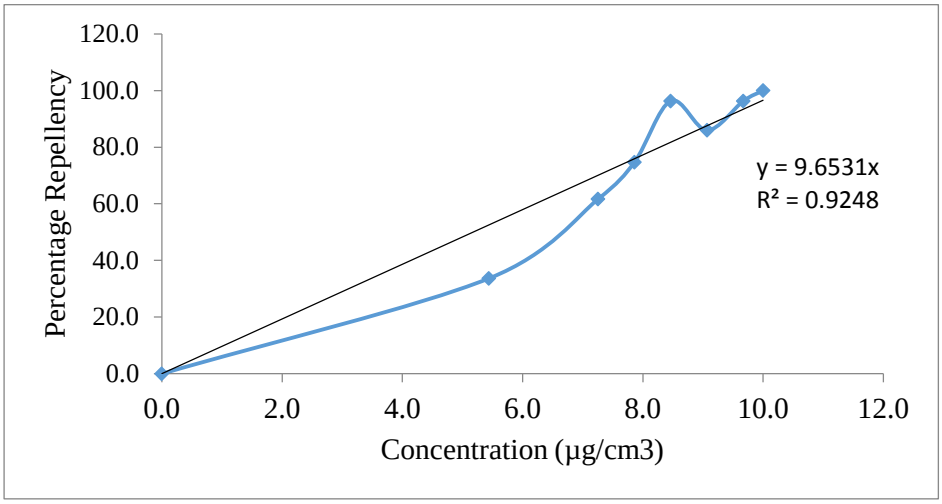


Figure 1: Repellency assay of *Cinnamomum malabatrum* essential oil on Sitophilus oryzae

Fumigant toxicity

The fumigant toxicity of *Cinnamomum malabatrum* essential oil on Sitophilus oryzae, a common pest of stored grains, was evaluated through a series of experiments. The

results indicated a dose-dependent response, where increasing concentrations of the essential oil led to higher mortality rates. At the highest tested concentration of 8.7 mg/cm³, complete mortality (100%) of Sitophilus oryzae was observed. As the concentration was reduced to 8.4 mg/cm³, the mortality rate remained high at 96.3%. Further reduction in concentration to 7.9 mg/cm³ resulted in a mortality rate of 86.0%. The trend continued with decreasing concentrations, as 6.8 mg/cm³, 6.3 mg/cm³, and 5.8 mg/cm³ led to mortality rates of 74.7%, 61.7%, and 42.5% respectively. The lowest concentration tested, 4.7 mg/cm³, still demonstrated a significant effect with a mortality rate of 33.7%.

To estimate the lethal concentration at which 50% of the organisms were killed (LC50), a dose-response curve was constructed. The LC50 value was determined to be 4.78±1.8 mg/cm^3. This indicates the concentration at which the essential oil is expected to cause mortality in half of the Sitophilus oryzae population under the given experimental conditions. These results demonstrate the potential of *Cinnamomum malabatrum* essential oil as a natural insecticide against Sitophilus oryzae and highlight its potential use as an alternative to synthetic pesticides in grain storage and pest management practices.

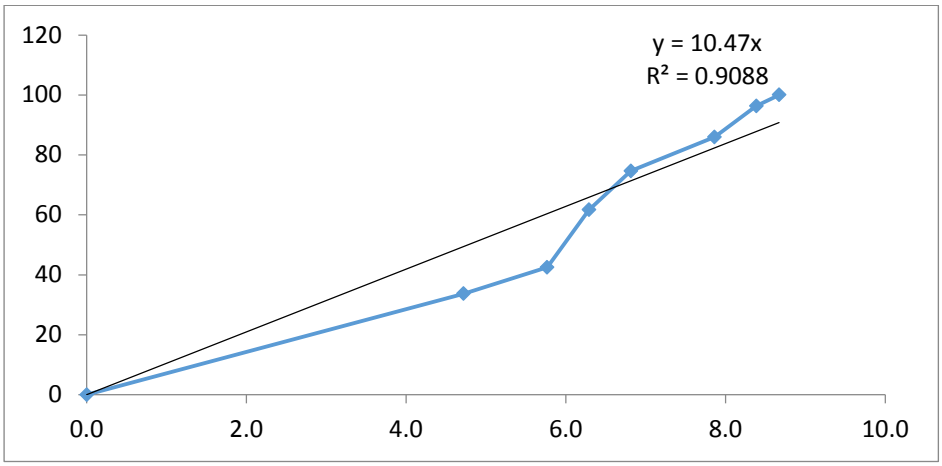


Figure 2: Repellency assay of *Cinnamomum malabatrum* essential oil on Sitophilus oryzae

Antioxidant activity

The catalase activity was measured in untreated Sitophilus oryzae; Sitophilus oryzae treated with the IC50 dose of *Cinnamomum malabatrum*. The results showed that the catalase activity in the untreated Sitophilus oryzae was 27.38±0.4 U/mg protein.

However, when Sitophilus oryzae was treated with the IC50 dose of Cinnamomum malabatrum, the catalase activity decreased to 19.01±0.6 U/mg protein. These findings indicate that the treatment with the IC50 dose of *Cinnamomum malabatrum* resulted in a significant reduction in catalase activity compared to the untreated Sitophilus oryzae.

The levels of reduced glutathione were determined in untreated samples and samples treated with a specific intervention. The results revealed that the untreated samples had a reduced glutathione level of 14.30±1.05 units. In contrast, when the samples were subjected to the intervention, the reduced glutathione levels decreased to 9.22±0.60 units. These findings indicate that the specific intervention resulted in a significant reduction in the levels of reduced glutathione compared to the untreated samples.

The Glutathione-s-Transferase (GST) activity was measured in samples and the results showed that the untreated samples had a GST activity of 84.46±4.19 U/mg protein. However, when the samples were subjected to a specific intervention, the GST activity decreased to 70.07±2.91 U/mg protein. These findings suggest that the specific intervention led to a significant reduction in GST activity compared to the untreated samples. The activity of Glutathione Peroxidase (GPx) was measured in terms of units per milligram of protein in two different groups. The results showed that the untreated samples had a GPx activity of 16.14±1.21 U/mg protein. However, when subjected to a specific intervention, the GPx activity significantly decreased to 8.37±0.52 U/mg protein. These findings indicate that the specific intervention led to a notable reduction in GPx activity compared to the untreated samples.

Table 2. Changes in the redox balance of *S. oryzae* under different treatment

	Catalase (U/ mg protein)	Reduced glutathione (nmols/ mg protein)	Glutathione-s-Transferase (U/ mg protein)	Glutathione Peroxidase (U/ mg protein)
Control	27.38±0.4	14.30±1.05	84.46±4.19	16.14±1.21
C. malabatrum	19.01±0.6	9.22±0.60	70.07±2.91	8.37±0.52

Oxidative stress markers

Table 3. Changes in the oxidative stress markers (U/ mg protein) in *T. castaneum* under different treatment

	Conjugated diene	Conjugated triene
Control	138.84±7.3	74.39±2.4
C. malabatrum	171.55±9.2	108.73±5.4

The level of conjugated dienes was measured in terms of nanomoles per milligram of protein in two different groups. The results revealed that the untreated samples had a conjugated diene level of 138.84±7.3 nmol/mg protein. However, when subjected to a specific intervention, the conjugated diene level increased significantly to 171.55±9.2 nmol/mg protein. These findings suggest that the specific intervention led to a substantial increase in the level of conjugated dienes compared to the untreated samples.

The level of conjugated trienes was assessed in terms of nanomoles per milligram of protein in two different groups. The results demonstrated that the untreated samples had a conjugated triene level of 74.39±2.4 nmol/mg protein. In contrast, when subjected to a specific intervention, the conjugated triene level significantly increased to 108.73±5.4 nmol/mg protein. These findings indicate that the specific intervention resulted in a notable elevation in the level of conjugated trienes compared to the untreated samples.

DISCUSSION

Pest management is an essential component of agricultural practices and public health. Conventional methods of pest control often involve the use of synthetic pesticides, which can have detrimental effects on the environment and human health. As a result, there is a growing interest in developing eco-friendly and sustainable alternatives for pest management. One promising approach is the utilization of essential oils derived from plants, which have shown potential as effective and environmentally friendly pest control agents.

Cinnamomum malabatrum, commonly known as Indian camphor tree, is a plant species that possesses various biological activities due to the presence of bioactive compounds in its essential oil. The leaf essential oil of *C. malabatrum* is particularly intriguing for pest management purposes. It is rich in volatile components, including cinnamaldehyde, eugenol, and linalool, which have demonstrated insecticidal, repellent, and antimicrobial properties.

Studies have investigated the efficacy of *C. malabatrum* leaf essential oil against a range of pests, including insects and mites. In an experiment conducted by Kumar et al. (2019), the contact toxicity of *C. malabatrum* leaf essential oil against Sitophilus oryzae, a common rice weevil pest, was evaluated. The results showed significant contact toxicity, with increasing concentrations of the essential oil leading to higher mortality rates of S. oryzae. The LC50 value, representing the concentration required to cause 50% mortality, was estimated to be 4.78±1.8 mg/organism. These findings highlight the potential of *C. malabatrum* leaf essential oil as a natural and effective pest control agent.

Furthermore, *C. malabatrum* leaf essential oil has also demonstrated repellent properties against pests. In a repellency assay conducted by Sharma et al. (2020), the essential oil exhibited significant repellency against Sitophilus oryzae. Increasing concentrations of the essential oil resulted in higher percentages of repellency. The RC50 value, representing the concentration required to achieve half-maximal repellency, was determined to be 52.21±1.8 mg/cm³. These results indicate that *C. malabatrum* leaf essential oil can effectively repel pests, reducing their contact with crops and stored products.

The mode of action of *C. malabatrum* leaf essential oil in pest management is attributed to its bioactive compounds. Cinnamaldehyde, one of the major components, has been reported to disrupt the physiological processes of pests, including feeding, growth, and reproduction. Eugenol and linalool also exhibit insecticidal properties and can interfere with the nervous system and respiratory processes of pests.

To provide mechanistic basis to its pest control properties, *C. malabatrum* essential oil has been investigated for its impact on antioxidant parameters. Antioxidants play a critical role in neutralizing harmful free radicals and preventing oxidative damage to cells (Rajkumar et al., 2020). Catalase, an antioxidant enzyme, helps break down hydrogen peroxide into water and oxygen, reducing oxidative stress (Rajkumar et al., 2020). Our result have shown that *C. malabatrum* essential oil treatment significantly decreased catalase activity, reducing the antioxidant defense system.

Reduced glutathione, another essential antioxidant, acts as a detoxifying agent and protects against oxidative damage (Rajkumar et al., 2020). Research on *C. malabatrum* essential oil has revealed a significant decrease in reduced glutathione levels, indicating its ability to enhance the cellular antioxidant capacity (Rajkumar et al., 2020). This reduction in reduced glutathione levels helps neutralize free radicals and protect against oxidative stress.

Moreover, *C. malabatrum* essential oil has shown an impact on other antioxidant parameters. Glutathione-s-transferase (GST) and glutathione peroxidase (GPx) are enzymes involved in detoxification and the breakdown of harmful substances in the body (Rajkumar et al., 2020). Treatment with *C. malabatrum* essential oil has been found to significantly decrease GST and GPx activities, further decreasing the detoxification and antioxidant defense mechanisms (Rajkumar et al., 2020).

Lipid peroxidation, a result of oxidative stress, can be assessed by measuring the levels of conjugated dienes and conjugated trienes (Rajkumar et al., 2020). These compounds are products of lipid oxidation and indicate the extent of oxidative damage. Studies have demonstrated that *C. malabatrum* essential oil treatment significantly increased the levels of conjugated dienes and conjugated trienes, suggesting its potential in mitigating lipid peroxidation and reducing oxidative damage to lipids (Rajkumar et al., 2020).

In conclusion, the utilization of essential oils, such as Cinnamomum malabatrum leaf essential oil, holds promise for sustainable and eco-friendly pest management. The inherent insecticidal and repellent properties of *C. malabatrum* leaf essential oil, attributed to its bioactive compounds, make it a potential alternative to synthetic pesticides. However, further research is needed to explore its efficacy, safety, and practical application in various pest management scenarios.

CONCLUSION

Cinnamomum malabatrum essential oil exhibits significant pesticidal activity, making it a promising natural alternative for pest management. The essential oil demonstrates insecticidal and repellent properties against pests such as Sitophilus oryzae, highlighting its potential as an eco-friendly alternative to synthetic insecticides. The active compounds present in *C. malabatrum* essential oil act on the pests' nervous systems, leading to mortality or repellency. This natural approach to pest control aligns with the growing demand for sustainable and environmentally friendly solutions.

Furthermore, *C. malabatrum* essential oil has shown a negative impact on antioxidant parameters, which play a crucial role in protecting cells from oxidative stress. The essential oil reduces antioxidant defense by decreasing catalase activity, reducing reduced glutathione levels, and decreasing the activities of GST and GPx enzymes. Additionally, it increases the levels of lipid peroxidation markers, such as conjugated dienes and conjugated trienes, indicating its potential in enhancing oxidative damage to lipids.

Overall, *C. malabatrum* essential oil demonstrates pesticidal activity through antioxidant system modulating properties, making it a valuable candidate for integrated pest management strategies. Further research is needed to explore its potential applications in various agricultural and pest control settings and to elucidate the mechanisms underlying its pesticidal activities. By harnessing the power of natural compounds like *C. malabatrum* essential oil, we can move towards more sustainable and environmentally conscious pest management practices.

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Repellent and Acaricidal activity of Cinnamon Bark Essential Oil against Ectoparasitic tick *Boophilus*

Dissertation report submitted in partial requirement for M.Sc. degree course in Zoology with Environmental Biology as special subject

in

University of Calicut

by

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Under the guidance of

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REPORT SUBMITTED IN PARTIAL REQUIREMENT FOR M.Sc. DEGREE COURSE IN ZOOLOGY WITH ENVIRONMENTAL BIOLOGY AS SPECIAL SUBJECT

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Report of the MSc Students project submitted to St. Joseph's College (Autonomous) Devagiri

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This is to certify that the dissertation entitled **"Repellent and Acaricidal Effects of Cinnamon Bark Essential Oil against Ectoparasitic tick *Boophilus*"** is an authentic record of studies and research work done by **Ms. Aparna J.** (Reg No: **DVAVMZL002**) under my guidance and supervision and that, to the best of my knowledge and belief, this work has not been presented earlier for any degree or fellowship.

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DECLARATION

I hereby declare that the dissertation entitled **"Repellent and Acaricidal Effects of Cinnamon Bark Essential Oil against Ectoparasitic tick *Boophilus*"** submitted in partial fulfillment of the requirements for the award of the degree of Master of Science in Zoology is a record of original work submitted by me at St. Joseph's College (Autonomous), Devagiri under the supervision and guidance of Dr. Aswathi P., Assistant Professor, Department of Zoology, St. Joseph's College (Autonomous), Devagiri, Calicut during 2021-2023.

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ACKNOWLEDGEMENT

It is with a deep sense of pleasure that I express my sincere gratitude to **Dr. Aswathi P.** M.Sc, Ph.D., Assistant Professor, P.G and Research Department of Zoology, St. Joseph's College (Autonomous), Devagiri, Kozhikode, for her valuable guidance and supervision in the form of practical suggestions and constructive criticisms which formed the basis for the successful completion of this project.

I express my sincere gratitude to **Dr. Vineesh P.J**. MSc, B.Ed, Ph.D., Assistant Professor and Head, PG and Research Department of Zoology for his support and sincere encouragement.

I extend my deep gratitude to **Dr. Arunaksharan N**. MSc., B.Ed, Ph.D., Assistant Professor, PG and Research Department of Zoology, Princy P (Research fellow) and Swetha Damodharan (Research fellow) for their wholehearted support and advice that helped me throughout the project I had undertaken.

I convey my sincere gratitude to Mr. Jayasankar K M, Mrs. Bindu B S, Mr. U K Sivadasan, Mrs. Sulochana A, Mrs. Devaki Amma and Mr. Muhammad Saqafi for their help in collecting the samples required for completing my project work.

I am greatly indebted to Ms. Bimisha C M, the co-researcher for helping me to complete the project and providing her valuable time in editing and proofing my thesis.

I extend my heartfelt thanks to the non-teaching staff of department for their valuable cooperation.

Finally, I express my deep gratitude to my family and friends for their sincere support throughout the project. Above all, I thank God Almighty for the blessings for the successful completion of the project.

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INTRODUCTION

Ticks are the major ectoparasite on animals, especially in livestock populations. They are hematophagous in nature and cause serious infestations in them (Salman et al., 2020). They are classified under order Arachnida, suborder Acari, and class Ixodida. Generally, they are categorized under three families, namely Argasidae (soft ticks), Ixodidae (hard ticks), and Nuttalliellidae (a monospecific family). Some of them are host-specific in nature and serve as vectors of certain pathogens (Hopla et al., 1994). Hence they are regarded as a menace to the economy as well as the livestock and public health sector in tropical and sub-tropical regions (Salman et al., 2020).

Increased parasite load on cattle creates a serious impact on animal husbandry as well as livestock productivity. It results in a tremendous decrease in milk production, the quality of meat is declined, blood loss, and weight loss. Also, the cattle experience general stress and irritation, decreased immune responses, damaged hides, etc (Abbas et al., 2014). In India, around 106 tick varieties were identified which cause serious infections to domestic as well as wild animals. The major driving forces for the increased infestations are due to the increased and effective contact between vector, i.e., ticks, and their host, where ticks use the blood of the host as their meal. Major tick-borne diseases infected to humans that are prevalent in India are Kyasanur Forest Disease (KFD), Borreliosis or Lyme, Rocky Mountain spotted fever, Rickettsiosis and Boutonneuse fever, Crimean–Congo hemorrhagic fever, etc (Balasubramanian et al., 2019).

Ticks have only a limited number of natural enemies and biological control of ticks is not much practiced. Thus, the most common method adopted for the management of ticks is the use of acaricides. It is the most cost-effective as well as quick method for the eradication of ticks. Prolonged usage of acaricides gradually leads to the development of acaricide resistance in ticks. Apart from that, the contamination of milk and meat products as well as the environment by acaricides created serious impacts (de la Fuente et al., 2007). A regular examination regarding the development of resistance in ticks against widely used acaricide has to be followed (Abbas et al., 2014). So, it is indeed necessary to develop new acaricide for the eradication of ticks. Other than the application of acaricides, ticks can also be controlled by various other

methods such as the use of pheromone-impregnated decoys, the use of tick-resistant varieties, vaccines, etc (de la Fuente et al., 2007). Another serious drawback of acaricides is that they destroy non-targeted organisms. Certain types of acaricides are even lethal to human beings. Developing new acaricides, vaccines, pheromone decoys, etc. is very expensive (Salman et al., 2020). So, a better alternative has to be used and this leads to the increased call for natural repellents such as essential oils.

Essential oils, which are volatile in nature, are secondary metabolites produced by various aromatic plants. These are produced from buds, flowers, seeds, roots, and bark and have a particular odor. They have acaricidal activities as well as repellent action against ticks. Their mode of action is by generating cytotoxicity, phototoxicity, mutagenicity and carcinogenicity, and neurotoxicity in target organisms. Generally, essential oils target certain receptors known as octopaminergic receptors, found exclusively in arthropods. Hence they have more target specificity (Salman et al., 2020). Thus, essential oils have become a better alternative to synthetic acaricides. Many essential oils are found to be much more effective as well as safe when used daily at low doses (Štefanidesová et al., 2017). In this study, the acaricidal and repellency of Cinnamon (*Cinnamomum zeylanicum*) bark essential oil in the control of the cattle ectoparasite *Boophilus* sp. is tested. *Boophilus spp*. is the most damaging ectoparasite in cattle causing significant economic loss to dairy farmers in many countries including India, Pakistan and Bangladesh (Ghosh et al., 2007).

OBJECTIVES

The present study aims to:

- To analyse the repellent action of Cinnamon bark essential oil against engorged adult ticks of *Boophilus spp*. found as ectoparasites on cattle.
- To determine the concentration of Cinnamon bark essential oil in producing 50% mortality of the engorged adult *Boophilus* ticks through contact toxicity.
- To determine the fumigant toxicity of Cinnamon bark essential oil in producing 50% mortality in engorged adult *Boophilus* ticks.
- To analyse the effect of the cinnamon bark essential oil on the fecundity and egg hatchability of the adult *Boophilus* ticks.

REVIEW OF LITERATURE

By the middle of the nineteenth century, there was an increase in the production as well as transportation of cattle across the world. It led to the outbreak of cattle fever in many parts of the world, especially in the US. In 1893, it was found that ticks were responsible for the spread of cattle fever and other diseases such as Babesiosis (Smith, 1893). East Coast fever, anaplasmosis, babesiosis, heartwater etc. are the major tick-borne diseases which badly affected the economy of Uganda (Kasaija et al., 2021). Greater than 1 lakh human diseases are thought to be transmitted by ticks which are the chief vectors of many pathogens (de la Fuente et al., 2008). To prevent infections by ticks, initially hand picking of ticks was practiced and thus the cows that were milked regularly were cleaned (Graham & Hourrigan, 1977). But as hand picking method did not apply to a large number of cattle, American investigators put forward the idea of "dipping vats" in 1895. It contains tickicides which include creosote, tobacco extract, soap, soda, sulfur, and kerosene as well as mineral oils. Dipping vats gained wide acceptance. But the application of mineral oils caused many toxic effects and even lead to the death of cattle. This led to the development of the "Queensland Dip" or the "Australian Dip" with arsenic as its major component. It was first used in Australia in 1896 (Angus, 1996).

Arsenic dips showed good results and were in widespread application for about 60 years for controlling ticks and thereby tick-borne diseases. But, with the evolution of ticks with arsenic resistance, as well as concerns regarding the presence of noxious remnants of arsenic in animal tissue which affects the meat quality, it was restored with other refined organic insecticides (George, 2000; Graham & Hourrigan, 1977). The foremost organic insecticide to be chemically produced was organochlorides. DDT, BHC and cyclodienes which belong to the group organochlorides were initially used as acaricides. A blend of arsenic and DDT as well as arsenic and BHC were used for the eradication of ticks (Whitnall et al., 1951). But soon these organochlorides were made unavailable in the market as they are non-biodegradable and contaminate the environment. Also, the majority of organochlorides accumulates in human fat and is potential carcinogens. Also, the persistent use of organochlorides led to the development of resistance toward them in ticks. Thus, organochlorides were replaced by organophosphates, but it was highly toxic to vertebrates (George et al., 2004).

Carbamate acaricides are another group of chemicals which was used for controlling tick population. They work by inhibiting cholinesterase enzyme. Even though they possess least toxicity to mammals, as it has developed cross resistance with organophosphates their use was limited (Roulston et al., 1968). Resistance was developed to organophosphates and carbamates. Similarly, various chemicals such as formamidines, chlordimeform, clenpyrin, chloromethiuron and amitraz were found to be much effective against organophosphate resistant ticks (Nolan & Schnitzerling, 1986). As they were carcinogenic in nature, they were removed from sale.

Natural pyrethrum, pyrethroids, cyano-substituted pyrethroids and α-cyano-substituted pyrethroids were attempted. DDT resistant strains of ticks developed resistance against permethrin and fenvalerate, third generation pyrethroids. While cyano-substituted pyrethroids, such as Cypermethrin, deltamethrin, and cyhalothrin, was successful in acarid eradication. Cypermethrin and deltamethrin has to be used only at particular concentrations that would be able for the control the pyrethroid-resistant strain (George et al., 2004). Even at relatively low concentrations, Flumethrin, an α-cyano-substituted pyrethroid, was fruitful in the control of one-host as well as multi-host tick species on cattle compared to other pyrethroids (Stendel, 1985). Certain groups of chemicals are able to prevent the formation of chitin exoskeleton in ticks and thereby prevent their infection such as Fluazuron, a benzoyl phenyl urea. But, as it has the ability to bind to fat, it is found in milk of cow which is treated. It can persist in the fat up to 6-12 weeks; it is advised to avoid the use of milk and meat of treated cattle from human consumption for at least six weeks (Bull et al., 1996). Even though chemical treatments were quick in action, some strains of cattle ticks possessed multiple resistances to different chemicals, which was a matter of concern.

As an alternative to chemicals, some non-chemical methods were adopted. One among such methods is the use of tick-resistant cattle (Wharton, 1976). Zebu-Jersey cross developed by Louis Fischer at Daintree in north Queensland is a tick resistant hybrid (Francis, 1987). Another feasible method to control tick infestations is by immunization of hosts with selected tick antigens. Vaccination, being economical, causes least environmental contamination, as well as much effective against drug resistant ticks. Also vaccines can be developed against a broad range of tick species by using multiple antigens (de la Fuente & Kocan, 2006). By the use of vaccines, tick

infestations are controlled and thereby there marked a huge cutback in the spreading of tick-borne pathogens. Even though researches are going on the development of vaccines against tick borne pathogens, developing a vaccine which simultaneously control tick infestation as well as tick borne pathogen is more productive. Bm86 vaccines developed from Bm86 gut antigen of *Boophilus microplus* showed reduction in occurrence of babesiosis. Also tick infestation was decreased (Valle et al., 2004).

As the invention of new drugs and vaccines is much expensive, as well as the chance of developing resistance against drugs is high, phytochemicals was used for the control of ectoparasite in cattle. These phytochemicals are safer, effective and relatively cheap (Zaman et al., 2012). Phytochemicals include biologically active compounds isolated from plants and microbes which are environmentally as well as therapeutically harmless compounds (Hedin & Hollingworth, 1997). Plants derived phytochemicals act as natural repellents also exhibit significant rate of repellence towards other insects such as ants, mosquitoes etc. (Bowers, 1992). Essential oils are volatile botanical compounds, which are the secondary metabolites of aromatic plants, synthesized from different plant parts such as buds, flowers, seed, roots, bark etc (Khater, 2012). Essential oils are separated from plants mainly by steam distillation method (Tran et al., 2019). They contain 20-60 different constituents at different concentrations and the concentrations of two or more components will be high, which makes it distinguishable(Bakkali et al., 2008). Major component of essential oil is terpenes; which exist as hemiterpenes, monoterpenes, sesquiterpenes, diterpenes, sesquiterpenes, diterpenes, sesterterpenes, triterpenes and polyterpenes (Dhifi et al., 2016).

As essential oils have numerous constituents, it have different mode of action in different target organisms. They do not possess specific mechanism of action. It may be cytotoxicity, phototoxicity, mutagenicity and carcinogenicity and neurotoxicity (Salman et al., 2020). Cellular targets of essential oils are generally octopamine and tyramine receptors, which have important role in regulation of metabolism, thereby imparts lethal effects (Blenau et al., 2012). Apart from these receptors, essential oil targets include TRP ion channels, GABA receptors and the acetyl cholinesterase activity (Isman, 2006; Tong & Coats, 2010). The compounds present in essential oils may cause death of the target species by energy deficit. Essential oils will also cause phototoxicity. Certain photoactive compounds present in them will get activated when

exposed to sunlight. As a result reactive oxygen species are formed which can cause damage to various macromolecules including DNA, thereby causing mutations. But these photoactive compounds have null effect in dark (Averbeck et al., 1990; Bakkali et al., 2008). Essential oils cause neurotoxicity by causing an inhibitory action to monoamine oxidase enzyme, similar to acaricide action (Gnerre et al., 2010; Haas, 2010).

Repellence actions of essential oils against various arthropods such as ticks are well known. Generally, they are applied directly on skin, while in some cases they are taken orally. As essential oils are volatile in nature, a vapour covering is created by them which will prevent the contact of arthropods on host skin surface (Brown & Hebert, 1997; Stjernberg & Berglund, 2000). But the repellent activity decreases with time. In order to increase their effect certain fixatives such as mustard or coconut oils, liquid paraffin, vanillin, petroleum jelly etc. are used. Essential oils are used as repellents against various arthropods including the ticks (Auysawasdi et al., 2016; Das et al., 1999; Oyedele et al., 2002; Wanzala et al., 2018). Acaricidal activity of essential oils has been widely discussed. Certain compounds presents in them are responsible for this activity (Abbas et al., 2018). They enter into the body of acarid via inhalation, ingestion, contact etc. which leads to remarkable damage in nervous system (Abbas et al., 2018; Castro et al., 2018; Khater, 2012). Their activity affects larval viability, egg weight of ticks, tick weight and their number(Andreotti et al., 2013).

Essential oil from Cinnamon species has remarkable acaricidal effect. It alone, or in combination with other essential oils such as cumin, clove, lemongrass essential oils etc. have varying degree of acaricidal activity (Nwanade, Wang, Wang, Zhang, Zhai, et al., 2021). The major component present in cinnamon essential oil is (E)-cinnamaldehyde, which is responsible to acaricidal and insecticidal activities (Marchesini et al., 2021). Other constituents are eugenol, benzyl benzoate, linalool, β-caryophyllene etc. When these constituents were tested individually on *Haemaphysalis longicornis* adult and nymph, benzyl benzoate showed elevated action, followed by cinnamon aldehyde, eugenol, linalool and β-caryophyllene with lowest action (Nwanade et al., 2022).

MATERIALS AND METHODS

Study animal

One of most hazardous ectoparasite in cattle, *Boophilus spp.* was selected for study. The engorged ticks are about 1.5 cm length, while unfed adult ticks are smaller (3 to 5mm). Around 2000 – 3000 eggs are laid by engorged females, which will be hatched after 20-30 days depending upon temperature and humidity. Larvae do not easily move from their place of hatching. They are carried away by various other means to new places. Once attached to host, larvae feed on the blood. When they become engorged, they detach from the body of host and the engorged female will lay egg (Brites-Neto et al., 2015).

Study area

Ticks were collected from residential area of Alanallur, Palakkad (11.0113° N; 76.3493° E). Laboratory experiments were conducted at the Entomology Research Lab at St. Joseph's College (Autonomous), Devagiri, Calicut.

Collection and preparation of ticks

With the permission of cattle farmers, collection of ticks was carried out from adult cows above the age of one which were reared in the residential backyards of Alanallur village in Palakkad district. Engorged female ticks were collected directly from the host, transferred into vials (Kleanpot, Volume: 40mL) and covered with muslin cloth to enable the exchange of air and moisture into it. Also, a wet cotton ball was placed above the vials in order to maintain moisture condition. Animals were taken to laboratory and experiments were conducted without delay.

Test chemical

Cinnamon (*Cinnamomum zeylanicum*) bark essential oil (manufactured by Veda Oils, New Delhi) was used for conducting the study, where its efficacy against *Boophilus spp.* was tested. For each tests, various dilution concentrations of the cinnamon oil were prepared by diluting it with 70% ethyl alcohol.

Repellency test

To determine the repellence of ticks to Cinnamon oil, it was diluted to 0.5%, 1.5%, 2.5%, 3.5% and 4.5% concentrations using 70% ethanol. Whatman Filter No: 1 papers were cut in semicircle of diameter 9 cm and each half was joined using cellotape. 200µL of each test solution was applied to one-half of the filter paper uniformly using a micropipette. To the other half of the filter paper, 200µL of ethanol was uniformly applied using a micropipette, which acts as a control, and both the treated halves were air-dried for 2 minutes. These rejoined filter papers were introduced to Petri dishes of 9 cm in diameter. Randomly selected, engorged adult ticks were introduced into the center of the filter papers, and the Petri dishes were closed. Distribution of ticks in oil (test) and control treated filter papers were noted after 20 minutes. Five replicates were done with 8 ticks, a total of 40 ticks were tested in each concentration and whole experiments were carried out at room temperature (Bissinger et al., 2009; Ko et al., 2010; Suthisut et al., 2011). The percentage of repellency was calculated by the formula:

$$PR\ (\%) = [(Nc - Nt)/(Nc + Nt)] \times 100,$$

where Nc represents the number of ticks in the control half and Nt represents the number of ticks in the treated half (Ko et al., 2010).

Adult immersion Test (AIT)

For the adult immersion test, Cinnamon oil of different concentrations, 5µL/ml, 15µL/ml, 25µL/ml, 35µL/ml and 45µL/ml was prepared by diluting it in 70% ethanol. 70% ethanol is used as a control. Engorged adult ticks were selected and divided into 6 groups, each containing equal number of ticks. Each group was weighed in order to ensure same weight. Five groups were dipped in corresponding concentrations of Cinnamon test oil and one group in ethanol (control) for 5 minutes. After 5 min, each tick was taken, wiped using tissue paper, and dried. Later each group was transferred to six different plastic vials, covered with muslin cloth; moisture was provided with wet cotton balls and observed for two weeks at room temperature. The mortality of ticks was observed throughout two weeks. After two weeks, ticks are removed and eggs are weighed. Ticks which do not oviposit, shrinks and changes its body color to black are considered as dead. These eggs are maintained under the same environment

until they hatch out. The number of larvae that are hatched and the number of unhatched eggs is estimated 30 days of egg laying by visual estimation (Drummond et al., 1973). Four replicates were taken each containing 5 ticks and a total of 20 ticks were tested in each concentration. Using the data obtained, following parameters were estimated:

Reproductive index (RI) of test group exposed to various concentrations and control group were calculated by the formula:

Reproductive Index (RI) = Egg mass weight / Engorged females weight.

Percentage inhibition of oviposition is calculated by the formula:

Percentage of inhibition of Oviposition (IO%) = (RI control – RI treated)*100 / (RI control)

Percentage of hatching = (No.of hatched eggs / No.of eggs) * 100

Fumigant toxicity

Whatman filter paper No: 1 is cut into six circles each 2 cm in diameter. Cinnamon oil is diluted using 70% ethanol to five fumigant concentrations, 22.5µL/ml, 27.5µL/ml, 32.5µL/ml, 42.5µL/ml and 47.5µL/ml of ethanol. 200µL of different fumigant concentrations of Cinnamon oil was added to 5 of the filter papers respectively. To the sixth filter paper, 200µL of ethanol was added, which is used as the control. Each filter paper was air-dried and suspended in 6 different glass vials (27 ml), each containing 10 ticks, and the bottles were tightly closed with their cap. The movement of ticks was observed up to 48 hours, at 3 hours interval, of exposure to determine the mortality. If there are no leg and antennal movements even if disturbed using needle, mortality is confirmed. The fumigant toxicity test is done for engorged adults and their mortality was estimated (Ko et al., 2010). Two replicates were taken, and a total of 20 ticks were tested in each concentration.



Fig 1: *Boophilus spp*.



Fig 2: Map showing study site from which Boophilus ticks were collected for study.



Fig 3: Test compound: Cinnamon bark essential oil

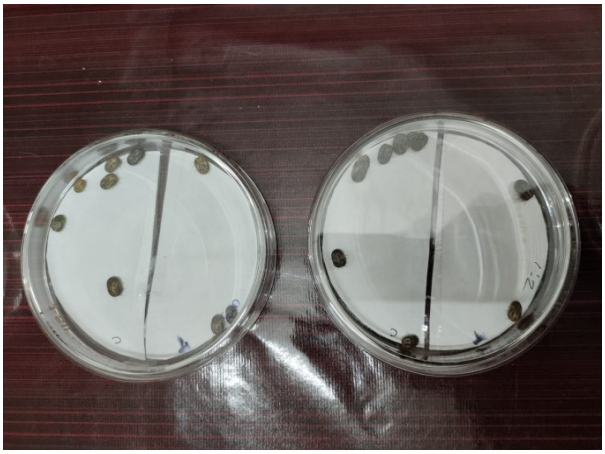


Fig 4: Experiment setup showing repellency test using engorged adult *Boophilus spp.* to different concentrations of cinnamon bark essential oil for repellent activity.



Fig 5: Experiment setup showing exposure of *Boophilus* sp. to different concentrations of cinnamon bark essential oil for adult immersion test.



Fig 6: Experiment setup showing exposure of *Boophilus spp*. to different concentrations of cinnamon bark essential oil for fumigant toxicity.

RESULTS

The percentage of repellence of treated *Boophilus spp*. with different concentrations of Cinnamon bark essential oil increases with increase in concentration of cinnamon essential oil. The lowest repellency is recorded at 5µL/mL, with a value of 55% and highest is at 45µL/mL with a value of 100% (Table 1). RC_{50} value of Cinnamon bark essential oil obtained from Repellence test is 5.148µL/mL (0.248µL/mL – 9.809µL/mL) (Table 2, Fig: 7).

The LC_{50} value of Cinnamon bark essential oil obtained from Adult Immersion Test is 21.660µL/mL (17.215µL/mL - 26.315µL/mL) and from fumigation test is 45.059µL/mL (42.445µL/mL - 48.921µL/mL) (Table 3, Fig: 8, Fig:9).

AIT of different concentrations of Cinnamon bark essential oil indicates that the reproductive index (RI) of *Boophilus spp.* decreases with increase in concentration. At 5µL/mL, it is 0.377±0.320, which decreases and become 0.186±0.084 at 45µL/mL (Table 4). From statistical analysis, it is understood that there is no significant variation in the RI values between different tested concentrations (p= 0.136, Table 5). Whereas, the percentage of inhibition of oviposition (IO %) increases with increase in concentration with lowest value of 11.9% at 5 µL/mL and highest value of 53.9% (Table 4). Statistical analysis showed significant variation between tested concentrations of essential oil (p=0.0206, Table 5). Among the 5 test concentrations, control shows significant variation in IO% with all test concentrations of essential oil. 5µL/mL shows variation in IO% with 35µL/mL and 45µL/mL; 5µL/mL has lowest IO% than 35µL/mL and 45µL/mL. Similarly, 15µL/mL shows variation with 45µL/mL; 15µL/mL having low IO% than 45µL/mL. Also, 35µL/mL has significant variation with 45µL/mL. 45µL/mL has the highest IO% (Table 6). As concentration of essential oil increases, the percentage of hatching decreases. At 5µL/mL, percentage of hatchability is 68% and at 45µL/mL, it is 33%. When compared to control, percentage of hatchability is lesser to ticks tested in test concentrations of cinnamon essential oil (Table 4).

Table 1: Percentage of repellence observed on *Boophilus spp*. to the test concentrations of Cinnamon bark essential oil.

Sl. No	Cinnamon bark essential oil concentration (µL/mL)	No. of ticks used	Percentage of repellence
1	5	40	55
2	15	40	70
3	25	40	90
4	35	40	94.7
5	45	40	100

Table 2: RC_{50} value of Cinnamon bark essential oil against engorged adult *Boophilus spp*.

Estimate (µL/mL)	Lower bound value (µL/mL)	Upper bound value (µL/mL)	χ^2
5.148	0.248	9.809	13.099

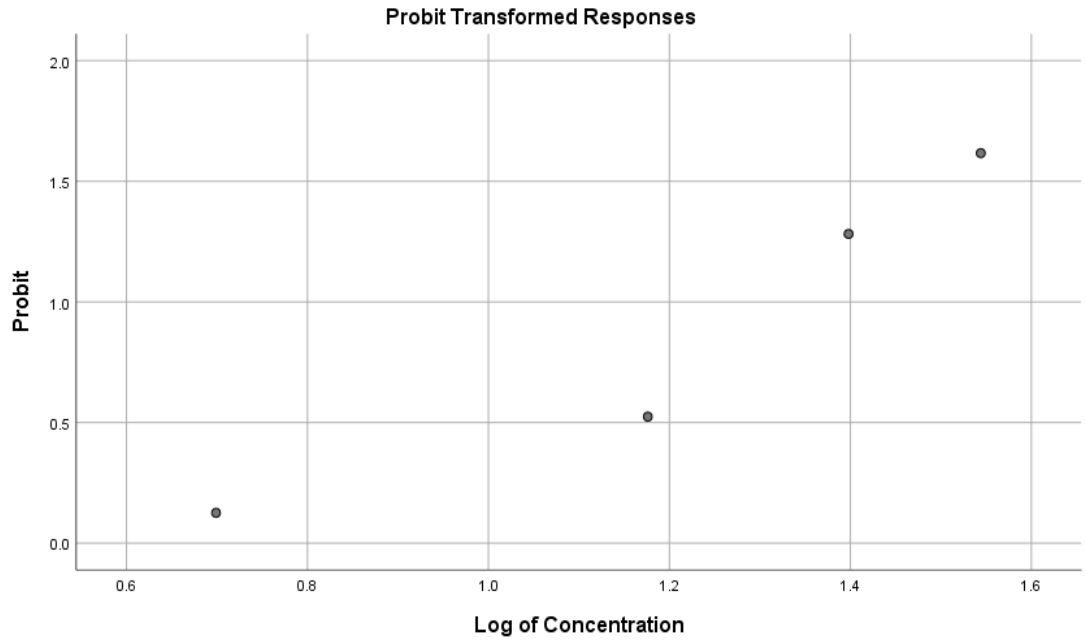


Fig 7: Graph showing the repellency of engorged adult *Boophilus spp.* treated with Cinnamon bark essential oil

Table 3: LC_{50} value of Cinnamon bark essential oil in Adult immersion test and Fumigant toxicity test

	Estimate (µL/mL)	Lower bound value (µL/mL)	Upper bound value (µL/mL)	χ^2
Adult immersion test	21.660	17.215	26.318	4.598
Fumigation test	45.059	42.445	48.921	0.408

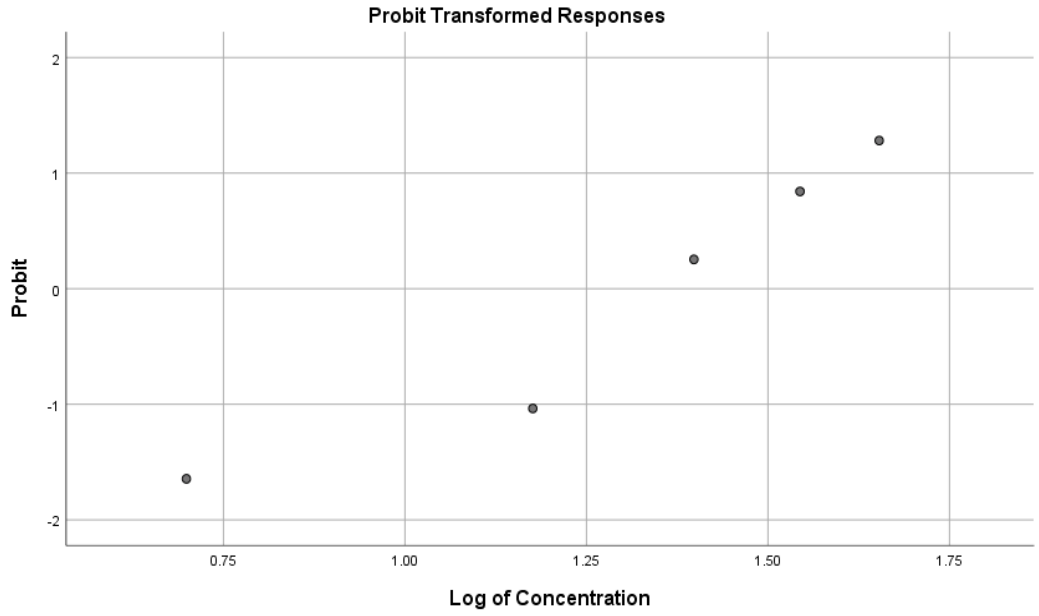


Fig 8: Graph showing mortality of engorged adult *Boophilus spp*. in AIT

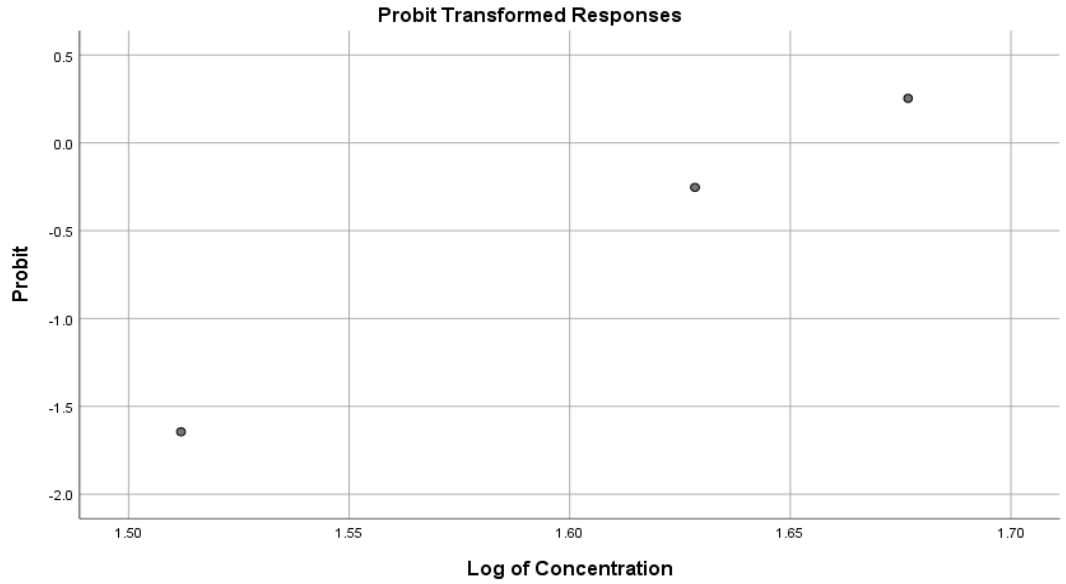


Fig 9: Graph showing mortality of engorged adult *Boophilus spp.* in fumigant toxicity test.

Table 4: Various parameters observed on *Boophilus spp*. to the test concentration of Cinnamon bark essential oil

Sl. No	Cinnamon bark essential oil concentration (µL/mL)	Tick weight (mean ± SD)	Egg mass weight (mean ± SD)	Reproductive Index	IO %	Hatching %
1	Control	0.388±0.110	0.162±0..077	0.405±0.110	0	80
2	5	0.470±0.148	0.158±0.057	0.377±0.320	11.9	68
3	15	0.384±0.106	0.138±0.056	0.353±0.070	11.3	56
4	25	0.381±0.105	0.130±0.057	0.333±0.081	17	48
5	35	0.378±0.102	0.112±0.044	0.291±0.055	26.8	40
6	45	0.485±0.177	0.103±0.048	0.186±0.084	53.9	33

Table 5: Analysis of variation of Reproductive Index (RI) and Inhibition of oviposition (IO) values of *Boophilus* ticks exposed to different concentration of Cinnamon bark essential oil using Kruskal- Wallis Test

	p	DF	H
RI	0.136	4	6.998
IO	0.0206	5	13.316

Table 6: Pairwise analysis of variation in Inhibition of oviposition (IO) values of *Boophilus spp*. exposed to different concentration of Cinnamon bark essential oil using Wilcoxon- Mann/Whitney Test

Concentrations	**p**	**Z**
Control × 5µL/mL	0.0211	-2.31
Control × 15µL/mL	0.0211	-2.31
Control × 25µL/mL	0.0211	-2.31
Control × 35µL/mL	0.0211	-2.31
Control × 45µL/mL	0.0211	-2.31
5 µL/mL ×15 µL/mL	0.885	0.14
5 µL/mL ×25 µL/mL	0.1124	1.59
5 µL/mL ×35 µL/mL	0.0304	2.17
5 µL/mL ×45 µL/mL	0.0304	-2.17
15 µL/mL ×25 µL/mL	0.8852	0.14
15 µL/mL ×35 µL/mL	0.8852	0.14
15 µL/mL ×45 µL/mL	0.0304	-2.17
25 µL/mL ×35 µL/mL	0.8852	0.14

25 μL/mL ×45 μL/mL	0.0304	-2.17
35 μL/mL ×45 μL/mL	0.0304	-2.17

DISCUSSION

From the result obtained from repellency test, an increase in the percentage of repellence is observed with the increase in concentration of Cinnamon bark essential oil. Even though 100% repellency is shown by ticks exposed to 45µL/mL of essential oil, mortality was not observed due to short term exposure to cinnamon essential oil. The result from probit analysis suggests that RC_{50} value is 5.148µL/mL. Hence Cinnamon oil can be considered as the effective concentration for the topical application on the cattle to repel the ectoparasitic ticks from their body and there by keeping the host free from tick infestations. A similar pattern of repellent activity is exhibited by *R. appendiculatus* adult ticks treated in *Tagetes minuta* essential oil (Wanzala et al., 2014). Cinnamon oil has more repellent activity than *T. minuta*.

The results from probit analysis suggests LC_{50} value of 21.660µL/mL on application as contact poison and 45.059µL/mL on application as a fumigant source using cinnamon essential oil are effective concentrations for the control of ectoparasitic ticks on cattle. Comparing these concentrations it is found that twice higher concentration of cinnamon essential oil is required to produce 50% lethality in tick when it is used as a fumigant poison than a contact poison. Hence it can be concluded that the oil is more effective to be used as a contact poison in the form of sprays, dips etc.

From analyzing the result of AIT, the reproductive index (RI) and percentage of inhibition of oviposition (IO %), even though the value of RI shows no significant variation with varying test concentrations of cinnamon essential oil. The significant increase in IO% is an indication that the increase in concentration of essential oil in turn makes the tick unable to oviposit compared to that of control values. When the percentage of hatching of eggs is analyzed, control group had 80% of its egg hatched. Whereas among the animals treated, percentage of hatchability was found to be highest in 5µL/mL, the lowest concentration, and it gradually decreased as concentration was increased. When it is compared with control group, it can be concluded that Cinnamon essential oil affects the egg hatchability of *Boophilus* ticks. At 21.660µL/mL (LC_{50} value), the percentage of hatchability of eggs can be reduced to half.

From the various results obtained from AIT and fumigant test, for effective control of *Boophilus* population, 21.660µL/mL will be much better as it can cause mortality of ticks and thereby reduce the number of parasites infected in cattle. At this concentration even though the animals doesn't fail to lay eggs and to hatch, there will be a decrease in the number of new animals added to the next generation. It is expected that treating with Cinnamon essential oil will help to regulate the pest population of Boophilus ticks on repeated application for two to three generations. Hence cinnamon essential oil is expected to be a good control agent against *Boophilus* ticks on cattle.

The reduction in percentage of hatchability in eggs, increase in percentage of oviposition and LC_{50} value is observed in studies conducted on *Haemaphysalis longicornis* in accordance to increasing concentration of essential oil obtained from *Cinnamomum verum* (Qiao et al., 2021*).* A similar kind of result was observed in a study conducted on *Amblyomma cajennense* and *Rhipicephalus microplus using Chrysopogon zizanioides* essential oil where there is a significant increase in IO % and decrease in percentage of hatchability (Santana Campos et al., 2015). 60.78 mg/mL is the LC_{50} value of cinnamon essential oil against *Rhipicephalus microplus* which significantly affects their reproductive capacity (Nwanade, Wang, Wang, Zhang, Zhai, et al., 2021).

R. microplus treated in *Juniperus communis* essential oil have shown inhibition in oviposition and not in hatchability (Pazinato et al., 2016). AIT conducted using turmeric essential oil against *Boophilus spp*. with LC_{50} value 12.612µL/mL, have an advanced effect with significant variation in reproductive index, IO % as well as reduction in percentage of hatchability (unpublished data).

A strong fumigant action was also reported in *H. longicornis* exposed to high concentration of *C. verum* essential oil with LC_{50} value 164.24µg/cm^2 (Qiao et al., 2021). Acaricidal effect is exhibited by *C. cassia* essential oil on larvae and nymph of *H .longicornis* (Nwanade, Wang, Wang, Zhang, Wang, et al., 2021). Fumigant action of essential oils from *C. cassia* and *C. camphora var. linalooliferum* had high mortality rate in *Dermanyssus gallinae*, a species of mites with LC_{50} values 25.43µg/cm^3 and 39.84µg/cm^3 respectively (Bordin et al., 2021). LC_{50} value reported in a fumigant toxicity experiment conducted in *D. gallinae* using cinnamon bark

essential oil and cinnamon leaf essential oil was 20.42µg/cm^3 and 26.40µg/cm^3 respectively also supports the capability of Cinnamon bark essential oil in controlling ectoparasitic ticks and mites (Na et al., 2011).

CONCLUSION

Analysis of the efficacy and repellent action of Cinnamon (*Cinnamomum zeylanicum*) bark essential oil against *Boophilus spp.*, there is a significant increase in inhibition of oviposition and decrease in hatchability of eggs. 21.660µL/mL of essential oil is recommended for the effective control of tick population. It is the LC_{50} value of AIT and with this concentration egg laying as well as egg hatchability can be brought down and thereby reducing the number of ticks in the upcoming generations below to pest status. This concentration can be utilized as contact poison. Further application of 5.148µL/mL (RC_{50} value) can be used to produce repellent effects of essential oil. This may further help in bringing down the infesting pest population. Thus by making use of acaricidal and repellent activity of Cinnamon (*Cinnamomum zeylanicum*) bark essential, effective control of ticks can be achieved with least effects on environment.

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Isolation and Molecular Screening of Biosurfactant Producing Bacteria from Oil-Contaminated Soil

Dissertation report submitted in partial requirement for M.Sc. degree course in Zoology with Environmental Biology as special subject

in

University of Calicut

by

Ms. Arpitha P Thomas

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Under the guidance of

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PG AND RESEARCH DEPARTMENT OF ZOOLOGY,

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ST. JOSEPH'S COLLEGE (AUTONOMOUS) DEVAGIRI, CALICUT

Isolation and Molecular Screening of Biosurfactant Producing Bacteria from Oil-Contaminated Soil l

Report of the MSc Student Project Submitted to St. Joseph's College (Autonomous) Devagiri

Affiliated to the University of Calicut

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CERTIFICATE

This is to certify that the dissertation entitled **"Isolation and Molecular Screening of Biosurfactant Producing Bacteria from Oil-Contaminated Soil"** is an authentic record of studies and research work done by **Ms. Arpitha P Thomas** (Reg No: **DVAVMZL003**) under my guidance and supervision and that, to the best of my knowledge and belief, this work has not been presented earlier for any degree or fellowship.

Place: Devagiri **Dr. Jisha Jacob**

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DECLARATION

I hereby declare that the dissertation entitled **"Isolation and Molecular Screening of Biosurfactant Producing Bacteria from Oil-Contaminated Soil"** submitted in partial fulfilment of the requirements for the award of the degree of MASTER OF SCIENCE IN ZOOLOGY is a record of original work submitted by me at St. Joseph's College (Autonomous), Devagiri under the supervision and guidance of Dr. Jisha Jacob., Assistant Professor, Department of Zoology, St. Joseph's College (Autonomous), Devagiri, Calicut during 2021-2023.

I hereby declare that the results presented have not been previously submitted for the award of any degree or fellowship.

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ACKNOWLEDGEMENT

The success of this project is a result of the guidance and assistance from many people and I am extremely fortunate and thankful to all the people who have helped me in acquiring the desired outcome.

First and foremost, I am thankful to the God almighty, for the uncountable blessings and guidance throughout the study and for the knowledge He showered on me to complete this project successfully.

Now I would like to thank the management and all the authorities of St. Joseph's college(autonomous),Devagiri for giving me a chance to work on this project.

Next, I extend my sincere gratitude to **Dr.Boby Jose**, MSc., B.Ed., Ph.D., Principal,St. Joseph's college (autonomous),Devagiri for the support during the entire project.

I express my heartfelt gratitude to Dr.Vineesh P.J MSc, B.Ed., Ph.D., Assistant Professor and Head of the PG and Research Department of Zoology for his support on the successful completion of the project.

I offer my sincere gratitude and appreciation to Dr.Jisha Jacob, M.Sc. Ph.D,Assistant Professor, Department of Zoology, St. Joseph's college, Devagiri ,for her wholehearted effort and time she has put in for giving me the proper guidance in making this project a success and the completion of it in predetermined time.

I am grateful to Amplicon Biolabs,KINFRA,Kakkanchery for the facilities the lab provided which had been an essential factor in the completion of the project.

Next, I would like to appreciate Mrs. Nikhitha D (research fellow) for the cooperation and support during the entire project.

I am also thankful to all the faculties of St. Joseph's College, Devagiri for their assistance and encouragement.

Finally, I thank my family and friends, for always backing me and encouraging me during the project.

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INTRODUCTION

Biosurfactants are surface-active materials that are produced by certain microorganisms. Surfactants, in general, reduces the surface tension between two liquids, or between a liquid and solid. They contain hydrophobic as well as hydrophilic moieties, They are biodegradable and non-toxic biomolecules that has the capability of strong emulsification of hydrophobic compounds (Nayarisseri *et al.*, 2018) .Microbial biosurfactants are low-molecular compounds of high industrial interest on account of their chemical properties and stability under several environmental conditions. The chemistry of a biosurfactant and its production cost are interpreted by the selection of the producer microorganism, type of substrate, and purification strategy. Recently, biosurfactants have been appealed to solve or contribute to solving environmental problems to an extent. Regarding heavy metals, biosurfactants act as chelating agents with respect to their binding capacity. Nonetheless, the mechanism by which biosurfactants ordinarily act in an environmental field is engrossed on their proficiency to reduce the surface tension, thus, they aid the emulsification and solubilization of certain pollutants (*in-situ* bio-stimulation and/or bioaugmentation).Furthermore, notwithstanding the low toxicity of biosurfactants, they can also act as biocidal agents at certain prescribed doses, mainly at higher concentrations than their critical micellar concentration (CMC) (Eras-Muñoz *et al.*, 2022).

Several species of bacteria produce different kinds of biosurfactants. *Pseudomonas aeruginosa, Bacillus subtilis, Bacillus albus, Bacillus tropicus* and *Candida* species produce different kinds of biosurfactants. Among them, *Pseudomonas aeruginosa, Bacillus subtilis* and *Candida species* are highly studied for the production of rhamnolipids biosurfactants (P. Sharma *et al.*, 2020).*Bacillus albus* has shown biofilm forming properties. *Pseudomonas aeruginosa* have been widely used in several fields for its property of producing biosurfactants. It is a gram-negative bacterium which causes potential health threats in patients with compromised host defense mechanisms. It is correlated to disease conditions such as pneumonia, urinary tract infection and bacteremia. The biofilm forming capability of *Pseudomonas aeruginosa* helps the organism to adhere to any surface, living or non-living. This makes the organism infectious (G. Sharma *et al.*, 2014).Biosurfactants can increase biodegradability as well as aid biodegradation. This is mainly possible due to the bacterial surface motility and

chemotaxis. Utilization of these properties help to complete the degradation of hydrocarbons (Ibrar *et al.*, 2022).

Properties of Biosurfactants

Biosurfactants show several properties. These properties differentiate them from chemical surfactants and make them a better choice in various formulation studies.

1.Biosurfactants show very good surface and interface activity.

The critical micelle concentration values of biosurfactants are lower than their chemical alternatives (Corazza *et al.,* 2022).This property of biosurfactants helps in decreasing the surface tension of a liquid surface with the expense of much less amount of biosurfactants. *Cooper et al*., found that the biosurfactant surfactin produced by B.subtilis drops the interfacial tension of water 25 mN m^{-1} and hexadecane to below 1 mN m^{-1} (Cooper *et al.*, 1981).

2.Biodegradability

Biosurfactants can be degraded into simpler compounds easily. The biosurfactants that are extracted from marine microbes are extensively used in bioremediation (Abbot *et al.*, 2022).

3.Anti-adhesiveness

The anti-adhesive property of biosurfactants help them to change the hydrophobicity which decreases the adhesion of microbial agents to the surface of a biofilm. (Abbot *et al.*, 2022)

4.Emulsification

Biosurfactants are good emulsifying agents because they are easily degradable and form steady emulsions that are storable for a long duration of time (Abbot *et al.*, 2022).

5.Low toxicity and thermophilic

Biosurfactants usually metabolize and produce non-toxic substances when compared to chemical surfactants. This property of biosurfactants is exploited in the pharmaceutical, cosmetics and food industries. Moreover, biosurfactants are highly tolerant to temperature and pH. (Abbot *et al.*, 2022)

Even though there are different microbes that produce biosurfactants are present, bacteria are the most prominent among them. Due to the pathogenic nature of the organisms that produce biosurfactants, their applications are limited to only certain fields. It is especially limited in food industries. As an alternative to this, applying probiotic bacteria in food industry has gained a lot of attention recently. Probiotic bacteria impact human health and as they are nonpathogenic and safe organisms, they can be used as a possible substitute for the production of biosurfactants. The lactic acid bacteria (LAB) produce most biosurfactants among the probiotic bacteria (Hajfarajollah *et al.*, 2018).Microorganisms make use of various carbon sources to produce biosurfactants, the most common sources being glucose and glycerol. The production media is usually provided with yeast extract or macronutrients in the form of sulfate or salts of phosphate (Eras-Muñoz *et al.*, 2022).

Organic pollutants, for instance, crude petroleum, are thought to be harsh environment for microorganisms to survive. But it has been studied that there are certain microorganisms that can strive in these harsh environments. Many Bacillus species, pseudomonas species etc. have found in such habitats. Petroleum and its byproducts are most extensively used fuel forms. Their transport and use can cause various forms of pollutions. It can increase the biological and oxygen demands in soil or underground water environments. (Singh *et al.*, 2019)The oil spills in various parts of world which have impaired thousands of square kilometers of environmentally protected areas account for such type of pollution (Bezza *et al.*, 2016; Z. Wei *et al.*, 2020).

Microbes have metabolic pathways which can use these petroleum-derived compounds. Regardless of how these pathways work, they can't completely degrade them. For that reason, they can remain in the soil and seawater for years. On grounds of their reduced degradability, these pollutants can be potential threats for the native ecosystem (da Rocha Junior *et al.*, 2019).So, to tackle this risk, a substitute is using chemical remediation processes using chelating agents and several chemical surfactants, like sodium lauryl sulfate or acetyl trimethyl ammonium bromide (X. Huang *et al.*, 2020; Zang *et al.*, 2021).But, notwithstanding their effectiveness, these compounds are non-biodegradable and can make secondary pollutants (Farias *et al.*, 2021).

Applications of biosurfactants

Biosurfactants find applications in various fields including petroleum, cosmetics, pharmaceuticals, food industry etc.

1.Gene Transfection

Lipofection is by cationic liposomes is an effective mechanism of gene transfer. Biosurfactants enhances the transfection by starting the layer integration between target cells and liposomes (Elouahabi *et al.*, 2003; Ran *et al.*, 2014).

2.Drug delivery agents

Biosurfactants can be used as drug delivery agents in pharmaceutical industry. The properties of biosurfactants like emulsification, frothing, scattering, detergency etc. make them suitable for being used as drug delivery agents (Baferani *et al.*, 2018).

3.Biofilm disruption

Biosurfactants can degrade the biofilms. For instance, the rhamnolipids produced by *Pseudomonas aeruginosa* can destruct many biofilms. Biofilms are a major source of disease and contamination in food industry. So, the biosurfactants can be employed to overcome this hurdles (H. Huang *et al.*, 2018).

4.Antioxidants

There are biosurfactants that possess antioxidant properties. They are used in pharmaceuticals. For example, Mannosyl erythritol lipid-C (MEL-C) has free radical ion scavenging activity and also exhibit highest antioxidant property. So, it can be used in cosmetics industry.RW1,another biosurfactant, is a natural alternative to antioxidants (Coelho *et al.*, 2020; Yalcin *et al.*, 2010).

5.Food industry

The emulsification property of biosurfactants is exploited in the food industry. Several amphiphiles, which are of low molecular weight are used to stabilize various delicacies and food products like beverages ,dressings, sauce etc. (Wei, 2019).Similarly, emulsifier lecithin is used in the process of manufacturing of cocoa powder (Atik *et al.*, 2020).Also, mayonnaise, salad, butter cream are also produced by incorporating emulsions into it. So bio-emulsifiers are a natural alternative to other emulsifiers. Manno proteins synthesized by yeast are effectively used in various food products. Similarly

many biosurfactants are used in cookie production (De Iseppi *et al.*, 2019).Microbial surfactants increase the shelf life of dough and enhances the texture and stabilize the fat of ice-cream (Nalini *et al.*, 2020).

6.Biodegradation

Surfactants make less soluble chemicals to more soluble and usable to microorganisms during the process of biodegradation. They degrade hydrocarbons, the low molecular weight ones being degraded first (Abbasian *et al.*, 2015).Biosurfactants also increase the solubility in water and facilitate enhanced mass transfer. Because of this, they are used in degradation of hydrocarbons (VanLoon *et al.*, 2017).

7.Bioremediation

Bioremediation is the process of breaking down of complex substances to fewer toxic forms. Usually during bioremediation, microbes are introduced to promote the degradation of pollutant. The process is enhanced by the use of biosurfactants, which will increase the desorption of pollutant, thereby increasing the decomposition of hydrocarbons (Zhang *et al.*, 2018).

8.Agriculture

Microbial surfactants, also called green or renewable surfactants are usually used in agriculture to improve the quality and enhance the production of crops. Several epiphytic as well as endophytic microbes are used for producing biosurfactants that help to increase seed germination (Saleh *et al.*, 2019) *Bacillus* and *Pseudomonas* belonging to the Rhizobacteria group secrete metabolites, which are having an array of applications including plant growth promoters, fungicidal agents like siderophores, phenazines and cyclic peptides.

REVIEW OF LITERATURE

Microorganisms synthesise a wide range of surface-active compounds (SAC) which are called as biosurfactants. They can be classified according to their molecular weight, physico-chemical properties and mode of action. According to Banat *et al* biosurfactants, which are low molecular SACs have the capability to reduce the surface tension at the air/water interfaces and the interfacial tension at oil/water interfaces. On the other hand the high-molecular-weight SACs, also called bio emulsifiers, are more effective in stabilising oil-in-water emulsions. (Banat *et al.*, 2010) According to Ward (2010), microbial biosurfactants are amphipathic molecules having typical molecular weights ranging from 500-1500 Da. They are made up of peptides, saccharides or lipids or in combinations of these. In biodegradation processes they mediate the processes such as solubilisation, mobilization and/or accession of hydrophobic substrates to microbes. Biosurfactants may be located on the cell surface of microbes or may be secreted into the extracellular medium and they facilitate uptake of hydrophobic molecules through direct cellular contact with hydrophobic solids or droplets or through micelle formation. They are involved in cell physiological processes such as biofilm formation and detachment. Also they facilitate diverse biofilm associated processes such as wastewater treatment and microbial pathogenesis (Ward, 2010).According to Marchant *et al (*2012).,biosurfactants can productively replace the chemical surfactants, produced from non-renewable resources, with alternatives produced from cheaper renewable feedstocks. They are important because they are less damaging to the environment yet are robust enough for industrial use. (Marchant *et al.*, 2012)

As studied by Santos *et al (*2016),biosurfactants are amphiphilic substances with hydrophilic tail and hydrophobic head. They identified that cyclic peptides, phosphates, carboxyl acids, or alcoholic groups are found in the hydrophilic region of biomolecules, while the hydrophobic zone generally gas long chain fatty acids, hydroxyl fatty acids, α-alkyl-β-hydroxyl fatty acids, etc. (Santos *et al.*, 2016)One among the attractive properties of microbial surfactants is their ability to decrease the surface tension; apart from that microbial surfactants also exhibit an array of properties such as emulsification, lubrication ability, phase dispersion, detergency etc. Biosurfactants find their applications in various fields like agriculture, pharmaceuticals, food, petroleum, cosmetics etc. (Sachdev *et al.*, 2013)

Goussous *et al.,* in 2017 and Wu *et al.*, in 2019 demonstrated that microbes can produce both high and low molecular weight biosurfactants. Low molecular weight biosurfactants usually include glycolipids like trehalose, fructose lipids and sophorolipids and peptidyl lipids include surfactin, polymyxin, and so on. Surfactin is produced by *Bacillus subtilis*, which can serve as a formidable inhibitor of bacterial and tumour growth, and are also can decrease the surface tension of the fluids. (Goussous *et al.*, 2017; Roy *et al.*, 2016; Wu *et al.*, 2019) Tian *et al* illustrated that the high molecular weight biosurfactants are amphiphilic polysaccharides which forms stable emulsions and generally do not lower interfacial tension. (Tian *et al.*, 2019)As the high molecular weight biosurfactants can form emulsions, they can strongly adhere to the surface of hydrophobic substances. As Martins *et al (*2016*)*,exemplified, a major share of the biosurfactants are obtained from *Arthrobacter, Bacillus subtilis, Pseudomonas aeruginosa, Acinetobacter calcoaceticus, Candida lipolytica, Corynebacterium, Nocardia, Bacillus licheniformis, Candida* spp. and so forth (Martins *et al.*, 2018; Santos *et al.*, 2016).

Rhamnolipids, as studied by Banat *et al (*2016)., belong to the class glycolipids and they are found to be produced by bacteria belonging to Pseudomonas species, more specifically *Pseudomonas aeruginosa. (Díaz De Rienzo et al., 2016)*The polymers like liposan, lipomannan, Emulsan, Alasan are produced from organisms like *Candida lipolytica,Candida tropicalis,Acinetobacter calcoaceticus,Acinetobacter radioresistens* respectively (Christofi *et al.*, 2002; Cirigliano *et al.*, 1985; Corbu *et al.*, 2019; Zosim *et al.*, 1982). Among microorganisms, bacteria are the largest group that produces a bulk of biosurfactants. Several bacteria species including Pseudomonas sp., Acinetobacter sp., Bacillus sp., and Arthrobacter species produces biosurfactants and are effectively studied. But the major hurdle is the pathogenicity. The pathogenic property of these bacteria hinders its applications in the food industry. Therefore, a more suitable alternative, the probiotic bacteria, have gained popularity. Declared that probiotic bacteria impact human health, putting in probiotics as non-pathogenic and safe organisms have attracted attention to produce biosurfactants recently. A major share of biosurfactants obtained from probiotic bacteria are related to Lactic acid bacteria (LAB) (Hajfarajollah *et al.*, 2018).

Abbot *et al* in 2022,instantiated that biosurfactant production can be effectively increased by manipulating the metabolic pathway of the producing organism and also by having a proper knowledge about the chemical structure of the biosurfactants (Abbot *et*

al., 2022).Zouari *et al (*2021) engaged this information to utilize the poultry farming waste and buttermilk as source of carbon and nitrogen, which was surmised to be efficient for creation of SPB1 lipopeptide (Zouari *et al.,* 2021).Bacteria from oil contaminated sites also produce biosurfactants. But one of the major stumbling blocks in the effective utilization of biosurfactants is their cost of production. Syldatk *et al (*2010) demonstrated that the most common reasons for meagre use of microbial surfactants are the use of expensive substrates, sparse product concentrations, scanty yields and formation of product mixtures in lieu of pure compounds (Syldatk *et al.,* 2010).The cost of biosurfactant production can be decreased by effectively utilizing minimal expense media (Makkar *et al.,* 2011).Another method to alleviate the cost of production, as illustrated by Lima *et al* in 2017 is modifying fermentation technologies and making use of renewable substrates and different microbial strains (Lima *et al.,* 2017; Rodríguez *et al.,* 2021).Several bacterial strains grow ideally in different range of temperature etc. So proper monitoring of these physical properties can make the biosurfactants produced from this bacterial strain more efficient. Abbot et al., found that the usage of agro-industrial waste and renewable resources for their production can constructively reduce the cost of production of biosurfactants. (Abbot *et al.,* 2022; Domínguez Rivera *et al.,* 2019)

The characterization of biosurfactants is mainly done through different spectroscopic methods. Janek *et al* (2013) employed ultra performance liquid chromatography-tandem mass spectrometer (UPLC-MS) method to spot the structures of biosurfactants produced from *Pseudomonas putida* BD2 isolate of Arctic bacterium (Janek *et al.,* 2013). *Ribeiro et al.* estimated the cytotoxic activity of a biosurfactant derived from the organism Candida *utilis* using MTT assay (3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). This test was performed by making use of mouse macrophage and fibroblast cells. These cells were maintained at 37 °C within a culture flask with 5% carbon dioxide (Ribeiro *et al.,* 2020).In 2020,Yang *et al.* delineated the utilization of biosurfactant generated from *B. subtilis* Y9 as an insecticidal metabolite. By performing column chromatography and preparative high performance liquid chromatography (HPLC) analysis, the biosurfactant property could be elucidated. (Ribeiro *et al.,* 2020) Luong *et al* (2018) performed similar characterization techniques for purification of trehalolipid biosurfactants which were obtained from *Rhodococcus erythropolis* S67 strain. The study was conducted at relatively low temperatures (10 °C and 26 °C) and the biosurfactants that formed were identified using mass spectrometric (MS) analysis (Luong *et al.,* 2018). In one of the recent researches,

Rosas-Galvan *et al (*2018) assessed the effect of carbon and nitrogen on the biosurfactants that are produced from *Serratia marcescens* and its isogenic SMRG-5 strains. Using gas chromatography-mass spectrometry (GC–MS) analysis ,the molecular structure of biosurfactants was elucidated (Rosas-Galván *et al.*, 2018).

According to Abbot *et al* (2022),microbial biosurfactants can be categorized based on their method of activity, sub-atomic weight, and general physicochemical properties .Generally, microbial biosurfactants are grouped based on their synthetic strategy and sub-atomic weight. Based on sub-atomic weight, they are classified as low atomic weight biosurfactants like glycolipids and lipopeptides as well as high atomic weight biosurfactants like polysaccharides, proteins and lipopeptides. In heterogeneous frameworks, they create an atomic interfacial coating that take hold of initial surface's wettability and surface vitality. Besides bringing down the fluids surface pressure, these can remarkably affect the interfacial conductance and mass exchange, as well as bring down the interfacial strain between diverse fluid stages on the interfacial limit existing between immiscible stages (Abbot *et al.*, 2022).

Biosurfactants find their applications in various fields. Raj *et al (*2021),studied that biosurfactants can be used extensively as alternatives to several pesticides that are now being used in the agricultural field. Pesticides are used indiscriminately all over the world. But the problem is that ,if they are used in excess, they have the potential to contaminate the soil and water bodies and as a result capable of producing negative effects on human health and the environment. Even though bioremediation is one of the most viable options to deal with these pollutants, there are certain limitations which reduces the application of bioremediation. Therefore, investigating more on the role of microbial biosurfactants in pesticide remediation is a promising approach. As biosurfactants are amphiphilic compounds, they can help to increase the bioavailability of pesticides, and speed up the bioremediation process. Biosurfactants can lower the surface area and interfacial tension of immiscible fluids and boost the solubility and sorption of hydrophobic pesticide contaminants. Biosurfactants have the property of biodegradability, low toxicity, high selectivity, and broad action spectrum under extreme pH, temperature, and saline conditions, as well as a low critical micelle concentration (CMC). All these factors can contribute to the process of pesticide remediation. Application of metagenomic and in-silico tools would help to characterize pesticide degrading microorganisms at a taxonomic and functional level, so that their isolation and thereby productive utilization can be ensured. (Raj *et al.*, 2021).

As encapsulated by Adu *et al* in 2020,another important usage of biosurfactants is in the cosmetics industry. Cosmetic and personal care products have got worldwide attraction in recent times. These products are globally used and is directly applied on skin generally. With a huge sum of money spent on cosmetics globally, these products have become an important part of the skincare. But the fact is that, majority of these products use chemical surfactants, of which many have been reported to have the potential to cause deteriorating effects such as allergic reactions and skin irritations to humans. So there occurs a need to substitute these chemical surfactants with biosurfactants which have less or no negative impacts on skin. Biosurfactants show properties like lower toxicity, skin compatibility and surface moisturizing effects, which are the main components in a skin care routine. (Adu *et al.*, 2020)Lipopeptides are one of the major biosurfactants used in cosmetic industry due to their surface properties and biological properties. Kanlayavattanakul *et al (*2010)., studies depict that cosmetics that contain lipopeptides are used in varying doses according to their beneficial surface properties, which mainly include anti-wrinkle, moisturizing and cleansing activities. Glycolipids like sophorolipids, rhamnolipids and mannosylerythritol lipids are also extensively used in cosmetics industry. Mannosylerythritol lipids (MELs) are produced by different basidiomycetous yeasts such as Pseudozyma. MELs exhibit excellent interfacial properties and versatile biochemical actions. These excellent properties that contribute to practical cosmetic ingredients include moisturization of dry skin, repair of damaged hair, activation of fibroblast and papilla cells and antioxidant and protective effects in skin cells. (Kanlayavattanakul *et al.*, 2010; Lourith *et al.*, 2009; Morita *et al.*, 2013)Biosurfactants are extensively used in agriculture also. Metabolic products called as green /renewable surfactants are widely used to improve the quality and quantity of crops. Seed germination is aided by epiphytic and endophytic microbes that have the capacity to produce biosurfactants. (Saleh *et al.*, 2019)

In 2018, Nayarisseri *et al.,* found that the water samples collected from oil contaminated sites of chilika lake, the largest brackish water lagoon in India contained microbes that produced biosurfactants. For the isolation of the microbial consortium, the samples were enriched by inoculating into sterile mineral salt medium (MSM).That is,1 ml sample was inoculated in 100 ml MSM. The screening was done by growing the bacterium in Erlenmeyer flask containing 100 ml MSM. The Flasks that contained sterilized mineral salt medium were inoculated with the bacterial culture which was

grown in crude oil containing nutrient agar plate. These culture flasks were maintained in a shaker for 7 days at 200 rpm and 30°C. After 7 days of incubation, the culture broth from each flask was centrifuged at 6000 rpm particularly and 4°C for 15 minutes. The supernatant was then filtered through a 0.45µm pore size filter paper, called as Millipore. This cell free culture broth was subjected to drop collapse assay, followed by oil spreading assay, emulsification assay, surface tension measurement and finally the bacterial cells were used for BATH assay. All the experiments for screening were performed in triplicates and the mean values obtained were used as results. (Carrillo *et al.*, 1996; Nayarisseri *et al.*, 2018)

Estefania *et al (*2022), mentioned about the source from which biosurfactants are produced by microbes. The main source is Carbon. Among the different carbon sources, the more frequently used are glucose and glycerol (Eras-Muñoz *et al.*, 2022).But, as pointed out by Ejike Ogbonna *et al (*2021)., ,glucose is obtained from food sources and is also extensively used as an industrial feedstock, it can increase the cost of production of biosurfactants (Ejike Ogbonna *et al.*, 2021).As an alternative, Wongsirichot *et al (*2021), suggested the utilization of carbon sources other than food sources, like, municipal waste, agricultural by products, food production waste etc (Wongsirichot *et al.*, 2021).Another case with biosurfactant production is that when complex substrates are used, impurities can be found in the final extract. For instance, fermentation using kerosene to produce a biosurfactant from Serratia sp. Caused large amount of impurities in the final extract (Borah *et al.*, 2019).

As detailed by Kumar *et al (*2021),fermentation processes are involved in biosurfactant production. Two types of fermentation-submerged fermentation (SmF) and solid-state fermentation (SSF) are involved. SSF have merits like avoiding inhibition of substrate, allowing use of industrial residues and waste products from agriculture, low energy consumption etc (Kumar *et al.*, 2021).But when comparing with SmF, SSF has certain disadvantages linked to substrate heterogenicity, difficulty in downstream processing etc (Sánchez *et al.*, 2015).Biosurfactant particular properties according to Malkapuram *et al (*2021),are usually signalized by collapse test, oil drop, surface tension, emulsification index, foaming index and critical micelle concentration (Malkapuram *et al.*, 2021).

For screening the biosurfactant from the isolate, Carillo *et al (*1996),had grown the bacteria in Erlenmeyer flask with MSM. The flask with MSM was properly sterilized and a loopful of inoculum was added in to it. The flasks were maintained in a rotary shaker

for 7 days at 200 rpm and 30°C.After 7 days, the culture broth from the Erlenmeyer flask was centrifuged at 6000 rpm and 4°C for 15 minutes. The supernatant was then filtered out using 0.45μm pore size filtered paper. After centrifugation, the cell free broth was tested using drop collapse test, oil spreading test, emulsification assay etc. All these experiments for screening were done in triplicates (Carrillo *et al.*, 1996).

Morikawa *et al (*2000)., described a procedure for oil spreading technique. In this method,20 ml of distilled water was added into a petri plate followed by 20 microlitre of crude oil to the surface of distilled water.Then,10 microlitre of cell free broth was added to this oil surface. If biosurfactant is present, then oil will be displaced producing oil free clearing zone. The diameter of this zone indicates the extent of biosurfactant activity of bacteria (Luo *et al.*, 2020; Morikawa *et al.*, 2000).

Bodour and Maier performed qualitative drop-collapse test. For this test crude oil was used (Batista *et al.*, 2006). Oil was applied to the culture in petri plates and after 48 hours, the culture was transferred to oil coated wells and drop size was observed after1 minute with a magnifying glass. The biosurfactant producing bacteria form a flat drop and this is considered as a positive result (Youssef *et al.*, 2004).The next assay for screening bacteria is emulsification assay. Bodour *et al* (2004).,conducted tests for emulsification. Many of the colonies from pure culture were then suspended in test tubes with mineral salt medium for 48 hours of incubation.Then,2ml petroleum was added. The mixture was then vortexed at high speed for 1 min and was kept undisturbed for 24 hours (Bodour *et al.*, 2004).

The emulsion index-E24 is calculated using the formula:

$$\text{E24} = \frac{\text{Total height of the emulsion layer}}{\text{height of the aqueous layer}} \text{x } 100$$

Biochemical characterization usually uses tests like catalase test, citrate test, MR-VP test etc. The biochemical tests, as reported by Nayarisseri *et al (*2018)., is best performed in 24-hour culture. The biosurfactant extraction is done by inoculating the bacteria into 100 ml medium. It is incubated at 25°C for 7 days in shaking condition-preferably in a shaking incubator. After 7 days, the cells were removed by centrifuging at 4000 rpm. The pH of supernatant was adjusted to 2,using 1M H2SO4.Then chloroform and methanol was added in the ratio 2:1.The mixture was shaken well and left undisturbed an overnight for proper evaporation. The white coloured sediment obtained is biosurfactant (Maulana *et al.*, 2022; Nayarisseri *et al.*, 2018).

Wishard *et al (*2014)., did the molecular characterisation by isolating the DNA, followed by 16S rRNA Sequencing (Wishard *et al.*, 2014).For DNA isolation,5 ml of overnight culture was centrifuged at 10000 rpm for 10 minutes. Later TE buffer (875βl) was added.10% SDS (100βl) and Proteinase K (100βl) was added to the cells. The mixture was incubated for 1 hour at 37 degree Celsius. Phenol-chloroform mixture (1ml) was added to vial and incubated at room temperature for 5 minutes. Later it was centrifuged at 10000 rpm for 10 minutes. The process was repeated. The supernatant was collected. To this supernatant,5M sodium acetate (100βl) was added. Isopropanol (2ml) was added. It was mixed till the DNA precipitated. Later, it was again centrifuged at 5000 rpm for 10 minutes. The supernatant was removed. Finally, ethanol (70%) was added and centrifuged. The process is done at 5000 rpm for 10 minutes. Later it is air dried and stored in TE buffer. The 16S rRNA sequencing was done. The amplified products were detached on 1% agarose gel in 1x TAE buffer. The purified PCR product was then sequenced using Sanger dideoxy sequencing technology (Nayarisseri *et al.*, 2020; Nayarisseri *et al.*, 2018)

AIMS AND OBJECTIVES

The study was based on isolation and screening of biosurfactants from oil contaminated soil and characterization of isolated organisms. The objectives include:

- Isolation of biosurfactant producing bacteria from oil contaminated soil
- Screening of isolated bacteria
- Characterization of the isolated strain

The characterization includes

- Morphological characterization
- Biochemical characterization
- Molecular characterization

MATERIALS AND METHODS

Collection of samples

Collection of oil contaminated Soil samples employed in this study were collected from workshops in Kuttikatoor and Iringadanpally, in the district of Kozhikode, Kerala. Top soil (about 1 cm from all the four corners of the workshop. Soil samples collected in aseptic sample bags with proper labelling were brought to the laboratory.

Isolation of bacteria

5g of soil sample was inoculated in 100 ml of Mineral Salt Medium (MSM) with 3ml kerosene oil added to the conical flask having capacity of 250 ml, as the carbon sources and then it was incubated for 72 hours at 30°C temperature. The samples then were serially diluted up to 10-6 dilution. 1 ml of 10- 5and -6 dilution was transferred to nutrient agar for spread culture. The plate was inverted and incubated at 30° C, for 72 hours. After incubation, morphologically of colonies were observed.

COMPONENTS	Weight (in gm)/litre of water
KH2PO4	1
MgSO4	0.5
FeSO4	0.01
NaNO3	1.5
CaCl2	0.002
(NH4)2SO4	1.5

Pure culturing

The isolated colonies were streaked on sterile nutrient agar plates to get pure culture. Well isolated colonies were taken for screening and observed for morphological characterization.

Screening of biosurfactant producing bacteria

The isolated colonies were taken and tested for the conformation of biosurfactant production, that comprises the following test.

Oil spreading technique:

In this method 2 µl of diesel is added to the surface of 15 ml of distilled water in a petri dish to form a thin oil layer. Then, 10 µl of culture or culture supernatant are gently placed on the centre of the oil layer. If biosurfactant is present in the supernatant, the oil is displaced and a clear zone is formed. The diameter of this clearing zone on the oil surface correlates to surfactant activity, also called oil displacement assay. The principle of this method was based on the ability of the biosurfactant to alter the contact angle at the oil-water interface. The surface pressure of the biosurfactant displaced the oil.

Emulsification assay:

Evaluating the emulsification capacity is a simple screening method suitable for a first screening by Cooper and Goldenberg 1987. Biosurfactants has the ability to emulsify various hydrocarbons. The emulsifying capacity was evaluated by an emulsification index (E24). The E24 of culture samples were determined by adding 2 ml of kerosene and 2 ml of the cell-free broth in test tube, the cell-free broth obtained after incubation period and centrifuge at 4000 rpm for 4 min, after that vortex the mixture at high speed for 2 min and allowed to stand for 24h. The E24 index is given as percentage of the height of emulsified layer (mm) divided by the total height of the liquid column (mm). The percentage of emulsification index calculated by using the following equation:

$$\text{E24} = \frac{\text{Total height of the emulsion layer}}{\text{height of the aqueous layer}} \text{x } 100$$

Lipase Test:

The isolates were streaked on tributyrin agar plates (Hi media) and incubated at 30°C for 24–48hr. After incubation the plates were observed for zone of clearing around colonies, it represents biosurfactant production.

Extraction of Biosurfactants

The culture was inoculated in 50ml of nutrient broth with 1ml of petrol. The culture was incubated at 25 C for 7 days with shaking condition. After incubation the bacterial cells were removed by centrifugation at 5000rpm, 4 C for 20 minutes. The supernatant was taken and the pH of the supernatant was adjusted to 2, using 1M HCL. Now add equal volume of chloroform: methanol (2:1). This mixture was shaken well for

mixing and left overnight for evaporation. White coloured sediments were obtained as a result i.e., the Biosurfactant.

Dry weight of biosurfactant

Sterile Petri plate was taken and the weight of the plate was measured. Now the sediment was poured on the plates. They were placed on the hot air oven for drying at 100 C for 30 minutes. After drying the plates were weighted. The dry weight of the biosurfactants was calculated by the

following formula:

Dry weight of biosurfactant = (Weight of the plate after drying - weight of empty plate)

CHARACTERIZATION OF ISOLATE

MORPHOLOGICAL CHARACTERIZATION

Cell morphology:

The cells were observed and the shape and colour of the cells were determined.

Gram staining:

Gram staining is a method that differentiates bacteria in two large group gram positive and gram negative. This method differentiates bacteria by the chemical and physical properties of their cell walls by detecting peptidoglycan, in gram positive it is present as a thick layer. A Gram-positive result in a purple/blue colour while a Gram-negative results in a pink/red colour.

Procedure:

- Prepare a thin film of smear on a clean glass slide.
- Gently flood smear with crystal violet and let stand for 1 minute.
- Gently wash with tap water.
- Gently flood smear with the gram's iodine mordant and let it stand for 1 minute.
- Gently wash with tap water
- Decolorize with 95% ethyl alcohol. Add reagent drop by drop until the alcohol runs almost clear, showing only a blue tinge.
- Gently wash with tap water.

- Counter stain with safranin for 30 seconds.
- Gently wash with tap water.
- Examine the slide under microscope.

Motility test:

Small amount of Vaseline was placed on the four corners of the cover slip. From the culture tube a loop full of culture was taken placed at the centre of the cover slip. Then a clean cavity slide was placed act with the cover slip with the depression over the drop containing bacteria. It was inverted quickly. So that the drop cannot run off to one slide and the drop hanging down from the cover slip containing the suspended bacteria. Edge of the drop was first focused under 10x and then in 45x objective.

Bio-Chemical Characterization:

Catalase Test

It is a common enzyme found in living organisms exposed to the oxygen. It has one of the maximum turnover numbers of all enzymes; one catalase molecule can convert millions of molecules of hydrogen peroxide to water and oxygen each second. The reaction of catalase in the decomposition of living tissue: $2\ H2O2 \rightarrow 2\ H2O + O2$

The main objective of this test is to determine whether microbes are able to produce catalase enzymes or not, it is a protective enzyme i.e., able to the dangerous chemical hydrogen peroxide.

- Transfer small amount of bacterial colony to a surface of clean, dry glass slide using a loop or sterile wooden stick.
- Place a drop of 3% H2O2 on to the slide and mix.
- A positive result is the rapid evolution of oxygen (within 5-10 seconds) as evidenced by bubbling
- A negative result is no bubbles or only a few scattered bubbles.

Oxidase Test:

- Place one OXIDASE disc onto a suitable surface. It is preferable to use young, fresh, cultures as older growth may produce unreliable results.
- Remove the chosen colony from the culture plate.

- Gently massage the colony onto the disc and observe for the development of a deep
- purple colour within 10 seconds.
- Alternatively prepare a suspension of test organism in sterile distilled or deionized water. Place 1 ml of suspension into sterile tube and one OXIDASE Disc. Gently shake the tube and leave at room temperature for 10 minutes and observe for development of a deep purple colour.

Indole Test:

The test determines the bacterial capability to breakdown the indole from tryptophan molecule. 24 hours pure culture was inoculated in tryptone broth. It was incubated for 24 hours at 30°C and then removed 2ml media and added in to the empty sterile test tube and added few drops of Kovac's reagent and agitate the tube for few minutes.

Positive: Within few minutes' cherry red colour form, Negative: No colour changes

MRVP:

This test is used to determine two things. The MR portion (methyl red) is used to determine if glucose that can be converted to acidic products like lactate, acetate, and formate. The VP portion is used to determine if glucose can be converted to acetoin. These tests are performed by inoculating a single loop full culture into MR and VP media and then allowing the culture to grow for 24hrs.

Methyl red test

- Inoculate the tubes containing MR-VP Broth with a pure culture of the microorganisms under investigation.
- Incubate at 37°C for up to 24 hours.
- Add about 5 drops of the methyl red indicator solution to the tubes.
- A positive reaction is indicated, if the colour of the medium changes to red within a few minutes.

Voges Proskauer test

- Inoculate MRVP broth with a pure culture of the organism.
- Incubate at 35°-37°C for a minimum of 48 hours in ambient air.
- Add 12 drops of Barritt's reagent A and B to the test tubes.
- Observe for the colour change in the broth medium.

Citrate test:

The citrate test notices the capability of an organism to consume citrate as the sole source of carbon and energy for growth and metabolism. The principle is based on the breakdown of citrate to oxaloacetate and acetate. Bacteria are inoculated on a medium containing sodium citrate and a pH indicator such as bromothymol blue. This medium also comprises inorganic ammonium salts, which are utilized source of nitrogen. Bacteria decompose the citrate and convert in to the enzyme citrase, which breaks down citrate to oxaloacetate and acetate. Oxaloacetate is further decomposed in to pyruvate and carbon dioxide (CO_2). Production of sodium bicarbonate ($NaHCO_3$) as well as ammonia (NH_3) from the use of sodium citrate and ammonium salts results in alkaline pH. This results in a change of the medium's colour from green to blue.

Procedure

- Inoculate **Simmons citrate agar** lightly on the slant by touching the tip of a needle to a colony that is 18 to 24 hours old
- Incubate at 35◦c to 37◦c for 18-24 hours. Some organisms may require up to 7 days of incubation due to their limited rate of growth on citrate medium
- Observe the development of blue colour; denoting alkalinization.

Urease Test:

The main objective of this test is to determine the ability of bacteria to degrade urea by means of urease enzymes. Culture was inoculated in urease broth for 48 hours and maintains temperature 37°C and then observed the colour, if a colour change from light yellow to pink then test is positive.

Procedure

Dissolve Stuart's urea broth ingredients in distilled water. Heat if necessary to dissolve the medium compleately.0

- Sterilize it by autoclaving at 15lbs pressure (120 ℃) for 15 minutes. Then cool to 55℃.
- Aseptically add 50 ml of sterile 40% Urea solution.
- Mix well and distribute in 5ml amounts into sterile test tubes. Use inoculation from an 18 to 24-hour pure culture to inoculate the broth. Then incubate at 37℃ for 48 hours to 7 days.

Starch hydrolysis:

Some bacteria can use starch as a source of carbon energy, starch hydrolysis test shows whether bacteria can use starch as sole carbon energy or not. The pure culture is streaked on starch agar plate and the inoculated plate is incubated for 24 hours and temperature is maintained 30-35℃. After then iodine reagent is added to flood the growth.

Molecular characterization:

DNA EXTRACTION

- The purpose of DNA purification from the cell / tissue is to ensure it performs well in subsequent downstream applications.eg. Polymerase Chain Reaction (PCR), microsatellite analysis etc. Ideally, the DNA should be free of contamination with Protein, Carbohydrate, Lipids, Other nucleic acid (i.e., DNA free of RNA), Tannins and phenolics.

GENOMIC DNA EXTRACTION FROM BACTERIA

- Inoculate 1 to 3 well isolated colonies of nutrient broth for overnight (12 to16 hours). After incubation transfer 1ml of the overnight culture is to sterile micro centrifuge tube and harvest the cells by centrifugation at 12,000 rpm for 10 minutes. Discard the supernatant and the resuspend the pellet in 1ml of 0.85% (W/V) NaCl solution and centrifuge as above. Discard the supernatant and at and add 600µl lysis buffer along with 7µl of proteinase-K. Vortex the mixture and incubate at 65ºC for 30 minutes centrifuge at 12,000 rpm for 10 minutes. Collect the supernatant and add equal volume of chloroform: isoamyl alcohol (24:1) with general mixing by inverting the tubes. Centrifuge the sample for 10 minutes at 12,000 rpm at RT. Collect the aqueous phase in another microcentrifuge tube without disturbing the interface at lower phase. $1/10^{th}$ volume of 3M Sodium

acetate ($p^{H\ 5.2}$) followed by equal quantity of ice-cold isopropanol, the DNA gets precipitated and centrifuge it again for 5 minutes at 12,000 rpm. Discard the supernatant and rinses the pellet with 70% alcohol, centrifuge at 12,000 rpm for 5 minutes followed by maintaining the tubes for drying in sterile hood. Resuspend that desiccated DNA samples in 50µl of DNA dissolving buffer (TE buffer)/ nuclease free water and store at -20ºC.

REAGENTS USED

0.85% (w/v) NaCl solution	
NaCl	0.85 g
Distilled water	90 ml

Add 0.8g NaCl to 90 ml of distilled water and make up the final volume to 100 ml, autoclave and store at 4ºC.

0.5M EDTA	
$Na_2EDTA\ 2H_2O$	4.65 g
Distilled water	20 ml
Adjust the P^H to 8.0 using 1M NaOH and make up the final volume to 25ml, autoclave and store at 4ºC.	

1M Tris (p^H 8.0)	
Tris-Cl	3.93 g
Distilled water	20 ml
Adjust the p^H to 8.0 using 1M HCl and make up the volume to 25ml, autoclave and store at 4ºC.	

20% SDS	
SDS	5.0 g
Distilled water	20ml
Make up the final volume to 25ml and store at 4ºC.	

0.1M Tris HCl (p^H 8.0)	
1M Tris HCl	15 ml
Distilled water	135 ml

Chloroform: Isoamyl alcohol (24:1)	
Chloroform	48 ml
Isoamyl alcohol	2 ml
Both of the components are mixed properly by inversion	
3M Sodium Acetate (pH 5.2)	
Sodium acetate	6.2 g
Distilled water	20 ml
Adjust the p^H to 5.2 using glacial acetic acid and make up the final volume to 25 ml, autoclave and store at 4ºC.	

TE buffer (DNA dissolving solution)	
10mM Tris	20µl from 1M Tris stock
1mM EDTA	4µl from 0.5M EDTA stock
Make up to 2ml and store at 4ºC.	
70% Isopropanol	

WORKING SOLUTION:

1. Nutrient broth

Peptone	1 g
Yeast extract	0.5 g
Sodium chloride	1 g
Distilled water	100 ml

Add the ingredients to 90 ml of deionized water, stir for complete solubility. Adjust the p^H to 7.0 with 5M NaOH and make up the volume to 100 ml using deionized water. Sterilized by autoclaving for 20 minutes at 15 lbs /sq. inch.

2. Lysis buffer

Stock	**Required stock volume**	**Final concentration**
0.5M EDTA (P^H 8.0)	5 ml	50 mM EDTA
1M Tris (p^H 8.0)	5 ml	100 mM Tris

20% SDS	7.5 ml	3% SDS
Distilled water	32.5 ml	
Total volume	50 ml	

The buffer solution stored at 4ºC.

3. Proteinase-k: Proteinase K was dissolved in distilled water at a concentration of 20 mg/mL; w/v)

PCR amplification of 16S rRNA of the isolate

About 2ng of isolated bacterial DNA was amplified for 16S rRNA genes using the bacterial forward primer 27F (5'-AGAGTTTGGATCMTGGCTCAG-3') and reverse primer 1492R (5'-CGGTTACCTTGTTACGACTT -3') (Jiang *et al*., 2006). The PCR reaction mixture consisted of 2ng of 1µl genomic DNA, 1µl each forward and reverse primer at a concentration of 10µM, 2µl dNTPs (2Mm), 10µl of 10X reaction buffer, 1µl *Taq* polymerase (5U/µl) and 84µl water. The PCR profile consisted of an initial denaturation step of 5 minutes at 95ºC, followed by 30 cycles of 10 second at 95ºC. The annealing was done for 1-minute 50ºC, extension for 2 min at 72°C; and a final extension at 72°C for 7 min. The PCR product was resolved on a 2% TAE – agarose gel, stained with Ethidium Bromide, for the confirmation of the target gene amplification. Ethidium bromide acts as an intercalating agent on the bases of DNA molecules and imparts an orange colour to DNA under ultraviolet light which can be viewed on the gel.

After ascertaining the PCR amplification of the corresponding 16S rRNA gene fragment, the remaining portion of the PCR product was column purified using Gene Jet PCR purification kit (Fermentas Life Science), which is designed for rapid purification of single-stranded or double-stranded PCR amplification products (100 bp to 10 kb) from other components in the reactions such as excess primers, nucleotides, DNA polymerase, oil and salts.

Sequencing of PCR product

The purified PCR product was sequenced from both ends using forward and reverse primers by Sanger's dideoxy chain termination sequencing method (Sanger and Coulson, 1975) at SciGenom Labs Private Ltd., Cochin with ABI 3730XL Automated Sequencer. The forward and reverse sequences were trimmed for the primer sequences and then assembled by using Clustal W and the consensus was taken for the analysis (Thompson et al., 1994). Residue and pair-wise distances were estimated using

the Clustal W tool of MEGA6 software. The final sequences were searched for its similarity using BLAST program of NCBI (*www.ncbi.nlm.nih.gov*)**.**

RESULTS

Isolation of Bacteria

By the serial dilution from MSM broth inoculated with soil sample, only one type of bacterial strain was observed which is taken for further study.

Pure culture of bacteria

The colonies obtained on serial dilution were streaked on nutrient agar plates were taken for further study.



Fig1: Pure culture of isolated bacteria

Screening of isolated colonies

The isolated strain was screened for the confirmation of biosurfactant producing bacteria in which the strains (MCCC 1A01406) showed good result in screening.

Oil displacement technique

Supernatant of isolated strains were added to the plate containing crude oil. It was added to the centre of oil layer. The strains displaced the oil and showing a clear zone (fig 4). The results were noted down and diameter of zone was measured. The bacterial strain was chosen for further screening.

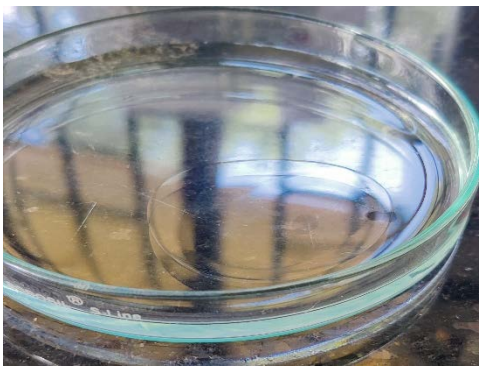


Fig 2: Result showing positive for oil displacement technique.

Table 1: The result of oil displacement technique

Bacterial strain	Diameter of clear zone (In cm)	Interpretation
MCCC 1A01406	1.00	Positive

Emulsification assay

The isolated strain showed positive result were tested for their abilities to emulsify crude oil and this study was taken for the study of emulsification assay. Test was done by the adding 2ml of crude oil and 1 ml of supernatant and kept overnight. After then results were noted down.

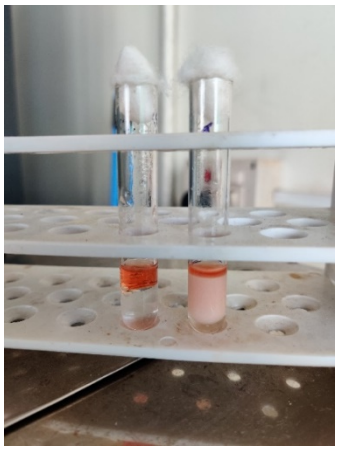


Fig 3: Result showing positive for emulsification assay.

Table 2 E24 index

Bacterial strain	Emulsified layer (cm)	Total liquid layer	E24 (%)
MCCC 1A01406	2.5	4.5	55.5%

Lipase Test

The isolated colonies when streaked on tributyrin agar plates after incubation showed, a defined zone of clearing around the colonies that represent biosurfactant production (fig 4).



Fig 5: Result showing positive for Lipase test.

Extraction of biosurfactant

The biosurfactants, were extracted by using acid-precipitation method at pH2 (fig 6).



Fig 6: Result showing extracted biosurfactant

Dry weight of extracted biosurfactant

Dry weight of extracted biosurfactant were calculated using the formula.

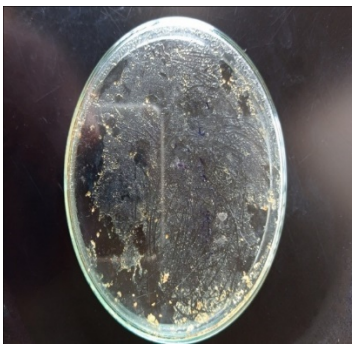


Fig 7: Dried

Dry weight of biosurfactant = (Weight of the plate after drying - weight of empty plate)

=11-9.2=1.8 grams

CHARECTERIZATION OF ISOLATED BACTERIA

Morphological characterization

Colony Morphology

The shape, size, elevation, margin and colour of the colony were observed in the culture plates. The observations were noted down.

Gram staining

Morphological characterization of isolated strain was done by Gram's staining. And it was found that the bacteria were Gram positive rod.

Table 3 Morphological characters of isolate.

Gram Staining	Cell Shape	Colony Shape
Negative	Rod shape	Small, circular, raised

Motility

By Hanging drop method, the bacterial isolate was observed motile.

Bio-Chemical Characterization

From the Gram's staining result, it was found that the strain was gram negative. So, for further characterization, different bio-chemical tests were performed to identify the species of strain.

Catalase Test.

No chemical reaction showed the formation of gas bubbles which confirm the organism is Catalase negative.

Oxidase Test

A deep purple colour was developed within 10 seconds, which indicates that the organism is Oxidase Positive (fig.9).



Figure 9: Result showing positive for Oxidase test

Indole test

After 24 hours incubation, in to the inoculated tryptone broth, Kovac's reagent was added. No coloured ring was in the samples, which indicates that the isolate was indole negative (fig.10) and also that the organism cannot produce indole by deamination of tryptophan.



Fig 10: Result showing negative for Indole test.

Methyl red test

There was no formation of any red colour at the surface, which indicates that methyl red test was negative for the isolate (fig 11). So, the organism is unable to utilize glucose and convert it into a stable acid like lactic acid, acetic acid or formic acid as their end product.



Fig 11: Result showing negative for methyl red test

Voges Proskauer test

There was no formation of any pink color at the surface. The isolate was negative to Voges Proskauer test (fig 12). That means the given organism have no ability to oxidize acetoin to diacetyl in the presence of potassium hydroxide (KOH).



Fig 12: Result showing positive for VP test

Citrate utilization test

Growth was observed on the slant surface. The slant turned green does not change colour. This indicates that the citrate utilization test was negative for the isolate. (Fig 13).



Fig 13: Result showing negative for citrate test

Urease test

There was no color change in the medium of isolate HM6 were observed, which indicates the isolate was negative to urease test (fig 14)



Fig 14:Result showing negative for urease test.

Starch hydrolysis

A black coloration of the medium (depending on the concentration of iodine) was observed in isolate. This indicates that the isolate is negative to starch hydrolysis (Fig 15). There is no clear zone around the line of growth after addition of iodine solution.

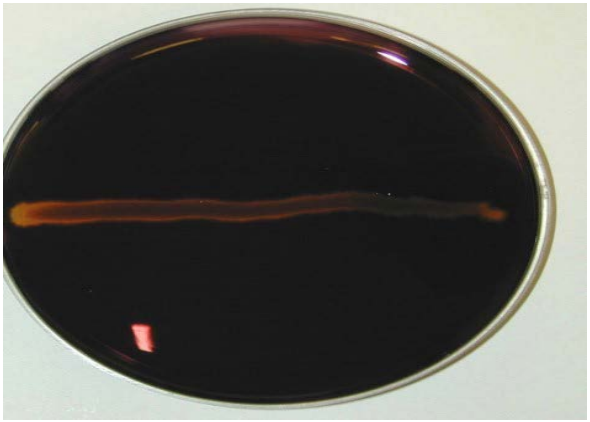


Fig 15: Result showing negative for starch hydrolysis

Table 4 Result of Biochemical Test

Biochemical Test	Result
Catalase	Negative
Oxidase	Positive
Indole	Negative
Methyl Red	Negative
VP	Positive
Citrate	Negative
Urease	Negative
Starch Hydrolysis	Negative

10. Molecular characterization:

The specific PCR amplification of 16S rRNA gene for identification of ***Bacillus tropicus*** Strain MCCC 1A01406 using 27F forward primer and 1492R reverse primer yielded a consequence single product of 879bp size. The strain was established by amplifying the 16S rRNA region and the sequence of the strain was subjected to BLAST. MCCC 1A01406 (***Bacillus tropicus***)

Table 5. *Bacillus tropicus* strain MCCC 1A01406 16S ribosomal RNA, partial sequence Sequence ID: NR_157736.1Length: 1509Number of Matches: 1

Alignment statistics for match #1				
Score	Expect	Identities	Gaps	Strand
1624 bits (879)	0.0	879/879 (100%)	0/879 (0%)	Plus/Plus

Query 1
GGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGT
GGGTAACC 60
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 65
GGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGT
GGGTAACC 124

Query 61
TGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACA
TTTTGAAC 120
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 125
TGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACA
TTTTGAAC 184

Query 121
CGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCC
GCGTCGCA 180
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 185
CGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCC
GCGTCGCA 244

Query 181
TTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACC
TGAGAGGG 240
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 245
TTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACC
TGAGAGGG 304

Query 241
TGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGC
AGTAGGGA 300
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 305
TGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGC
AGTAGGGA 364

Query 301
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GGCTTTCG 360
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 365
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GGCTTTCG 424

Query 361
GGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGG
CACCTTGA 420
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 425
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CACCTTGA 484

Query 421
CGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAAT
ACGTAGGT 480
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 485
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ACGTAGGT 544

Query 481
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Query 541
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Query 601
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Sbjct 785
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Query 781
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GCCGCAA 840
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Sbjct 845
TCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACG
GCCGCAA 904

Query 841 GGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGC
879
||||||||||||||||||||||||||||||||||||
Sbjct 905 GGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGC 943

The BLAST results of our strain MCCC 1A01406 shows 98.45% sequence similarity to *Bacillus tropicus*. These results confirm that our strain is *Bacillus tropicus*. Nucleotide BLAST results of *Bacillus tropicus* shown above. *Bacillus tropicus* is rod-shaped, bacteria. and has the ability to tolerate the petrol concentrations and grow on them. Hence, this strain can be used in cleaning oil polluted sites.

DISCUSSION

Biosurfactant-producing microorganisms are a diverse group of microbes that possess the ability to synthesize and secrete biosurfactants, which are surface-active compounds (Banat et al., 2010). These microorganisms, including bacteria, yeast, and fungi, produce biosurfactants with unique chemical structures and properties. Biosurfactants have gained significant attention due to their broad range of applications in various industries, such as bioremediation, agriculture, food, and pharmaceuticals (Banat et al., 2010). They exhibit excellent emulsifying, foaming, and wetting properties, making them effective in enhancing oil recovery, biodegradation of pollutants, and controlling pests and diseases in crops (Banat et al., 2010; Rodrigues et al., 2006). Moreover, biosurfactants are considered eco-friendly alternatives to synthetic surfactants as they are biodegradable, non-toxic, and often derived from renewable resources (Desai & Banat, 1997). Their potential significance lies in their ability to improve the efficiency and sustainability of industrial processes while reducing environmental impact (Banat et al., 2010).

The necessity for oil degrading bacteria might have arisen from the fact that introduction of efficient oil degraders would be essential in order to effectively degrade the oil mixture. The oil spreading technique shows the clearing zone having a diameter of 1 cm. This confirms the presence of biosurfactant producing microorganism in the sample. The emulsification index of 55.5% depicts the ability of the bacterium to emulsify the crude oil. The biosurfactant produced by the bacteria was extracted using acid precipitation method and the obtained biosurfactant was dried and the weight was 1.8grams.The bacteria was tested negative in gram staining. The biochemical characterization included assays like catalase test, oxidase test, indole test, MR-VP test, Citrate test, urease test and starch hydrolysis test. Among these, oxidase and Voges Proskauer tests were found positive. These characterizations made it easier to analyse the character of the isolated bacterium. The molecular characterization was done using the specific amplification of 16s rRNA gene for identification of the strain of the isolated bacteria. The process was done using 27F forward primer and 1429R reverse primer. The strain was established by amplifying the 16s rRNA and the sequence of strain was subjected to blast. The obtained result depicted that the isolated strain was *Bacillus tropicus* MCCC 1A01406.The isolation helped in studying more about the biosurfactant production by *Bacillus tropicus*. The applications of the isolated strain in various fields including agriculture and cosmetics can be a very promising alternative to the synthetic surfactants. More studies

related to the nature of the isolated biosurfactant can be fruitful for several bioremediation activities. Due to the petroleum degrading properties, this bacterium could be used for in situ bioremediation of oil spills and leakages that occur in terrestrial and marine ecosystem during the exploration, wide scale production, refining, transport, storage, and disposal of petroleum.

CONCLUSION

The study aimed at isolation of biosurfactant producing bacteria from oil contaminated soil samples. It also deals with the characterization of biosurfactant producing organisms. The selected *Bacillus tropicus* colonies were sub cultured and confirmed by biochemical characterization and molecular study.

Biosurfactants producing bacterial strain were isolated and screened from oil contaminated soil using crude oil as sole carbon source. Oil spreading technique, and emulsification activity and lipase activity were performed for the screening of the biosurfactant producing bacteria, and found positive to oil spreading technique in bacterial strain MCCC 1A01406 Strain and has high emulsifying activity. The isolated bacterial strain MCCC 1A01406 showed Gram's negative, and they are in rod shape. Molecular study included genomic DNA isolation by agarose gel electrophoresis and PCR amplification of 16srRNA of isolates.

The unique properties of biosurfactant (Microbial Surface-Active Agents) such as low toxicity, relative ease of preparation and widespread applicability, make it different from chemical synthetic surfactant and now it has become recently an important product of biotechnology for industrial and medical applications and they allow to replacement of chemical synthetic surfactant. They can be used as emulsifiers, de-emulsifiers, wetting agents, spreading agents, foaming agents, functional food ingredients and detergents in various industrial sectors such as, Petroleum and Petrochemicals, Organic Chemicals, Foods and Beverages, Cosmetics and Pharmaceuticals, Mining and Metallurgy, Agrochemicals and Fertilizers, Environmental Control and Management, and many others.

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Acaricidal activity of *Curcuma longa* Essential Oil against the Cattle Ectoparasitic ticks of *Boophilus* species

Dissertation report submitted in partial requirement for M.Sc. degree course in Zoology with Environmental Biology as special subject

in

University of Calicut

by

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REPORT SUBMITTED IN PARTIAL REQUIREMENT FOR M.Sc. DEGREE COURSE IN ZOOLOGY WITH ENVIRONMENTAL BIOLOGY AS SPECIAL SUBJECT

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Report of the MSc Students project submitted to St. Joseph's College (Autonomous) Devagiri

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This is to certify that the dissertation entitled **"Acaricidal activity of *Curcuma longa* Essential Oil against the Cattle Ectoparasitic ticks of *Boophilus* species"** is an authentic record of studies and research work done by **Ms. Bimisha C M.** (Reg No: **DVAVMZL004**) under my guidance and supervision and that, to the best of my knowledge and belief, this work has not been presented earlier for any degree or fellowship.

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DECLARATION

I hereby declare that the dissertation entitled **"Acaricidal activity of *Curcuma longa* Essential Oil against the Cattle Ectoparasitic ticks of *Boophilus* species"** submitted in partial fulfillment of the requirements for the award of the degree of Master of Science in Zoology is a record of original work submitted by me at St. Joseph's College (Autonomous), Devagiri under the supervision and guidance of Dr. Aswathi P., Assistant Professor, Department of Zoology, St. Joseph's College (Autonomous), Devagiri, Calicut during 2021-2023.

I hereby declare that the results presented have not been previously submitted for the award of any degree or fellowship.

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ACKNOWLEDGEMENT

It is with a deep sense of pleasure that I express my sincere gratitude to **Dr. Aswathi P.** M.Sc, Ph.D., Assistant Professor, P.G and Research Department of Zoology, St. Joseph's College (Autonomous), Devagiri, Kozhikode, for her valuable guidance and supervision in the form of practical suggestions and constructive criticisms which formed the basis for the successful completion of this project.

I express my sincere gratitude to **Dr. Vineesh P.J**. MSc, B.Ed, Ph.D., Assistant Professor and Head, PG and Research Department of Zoology for his support and sincere encouragement.

I extend my deep gratitude to **Dr. Arunaksharan N**, MSc, B.Ed., PhD, Assistant Professor, PG and Research Department of Zoology, Princy P (Research fellow) and Swetha Damodharan (Research fellow) for their wholehearted support and advice that helped me throughout the project I had undertaken.

I convey my sincere gratitude to Ms. Aparna J, Ms. Bijisha C M, Ms. Bijila, Ms. Bincy C M, Ms. Akshaya K M, and Mrs. Kallyani N M for their help in collecting the samples required for completing my project work.

I am greatly indebted to Ms. Aparna J, the co-researcher for helping me to complete the project and providing her valuable time in editing and proofing my thesis.

I extend my heartfelt thanks to the non-teaching staff of department for their valuable cooperation.

Finally, I express my deep gratitude to my family and friends for their sincere support throughout the project. Above all, I thank God Almighty for the blessings for the successful completion of the project.

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INTRODUCTION

Ticks are harmful blood sucking ecto parasites of mammals, birds and reptiles. They act as vectors and transmit diseases to humans and animals. Ticks belong to phylum, *Arthropoda* and order *Acarina* (Rajput et al., 2006). They belongs to three families: *Ixodidae* (hard ticks, 720 species), *Argasidae* (soft ticks, 186 species), and *Nuttalliellidae* (1 species) (Selles et al., 2021).

Ticks cause great economic loss to livestock in the world and produce paralysis or toxicosis, and cause physical damage to livestock leading to decreased meat and milk production. The major losses are mainly due to their ability to transmit protozoan, rickettsial and viral diseases of livestock. Tick-borne protozoan diseases (e.g. Theileriosis and Babesiosis), rickettsial diseases (e.g. Anaplasmosis) , cowdriosis and tick-associated dermatophilosis are the major health and management problems in livestock industry (Rajput, Hu et al. 2006). Tick can cause blood loss and skin damage at the site of attachment (Selles et al., 2021) and irritation or anaemia and tick worry in case of heavy infestations. Many cases conditions may lead to dermatitis in cattle.

There are lots of methods to control ticks and tick-borne diseases. One of the major control methods is the use of chemical acaricides. There are several side effects and drawbacks while using chemical acaricides such as the presence of residues in the milk and meat, the development of chemical resistant tick strains, health hazards caused on the animals, human beings and the environment (Rajput et al., 2006). Increased production cost of chemical acaricides is also a disadvantage. In recent years, there has been growing concern among scientists and the general public regarding the environmental issues arising from excessive pesticide usage (Koul et al., 2008).

Chemicals derived from plants are an important source of insecticides as various plant extracts have been used by humans for control of insects since the time of ancient Romans. Natural plant extract or essential oil exhibit low mammalian toxicity and low persistence in the environment (Raja et al., 2001; Papachristos and Stamopoulos, 2002).

Essential oils can be described as volatile oils that possess potent aromatic components, contributing to the characteristic odour, flavour, or scent of a plant. They are the result of plant metabolism and are often referred to as volatile plant secondary metabolites. Generally, these oils exist in a liquid state at normal room temperature and readily

undergo transition from a liquid to a gaseous state at room temperature or slightly elevated temperatures without undergoing any decomposition. For more than 5,000 years, essential oils have been utilized for diverse applications, encompassing personal grooming (such as fragrances and beauty products), culinary uses, household maintenance, protection against insects for both humans and animals (including domestic and farm animals), and as beneficial substances to support the treatment of different ailments (Sharifi-Rad et al., 2017). The use of natural products, plant extracts and oils has been also identified as an efficient alternative method to control ticks (Dos Santos et al., 2017).

Turmeric (*Curcuma longa L*.) essential oil is one of the effective oil to control tick with its insecticidal, anti-inflammatory, antioxidant, anticancers, anti-rheumatic and antibiotic properties. Characteristic yellow colour in turmeric is due to curcuminoids. Curcumin, also known as diferuloyl methane (hydrophobic polyphenol), was studied extensively for the biological properties. *Curcuma longa* contains a range of bioactive compounds such as alpha-pinene, beta-Pinene, caryophyl lene, eugenol, limonene turmerone and ar-turmerone with insecticidal, pesticidal or insect repellent activity which is used to control ticks. The major phyto-constituents of *Curcuma longa* consist of curcuminoids (1% - 6%), volatile oils (3% - 7%), fiber (2% - 7%), mineral matter (3% - 7%), protein (6% - 8%), fat (5% - 10%), and carbohydrates (60% - 70%). Of the curcuminoids, 60% - 70% of weight is curcumin and 20% - 27% are dimethoxy curcumin and 10% - 15% are bisdemethoxycurcumin (Perera, et al. 2022). As a non-toxic natural material, *Curcuma longa* offers new option for organic pest control and replacement of synthetic pesticides.

This study is mainly concerned with testing the efficacy of *Curcuma longa* essential oil in controlling cattle ectoparasitic ticks. By investigating the potential of *Curcuma longa* essential oil as a natural alternative for cattle tick control, this project aims to contribute to the development of sustainable and eco-friendly solutions in the livestock industry. The findings of this study have the potential to support farmers, veterinarians, and researchers in implementing effective and safe tick management strategies, ultimately enhancing animal welfare, productivity, and environmental sustainability.

OBJECTIVES

The current study aims to analyse

1. The efficacy of *Curcuma longa* essential oil in producing repellency in nymph, and adult of Boophilus species ticks.
2. Determine the LC_{50} value of *Curcuma longa* essential oil against *Boophilus* ticks by using adult immersion test (AIT).
3. To determine the effect of *Curcuma longa* essential oil, altering the RI, oviposition and hatchability of eggs laid by the ticks exposed to different concentration of essential oil.

REVIEW OF LITERATURE

Plant extracts and plant derived essential oils are used as ecofriendly acaricides, pesticides and insecticide. A wide range of activities, including insecticidal, antifeedant, repellent, oviposition deterrent, growth regulation, and antivector properties, can be observed in numerous plant essential oils against both pest insects and plant pathogenic fungi (Koul et al., 2008). The utilization of plant extracts by farmers for pest management has been recognized, particularly in the traditional control of ticks (Adenubi et al., 2016). The emergence of resistance to acaricides and environmental pollution has increased the demand for environmentally friendly pesticides and herbal acaricides (Nazim et al., 2021).

The productivity of livestock and agricultural products is significantly impacted by ticks and tick-borne diseases (De Meneghi et al., 2016). The presence of tick species causes significant financial hardships for livestock owners due to the direct impact of blood-feeding, injection of toxins, and the indirect transmission of pathogens. Unfortunately, the uncontrolled and indiscriminate use of chemical agents to eradicate ticks has led to the development of resistance in field tick populations against nearly all commonly used chemicals employed for their management (Kumar et al., 2020).

The acaricidal activity of several plant extracts and essential oils has been investigated, and they are also utilized for controlling cattle ticks. Neem seed (*Azadirachta indica*) extract can significantly control *Hyalomma anatolicum excavatum.* It increases the hatching rate of eggs and cause incomplete development. Increased concentration of extract increases the mortality rate in larva and 4% of adults were malformed by using Neem extract (Abdel-Shafy & Zayed, 2002). Acaricidal effect of *Allium sativum* and *Allium cepa* oils on *Boophilus annulatus* was studied. 10% of garlic oil prevents hatching of egg and cause colour change and malformation in shape. 86% of adult ticks were killed by 10 -20% of onion oil after 72 hours of treatment. At concentrations above 5% of onion and garlic oil induce acaricidal effect (Aboelhadid et al., 2013). *Melia* azedarach with significant insecticidal properties is used to control *Dermanyssus gallinae. Melia azedarach* has significant insecticidal properties. *M. azedarach* fruit extract is highly toxic to *D. gallinae*. 100% of mites were killed by using 10% concentration. Acaricidal activity of fruit extract is due to several components such as meliartenin, meliacaprin, meliacin, meliantrol, melianol, salanin, nimbin, and azaderachine. They act as

antifeedent growth regulator, repellent or inhibiting egg production and embryogenesis (Sariosseiri et al., 2018). *M. azedarach* ripe fruit extract is also used to control or inhibit egg production and embryogenesis in *Boophilus microplus.* Less polar fruit extract has higher efficacy (Borges et al., 2003). Essential oil of *Tagetes minuta* has both inhibitory and repellent activities against *Hyalomma rufipes (Nchu et al., 2012) T. minuta* also controls *Rhipicephalus (Boophilus) microplus, Rhipicephalus sanguineus, Amblyomma cajennense and Argas miniatus* (Garcia et al., 2012). Essential oil of *Gynandropsis gynandra* has repellent activity on *Rhipicephalus appendiculatus.* Most repellent components in *G. gynandra* against *R. appendiculatus* includes m-Cymene, nonanal, 1-aterpineol, b-cyclocitral, nerol, trans-geraniol, carvacrol, b-ionone, trans-geranylacetone, and nerolidol (Lwande et al., 1999). Crude-extract of tamarind fruits exhibit acaricidal properties against *Boophilus microplus*. Acaricidal activity is due to two major organic acids such as oxalic acid and tartaric acid present in the tamarind. These components cause patchy lesions, swelling in the tick's skin and high delayed mortality (Chungsamarnyart & Jansawan, 2001). Lemon grass and citronella grass oil were treated against *Boophilus microplus* (Chungsamarnyart & Jiwajinda, 1992). Acaricidal activities of peel oil from three species of citrus were studied through dipping method. Peel oil from mature fruits of *Citrus sinensis* and *Citrus maxima* have high larvicidal activity. Direct application of peel oil from citrus fruit is also highly effective to control *Boophilus microplus.* Peel oil from immature and mature fruits of *Citrus maxima* and *Citrus reticulata* exhibit highest acaricidal activity on both larva and engorged female tick. *Citrus reticulata* is three times effective than lemon and citronella grass oil (Chungsamarnyart & Jansawan, 1996). Essential oil of *Origanum minutiflorum* has acaricidal property due to its component carvacrol. 10µl/L concentration essential oil of *O. minutiflorum* cause 100% mortality in adult *Rhipicephalus turanicus* at 120 minutes (Cetin et al., 2009). Acaricidal activity of *Eucalyptus citriodora* and *Cymbopogon nardus* were studied on larvae of *Amblyomma cajennense* and *Anocentor nitens.* 6.5% concentration of *E.citriodora* has 10.8 % mortality rate in *A.cajennense.* Essential oil of *C. nardus* is more effective than *E. citriodora* (Clemente et al., 2010). Efficacy of *Piper tuberculatum* fruit extracts was studied on *Rhipicephalus microplus* through adult immersion test and larval packet test (Lima Ada et al., 2014). Control of *Boophilus microplus* using oleoresinous extract of *Copaifera reticulate* was studied. 0.35% concentration of *C. reticulate* has 92 % acaricidal activity against larvae of *B. microplus.* Acaricidal activity of *C. reticulate is* due the group of phytochemicals including Terpenic

acids, sesquiterpenes and diterpenes (de Freitas Fernandes & Freitas, 2007). *Chamaecyparis nootkatensis* has acaricidal properties against nymphal ixodes Scapularis. Fourteen natural components were extracted from hardwood of *C. nootkatensis* and conducted bioassay (Dietrich et al., 2006). Studies reported that non alkaloid and alkaloid compounds of *Leucas indica* have acaricidal activity against *Rhipicephalus annulatus*, detected through adult immersion test. Efficacy of 66.67 % mortality and 55.16 % inhibition of fecundity were caused by 50 mg/ml concentration (Divya et al., 2014). Acaricidal activity of *Sapindus saponaria* was studied against *Boophilus microplus* by extracting the crude ethanol from stem peel. LC_{50} and LC_{90} values of crude ethanol extract of *S. saponaria* against larvae were 1,258 and 6,360 ppm respectively (Fernandes et al., 2005). Five essential oils from *Ocimum gratissimum L., O. urticaefolium* and *O. canum* were used to control larvae of *Rhipicephalus microplus* (Hüe et al., 2015). Acaricidal property of extract of shrub *Cleome monophylla* against *Rhipicephalus appendiculates* was investigated through tick climbing repellency bioassay and found that has significant efficiency for controlling *R. appendiculates*. 1-α-terpeneol and 2-dodecanone are the most active components which cause repellency against *R.appendiculates. C. monophylla* also exhibit repellency against maize weevil, *Sitophilus zeamais* (Ndungu et al., 1995). The efficacy of essential oil of *Lippia javanica* on *Hyalomma marginatum rufipes* has been investigated.

Curcuma, a *genus* known for its medicinal uses, has been utilized for many centuries. It consists of around 133 species distributed globally. Traditionally, *C. longa* has been used in Ayurvedha, unani and siddha medicine as a household remedy for numerous ailments. Curcumin, the primary active yellow compound found in turmeric, exhibits a broad range of biological effects. These effects encompass anti-inflammatory, antioxidant, anticarcinogenic, antimutagenic, anticoagulant, antifertility, antidiabetic, antibacterial, antifungal, antiprotozoal, antiviral, antifibrotic, antivenom, antiulcer, hypotensive, and hypocholesteremic activities (Chattopadhyay et al., 2004). The main component found in the volatile oil of turmeric is aromatic turmerone which was noted for its mosquito repellent properties and potential effectiveness in treating respiratory diseases and dermatophilosis (Naz et al., 2010). *Curcuma longa* possesses a variety of bioactive compounds, some of which exhibit insecticidal, pesticidal, or insect repellent properties. Being a non-toxic natural substance, turmeric presents a promising alternative for organic pest control, offering a substitute for synthetic pesticides derived from mineral oil.

The primary constituents of *Curcuma longa* include curcuminoids, volatile oils, fibre, mineral matter, protein, fat, and carbohydrates. (Nelson et al., 2017). *Curcuma longa* exhibits notable repellent activity against ticks in dogs. The acaricidal activity of a major extract from *Curcuma longa* against *Rhipicephalus sanguineus* was studied (Perera et al., 2022). The findings revealed that *Curcuma longa* oil exhibited dual capabilities, effectively preventing ticks from climbing and displaying longer residual activity compared to other oils. The *Curcuma longa* essential oil suspension can be utilized as a dog repellent even at low concentrations. Where the host experienced a reduced likelihood of tick attachment or feeding during the trial, in comparison to the dogs in the negative control and untreated control groups. This finding strongly suggests that the turmeric suspension effectively repelled ticks through contact (Goode et al., 2018). Turmerone and ar-turmerone, the main compounds present in the oil derived from turmeric rhizome powder, demonstrate strong repellent properties against pests commonly found in stored grains. Studies have shown that turmeric oil can effectively protect wheat grains against the red flour beetle, *T. castaneum* (Chahal et al., 2005). The larvicidal and repellent effects of essential oils and extracts derived from *Curcuma longa*, as well as the compounds ar-turmerone and curcuminoids, were assessed against mosquitoes. Among the tested compounds, ar-turmerone demonstrated the highest efficacy as a larvicide and biting deterrent specifically targeting *Aedes aegypti* and *Anopheles quadrimaculatus* (Ali et al., 2015). The insecticidal properties of essential oil derived from *Curcuma longa* leaves, as well as its main component α-phellandrene, were investigated in order to assess their impact on *Lucilia cuprina* larvae (Chaaban et al., 2019). Insecticidal activity of *Curcuma longa* and *Allium sativum* extracts against red flour beetle, *Tribolium castaneum* were investigated (Ali et al., 2014). *Curcuma longa* essential oil was studied for its antifungal properties (Dhingra et al., 2007). Extensive investigations were carried out to study the in vitro antifungal activity and potential mechanisms, as well as the effectiveness in inhibiting aflatoxin production, of a natural essential oil obtained from *Curcuma longa L.* against *Aspergillus flavus* (Hu et al., 2017). The inhibitory effects of essential oil derived from *Curcuma longa* were examined against *Staphylococcus aureus, Pseudomonas aeruginosa, Candida albicans,* and *Aspergillus niger* using the inhibition zone diameter test to assess antimicrobial activity (Singh et al., 2011). The effectiveness and safety of an ethanolic extract derived from *Curcuma longa* were evaluated as a treatment for sand tampan ticks in a rabbit model (Abdel-Shafy et al., 2019). The contact and fumigant toxicity of the essential oil obtained from *Curcuma*

longa leaves were examined, along with its impact on progeny production, in three species of stored-product beetles: *Rhyzopertha dominica F*, *Sitophilus oryzae L*., and *Tribolium castaneum Herbst* . The oviposition-deterrent and ovicidal effects of the leaf oil of *C. longa* were also assessed *against T. castaneum*. In contact and fumigant toxicity tests, the oil exhibited insecticidal properties. The adult *R. dominica* beetles displayed high susceptibility to the contact action of the *C. longa* leaf oil, with an LD_{50} value of 36.71µg/mg. On the other hand, in the fumigant assay, the adult *S. oryzae* beetles demonstrated high susceptibility, with an LC_{50} value of 11.36 mg/liter air. In *T. castaneum*, the oil derived from *C. longa* significantly decreased oviposition and resulted in a reduction of 72% and 80% in egg hatching, respectively (Tripathi et al., 2002). Three species of *Curcuma*, namely *Curcuma aeruginosa*, *Curcuma aromatica*, and *Curcuma Xanthorrhiza*, were investigated to study the repellent activity in mosquito. The effectiveness of ethanolic extract of *Curcuma longa* as an acaricide was investigated on the second nymphs of *Ornithodoros savignyi*, a soft tick found in various parts of Africa, including Egypt, predominantly infests camels, cattle, donkeys, and cows.

MATERIALS AND METHODS

Study animal

Boophilus species are one-host hard ticks, widely distributed in subtropical and tropical areas in the world. They are most damaging cattle parasite and cause reduction in weight, milk production in the host animal and transmit tick born diseases. These ticks follow a one-host life cycle, where larvae, nymphs, and adults feed and undergo development on a single host After completing their feeding and development stage, they lay eggs, which then incubate and give rise to larval life in environments away from their host (Murrell & Barker, 2003).

Study site

Ticks were collected from residential area near Atholi village in Kozhikode district, Kerala. Experiments were conducted at the Entomology research lab at St. Joseph's College (Autonomous), Devagiri, Kozhikode.

Collection and preparation of ticks

Ticks of *Boophilus* spp. were collected directly from infested animals with the guidance of farmers. Adult and nymphal stages of tick were collected. Collected ticks were transferred into vials and covered with a cotton cloth to allow air and moisture exchange through it. Placed a wet cotton at the top of vial to provide moisture to collected animals. Experiments were performed at laboratory using collected ticks without much delay after collections were performed.

Test compound

*Curcuma longa L.*essential oil was selected as the test compound and was purchased from Veda oils (Wazirpur industrial area, New Delhi). Major constituent in the oil is turmeric root, extracted by steam distillation. Different concentrations of *Curcuma longa* essential oil were prepared in laboratory and used to test the efficacy against *Boophilus* species using repellency test and adult immersion test.

Repellency test

Repellent activity of *Curcuma longa L*. oil on adult and nymph of *Boophilus* spp. can be determined by repellency test using Petri dishes, filter paper, ethanol, and essential oil. Petri dishes of 9 cm in diameter were taken. Essential oil of *c L*. was diluted in ethanol to different concentrations (15µl/ml, 25µl/ml, 35µl/ml, and 45µl/ml respectively). Ethanol was used as the control. Filter papers of 9 cm in diameter were cut into half. The two halves were attached to each other with cello tape. Using micropipette, one half of the filter paper was uniformly applied with 200 µl essential oil (test) and the other half was treated with 200µl ethanol (control). Both the tested half and the control half were air–dried to evaporate the solvent completely. The remade filter paper placed in petri dish. Ten engorged female ticks were released into the cellotape part i.e., the center of each filter-paper disc and a cover was placed on each Petri dish. The movement of each tick after 20 minutes was observed. Repellency test was performed in adult and nymphal stages of *Boophilus spp*. In adult ticks five replicates were performed for each (25, 35 and 45µl/ml) concentration of the test solution, with each replicate containing ten ticks, resulting in a total of 50 ticks in each concentration. In nymphal stage, four replicates were performed with each concentration (15, 25 and 35µl/ml) containing 7 ticks (Ko et al., 2010). Percentage of repellency of *Curcuma longa* essential oil at each concentration were calculated using the formula

Percentage of Repellency = $((N_C - N_t) / (N_C + N_t)) \times 100$

Where, N_C - Number of ticks in control.

N_t -Number of ticks in oil

Adult immersion test

Collected engorged Boophilus ticks were grouped into five groups of similar size and were weighed and immersed in different concentrations (5µl/ml, 15µl/ml, 25µl/ml, 35µl/ml, and 45µl/ml, respectively) of *Curcuma longa L*. essential oil for 5 minutes, drained, and dried on filter paper. Dried ticks were transferred into transparent vials to observe the mortality, fecundity and egg hatchability. The vial was closed with cotton cloth and a wet cotton was placed on top of the vial to provide moisture. Ethanol was taken as control; control was also treated similarly. Four replicates

were performed for each concentration of the test solution, with each replicate contained five ticks, resulting in a total of 20 ticks in each concentration. The egg mass of each group was weighed after completion of egg laying. Hatched and unhatched eggs were counted. Tick mortality, percentage of egg hatching, reproductive index and hatchability percentage of egg of ticks in different concentrations of oil were calculated from the adult immersion test (Drummond et al., 1973).

Reproductive index (RI) = Egg mass weight/ engorged females weight

Percentage of inhibition of oviposition (IO) = ((RI control-RI Treated)/(RI Control*100))

Percentage of hatching = $\frac{\text{No. of hatched eggs} * 100}{\text{No. of total eggs}}$



Figure 1: *Boophilus spp*.- a common ectoparasitic tick on cattle.



Figure 2: *Curcuma longa* essential oil.



Figure 3: Experimental setup showing repellency test using *Curcuma longa L.* essential oil against *Boophilus spp.*



Figure 4: Different concentrations of *Curcuma longa L*. essential oil used for the adult immersion test.



Figure 5: *Boophilus* ticks after the adult immersion test

RESULTS

REPELLENCY TEST

Repellent activity of *Curcuma longa* essential oil against *Boophilus species* increases with increasing concentration. In adult *Boophilus* species, highest concentration (45µl/ml) of *Curcuma longa* essential oil cause 96 % repellency after 20 minutes of treatment. 84 % of repellency was detected in lowest concentration (15µl/ml) of essential oil (Table: 1). From probit analysis, the RC_{50} value of *curcuma longa* essential oil against *Boophilus spp.* was 11.481µl/ml with a lower bound value of 1.368µl/ml and an upper bound value of 17.568µl/ml (table 2).

In nymph of *Boophilus spp*., the Rc50 value is 5.375µl/ml, with a lower bound value of 0.019µl/ml and an upper bound value of 10.965µl/ml. The highest concentration (35µl/ml) showed 84% repellency, while the lowest concentration (15µl/ml) showed 71.4286% repellency. Among the RC_{50}values, the RC_{50} value of the nymphal stage is lower than that of the adult stage (Table 2).

ADULT IMMERSION

The LC_{50} value of *Curcuma longa L*. essential oil against *Boophilus* is 12.61µl/ml, with a lower bound value of 8.68µl/ml and an upper bound value of 16.42µl/ml (Table 2, Figure 3). The reproductive index of treated *Boophilus* with various concentrations of *Curcuma longa* essential oil declines with an increase in concentration. The lowest value of the reproductive index, 0.013±1.001 was detected at a 45µl/ml concentration of *Curcuma longa* essential oil and the highest value, 0.383±0.000, at 5µl/ml (Table 3). The reproductive index values showed significant variation among the various tested concentrations of *Curcuma longa* essential oil (p=0.00, Table 4). Control group showed significant variation with all the test concentrations and the reproductive index of control was higher than all test concentrations. 5µl/ml showed significant variation with 15µl/ml, 25 µl/ml, 35µl/ml, and 45µl/ml. The RI value at 5µl/ml is higher than that of 15µl/ml, 25 µl/ml, 35µl/ml and 45 µl/ml. Test concentration of 15 µl/ml showed significant variation with 45µl/ml whereas did not show any variation with 25µl/ml and 35µl/ml. the RI value of 15µl/ml was higher than that of 45µl/ml. Test concentration of 25µl/ml showed significant difference with 45µl/ml and didn’t show any variation with 35µl/ml. The test

concentration of 45µl/ml exhibited the lower RI value when compared to 25µl/ml. 35 µl/ml showed significant variation with 45µl/ml. and was higher than 45µl/ml (Table 3, 5).

The percentage of inhibition of oviposition of treated Boophilus increases with concentration of *Curcuma longa L*. essential oil (Table 3). There is significant variation between values of IO % at different concentrations (p=0.0010, Table 4). Control group showed significant variation with all test concentrations and had the least IO% value. The IO % value at 5µl/ml concentration showed significant variation with 15µl/ml, 25 µl/ml, 35 µl/ml and 45µl/ml. Among these test concentrations 5µl/ml exhibited lower IO % value than 15µl/ml, 25µl/ml, 35µl/ml and 45µl/ml. The IO % value at 15 µl/ml concentration showed significant variation with 45µl/ml and didn't show any variation with 25µl/ml and 35µl/ml. The IO % value at 15µl/ml is 0.004±0.0025, which is lower than 45µl/ml. Hence 45µl/ml concentration showed higher IO% value when compared to 15µl/ml. The IO % value at 25 µl/ml concentration showed significant variation with 45µl/ml and didn't show any variation with 35µl/ml. The IO % value at 25µl/ml is 0.005±0.002, which is lower than 45µl/ml. The IO % value at 35 µl/ml concentration showed significant variation with 45µl/ml and is lower than 45µl/ml (Table: 3 & 6).

The percentage of hatching decreased with increasing test concentration. The lowest test concentration showed 81% hatching, while the highest test concentration showed 30% hatching (Table : 3).

Table 1: Percentage of repellency detected on *Boophilus* species at different concentrations of *Curcuma longa* essential oil.

	Adult (not engorged)		
Sl.No	Concentration (µl/ml)	No.of ticks	Percentage of repellency
1	25	50	84
2	35	50	92
3	45	50	96
	Nymph		
Sl.No	Concentration (µl/ml)	No.of ticks	Percentage of repellency
1	15	28	71.42857
2	25	28	78.57142
3	35	28	85.71428

Table 2: LC_{50} and RC_{50} values of *Boophilus* ticks against *curcuma longa* essential oil obtained from AIT and repellency test.

		Estimate(µl/ml)	Lower bound(µl/ml)	Upper bound(µl/ml)
LC_{50}		12.612	8.680	16.417
RC_{50}	Adult	11.481	1.368	17.568
	Nymph	5.735	0.019	10.965

Table 3: Various parameters observed on exposure of *Boophilus* to the test concentrations of *Curcuma longa* essential oil.

Sl no.	concentration of turmeric oil	Tick weight (mean±SD)	Egg mass weight (mean±SD)	Reproductive index (RI)	Inhibition of oviposition % (IO%)	Hatching %
1	control	0.558±0.166	0.256±0.074	0.46±0.008	0	90
2	5µl/ml	0.446±0.193	0.171±0.074	0.383±0.0004	0.0017±0.00014	81
3	15µl/ml	0.5415±0.158	0.151±0.091	0.264±0.1096	0.004±0.0025	70
4	25µl/ml	0.529±0.149	0.126±0.0102	0.227±0.102	0.005±0.002	59
5	35µl/ml	0.517±0.150	0.063±0.062	0.110±0.097	0.008±0.002	48
6	45µl/ml	0.449±0.194	0.006±0.003	0.013±1.001	0.009±5.022	30

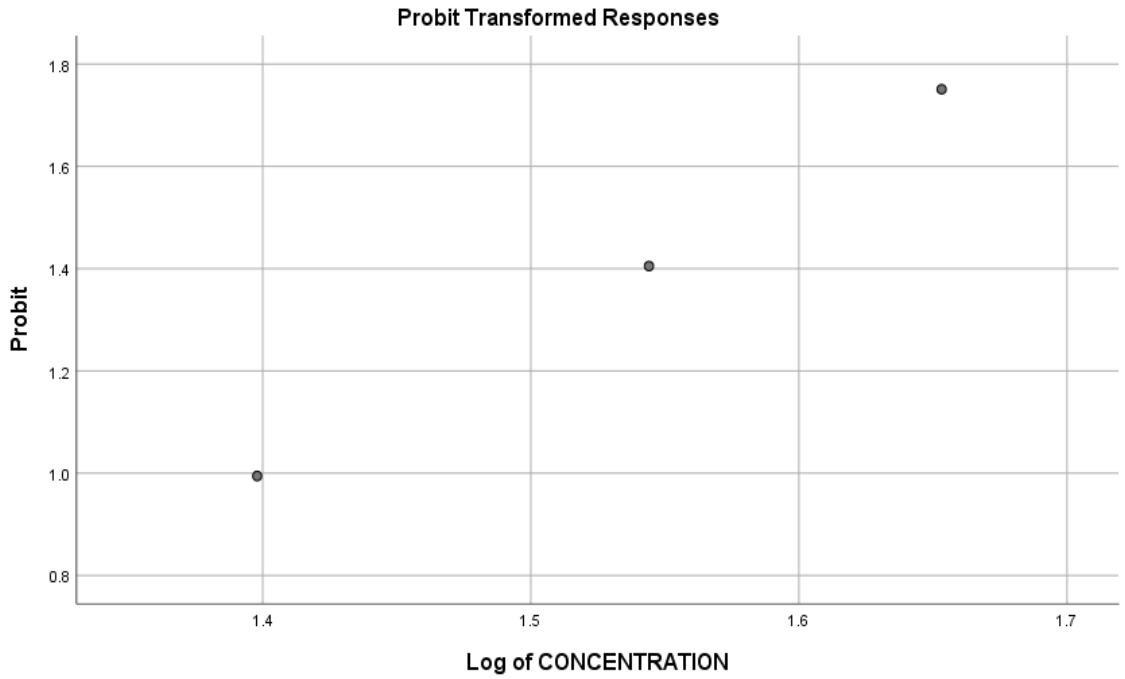


Fig 1: graph showing repellency of adult *Boophilus spp*. in repellency test.

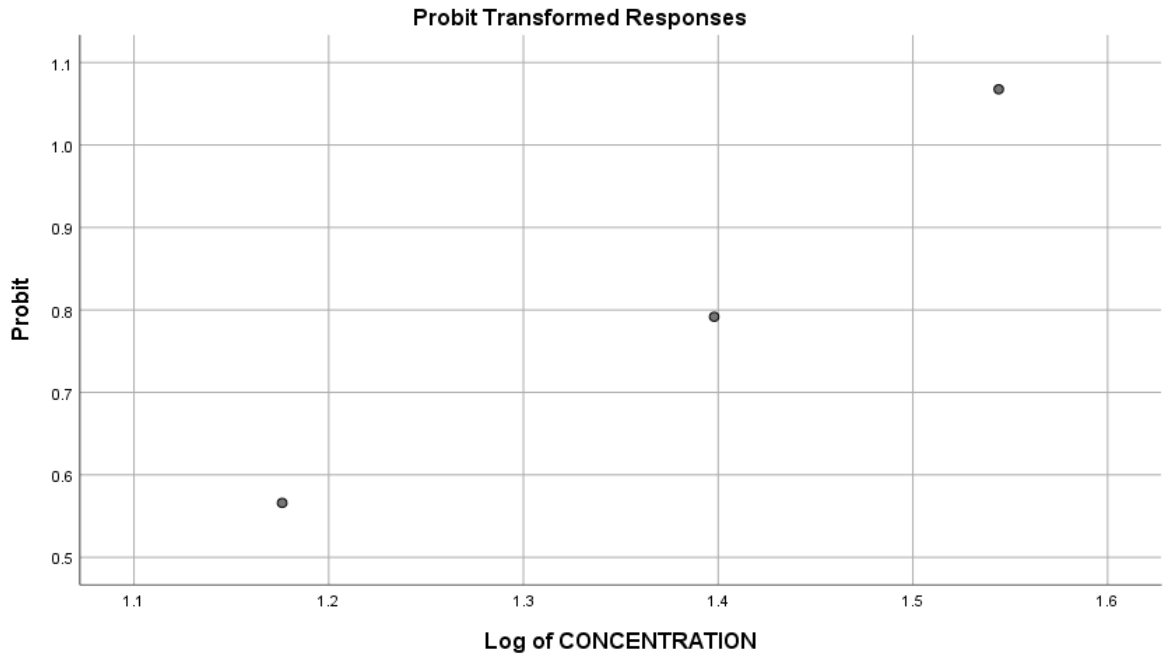


Fig 2: graph showing repellency of nymph of *Boophilus spp*. in repellency test.

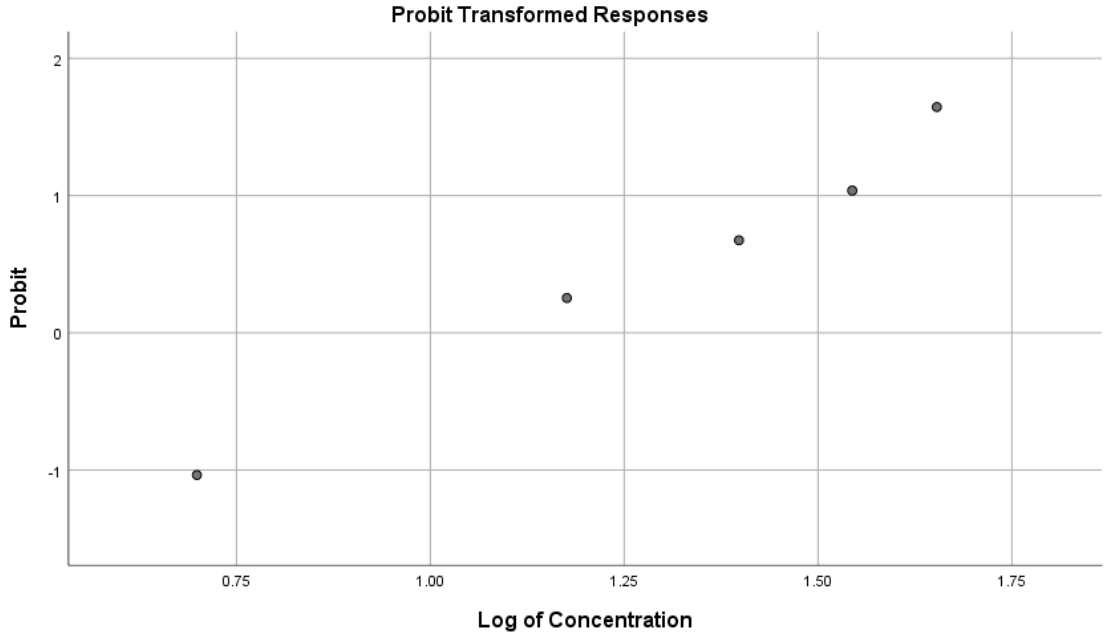


Figure 3: graph showing mortality of *Boophilus spp.* in adult immersion test

Table 4: Analysis of variation in reproductive index and inhibition of oviposition of *Boophilus* exposed to different concentration of *Curcuma longa* essential oil using Kruskal-Wallis Test.

	p	DF	H
Reproductive index	0.0010	5	20.560
Inhibition of oviposition	0.0010	5	20.596

Table 5: Pair wise analysis of variation in reproductive index values of *Boophilus* exposed to different concentration of *Curcuma longa* essential oil.

	p	Z
Control × 5µl/ml	0.0265	2.22
Control × 15 µl/ml	0.0304	2.17
Control × 25 µl/ml	0.0294	2.18
Control × 35 µl/ml	0.0304	2.17
Control × 45 µl/ml	0.0265	2.22
5 µl/ml × 15 µl/ml	0.0265	2.22
5 µl/ml × 25 µl/ml	0.0256	2.23
5 µl/ml × 35 µl/ml	0.0265	2.22
5 µl/ml × 45 µl/ml	0.0265	2.22
15 µl/ml × 25 µl/ml	0.3094	1.02
15 µl/ml × 35 µl/ml	0.3123	1.01
15 µl/ml × 45 µl/ml	0.0265	2.22
25 µl/ml ×35 µl/ml	0.3094	1.02
25 µl/ml ×45 µl/ml	0.0256	2.23
35 µl/ml ×45 µl/ml	0.0265	2.22

Table 6: Pair wise analysis of variation in inhibition of oviposition values of *Boophilus* exposed to different concentration of *Curcuma longa* essential oil.

	p	Z
Control × 5µl/ml	0.0211	-2.31
Control × 15 µl/ml	0.0202	-2.32
Control × 25 µl/ml	0.0211	-2.31
Control × 35 µl/ml	0.0202	-2.32
Control × 45 µl/ml	0.0202	-2.32
5 µl/ml × 15 µl/ml	0.0294	-2.18
5 µl/ml × 25 µl/ml	0.0304	-2.17
5 µl/ml × 35 µl/ml	0.0294	-2.18
5 µl/ml × 45 µl/ml	0.0294	-2.18
15 µl/ml × 25 µl/ml	0.3094	-1.02
15 µl/ml × 35 µl/ml	0.3065	-1.02
15 µl/ml × 45 µl/ml	0.0284	-2.19
25 µl/ml ×35 µl/ml	0.3094	-1.02
25 µl/ml ×45 µl/ml	0.0294	-2.18
35 µl/ml ×45 µl/ml	0.0284	-2.19

DISCUSSION

Through the analysis of the repellency test results, it was found that the repellent effect of *Curcuma longa* essential oil against *Boophilus* ticks increases proportionally with the concentration of the oil. The RC_{50} value of 11.481µl/ml is expected to produce effective repellency on the adult *Boophilus* ticks which can be used for topical application on cattle to regulate the tick population. 5.735µl/ml is the RC_{50} of nymph of *Boophilus spp*. which is lower than the RC_{50} value of adult. Hence it can be concluded that nymph of *Boophilus* can be controlled by applying very low concentration (5.735µl/ml) compared to adult.

Upon analysing the adult immersion test results, the percentage of mortality rate of treated *Boophilus* ticks increased in correlation with the concentration. The effective concentration for producing 50% mortality in the *Boophilus* ticks was identified to be 12.612µl/ml. Hence it is concluded that *Curcuma longa* essential oil is effective in controlling the Boophilus ticks on application of 15µl/ml concentration.

In a study of acaricidal activity of cinnamon essential oil against Boophilus showed LC_{50} value of 21.660µl/ml, this value is higher than LC_{50} value of *Curcuma longa*. Hence *Curcuma longa* essential oil was found to be more efficient than cinnamon oil in controlling *Boophilus* species. This higher efficiency could be attributed to the specific chemical composition of *Curcuma longa* oil, which may contain compounds that have a stronger repellent or insecticidal effect on these particular ticks. The essential oil component thymol, extracted from plant families *Lamiaceae* and *Verbenaceae*, and the chemical cypermethrin showed an LC_{50} value of 52.7 µg/mL against *Boophilus microplus* (Tavares et al., 2022). This value is higher than the LC_{50} value of turmeric essential oil. Hence, it can be concluded that 15 µl/mL of *Curcuma longa* is more effective than the combination of cypermethrin and thymol. *Curcuma longa* can be used as a replacement for this combination.

Nazim et al. studied the efficacy of ethanolic, acetone and chloroform extracts of *C. longa* against unfed larvae of *Rhipicephalus (Boophilus) microplus.* Among these extract, ethanolic extract was more effective and showed LC_{50} value of 2.34%. The ethanolic extract's higher acaricidal activity is likely due to its greater yield of Curcumin compared to the acetone and chloroform extracts (Nazim et al., 2021). The essential oil extracted

from C. longa leaves was tested against *Lucilia cuprina*, the Australian blowfly, resulting in a significant 96.22% reduction in adult emergence (Chaaban et al., 2019). In a study of Larvicidal and Biting Deterrent Activity of Essential Oils of *Curcuma longa*, Ar-turmerone, and Curcuminoids Against *Aedes aegypti* and *Anopheles quadrimaculatus* . Ar-turmerone is a major component in the rhizome oil of *Curcuma longa*, which exhibited LC_{50} values of 2.8 and 2.5 ppm against larvae of *Anopheles quadrimaculatus* and *Aedes aegypti*, respectively. The leaf essential oil of *Curcuma longa* showed LC_{50} values of 1.8and 8.9 ppm against larvae of *Anopheles quadrimaculatus* and *Aedes aegypti*, respectively(Ali et al., 2015). Hence *Curcuma longa* essential oil is effective to control different pests.

A decrease in the reproductive index of treated *Boophilus* ticks was observed with an increase in the concentration of *Curcuma longa* essential oil. This makes it clear that the exposure to *Curcuma longa* essential oil causes a notable reduction in the tick's ability to convert live weight into egg mass. Additionally, as the essential oil concentration increases, there is a notable rise in the percentage inhibition of oviposition in ticks. This observation further reinforces the idea that ticks treated with essential oils are less capable of laying eggs compared to the control group. In15µl/ml the inhibition of oviposition is 0.004±0.0025. As the concentration of *Curcuma longa* essential oil increased, there was a corresponding decrease in the percentage of hatching of the eggs produced by treated females. Control group showed 90% of hatchability while the effective 12.612 µl/ml concentration of oil showed a hatchability around 70%. Hence, 12.612µl/ml concentration of *Curcuma longa* essential oil is expected to reduce the hatchability of eggs produced by treated females by about 70%. Hence we can assume that the concentration of 12.612µl/ml will produce 50 % mortality of the adult ticks and further reduce their reproductive potential, inhibit the egg laying capacity by about, 0.004±0.0025 and the hatchability of the eggs laid by the treated ticks by about 70%. This significantly lower the number of the viable next generation ticks produced by an existing population of *Boophilus* ticks exposed to test essential oil. There by *Curcuma longa* essential oil is found to be an effective and safer control against the *Boophilus* ticks infesting the host. *Curcuma longa* has been used in Ayurvedic medicine since ancient times, with various biological applications. *Curcuma longa* is a non-toxic, highly promising natural medicinal plant. It is extensively used as a spice, food preservative and coloring material in India,

China and South East Asia. *Curcuma longa* is enriched with many useful phytoconstituents which are responsible for its efficacy. Curcumin, the primary yellow bioactive compound found in turmeric, exhibits a diverse range of biological actions. These actions encompass anti-inflammatory, antioxidant, anticarcinogenic, antimutagenic, anticoagulant, antifertility, antidiabetic, antibacterial, antifungal, antiprotozoal, antiviral, antifibrotic, antivenom, antiulcer, hypotensive, and hypocholesteremic activities (Chattopadhyay et al., 2004). Resistance to essential oil-based pesticides is expected to develop at a slower pace due to the complex mixtures of constituents found in these oils. Pesticides derived from plant essential oils offer several significant advantages. One notable benefit is their lower environmental risk compared to current synthetic pesticides, primarily due to their volatile nature. Predator, parasitoid and pollinator insect populations will be less impacted because of the minimal residual activity (Koul et al., 2008). The efficacy of *Curcuma longa*, is widely recognized worldwide, both as a curative and preventive measure. *Curcuma longa*, a widely available natural product, surpasses the availability of chemical pesticides. As a common spice and medicinal plant cultivated in numerous regions worldwide, *Curcuma longa* serves as a readily accessible natural alternative due to its easy sourcing.12.612µl/ml concentration of turmeric essential oil is effective to control Boophilus ticks. It is expected that *Curcuma longa* may find application as an acaricide in the near future to control various cattle ticks.

CONCLUSION

By analyzing the efficacy of *Curcuma longa* essential oil against *Boophilus*, it has become evident that *Curcuma longa* essential oil exhibits repellent and acaricidal effects on Boophilus ticks. A concentration of 15µl/ml of *Curcuma longa* oil effectively killed 50% of the tick population, while also negatively impacting their reproductive capacity and increasing the inhibition of oviposition in the surviving ticks and it also exerted a detrimental effect on the hatchability of the eggs layed by exposed ticks. Hence *Curcuma longa* essential oil is effective to control the ectoparasitic ticks of genus *Boophilus*.

Curcuma longa essential oil is widely available and eco-friendly, and it does not cause any harmful effects to non-targeted animals and the environment, making it a promising potential alternative acaricide to chemical acaricides.The findings of this study have the potential to support farmers, veterinarians, and researchers in implementing effective and safe tick management strategies, ultimately enhancing animal welfare, productivity, and environmental sustainability. However, further research is required to identify the specific components in turmeric essential oil that impart its acaricidal properties.

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DIVERSITY OF ROVE BEETLES (COLEOPTERA: STAPHYLINIDAE) IN THE FAECAL MATTER OF THREE DOMESTIC ANIMALS

Dissertation report submitted in partial requirement for M.Sc. degree course in Zoology with Environmental Biology as special subject

in

University of Calicut

by

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REPORT SUBMITTED IN PARTIAL REQUIREMENT FOR M.Sc. DEGREE COURSE IN ZOOLOGY WITH ENVIRONMENTAL BIOLOGY AS SPECIAL SUBJECT

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Report of the Students project submitted to St. Joseph's College (Autonomous) Devagiri

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CERTIFICATE

This is to certify that the dissertation entitled **"DIVERSITY OF ROVE BEETLES (COLEOPTERA: STAPHYLINIDAE) IN THE FAECAL MATTER OF THREE DOMESTIC ANIMALS"** is an authentic record of studies and research work done by **Ms. Emil James** (Reg No: **DVAVMZL005**) under my guidance and supervision and that, to the best of my knowledge and belief, this work has not been presented earlier for any degree or fellowship.

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DECLARATION

I hereby declare that the dissertation entitled **"DIVERSITY OF ROVE BEETLES (COLEOPTERA: STAPHYLINIDAE) IN THE FAECAL MATTER OF THREE DOMESTIC ANIMALS"** submitted in partial fulfillment of the requirements for the award of the degree of MASTER OF SCIENCE IN ZOOLOGY is a record of original work submitted by me at St. Joseph's College (Autonomous), Devagiri under the supervision and guidance of Dr. Vineesh P. J., Assistant Professor, Department of Zoology, St. Joseph's College (Autonomous), Devagiri, Calicut during 2021-2023.

I hereby declare that the results presented have not been previously submitted for the award of any degree or fellowship.

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ACKNOWLEDGEMENT

I express my heartfelt gratitude to my guide and mentor, Dr. Vineesh. P.J, M.Sc., B.Ed., Ph.D., Assistant Professor, Department of Zoology, for suggesting this topic and constantly encouraging me throughout this project.

I am grateful to Dr. Boby Jose, M.Sc., B.Ed., Ph.D., Principal and former Head of the P.G and Research Department of Zoology.

I am indebted to the research scholars of the Department, Kavyamol P.M., and Vineetha V.P. for specimen identification.

I express my heartfelt thanks to the non-teaching staff of the Department for their sincere help.

I express my gratitude to my classmates, for having been strong shoulders that I could always lean on for support.

I would like to express my heartfelt appreciation to my parents and siblings for their help and support.

Above all let me bow down before God the Almighty, who has been my guiding light and source of wisdom throughout my life.

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INTRODUCTION

The world of insects is a fascinating realm, harboring a remarkable array of creatures that play essential roles in various ecological processes. Among these intriguing insects are the Staphylinidae beetles, commonly known as rove beetles, which exhibit an astonishing diversity in their morphology, behavior, and ecological interactions. More species of beetles are currently recognized than any other insect order. With approximately 63,000 species currently recognized across the globe and most likely more than 75% of tropical species remaining undocumented, Staphylinidae is one of the largest families of beetles as it is currently organized (Stocker, Barthold, & Betz. 2022). They are typical members of the soil biota, living in the litter and deeper soil layers where they prey on a range of soil creatures. However, rove beetles are a very appealing study group for comparative research because other habitat preferences and feeding kinds are well documented among them. Currently, the Staphylinidae comprise 32 subfamilies and 167 tribes (Irmler *et al*., 2018).

Several species of rove beetles (Coleoptera: Staphylinidae), both predators and scavengers/coprophages, are among the most frequent visitors of cattle dung. Rarely do they have a dung-specific habitat; instead, they frequent or occupy diverse decomposing materials. But since they frequently consume a sizable amount of the young flies that live in dung, predatory rove beetles have received extensive study in the cattle dung community for years. (Cabrera & Mariana. 2003).

Dung pats are used by some of the fauna as a breeding ground, while others go there to parasitize or prey on the coprophagous fauna. The competing dung-burying scarabs, the Staphylinidae and Histeridae predators (Coleoptera), and the parasitoids (Hymenoptera) are the most significant biotic mortality factors of the dung breeding Diptera (Guimarães & Mendes. 1998). Dung breeding flies decay the dung. On the other hand, when adults, the horn fly *Haematobia irritans*, the face fly *Musca autumnalis*, and the bush fly *Musca vetustissima* are important pests of cattle and can cause expensive economic losses. Many researchers have reported the Staphylinidae beetle diversity in cattle dung and their importance as natural controllers of the pest (Wingo *et al*. l974).

Their interactions within the dung community, including competition for resources and utilization of different dung microhabitats, contribute to the overall efficiency of dung decomposition and nutrient utilization. While some studies have explored the diversity of

Staphylinidae beetles in dung ecosystems, there are still notable research gaps that merit further investigation. Specifically, a comprehensive comparative analysis of Staphylinidae beetle diversity in different types of dung, such as cow dung, pig dung, and hen droppings, remains largely unexplored. With an emphasis on their abundance, distribution, and ecological importance throughout dung ecosystems, this research attempts to dig into the fascinating world of Staphylinidae beetles in the faecal deposition of three different domestic animals (hen, pig, and cow).

OBJECTIVES

1. Identification of Staphylinidae beetles (Species/ Genera) associated with the faecal matter of Hen, Pig, and Cow.
2. Comparison of abundance and species diversity of Staphylinidae beetles associated with the faecal matter of Hen, Pig, and Cow.
3. Feeding Guild composition of Staphylinidae associated with the faecal matter of Hen, Pig, and Cow.

REVIEW OF LITERATURE

INTERNATIONAL STATUS

According to Herman (2001), more than 62,290 species with 3418 genera were described in the family Staphylinidae. Blume (1985) and Hunter et al (1986) recorded the dung associated Staphylinidae. Koler *et al*., (2002) carried out a work in order to determine the local Staphylinidae species due to these predators and or parasites importance on the natural control of the dung associated bovine parasites. A total of 13215 specimens were collected, belonging to 34 species. Caballero *et al*., (2007) evaluated changes in levels of diversity of copro-necrophilous staphylinids attracted to piles of cattle dung and rat carrion in four contrasting habitats (continuous oak forest, oak forest patches, savannah, and ravines) in southeastern Mexico during 2005 and 2006. In a study conducted by Caberra *et al*., (2009) in two locations in temperate and subtropical, the most prevalent predatory rove beetles were Philonthus Stephens specimens, and field and lab data indicate that they are the group of predators that have the most impact on the community of dung breeding flies. Yamamoto *et al*., (2014) reported that the richest subfamily in terms of species number was Aleocharinae, followed by Staphylininae and Paederinae in the dung of sika deer in coniferous forests of southwest Japan.

Dekeirsschieter *et al*., (2013) monitored the presence of adult rove beetles (Coleoptera, Staphylinidae) on decaying pig carcasses in a forest biotope during four seasons (spring, summer, fall, and winter). A total of 23 genera comprising 60 species of rove beetles were collected from pig carcasses. Peris *et al*., (2014) reported two novel genus and four novel species: *Penarhytus tenebris* belongs to the supertribe Pselaphitae, while *Cretasonoma corinformibus* belongs to the supertribe Faronitae in the early Cretaceous Spanish amber. According to Klimaszewski, *et al*., (2018) communities of staphylinids can be used as bioindicators of the environmental status and particularly of human influence on ecosystems. Salnitska and Solodovnikov (2019) published the first inventory of the fauna of the rove beetle genus *Quedius* in the Russian Federation. Brunke *et al*., (2019) reported that 153 non-native Staphylinidae beetles have become established in Canada. Paliy *et al*., (2020) identified 39 species of staphylinids, belonging to 5 subfamilies: Oxytelinae, Steninae, Tachyporinae, Aleocharinae, Staphylininae in livestock biocenoses of forest-steppe and steppe Ukraine.

NATIONAL STATUS

Cameron (1930, 1931a, b, 1932, and 1939 a, b) described 3000 species from 32 genera in India. Very few works after Cameron reported on India. Five subfamilies, 12 genera, and 21 species were identified by Biswas (1986) at Silent Valley National Park in Kerala. Six species of Staphylinidae associated with stored goods were identified by Basak and Pal in 2006. Staphylinidae beetles are the most prevalent in Kerala's Western Ghats ecosystem (Sabu. 2006). While conducting a study on the insect diversity of the Sunderban Biosphere Reserve, 12% of Staphylinidae were reported (Mitra *et al*., 2015). A Study on the diversity and abundance of coleopterans in Gujarat recorded five species *Ocypus brunnipes* 36 *Paederus riparius* 28 *Quedius sp*. 39 *Siagonium sp* (Thakkar, Bhumi & Parikh, Pragna. 2016). New records of 22 species from Mizoram (Sar, & Ilango. 2016).

MATERIALS AND METHODS

STUDY AREA

The study was conducted at Peravoor in Kannur district. It is located at 11.8962°N 75.7342°E.

PREPARATION OF FAECAL PATS AND COLLECTION OF SAMPLES

Fresh faecal matter from adult cattle, pigs, and hens was collected. Each composite area sample consists of 200g of manure from ten samples within an area. Ten samples of each animal are placed one meter apart and left undisturbed for five days (Figure 1-3). Beetles are collected from the faecal pats after five days by the floating method. Gather the feces and the ground beneath it into a bucket following the exposure period. Dig up the dung pat and the soil beneath it with a spade, ideally as a single cube (at least 10 cm deep), and quickly place it in the bucket. Bring the bucket to a location where water is available. Add water to the bucket and made the beetles float. Most of the beetle's float and can be collected using a brush and put into the labelled collecting bottle containing 70% alcohol. This can be stored for further identification and studies (Krell, Frank-Thorsten. (2007)).



Figure 1: Hen droppings



Figure 2: Pig dung



Figure 3: Cow dung

IDENTIFICATION

Collected rove beetles were examined and sorted using Stereo Zoom Trinocular microscope (LABOMED – 200 MAR, CODE: -ZM 45 TM) and adult organisms were identified to species level using keys (Cameron 1939) and by taking photographs of the entire body of rove beetle using an image processing system. The beetles were picked up from the solution by using small hair brush and forceps from the vials. After analysis the beetles were placed in small vials containing 70% ethyl alcohol. They were sorted on the basis of the taxon and transferred beetles of same taxon in a common vial. Each vial

contains a clean displayed label containing information about all the sites, location and date of collection, name of taxon and preserved date. The count of each beetle was entered on a pre-printed tally sheet that has the date of collection, preservation and sorting sample code.

DATA ANALYSIS

Collected beetles were identified, and the mean abundance and standard deviation for each sub-family were calculated on an Excel sheet. The normality of data was checked and parametric test for analysis were opted. Differences in staphylinidae beetle community in three faeces were analyzed with ANONA. Traditional alpha diversity indices namely Margalef's richness(d), Pielou's evenness(j), Simpson's dominance(λ), and Shannon's diversity index(H') were estimated (Magurran. 2004). All statistics and diversity estimation was done with PAST free software (Hammer et al., 2001).

RESULT

Three different livestock animal dung, a total of 1781 staphylinidae beetles, belonging to 5 subamiles *viz*. Staphylininae, Paederinae, Oxytelinae, Aleocharinae, and Tachyporinae were collected from the study site. With regard to the abundance, Oxytelinae was the most abundant and Tachyporinae was the least abundant. The abundance order of the subfamily Staphylinidae in the faceal matter of hen is Oxytelinae > Staphylininae > Aleocharinae > Paederinae. In pig dung, it is in the order Oxytelinae > Staphylininae > Paederinae > Aleocharinae. And in cow dung, the order of abundance is Oxytelinae > Staphylininae > Paederinae > Aleocharinae > Tachyporinae (Figure 1)

By comparing the diversity of staphylinidae beetle in the faecal matter of three domestic animals, the highest Shannon diversity in the subfamily level was recorded in Hen, (H'=1.369). The lowest diversity was observed in Cow dung (H'= 0.8044) (Table 1).

The species belonging to the subfamily Staphylinidae fall under the categories of saprophage, mixed, and predator species. Saprophage is the most prevalent feeding guild, followed by predators then mixed Subfamilies. Oxytelinae is a saprophage. Paederiane, Tachyporinae and Staphylininae are predators. Subfamily Aleocharinae is a mixed feeding guild variation. (Figure 2).

A total of thirteen species of staphylinidae were identified, which belong to nine different genera and four tribes. Subfamily oxytelinae consists of only one tribe namely Oxytelini it includes 3 genera namely *oxytelus*, *anotylus* and *platystethus*. *Oxytelus* with 2 species, anotylus and platystethus with single species. Paederini it consist of 4 genera, namely Lithocharis with two species, medon, thinocharis and stilicus with single species. Subfamily staphylininae consists of single genera philonthus with 2 species. Subfamily Aleocharinae contains sinle genera pronomaea with two species (Table 2)

Species were collected from this faecal matter of three domestic animals. *Oxytelus incisus* was found to be the most abundant and *Thinocharis pygmaea* was the least abundant. (Figure 3). Highest species diversity is found in the faecal matter of hen ((H'=1.99) and the least diversity was observed in cow dung (H'=0.987) (Table 3)

Table 1: Alpha diversity values of subfamilies of Staphylinidae in the faecal matter of hen, pig, and cow.

Sample	Shannon diversity (H')	Margalef's species richness (d)	Pioelou's Evenness (j)	Simpson's dominance (λ)
Hen	1.369	1.137	0.9832	0.259
Pig	0.9122	0.7708	0.6224	0.5307
Cow	0.9122	0.8543	0.4471	0.6225

Table 2: List of identified Staphylinidae specimens

SUBFAMILY	TRIBE	SPECIES NAME
Oxytelinae	**Oxytelini**	*Oxytelus incisus*
		Anotylus occultatus
		Platystethus sp
		Oxytelus lividus
Paederinae	**Paederini**	*Lithocharis uvida*
		Stilicus rufesciens
		Medon sp
		Thinocharis pygmae
		Lithocharis vilis
Staphylininae	**Staphylinini**	*Philothus fimitarus*
		Philonthus incultus
Aleocharinae	**Pronomaeini**	*Pronomaea thaxiteri*
		Pronomaea bramina

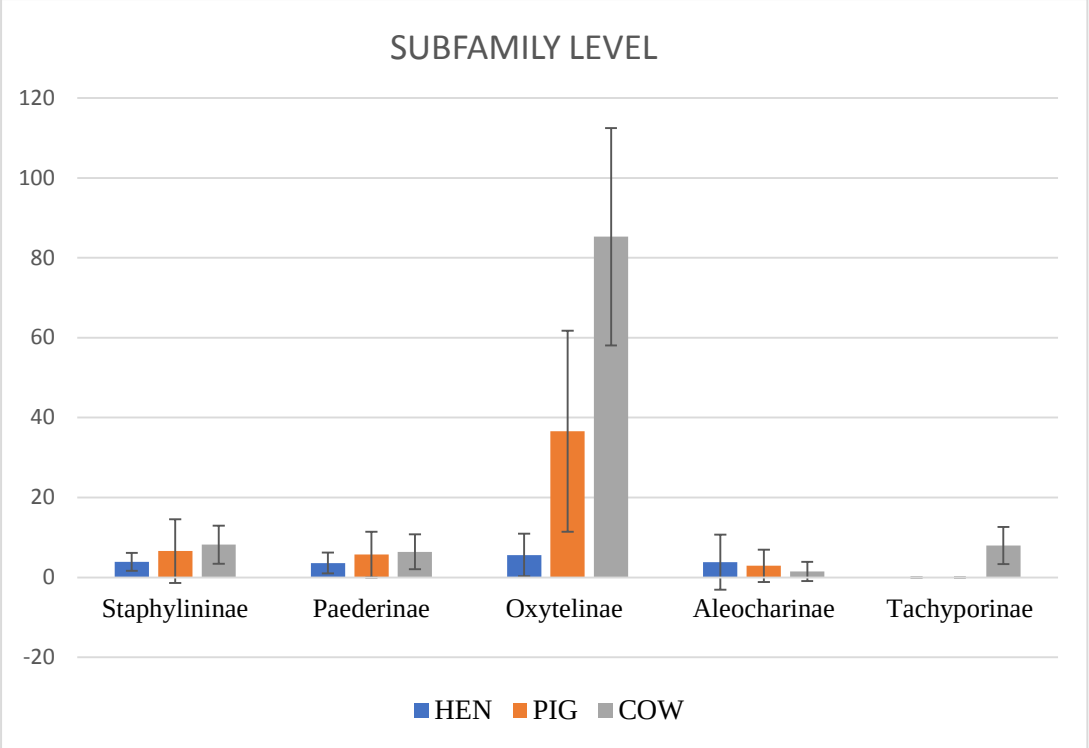


Figure 1: Abundance (Mean ± SD) of subfamily collected from the faecal matter of hen, pig, and cow.

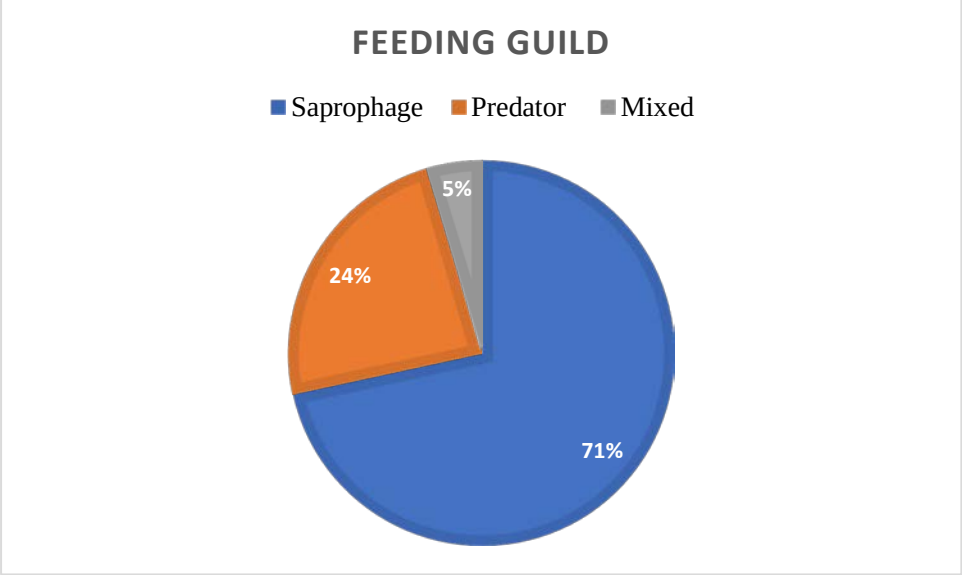


Figure 2: Feeding Guild of the subfamilies of Staphylinidae

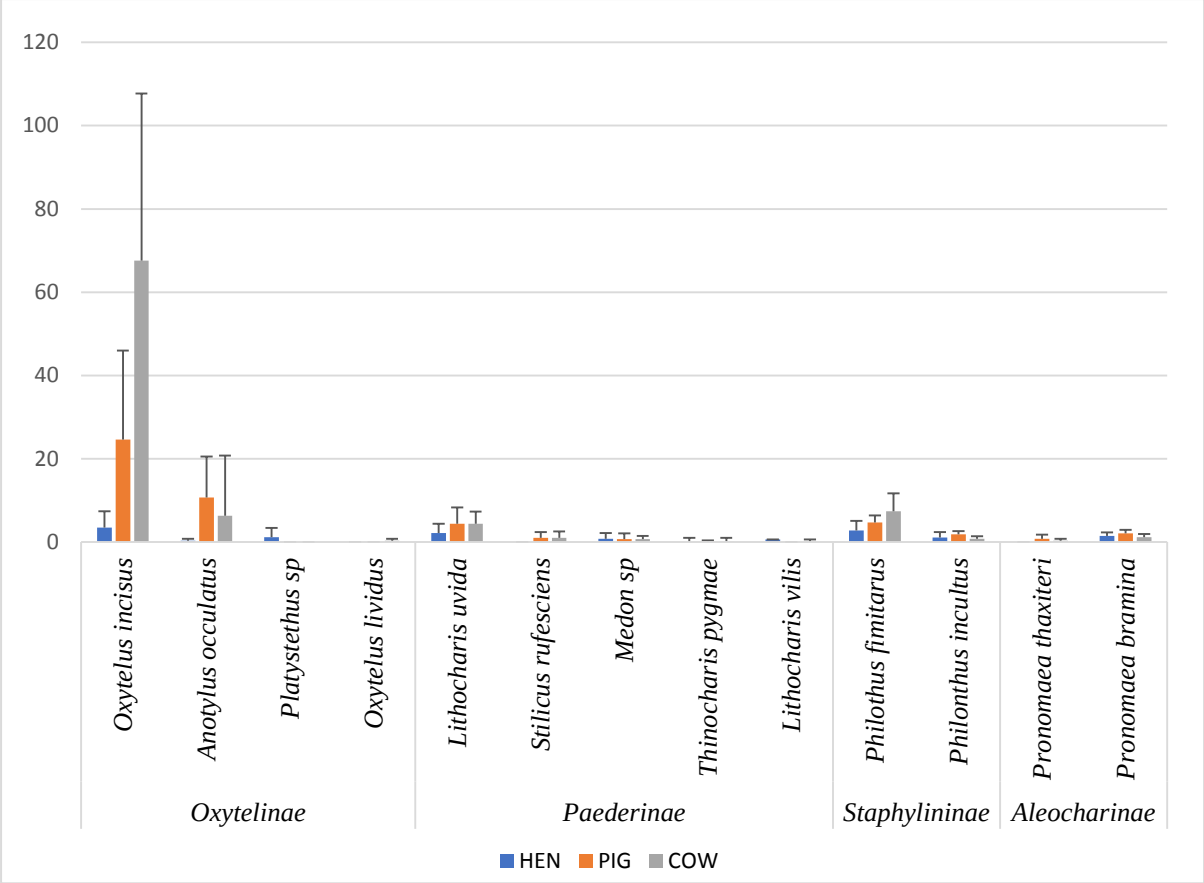


Figure 3: Abundance (Mean ± SD) of species collected from faecal matter of three different domestic animals

Table 3: Alpha diversity values of identified species collected from faecal matter of three different domestic animals.

Sample	Shannon diversity (H')	Margalef's species richness (d)	Pioelou's Evenness (j)	Simpson's dominance (λ)
Hen	1.99	3.474	0.8128	0.8422
Pig	1.578	2.351	0.4844	0.7035
Cow	0.987	2.245	0.2439	0.4226

TAXONOMIC DESCRIPTION

Plate No: 1



Oxytelus incisus

Sl No.	**:**	**1**
Locality	**:**	**Peravoor**
Habitat	**:**	**Agroecosystem**
Collector	**:**	**Emil James**
Guild	**:**	**Detritivore**
Class	**:**	**Insecta**
Order	**:**	**Coleoptera**
Family	**:**	**Staphylinae**
Subfamily	**:**	**Oxytelinae**
Tribe	**:**	**Oytelini**
Genus	**:**	**Oxytelus**
Species	**:**	***Oxytelus incisus***

Description

Smaller and notably more elongated. The sides of the thorax are not crenulated. The color of the thorax and abdomen is red. The last eight joints of the antennae are brown, the others testaceous. Head with a well-marked fovea.

TAXONOMIC DESCRIPTION

Plate No: 2



Anotylus occulatus

Sl No.	**:**	**2**
Locality	**:**	**Peravoor**
Habitat	**:**	**Agroecosystem**
Collector	**:**	**Emil James**
Guild	**:**	**Detritivore**
Class	**:**	**Insecta**
Order	**:**	**Coleoptera**
Family	**:**	**Staphylinae**
Subfamily	**:**	**Oxytelinae**
Tribe	**:**	**Oytelini**
Genus	**:**	**Anotylus**
Species	**:**	***Anotylus occulatus***

Description

Opaque, black, the elytra brownish, abdomen a little more shining. Antennae black, the first two joints more or less testaceous. Legs testaceous.

TAXONOMIC DESCRIPTION

Plate No: 3



Platystethus sp

Sl No.	**:**	**3**
Locality	**:**	**Peravoor**
Habitat	**:**	**Agroecosystem**
Collector	**:**	**Emil James**
Guild	**:**	**Detritivore**
Class	**:**	**Insecta**
Order	**:**	**Coleoptera**
Family	**:**	**Staphylinae**
Subfamily	**:**	**Oxytelinae**
Tribe	**:**	**Oytelini**
Genus	**:**	**Platystethus**
Species	**:**	***Platystethus sp***

Description

Body shiny and brownish latericeous; maxillary palpi, antennae, and legs lighter; elytra yellowish pale, transparent, with base and apex infuscate.

TAXONOMIC DESCRIPTION

Plate No: 4



Oxytelus lividus

Sl No.	**:**	**4**
Locality	**:**	**Peravoor**
Habitat	**:**	**Agroecosystem**
Collector	**:**	**Emil James**
Guild	**:**	**Detritivore**
Class	**:**	**Insecta**
Order	**:**	**Coleoptera**
Family	**:**	**Staphylinae**
Subfamily	**:**	**Oxytelinae**
Tribe	**:**	**Oytelini**
Genus	**:**	**Oxytelus**
Species	**:**	***Oxytelus lividus***

Description

The elytra are pale testaceous, and the sides are infuscate (save at the humeral angle). The first or first and second joints are testaceous, while the antennae are black and infuscate below. Legs are palpi testaceous.

TAXONOMIC DESCRIPTION

Plate No: 5



Lithocharis uvida

Sl No.	**:**	**5**
Locality	**:**	**Peravoor**
Habitat	**:**	**Agroecosystem**
Collector	**:**	**Emil James**
Guild	**:**	**Predator**
Class	**:**	**Insecta**
Order	**:**	**Coleoptera**
Family	**:**	**Staphylinae**
Subfamily	**:**	**Paederinae**
Tribe	**:**	**Paederini**
Genus	**:**	**Lithocharis**
Species	**:**	***Lithocharis uvida***

Description

Head black, thorax ferruginous red, elytra ochraceous, more or less broadly infuscate on the disc. Abdomen brown, the posterior margins of the segments and the apex reddish. Atennae reddish-testaceous.

TAXONOMIC DESCRIPTION

Plate No: 6



Medon sp

Sl No.	**:**	**6**
Locality	**:**	**Peravoor**
Habitat	**:**	**Agroecosystem**
Collector	**:**	**Emil James**
Guild	**:**	**Predator**
Class	**:**	**Insecta**
Order	**:**	**Coleoptera**
Family	**:**	**Staphylinae**
Subfamily	**:**	**Paederinae**
Tribe	**:**	**Paederini**
Genus	**:**	**Medon**
Species	**:**	***Medon sp***

Description

Labrum bidentate in the middle or at least trisinuate; prosternal epimera present, the stigma concealed. Eyes shorter than the temples.

TAXONOMIC DESCRIPTION

Plate No: 7



Lithocharis vilis

Sl No.	**:**	**7**
Locality	**:**	**Peravoor**
Habitat	**:**	**Agroecosystem**
Collector	**:**	**Emil James**
Guild	**:**	**Predator**
Class	**:**	**Insecta**
Order	**:**	**Coleoptera**
Family	**:**	**Staphylinae**
Subfamily	**:**	**Paederinae**
Tribe	**:**	**Paederini**
Genus	**:**	**Lithocharis**
Species	**:**	***Lithocharis vilis***

Description

The head transversely subquadrate, as broad as the thorax, more shining, the eyes larger, the thorax a little less closely punctured

TAXONOMIC DESCRIPTION

Plate No: 8



Stilicus rufesciens

Sl No.	**:**	**8**
Locality	**:**	**Peravoor**
Habitat	**:**	**Agroecosystem**
Collector	**:**	**Emil James**
Guild	**:**	**Predator**
Class	**:**	**Insecta**
Order	**:**	**Coleoptera**
Family	**:**	**Staphylinae**
Subfamily	**:**	**Paederinae**
Tribe	**:**	**Paederini**
Genus	**:**	**Stilicus**
Species	**:**	***Stilicus rufesciens***

Description

Head and thorax dull red, elytra copper bronze, slightly shining, with apical margins and postereo-external angles testaceous. Antennae, palpi, and legs reddish testaceous.

TAXONOMIC DESCRIPTION

Plate No: 9



Thinocharis pygmaea

Sl No.	**:**	**9**
Locality	**:**	**Peravoor**
Habitat	**:**	**Agroecosystem**
Collector	**:**	**Emil James**
Guild	**:**	**Predator**
Class	**:**	**Insecta**
Order	**:**	**Coleoptera**
Family	**:**	**Staphylinae**
Subfamily	**:**	**Paederinae**
Tribe	**:**	**Paederini**
Genus	**:**	**Thinocharis**
Species	**:**	***Thinocharis pygmaea***

Description

Greasy lustrous, head black, thorax reddish brown, elytra brownish yellow, abdomen brown. Antennae, palpi, and legs yellow. Head is little longer than, subovate, as broad as the thorax. Elytra closely but less finely punctured.

DISCUSSION

Staphylinidae is considered to be the most important predator of dung (Irmler *et al*., 2018). The findings revealed notable differences in the diversity of Staphylinidae beetles among the three animal species. In the faecal matter of cows, we observed higher species richness and abundance compared to that of hens and pigs. This result aligns with previous studies highlighting the importance of cow dung as a favorable habitat for Staphylinidae beetles due to its higher organic content and nutrient availability. (Koller *et al*., 2002). Oxytelinae was the most abundant subfamily among the Staphylinidae beetles that were collected. The abundance of Staphylinidae beetles in a specific order: Oxytelinae > Staphylininae > Paederinae > Aleocharinae > Tachyporinae. Among the species in the Oxytelinae *Oxytelus incisus* is most abundant.

By comparing the diversity of Staphylinidae beetle in the faecal matter of three domestic animals, the highest Shannon diversity in the subfamily level was recorded in Hen, (H'=1.369). The lowest diversity was observed in Cow dung (H'= 0.8044)

Among the Staphylinidae subfamilies, oxytelinae is a large and widespread subfamily (Herman, 2001). Aleocharinae has the lowest abundance, indicating that they prefer a specific habitat. The prevalence of predatory subfamilies like as Staphylininae, Paederinae, and Aleocharinae may be linked to a significant number of prey resources such as fly larvae connected with grazing cow dung. Dipteran larvae were discovered to be suitable prey for predatory Staphylinidae (Ashe, 1984). Aleocharinae is the least well-known and taxonomically challenging family. It is incredibly little, measuring only 3-5mm in length. Furthermore, many of the Aleocharinae species that have been described are only likely to make up a small part of the subfamily's total diversity (Arnett, 1968).

It is worth noting that our study has certain limitations. First, the sampling was conducted in a specific geographical region, which may have influenced the observed diversity patterns. Further investigations encompassing different regions and climates would provide a more comprehensive understanding of the diversity of Staphylinidae beetles in faecal matter. The findings of this study contribute to the existing knowledge on Staphylinidae beetles and their association with animal waste systems.

CONCLUSION

Our study has provided valuable insights into the presence and diversity of Staphylinidae beetles in the faecal matter of hen, pig and cow. The study confirmed the presence of Staphylinidae beetles in the faecal matter of cows, hens, and pigs, highlighting their ability to inhabit and utilize diverse environments. This finding emphasizes the importance of considering faecal matter as a potential ecological niche for various organisms. We observed that hen faecal matter hosted a higher diversity of Staphylinidae beetles compared to the faecal matter of pig and cow. These variations might be attributed to differences in diet, physiology, and habitat preferences among the animal species. Another important outcome was the identification of specific Staphylinidae beetle species associated with each animal's faecal matter. By conducting taxonomic analyses, we were able to determine the presence of distinct beetle species in the faeces of cows, hens, and pigs. This information adds to the existing knowledge of Staphylinidae beetle distribution and highlights the potential role of these insects in the faecal matter. Moreover, findings suggest that the presence of Staphylinidae beetles in the faecal matter may have broader ecological implications. These beetles are known predators and scavengers, and their presence in the faeces of these animals indicates their potential role in controlling other insect populations or contributing to the decomposition of organic matter. Further studies can build upon these findings to explore the functional roles of Staphylinidae beetles in nutrient cycling, decomposition, and ecosystem dynamics within agricultural and ecological contexts.

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Uncovering Biosurfactant-Producing Bacteria from Oil-Contaminated Soil: Isolation and Molecular Analysis

Dissertation report submitted in partial requirement for M.Sc. degree course in Zoology with Environmental Biology as special subject

in

University of Calicut

by

Ms. Lakshmi Krishnakumar

Reg No: DVAVMZL006

Under the guidance of

Dr. Jisha Jacob

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PG AND RESEARCH DEPARTMENT OF ZOOLOGY,

ST. JOSEPH'S COLLEGE (AUTONOMOUS), DEVAGIRI

KOZHIKODE -08

2021-2023

Uncovering Biosurfactant-Producing Bacteria from Oil-Contaminated Soil: Isolation and Molecular Analysis

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REPORT SUBMITTED IN PARTIAL REQUIREMENT FOR M.Sc. DEGREE COURSE IN ZOOLOGY WITH ENVIRONMENTAL BIOLOGY AS SPECIAL SUBJECT

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2021-2023

ST. JOSEPH'S COLLEGE (AUTONOMOUS) DEVAGIRI, CALICUT

Uncovering Biosurfactant-Producing Bacteria from Oil-Contaminated Soil: Isolation and Molecular Analysis

Report of the MSc Students project submitted to St. Joseph's College (Autonomous) Devagiri

Affiliated to the University of Calicut

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Program: M.Sc. Zoology

Semester: FOURTH

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CERTIFICATE

This is to certify that the report entitled **"Uncovering Biosurfactant-Producing Bacteria from Oil-Contaminated Soil: Isolation and Molecular Analysis"** is an authentic record of studies and research work done by **Ms. Lakshmi Krishnakumar (Reg No: DVAVMZL006**) under the guidance and supervision of **Dr. Jisha Jacob** Assistant Professor, Department of Zoology, St. Joseph's College, Devagiri, Calicut. The work has been carried out for the partial fulfillment of the requirements for the award of the degree of Masters of Science in Zoology.

Place: Devagiri **Dr. Vineesh P. J., MSc. B.Ed, Ph.D**

Date: **Head of the Department**

CERTIFICATE

This is to certify that the dissertation entitled **"Uncovering Biosurfactant-Producing Bacteria from Oil-Contaminated Soil: Isolation and Molecular Analysis"** is an authentic record of studies and research work done by **Ms. Lakshmi Krishnakumar (Reg No: DVAVMZL006**) under my guidance and supervision and that, to the best of my knowledge and belief, this work has not been presented earlier for any degree or fellowship.

Place: Devagiri **Dr. Jisha Jacob**

Date:

DECLARATION

I hereby declare that the dissertation entitled **“Uncovering Biosurfactant-Producing Bacteria from Oil-Contaminated Soil: Isolation and Molecular Analysis "** submitted in partial fulfillment of the requirements for the award of the degree of MASTER OF SCIENCE IN ZOOLOGY is a record of original work submitted by me at St. Joseph’s College (Autonomous), Devagiri under the supervision and guidance of Dr. Jisha Jacob, Assistant Professor, Department of Zoology, St. Joseph’s College (Autonomous), Devagiri, Calicut during 2021-2023.

I hereby declare that the results presented have not been previously submitted for the award of any degree or fellowship.

Place: Devagiri **Lakshmi Krishnakumar**

Date:

ACKNOWLEDGEMENT

The successful accomplishment of our endeavour required a lot of guidance and assistance from many people and we are extremely fortunate to have got this all along the course of our project work.

First, we thank the Almighty for showering His blessings on us and helping us to submit our project on time.

We express our sincere thanks to **Dr. Boby Jose**, M.Sc., B.Ed., Ph.D, Principal, St. Joseph's College,Devagiri Calicut for providing research facilities to carry out this investigation.

I express my sincere gratitude to **Dr. Vineesh P.J.** MSc, B.Ed, Ph.D., Assistant Professor and Head, PG and Research Department of Zoology for his support and sincere encouragement

We take this opportunity to express our profound gratitude and deep regard to our guide **Dr. Jisha Jacob**, M.Sc. Ph.D,Assistant Professor, Department of Zoology, St. Joseph's college, Devagiri for her exemplary guidance, monitoring and constant encouragement throughout the course of our project.

We acknowledge with gratitude the immense support rendered by all the staff of Amplicon Biolabs KINFRA, Kakkanchery for all valuable help in the completion of this project.

I express my heartfelt thanks to the research scholar of the Department Nikhitha. D for her sincere help.

We are grateful to other faculty members of the Department,for their sincere support, co-operation and guidance throughout the course of this project.

I express my gratitude to my classmates, for having strong shoulders that I could always lean on for support.

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INTRODUCTION

The growing concern about food shortage and overpopulation has led to many discoveries in the field of science, an important one being the discovery of biosurfactants. Biosurfactants are considered as high-value products in the market due to their various roles. Studies regarding biosurfactants started around the 1960s, and since then they are used in many industries. These are amphiphilic molecules that can partition at the liquid/solid, liquid/liquid and liquid/gas interface. These properties are exploited by scientists and biosurfactants are thus used in foam formation, emulsification, dispersal and as carrier molecules also.

Biosurfactants are surface-active compounds that are produced at the microbial cell surface or excreted and reduce the surface and interfacial tension. These are amphiphilic compounds with hydrophilic and hydrophobic moieties that confer on the organism the ability to accumulate between fluid phases thereby reducing its surface tension. The hydrophilic domain can be carbohydrates, amino acid, phosphate group or any other molecule; whereas hydrophobic domain can be a long chain fatty acid. Based on their chemical structure and microbial origin they are classified as Glycolipids, Phospholipids, Polymeric Biosurfactants and lipopeptides (surfactin). The best-known glycolipids are rhamnolipids, sophorolipids and trehalolipids. Biosurfactants are synthesised by microbes like bacteria, yeast and fungi.

Biosurfactants are chemically diverse and this property allows them to be used widely in biological systems. The nutritional parameters regarding biosurfactants need to be studied and the characteristics of these molecules need to be studied in more depth. (Sanches *et al*. 2021). The distinct properties of biosurfactants when compared to their chemically synthesised forms made them suitable for commercial applications. The distinctive features of microbial surfactants are their surface activity, tolerance to a wide variety of pH, temperature and ionic strength, biodegradability, low toxicity, emulsifying and demulsifying ability and antimicrobial activity (Chakrabarti, 2012).

Amphiphilic biomolecules of high molecular weight are often referred to as Bioemulsifiers (Perfumo et al.,2010), but they are generalised under the term biosurfactant.The primary role of biosurfactants in oil biodegradation is to increase the collision frequency between oil and microbes. (Hua,F & Wang,H.Q, 2014).

The process of dispersing two or more immiscible liquids together to form a semi stable mixture is known as emulsification. Bioemulsifiers can reduce the surface tension of oil-water interface. Bioemulsifiers accumulate at the interphase between two immiscible phases, thereby reducing its surface tension which causes the increased solubility and emulsification of the immiscible phases. Hence biosurfactants can be used in the degradation of hydrocarbons in contaminated water and soil. The biosurfactants can convert the insoluble substrate into their soluble form which can then be utilised by microbes for their metabolism. (Rodrigues,L. *et al*. 2006).

Properties of Biosurfactants

Biosurfactants are of increasing interest for commercial use because of it's increasing span of available sources.

1. Surface and interfacial activity

Lower molecular weight biosurfactants can produce the surface and interfacial tension. Among them trehalolipids, cellobiose lipids,mannosylerythritol lipids and rhamnolipids show greater activity. (Jahan, R. *et al*. 2020). Surfactin shows a surface activity from 72 mN/m to 27± 2mN/m . (Alvarez, V.M et al. 2015) and interfacial tension to 3.79 ± 0.27 mN/m and 0.32 ± 0.02 mN/m (Al-Wahaibi Y., *et al.* 2014) Biosurfactants show greater interfacial activity when compared to chemical surfactants (Pereira *et al*. 2013).

2. Self assembly

Biosurfactants can self-assemble through Vanderwaals interaction and weak hydrophobic effect. (Desai, J.D., & Banat, I.M, *et al*. 1997)

3. Emulsification

The biosurfactants can reduce surface tension, by enhancing emulsification and formation of micelle. (Sah,D., *et al.* 2022). The biosurfactant production of 15 most efficient bacteria when analysed,displays high stability and emulsification capacity (E 24>48%) to reduce the surface tension from 49.40 to 34.50 mN/m. (de Oliveira,E.M., *et al.* 2021).

4. Low toxicity

Most biosurfactants have low toxicity (Sarubbo *et al.* 2013). But very little information is available about the toxicity of biosurfactants to plants and microbes in the environment. (Lima et al., 2011; Santos et al., 2017; Sharma *et al*. 2014).

5. High biodegradability

Biosurfactants are highly biodegradable and this property has led to its use in petroleum, food processing, health and cosmetic industries. (Santos *et al.* 2016). This causes less harm to the environment than chemical surfactants.

6. High selectivity and specificity at extreme pH, temperature and salinity

Most of the biosurfactants are resistant towards extreme pH and temperature. Studies have proved that B. licheniformis to be resistant to temperature up to 50°C and pH between 4.5-9. This property has enabled them to be used in many industrial processes involving exposure to high temperature, pH and pressure. (Cameotra and Makkar 2004).

7. Antioxidant

Antioxidant activity of biosurfactants when studied in vitro using 2,2-diphenyl-1-picryl hydrazyl (DPPH) and hydroxyl radicals at a biosurfactant concentration of 5mg/ml showed scavenging of DPPH and hydroxyl radicals between 69.1 -73.5 % and 63.3-69.8 % respectively. (Giri, S. S *et al.* 2019). This shows the excellent antioxidant property of biosurfactants.

8. Anti-adhesive

   Biosurfactants can be used as anti-adhesive agents against several pathogens, and hence they are suitable anti-adhesive coating agents for medical insertional materials. (Rodrigues,L.R.,&Teixeira,J.A. 2010)

Applications of Biosurfactants

Biological surfactants are world wide accepted as sustainable greener alternatives. Biosurfactants show the same functions of synthetic analogues having the capability to synergize with other molecules, thereby increasing their performance; thus making them available in markets. Recently their use was proposed to help fight against Covid 19.(

Banat, I.M *et al.* 2021) The potential application of biosurfactants include enhanced oil recovery, through detergents, food emulsifiers, antibiotics, antifungal agents and in mineral processing industries. Although the market demand of these products are on a rise, the industry is still minor when compared to synthetic surfactant market, because biosurfactant extraction is very expensive.

One of the emerging branches of biotechnology, i.e. Nanobiocatalysis is the combination of Nanotechnology and biocatalysis. It involves the use of immobilised enzymes in industries. Due to their characteristic properties, they are used in large and modifiable surfaces and easy recovery. Further their properties were studied using magnetic CLEAs of CALB.(Lopez, C. *et al.* 2014).

1. Biomedical Application

Biosurfactants also have antimicrobial properties and biofilm disruption ability. Sophorolipids was able to disrupt biofilms formed by different bacterial cultures, indicating the promising use of sophorolipids in biomedical applications as adjuvants to other antimicrobials against pathogens through growth inhibition or by disruption of biofilms.(Diaz De Rienzo,M.A., *et al.* 2015). Biosurfactants can be used as anti-adhesive agents against several pathogens and hence they are suitable anti-adhesive coating agents for medical insertional materials (Rodrigues, L.R., Teixeira, J.A 2010).They have many advantages over chemical surfactants because of their eco-friendly nature (Saikia. R, R., Deka, S., Sarma, H. 2021).

2. Cosmetics

The antimicrobial and antitumor capacity of glycolipids and their surface activity has enabled them to be applied in food,pharmaceutical and cosmetic industries. (Zago, E *et al.* 2021).Chemical surfactants cause negative effects on skin health. Hence as an alternative biosurfactants with properties like low toxicity, skin compatibility, protection and surface moisturising effects are useful in skincare. They are currently being used in cosmetics and personal skincare pharmaceuticals. (Adu, S. A., *et al*. 2020). Glycolipids and lipopeptides are utilised in cosmetics due to their detergency, emulsifying, foaming and skin hydrating properties (A. Varvaresou and K. Iakovou 2015). They are used as cleanser in shampoo,body cleanser, body washer and in anti dandruff shampoo formulations also.

3. Petroleum industry

Biosurfactants are assuring molecules for petroleum industries.Petroleum is a primary energy source. Microbial enhanced oil recovery (MOER) is a technology used to improve residual crude oil production from oil wells (Patel, J *et al*. 2015). They use significant amounts of synthetic surfactants as mobilising agents to increase the availability or recovery of hydrocarbons, their extraction, cleaning, treatment and transportation. Biosurfactants have several applications in oil processing industries and petrochemical product formulations such as emulsifying agents, biocides,anticorrosives,fuel formulations, extraction of bitumen from tar sands etc. Biosurfactants are valuable tools that can transform and update petroleum biotechnology. (De Almeida, D.G., *et al.* 2016). Biosurfactants due to their biodegradability and low toxicity can be applied in environmental bioremediation,petroleum industry and enhanced oil recovery (Bouassida, M., *et al.* 2018).

4. Food and healthcare

Sophorolipids are one of the attractive biosurfactants showing different biological and physicochemical properties due to their antimicrobial action against both gram positive and gram negative microorganisms in the food and healthcare sector . Hence they can be used in food,agriculture, anticancer,antimicrobial, pharmaceutical and cosmetic industries. (Cho, W.Y *et al.* 2022)

5. Drugs

Biosurfactants have been employed as a drug delivery system to improve the bioavailability of a good number of drugs. (Bjerk, T.R *et al.* 2021). Biosurfactants are promising drug delivery systems in relief of symptoms associated with SARS-CoV-2 acute respiratory distress syndrome (Ceresa,C., *et al.* 2021). They have the ability to create micelles that enhance encapsulation of bioactives. They are used as drug delivery systems to improve bioavailability of many low aqueous solubility drugs.(Bjerk, T.R *et al.* 2021).

6. Oil recovery

Biosurfactants enhance emulsification of hydrocarbons, thereby solubilising them and increasing their availability for degradation of microbes.(Shulga, A *et al.* 1999) .So they

play a crucial role in enhanced oil recovery and can be considered for other potential applications in protection of the environment also. (Rosenberg E and Ron E. Z 2001)

7. Bioremediation

Marine fungi are also capable of producing biosurfactants. They are potential aspirants for novel enzymes, bioremediation, secondary metabolites production etc. (Damare, S. *et al.* 2012).

Biosurfactants are also used in pollution control due to their emulsifying/demulsifying activity. They are of great use in the textile industry, in the preparation of fibres,dyeing,printing and in emulsion polymerization, latex adhesive, foam polymers, pulp treatment, concentration of ore,cutting,casting and forming due to their wide range of properties.

8. Therapeutics

Biosurfactants are employed as sustainable, non toxic and safe surface cleaners or disinfectants in a wide range of physiological and environmental conditions. The amphiphilic micellar property of biosurfactants makes them useful as therapeutic carriers.(Raza, Z.A *et al.* 2022). Due to their surface activity, they interact with cell membranes of many organisms, and are viewed as potential cancer therapeutics. (Gudina, E.J *et al.* 2015).

9. Agriculture

Biosurfactants are investigated in many areas like increasing feed digestibility in agriculture,improval of seed protection, fertility and control of plant pathogens. (Naughton, P.J *et al*. 2019). Trehalolipids, rhamnolipids,cellobiose etc are used as biopesticides to protect stored products and control plant diseases. They also help in preserving plants and plant crops from pest invasion. (Mnif, I.,& Ghribi, D. 2016).

10. Nanotechnology

Microbial derived molecules are used in nanomaterial synthesis. Biosurfactants are good alternatives in nano sized material preparation. The amphiphilic and self assembly property of biosurfactants along with their biodegradability, low eco-toxicity,biocompatibility and structural diversity are potential advantages over chemical surfactants (Nitschke,M.,& Marangon, C.A. 2022).

11. Cancer treatment

Biosurfactants can interact with lipid membranes of microbes and can alter their properties. Hence due to their antimicrobial, antibiofilm and antiviral,immunomodulatory effects they are being used in anti-cancer treatment. (Ceresa,C *et al.* 2021). Biosurfactants have significant activity in inhibiting multiple tumour types,especially surfactin, iturin and fenglycin lipopeptide biosurfactants produced from species belonging to Bacillus genes. They are cytotoxic against some tumour types such as breast,colon,leukaemia and hepatoma. (Rofeal, M., 2021). A newly isolated B. amyloliquefaciens strain fiply 3A is found to produce a cyclic lipopeptide. It shows a dose dependent killing of three different human cancer cell lines, A549 (alveolar adenocarcinoma), A498 (renal carcinoma) and HCT-15 (colon adenocarcinoma), while not affecting the normal cell line L-132 (pulmonary epithelial cells) when analysed using MTT assay and FACS analysis. Further an increase in reactive oxygen species formation in the lipopeptide treated cell population was observed. Apoptosis is a major mechanism of cell death in lipopeptide treated cells. Lipopeptide treatment induced DNA damage in the treated cells, was indicated by TUNEL assay. (Hajare,S.N., Subramanian,M.,Gautam,S.,& Sharma,A. 2013).

AIM AND OBJECTIVE

The study was based on isolation and screening of biosurfactants from oil contaminated soil and characterization of isolated organisms. The objectives include:

- Isolation of biosurfactant producing bacteria from oil contaminated soil
- Screening of isolated bacteria
- Characterization of the isolated strain

The characterization includes

- Morphological characterization
- Biochemical characterization
- Molecular characterization

REVIEW OF LITERATURE

Biosurfactants are economically the most important compounds of the 21st century. Microorganisms synthesise a wide variety of surface active compounds(SAC), called as biosurfactants. They are classified according to their molecular weight, physico-chemical properties and mode of action. The low molecular weight SAC can reduce interfacial tension at oil –water interface, whereas high molecular weight SAC's called bioemulsifiers are effective in stabilising oil in water emulsions (Banat, I.M. 2010). According to Estefanía Eras- Muñoz (2022), microbial biosurfactants are low molecular weight surface active compounds of very high industrial application due to their peculiar chemical characteristics and stability under several environmental factors. The chemistry and cost of production of a biosurfactant are defined by the selection of microbe, substrate type and the purification strategy. Presently biosurfactants have been applied in solving some environmental problems, the most referred one is the bioremediation of contaminated soils with pollutants like hydrocarbons and heavy metals. In heavy metals biosurfactants act as chelating agents due to their binding capacity. They reduce surface tension thereby facilitating the emulsification and solubilisation of pollutants. Biosurfactants are less toxic, in that they also act as biocidal agents at certain doses. A new branch of science also found that biosurfactant production can be made using alternative substrates like organic waste and solid state fermentation with its increased applicability in the present economy. (Eras-Muñoz E, Farré. A, Sánchez. A, Font. X, Gea .T) .But the inefficiency in bioprocessing of biosurfactants has accelerated the commercial production of these compounds. Now many works are being done on the usage of low cost substrates for biosurfactant production. The use of nanoparticles and the coproduction of biosurfactants along with other compounds like enzymes are also on the rise. This would go a major way in making biosurfactants, one of the commercially important compounds of the current century.

The studies conducted by Ward O. P(2010), shows that microbial biosurfactants have a typical molecular weight of 500 to 1500 Da, made of peptides, saccharides or lipids or their combinations. They mediate solubilisation and mobilisation of hydrophobic substrates to microbes in the biodegradation process. They are also associated with wastewater treatment and microbial pathogenesis. In bioremediation, biosurfactants

facilitate the release of contaminants from soil, but soil also binds surfactants strongly, which makes their role in contaminant desorption complex. (Ward O. P). Biosurfactants were also studied by Naughton P. J *et al.*(2019) in numerous areas like increasing the feed digestibility in agriculture, improving seed protection and fertility, control of plant pathogens, antimicrobial activity, wound healing, dermatological care, anticancer treatment, drug delivery System and oral cavity care.(Naughton, P. J., Marchant, R., Naughton, V., & Banat, I. M.). Biosurfactants are molecules either produced by microorganisms on their cell surface or secreted extracellularly. They can form a thin film on the surface of microorganisms, and help in attachment or detachment to other cell surfaces. They can also regulate the sensing and motility of bacteria.(Sharma, J., Sundar, D., & Srivastava, P. 2021).

Based on the study conducted by Jahan R *et al*. (2020) on low molecular weight and high molecular weight biosurfactants of microbial origin, they were found to be environmentally safe and biodegradable making them useful in cosmetics to oil recovery. Biosurfactants adsorb at various interfaces and self-assemble in aqueous solution, resulting in properties like decreased surface and interfacial tension, low critical micellization concentration (CMC) and ability to solubilize hydrophobic compounds. (Jahan, R., Bodratti, A. M., Tsianou, M., & Alexandridis, P.). Sachdev D.P and Cameotra S.S (2013) stated that Biosurfactants that are produced by bacteria, yeast and fungi could also serve green surfactants, because these are less toxic and eco-friendly and have great potential to be commercially produced for wide applications in pharmaceutical, food and cosmetic industries. These biosurfactants also have a great role in the agricultural industry. Many rhizosphere and plant associated microbes produce biosurfactants that play a major role in motility, signalling and in biofilm formation which indicates that biosurfactant mediates microbe interaction. In agriculture, these are used for the elimination of pathogens in plants and to increase the bioavailability of nutrients. Biosurfactants can also be used to improve agriculture or soil quality by soil remediation. They can also replace the harsh surfactant that is used at present.(Sachdev DP, Cameotra SS.)

Ibrar, M. *et al.* (2022) biosurfactants increase the bioavailability and help in biodegradation which are linked to bacterial surface motility and chemotaxis. Therefore, the disturbance of biosurfactant production and chemotaxis of degrading microorganisms

is a possible approach for the complete degradation of hydrocarbons. Hence, biosurfactants and chemotaxis are closely linked in bioremediation. (Ibrar M, Khan S, Hasan F, Yang X.). Patel. S *et al*. (2019)suggests that the effective degradation of crude oil spreads on water can also be treated with bio surfactants. They are capable of reducing the interfacial tension thereby dispersing the oil particles, and degrading them into non-toxic debris. The trio of rhamnolipids, sophorolipids and surfactin has been characterised well, and among the microbes only Pseudomonas, Bacillus and candida have been evaluated broadly. Hence, biosurfactants can be utilised for oil spill management.(Patel, S., Homaei, A., Patil, S., & Daverey, A.) Sharma. J *et al.* (2021) in his studies indicates that Pseudomonas aeruginosa, Bacillus subtilis and candida sp. are the most important classes of microorganisms involved in the production of Rhamnolipids biosurfactant. Rhamnolipids are unique in their surface activity and have gained interest in various other industrial applications also. Due to their high biodegradability and renewability at extreme conditions, microbial biosurfactants are more advantageous than chemical biosurfactants. (Sharma, J., Sundar, D., & Srivastava, P.).

Chafale A and Kapley A (2022) showed that biosurfactants are actively involved in microbial enhanced oil recovery. This is an efficient process to recover oil from oil reservoirs and waste sledges. Microbial metabolites are also effective in oil recovery from waste oily sludges generated as a byproduct of petroleum production(Chafale. A, Kapley.A). Bhadra.S *et al.* (2022) suggests that biosurfactants are preferred to be used in the removal of pollutants from nature. These amphipathic molecules can be synthesised inexpensively by using cheap substrates like agriculture and industrial wastes. Biosurfactants can also be used to remove organic pollutants and harmful microbial aggregates. Biosurfactants are also involved in the remediation of xenobiotics, removing biofilms and various types of hydrocarbons like crude oil,aromatic hydrocarbons, naphthene, petroleum products and n-alkanes etc. This property of biosurfactants is very important, as biofilms are of great regard due to their role in environmental impact and public health.(Bhadra S, Chettri D, Kumar Verma A.).

The increased industrial sustainability of bio surfactants has pushed it to the top of many companies .Biosurfactants can possibly replace chemical surfactants. They are also attractive because they are less damaging to the environment and can be industrially utilised. The most promising biosurfactants at present are the glycolipids ,sophorolipids

and mannosylerythritol.(Marchant, R., & Banat, I.M. 2012). Raj, A. *et al*. (2021) investigated the role of microbial biosurfactants in pesticide remediation is an assuring approach. Biosurfactants are compounds that help to increase the bioavailability of pesticides and thereby speeds up the bioremediation. They can lower the surface area and interfacial tension of immiscible liquids and raise the solubility of hydrophobic pesticide contaminants. They are highly biodegradable, less toxic, highly selective and have a broad action spectrum under extreme conditions. The role of biosurfactants in the biological remediation of pesticides has received great attention.(Raj, A., Kumar, A., & Dames, J. F.) . The growing use of biosurfactants in detergents, personal care products and oil field chemicals is triggering the growth of the market in many applications by strict regulations and by increasing the consumer demand for 'green and sustainable' products. Microbial biosurfactants are biologically produced types of bio- based surfactants which are rapidly expanding from a scientific basis to an industrial reality. (Roelants, S. L. K. W., & Soetaert, W. 2022).

T. O Femi-Ola *et al*. (2015) screened bacterial isolates from wastewater oil contaminated soil samples from eight different sites, using conventional biosurfactant screening tests, 22 bacterial isolates were recovered. The dominant species among them were Bacillus and Pseudomonas, with an occurrence rate of 22.73% each, which confirms their ability to produce biosurfactants that reduce surface tension, leading to increased solubility and emulsification of oils (TO Femi-Ola, OA Oluwole, TO Olowomofe, H Yakubu). Dhail. S(2012) studied biosurfactant producing microbes from oil- spilled seawater collected from harbours and docks from Arabian sea, Pseudomonas species showed high salt tolerance and successful production of biosurfactant in a vast range of conditions. The application of these bacteria in microbial enhanced oil recovery was also investigated and the biosurfactant detected was glycolipid in nature. Here haemolysis was used as a criterion for primary isolation of biosurfactant producing bacteria. (Dhail, S.). In the studies conducted by Shahwar, D. E *et al.* (2019) biosurfactant production potential of bacteria isolated from produced water and produced water contaminated soil samples were evaluated and among the 47 isolated bacteria's, five were selected on the basis of high optical densities of 0.7, 70% oil and grease reduction potential.(Dur-e-Shahwar., Sheikh, R.A. and Jamil, N.).

K.A Hassan *et al*. (2018) conducted the screening of biosurfactant producing bacterial strains from engine oil contaminated sites, by using haemolytic assay method and foam capacity test, three bacterial isolates tested positive and when the optical density was measured all isolates displayed mesophilic characteristics (4°C, 37°C, 42°C). (Kawo Abdullahi Hassan, Yahaya Sani and Olawore Yemisi Ajoke). S O. Adebajo *et al.* (2018) collected soil samples from automobile shops in Abeokuta, Ogun state, bacteria were isolated and screened by different stepwise processes like haemolytic test, drop collapse test, bacterial adhesion to hydrocarbon test etc. Among the 14 isolates tested positive for drop- collapse test, Pseudomonas aeruginosa had the maximum emulsification capacity (65%) while the least was exhibited by Proteus mirabilis (43%).(S.O. Adebajo A.K. Akintokun S.E). Ewida, A.Y.I *et al.* (2019) also studied The water samples from three different environments including Nile river where some oil spills from ship fixation remains and when screened for biosurfactant production, bacterial strains from these area showed high potential for biosurfactant production and the best biosurfactant producing isolate was identified by 16srRNA technique as Pseudomonas protegens and the biosurfactant was a rhamnolipid group. (Ewida, A.Y.I., *et al.*).

Uyar, E and Saglam, O (2021) isolated 107 bacterial strains from used motor oil contaminated soil samples from auto repair shops. The evaluation of isolates was done by hemolytic assay, oil displacement assay, drop collapse assay and parafilm M test. The potential biosurfactant producers were characterised by 16S rDNA based molecular tools. It was identified as Proteus mirabilis, Klebsiella pneumoniae, Enterobacter cloacae, Micrococcus sp., Citrobacter sp., and Bacillus sp. The analysis showed that biosurfactants produced by P. mirabilis SLM- B52 have the highest surface tension reduction capacity. The emulsifying activity of CFS from P. mirabilis was also described for the first time in this study. Thus the biosurfactants from bacterial strains have biodegradation capacity on hydrocarbons.(Uyar, E., & Sağlam, Ö.). Mulugeta K *et al.*(2022) in the studies screened biosurfactant-producing bacteria from the alkaline lake of Ethiopia, and the potential bacterial strain and their produced biosurfactant were characterised. In the screening, the most potent producer of biosurfactant was assigned as CS1 and identified as Serratia sp. The CS1-extracted biosurfactants were characterised by FTIR, and the type of biosurfactant produced was a glycolipid. The crude biosurfactant stability was also studied. The extracted biosurfactants were tested in commercial detergents, and its efficiency also increased. This study suggests that the isolated S.marcescens CS1 strain

has the potential to produce biosurfactants that are viable and can replace the use of synthetic chemicals in commercial detergents.(Mulugeta, K., Kamaraj, M., Tafesse, M. *et al.*).

Zargar, A.N *et al*.(2022) conducted The isolation and chemical characterization of the biosurfactant produced by biodesulfurization bacteria Gordonia sp. IITR100 was studied and biosurfactant production was determined by oil spreading, drop-collapse, emulsion index, and BATH assay. The biosurfactant was identified as a glycolipid by LCMS and GCMS analysis. The chemical structure was confirmed by FTIR and NMR studies. The emulsion formed by the biosurfactant was stable between temperatures of 4-30˚C. It can also reduce the surface tension of the aqueous media. The biosurfactant produced can be used in petroleum, detergents, soaps,beverage industry etc. (Zargar AN, Mishra S, Kumar M, Srivastava P) . Dhasayan. A *et al.* (2014) isolated Halomonas sp. producing biosurfactant MB-30 was from *Callyspongia diffusa*, and its effectiveness in crude oil recovery was investigated. The biosurfactant produced by MB-30 reduced the surface tension. The chemical structure of biosurfactants was glycolipid in nature and it was identified by IR spectroscopy and proton magnetic resonance spectroscopy. The emulsification activity of MB-30 biosurfactant was tested with different hydrocarbons, and maximum emulsification activity was exhibited with crude oil followed by kerosene.Thus, the stable emulsifying biosurfactant produced by Halomonas sp. MB-30 can be used for in situ biosurfactant mediated enhanced oil recovery process and hydrocarbon bioremediation in extreme environments. (Dhasayan, A., Kiran, G.S. & Selvin, J).

Pseudomonas strains isolated from oil contaminated soils were screened for biosurfactant production by Zouari *et al.*(2019) from which three Pseudomonas isolates were selected and they were identified as members of the P. putida. The chemical nature of produced biosurfactants was analysed by thin layer chromatography and MALDI-ToF mass spectrometry and identified as lipopeptides belonging to the syringafactin family. The activity of this biosurfactant was stable over a high pH range and at high salinity. Tests in contaminated sand micro bioreactors showed that the three strains are able to degrade diesel. These results suggest the potential of these bacterial strains in hydrocarbon bioremediation..(Zouari, O., Lecouturier, D., Rochex, A. *et al.*). Farias, B. C. S *et al*. (2018) found that cyclic lipopeptides are biosurfactants produced by Bacillus species and their

synthesis is under genetic and environmental control. This study evaluated the effect of low and high NaCl concentrations on the composition and surface activity of CLPs produced by Bacillus strains TIM27, TIM49, TIM68, and ICA13 towards microbial enhanced oil recovery. The strains were evaluated in mineral medium containing varying concentrations of NaCl, and their surface tension and emulsification activity were also monitored. Based on the analysis of 16S rDNA, TIM27 and TIM49 were assigned to Bacillus subtilis, TIM68 to Bacillus vallismortis, and ICA13 to Bacillus amyloliquefaciens. The ESI-MS analysis showed that the strains produced a mixture of CLPs, which presented variable CLP profiles at low and high NaCl concentrations. Surfactins were produced at high concentrations and iturins during low concentration. These findings suggest that the CLPs under the optimal conditions can be useful in microbial enhanced oil recovery.(Farias, B.C.S., Hissa, D.C., do Nascimento, C.T.M. *et al.*).

Mohanram *et al.* (2016) in their study isolated oil- degrading marine bacteria using a modified Bushnell- Haas medium from oil polluted sites of Mumbai harbour. About nineteen bacterial strains showed positive results for surface active agent screening methods by biochemical and nucleic acid sequencing methods. Hence many surface active agent producing bacteria were found in oil polluted water and they belong to genera Acinetobacter, Bacillus, Halomonas, Pseudomonas, Serratia, Comamonas, Alcanivorax etc.(Mohanram, R., Jagtap, C., & Kumar, P.). Cai, Q *et al.(*2015) identified and characterised biosurfactant producing bacteria belonging to four genera, Bacillus, Rhodococcus, Halomonas and Pseudomonas. Samples were collected from offshore oil and gas platforms in North Atlantic Canada- crude oil, formation water, seawater sample and drilling mud. Diverse isolates were found with unique properties like effective reduction of surface tension, production of biosurfactant at high rate and emulsion stabilisation etc and all of these were site and medium specific. They are also found to be highly effective in offshore oil spill control, soil washing treatment of petroleum contaminated sites and oil recovery.(Cai, Q., Zhang, B., Chen, B., Song, X., Zhu, Z., & Cao, T.). Piotrowska-Seget,Z. *et al.*(2016) conducted studies in which isolates from heavily contaminated soil with petroleum hydrocarbons were characterised and most of them belong to genera Rahnella, Serratia and Proteus. The isolates were screened for their biosurfactant producing ability using oil spreading, blood agar, methylene blue agar and emulsification index. Among the studied bacteria, they were classified into some categories based on their potential in production of biosurfactant, bioaugmentation of pH

contaminated soil and plant growth promotion. Thus they are considered as promising agents in microbe assisted phytoremediation. (Pacwa-Płociniczak, M., Płociniczak, T., Iwan, J., Żarska, M., Chorążewski, M., Dzida, M., & Piotrowska-Seget, Z.).

The studies of Jamal P *et al. (*2014), was based on isolation and identification of biosurfactant producing bacteria from degraded Palm kernel cake under liquid state fermentation. Potential bacterial strains were isolated from degraded PKC and were screened using drop collapse assay and analysis of surface tension activity. The best results were obtained from Providencia alcalifaciens SM03 bacterial strain and hence it is selected as the most important biosurfactant producing strain. (Jamal, P., Mir, S., Alam, M. Z., & Wan Nawawi, W. M.). Fan, F *et al.*(2018) studied the nitrate reducing bacterial strain from offshore produced water samples, when screened, were identified as Pseudomonas stutzeri. Their biosurfactant producing ability was investigated when fed on either glucose or glycerol media.P stutzeri reduced medium surface tension significantly. The biosurfactant product was identified as a mixture of lipopeptides and glycolipids and it is stable over a wide range of temperature, pH and salinity. Hence it is effective in offshore sourcing control and oil recovery.(Fan, F., Zhang, B., Morrill, P. L., & Husain, T.).

Nayarisseri. A *et al.* (2020) investigated bacterial strains isolated from brackish river water when examined using hemolysis, oil spread assay, BATH, E24 etc the lipopeptide biosurfactant producing Microbacterium spp was identified that could produce 0.461g biosurfactant in 100 ml of medium throughout a life cycle.Microbacterium also has powerful tension reducing property.(Nayarisseri, A., Khandelwal, R., & Singh, S. K.). S. Habib *et al.* (2023)found that petroleum hydrocarbons cause soil pollution, the isolates selected from here when screened for biosurfactant production, Bacillus albus S2i, Paraclostridium benzoelyticum Pb4 and Proteus mirabilis Th1 was identified based on 16S rRna sequence analysis. These bacteria also exhibited positive activity towards lipase activity, surface activity, hydrophobicity and hydrocarbon degradation.(Habib, S., Ahmed, A., Haque, M.F.U. *et al.)*

Phulpoto I . A *et al.*(2020) characterised the biosurfactant produced by Bacillus nealsonii S2MT from lake sediment. It produced biosurfactants with excellent emulsifying potential. The biosurfactant was cyclic lipopeptides relating to surfactin like form. This is highly efficient and acceptable in soil remediation when compared to synthetic surfactants, as the extracted biosurfactants were highly stable at a variety of

environmental factors.(Phulpoto IA, Yu Z, Hu B, Wang Y, Ndayisenga F, Li J, Liang H, Qazi MA)Similarly, Derguine. L (2021) also isolated and studied properties of Rhodotorula sp. strain YBR from an oilfield in Algeria, using wastewater from olive oil mills. Screening of biosurfactants was done by emulsification index 24, measurement of surface tension, oil spreading technique and it showed the presence of peptides, carbohydrates and lipids in its structure. Thus the produced biosurfactant is of great application in remobilization of hydrocarbons from polluted soil.(Derguine-Mecheri L, Kebbouche-Gana S, Djenane D.)

Ayangbeuro A. S *et al.* (2020) conducted the genomic analysis of Bacillus cereus NWUAB01 isolated from mining soil and its heavy metal resistance was studied on Luria- Bertani agar. The biosurfactant produced was evaluated for metal removal in contaminated soil. The biosurfactant was a lipopeptide with metal complexing properties. Its genome also contains biopolymer synthesis genes. The biosurfactant has high metal removal efficiency for Pb, Cd and Cr. This property can be utilised for biotechnological purposes. (Ayangbenro A.S, Babalola O.O.). Hsieh F.C *et al*.(2004) conducted research based on PCR screening surfactin production genetic locus(sfp), which is responsible for surfactin biosurfactant production in Bacillus subtilis. Several other techniques like hemolysis zone assay, quantitative HPLC and NMR also provided correct results. Among the 15 selected standard strains, Bacillus amyloliquefaciens S13 and Bacillus subtilis S15 have high productivity of surfactin. The PCR method is of high use to find different species or genera containing homologous genes.(Hsieh FC, Li MC, Lin TC, Kao SS.). Biosurfactant producing bacteria was found in the biopurification systems for pesticide treatments. From the isolates C11 and C27 were identified as B. amyloliquefaciens and Streptomyces lavendulae by MALDI- TOF. The maximum biosurfactant production was similar for both strains (46 and 45 mN/m). The biosurfactant produced was characterised as lipopeptide and glycolipid. This potential of biosurfactants can be used in improving pesticidal bioavailability.(Diez MC, Llafquen C, Fincheira P, Lamilla C, Briceño G, Schalchli H 2022).

Ndlovu. T (2017) studied on Bacillus amyloliquefaciens ST34 and Pseudomonas aeruginosa ST5, isolated from wastewater, were screened for biosurfactant production by UPLC coupled with ESI-MS. B.amyloliquefaciens produced C13-16 surfactin analogues and P. aeruginosa, di and mono rhamnolipid congeners. They also showed antimicrobial

activity against a broad spectrum of pathogenic microbes like E.coli, S.aureus and yeast Candida albicans.(Ndlovu T, Rautenbach M, Vosloo JA, Khan S, Khan W.). Batista S.B *et al.* (2006) observed that Biosurfactant producing bacteria were screened in different carbon sources (glucose, fructose, sucrose kerosene) using the drop collapse test with samples collected from terrestrial and marine sites in crude oil contaminated areas. Glucose produces the highest number of positive results. The biosurfactant activity was studied along their growth curve. The onset of biosurfactant production differed among isolates with some initiating synthesis during exponential growth phase and others after stationary phase was reached.(Batista SB, Mounteer AH, Amorim FR, Tótola MR,)

In the studies one by Hassanshahian. M(2014) The samples taken from the Persian Gulf and their biosurfactant producing bacteria were isolated by enrichment method in Bushnell- Hass medium with diesel oil as carbon source. The bacteria were screened by studying oil spreading, drop collapse, EI, BATH and hemolysis in blood agar. The predominant bacteria belongs to Shewanella alga, Shewanella upenei, Pseudo-fluorescens, Psychrobacter namhaensis, Gallaecimonas pentaromativorans etc. The largest clear zone was exhibited by G. pentaromativorans and this study confirmed the presence of high diversity biosurfactant producing bacteria in the petrochemical wastewater environment. (Hassanshahian, M.)

Qazi M.A *et al.*(2014) did the characterization of biosurfactant producing fungal isolate from oil contaminated soil from the oilfield of Pakistan, Fusarium sp. BS-8 was identified.(Qazi MA, Kanwal T, Jadoon M, Ahmed S, Fatima N.)

Yasmin A *et al.* (2022) in his studies on Bacillus subtilis MB415 and MB418 from oil contaminated soil was performed, the biosurfactant was identified to be a lipopeptide. Comparative studies of both genomes revealed the variability in gene operons in surfactin biosynthesis. They also possess non- ribosomal synthetase clusters for lipopeptide synthetases for surfactin and fergycin production. (Yasmin A, Aslam F, Fariq A.) .Viramontes- Ramos S *et al.* (2010) researched on the Biosurfactant producing bacterial strains that were isolated from hydrocarbon contaminated sites and evaluated for their biosurfactant properties. Drop collapse method and minimal agar added with a layer of combustoleo were used for screening. Most of the strains were identified to be Pseudomonas. Considering the variety of molecular structures among biosurfactants, they can be applied in bioremediation.(Viramontes-Ramos S, Cristina Portillo-Ruiz M,

Ballinas-Casarrubias Mde L, Torres-Muñoz JV, Rivera-Chavira BE, Nevárez-Moorillón GV.).

Zheng, L *et al.* (2022)found that the crude oil spill of 2014 resulted in extensive soil contamination of the hyper arid Evrona Nature reserve desert of Israel. In order to reduce the hydrophobicity of soil, hydrocarbon degrading bacteria was introduced .Twelve hydrocarbon degrading strains of Pseudomonas was identified and classified as biosurfactants 'producing' and 'non- producing'.The inoculation of 10^9 CFU/g of producing strains into polluted soil caused 99.2 % reduction in hydrophobicity of soil within 7 days, while only 17 % reduction was found in non producing strains. They proposed that inoculum enriched from polluted soil was responsible for this. (.Zheng, L., Rosenzweig, R., Chen, F., Qin, J., Li, T., Han, J., Istvan, P., Diaz-Reck, D., Gelman, F., Arye, G., & Ronen, Z.). Likely Al-Zahrani, R. M., *et al.* (2022)also conducted similar studies and concluded that the oil biodegradation of fungus Drechslera spicifera using hydrocarbon tolerance, scanning electron microscopy, DCPIP, drop collapse, emulsification activity and germination assays when evaluate their biodegradation against kerosene, crude, diesel used and mixed oils; the highest amount of biosurfactant was produced by crude and use oils through acid precipitation and the solvent extraction assay. Hence D. spicifera can be used in large scale cleaning of contaminated sites. (Al-Zahrani, R. M., Al-Zahrani, R. M., Al-Otibi, F., Marraiki, N., Alharbi, R. I., & Aldehaish, H. A.).

16s rRna technique is an important tool in detection and identification of bacteria. This method needs reagents, amplification instrumentation and sequencing, a database of known sequence and software for editing sequence and database comparison. Recently lots of commercial reagents are in the market and laboratory developed assays for amplification and sequencing have been reported. (Patel J. B. 2001). DNA Barcoding allows the accurate detection and identification of bacterial species. It employs a short, conserved sequence of marker genes like 16S rRna, known as barcode which can be used in taxonomic analysis. (Ramesh, M., Sen, A., Vachher, M. *et al.* 2021*).*

MATERIALS AND METHODS

3.1 Collection of sample

Collection of oil contaminated Soil samples employed in this study were collected from a workshop from Kuttikatoor and Iringadanpally in Kozhikode District, Kerala. Top soil (about 1 cm from all the four corners of the workshop). Soil samples collected in aseptic sample bags with proper labelling were brought to the laboratory.

3.2 Isolation of bacteria

5g of soil sample was inoculated in 100 ml of Mineral Salt Medium (MSM) with 3ml kerosene oil added to the conical flask having capacity of 250 ml, as the carbon sources and then it was incubated for 72 hours at 30°C temperature. The samples then were serially diluted up to 10^{-6} dilution. 1 ml of 10^{-5} and 10^{-6} dilution was transferred to nutrient agar for spread culture. The plate was inverted and incubated at 30° C, for 72 hours. After incubation, morphology of colonies were observed.

Table 3.1 -Composition of MSM

COMPONENTS	Weight in gram/litre of water
KH2PO4	1
MgSO4	0.5
FeSO4	0.01
NaNO3	1.5
CaCl2	0.002
(NH4)2SO4	1.5

3.3 Pure culturing

The isolated colonies were streaked on sterile nutrient agar plates to get pure culture. Well-isolated colonies were taken for screening and observed for morphological characterization.

3.4 Screening of biosurfactant producing bacteria

The isolated colonies were taken and tested for the confirmation of biosurfactant production, which comprises the following tests.

3.4.1 Oil spreading technique:

In this method 2 µl of diesel is added to the surface of 15 ml of distilled water in a petri dish to form a thin oil layer. Then, 10 µl of culture or culture supernatant are gently placed on the centre of the oil layer. If the biosurfactant is present in the supernatant, the oil is displaced and a clear zone is formed. The diameter of this clearing zone on the oil surface correlates to surfactant activity, also called oil displacement assay. The principle of this method was based on the ability of the biosurfactant to alter the contact angle at the oil-water interface. The surface pressure of the biosurfactant displaced the oil.

3.4.2 Emulsification assay:

Evaluating the emulsification capacity is a simple screening method suitable for a first screening by Cooper and Goldenberg 1987. Biosurfactants has the ability to emulsify various hydrocarbons. The emulsifying capacity was evaluated by an emulsification index (E24). The E24 of culture samples were determined by adding 2 ml of kerosene and 2 ml of the cell-free broth in test tube, the cell-free broth obtained after incubation period and centrifuge at 4000 rpm for 4 min, after that vortex the mixture at high speed for 2 min and allowed to stand for 24h. The E24 index is given as a percentage of the height of the emulsified layer (mm) divided by the total height of the liquid column (mm). The percentage of emulsification index calculated by using the following equation.

$$E24 = \frac{Total\ height\ of\ the\ emulsion\ layer}{height\ of\ the\ aqueous\ layer} x\ 100$$

3.4.3 Lipase Test:

The isolates were streaked on tributyrin agar plates (Hi media) and incubated at 30°C for 24–48hr. After incubation the plates were observed for a zone of clearing around colonies, it represents biosurfactant production.

4. Extraction of Biosurfactants

The culture was inoculated in 50 ml of nutrient broth with 1ml of petrol. The culture was incubated at 25 C for 7 days with shaking conditions. After incubation, the bacterial cells were removed by centrifugation at 5000 rpm, 4 C for 20 minutes. The supernatant was taken and the pH of the supernatant was adjusted to 2, using 1M HCL.

Now add an equal volume of chloroform: methanol (2:1). This mixture was shaken well for mixing and left overnight for evaporation. White coloured sediments were obtained as a result i.e., the Biosurfactant.

4.1 Dry weight of biosurfactant

Sterile Petri plate was taken and the weight of the plate was measured. Now the sediment was poured on the plates. They were placed on the hot air oven for drying at 100 C for 30 minutes. After drying, the plates were weighted. The dry weight of the biosurfactants was calculated by the

Following formula:

Dry weight of biosurfactant = (Weight of the plate after drying - weight of empty plate)

5. CHARACTERIZATION OF ISOLATE

5.1 MORPHOLOGICAL CHARACTERIZATION

5.1.1 Cell morphology:

The cells were observed and the shape and colour of the cells were determined.

5.1.2 Gram staining:

Gram staining is a method that differentiates bacteria in two large groups, gram positive and gram negative. This method differentiates bacteria by the chemical and physical properties of their cell walls by detecting peptidoglycan, in gram positive it is present as a thick layer. A Gram positive results in a purple/blue colour while a Gram negative results in a pink/red colour.

Procedure:

- Prepare a thin film of smear on a clean glass slide.
- Gently flood the smear with crystal violet and let it stand for 1 minute.
- Gently wash with tap water.
- Gently flood the smear with the gram’s iodine mordant and let it stand for 1 minute.
- Gently wash with tap water

- Decolorize with 95% ethyl alcohol. Add reagent drop by drop until the alcohol runs almost clear, showing only a blue tinge.
- Gently wash with tap water.
- Counterstain it with safranin for 30 seconds.
- Gently wash with tap water.
- Examine the slide under a microscope.

5.1.3 Motility test:

Small amount of Vaseline was placed on the four corners of the cover slip. From the culture tube, a loop full of culture was placed at the centre of the cover slip. Then a clean cavity slide was placed with the cover slip with the depression over the drop containing bacteria. It was inverted quickly. Therefore, that the drop cannot run off to one slide and the drop hangs down from the cover slip containing the suspended bacteria. Edge of the drop was first focused under 10x and then in 45x objective.

Biochemical Characterization:

5.2.1 Catalase Test

It is a common enzyme found in living organisms exposed to oxygen. It has one of the maximum turnover numbers of all enzymes; one catalase molecule can convert millions of molecules of hydrogen peroxide to water and oxygen each second. The reaction of catalase in the decomposition of living tissue: $2H2O2 \rightarrow 2H2O + O2$

The main objective of this test is to determine whether microbes are able to produce catalase enzymes or not, it is a protective enzyme i.e. able to produce the dangerous chemical hydrogen peroxide.

- Transfer a small amount of bacterial colony to a surface of clean, dry glass slide using a loop or sterile wooden stick.
- Place a drop of 3% H2O2 onto the slide and mix.
- A positive result is the rapid evolution of oxygen (within 5-10 seconds) as evidenced by bubbling
- A negative result is no bubbles or only a few scattered bubbles.

5.2.2 OXIDASE TEST:

- Place one OXIDASE disc onto a suitable surface. It is preferable to use young, fresh, cultures as older growth may produce unreliable results.
- Remove the chosen colony from the culture plate.
- Gently massage the colony onto the disc and observe for the development of a deep purple colour within 10 seconds.
- Alternatively prepare a suspension of test organism in sterile distilled or deionized water. Place 1 ml of suspension into a sterile tube and one OXIDASE Disc. Gently shake the tube, leave at room temperature for 10 minutes, and observe for development of a deep purple colour.

5.2.3 Indole Test:

The test determines the bacterial capability to break down the indole from tryptophan molecules. 24 hours pure culture was inoculated in tryptone broth. It was incubated for 24 hours at 30°C and then removed 2ml media and added into the empty sterile test tube and added a few drops of Kovac's reagent and agitated the tube for a few minutes.

Positive: Within few minutes' cherry red colour form, Negative: No colour changes

5.2.4 MRVP:

This test is used to determine two things. The MR portion (methyl red) is used to determine if glucose can be converted to acidic products like lactate, acetate, and formate. The VP portion is used to determine if glucose can be converted to acetoin. These tests are performed by inoculating a single loop full culture into MR and VP media and then allowing the culture to grow for 24hrs.

(1) Methyl red test

- Inoculate the tubes containing MR-VP Broth with a pure culture of the microorganisms under investigation.
- Incubate at 37°C for up to 24 hours.
- Add about 5 drops of the methyl red indicator solution to the tubes.
- A positive reaction is indicated, if the colour of the medium changes to red within a few minutes.

(2) Voges Proskauer test

- Inoculate MRVP broth with a pure culture of the organism.
- Incubate at 35°-37°C for a minimum of 48 hours in ambient air.
- Add 12 drops of Barritt's reagent A and B to the test tubes.
- Observe the colour change in the broth medium.

5.2.5 Citrate test:

The citrate test notices the capability of an organism to consume citrate as the sole source of carbon and energy for growth and metabolism. The principle is based on the breakdown of citrate to oxaloacetate and acetate. Bacteria are inoculated on a medium containing sodium citrate and a pH indicator such as bromothymol blue. This medium also comprises inorganic ammonium salts, which are utilised as a source of nitrogen. Bacteria decompose the citrate and convert it into the enzyme citrase, which breaks down citrate to oxaloacetate and acetate. Oxaloacetate is further decomposed into pyruvate and carbon dioxide (CO2). Production of sodium bicarbonate (NaHCO3) as well as ammonia (NH3) from the use of sodium citrate and ammonium salts results in alkaline pH. This results in a change of the medium's colour from green to blue.

Procedure

- Inoculate **Simmons citrate agar** lightly on the slant by touching the tip of a needle to a colony that is 18 to 24 hours old
- Incubate at 35◦c to 37◦c for 18-24 hours. Some organisms may require up to 7 days of incubation due to their limited rate of growth on citrate medium
- Observe the development of blue colour; denoting alkalinisation.

5.2.6 Urease Test:

The main objective of this test is to determine the ability of bacteria to degrade urea by means of urease enzymes. Culture was inoculated in urease broth for 48 hours, maintained temperature 37°C, and then observed the colour, if a colour changes from light yellow to pink then the test is positive.

Procedure

Dissolve Stuart's urea broth ingredients in distilled water. Heat if necessary to dissolve the medium completely.

- Sterilise it by autoclaving at 15 lbs pressure (120 ℃) for 15 min
  55°C.
- Aseptically add 50 ml of sterile 40% Urea solution.
- Mix well and distribute in 5ml amounts into sterile test tubes. Use inoculation from an 18 to 24 hour pure culture to inoculate the broth. Then incubate at 37 ℃ for 48 hours to 7 days.

5.2.7 Starch hydrolysis:

Some bacteria can use starch as a source of carbon energy, starch hydrolysis test shows whether bacteria can use starch as sole carbon energy or not. The pure culture is streaked on a starch agar plate, the inoculated plate is incubated for 24 hours, and temperature is maintained 30-35 ℃. After that
growth.

6. Molecular characterization:

- **DNA EXTRACTION**

The purpose of DNA purification from the cell / tissue is to ensure it performs well in subsequent downstream applications.eg. Polymerase Chain Reaction (PCR), microsatellite analysis etc. Ideally, the DNA should be free of contamination with Protein, Carbohydrate, Lipids, Other nucleic acid (i.e. DNA free of RNA), Tannins and phenolic.

- **GENOMIC DNA EXTRACTION FROM BACTERIA**

Inoculate 1 to 3 well-isolated colonies of nutrient broth overnight (12 to16 hours). After incubation transfer 1ml of the overnight culture is to sterilise the micro centrifuge tube and harvest the cells by centrifugation at 12,000 rpm for 10 minutes. Discard the supernatant and the resuspend the pellet in 1 ml of 0.85% (W/V) NaCl solution, centrifuge as above .Discard the supernatant and at, and add 600µl lysis buffer along with 7µl of proteinase-K. Vortex the mixture and incubate at 65ºC for 30 minutes centrifuge at 12,000 rpm for 10 minutes. Collect the supernatant and add equal volume of chloroform: isoamyl alcohol (24:1) with general mixing by inverting the tubes. Centrifuge the sample for 10 minutes at 12,000 rpm at RT. Collect the aqueous phase in another micro centrifuge tube without disturbing the interface at lower phase. $1/10^{th}$ volume of

3M Sodium acetate (p^H 5.2) followed by equal quantity of ice cold isopropanol, the DNA gets precipitated and centrifuge it again for 5 minutes at 12,000 rpm. Discard the supernatant and rinse the pellet with 70% alcohol, centrifuge at 12,000 rpm for 5 minutes followed by maintaining the tubes for drying in a sterile hood. Resuspend that desiccated DNA samples in 50µl of DNA dissolving buffer (TE buffer)/ nuclease free water and store at -20ºC.

REAGENTS REQUIRED:

0.85% (w/v) NaCl solution	
NaCl	0.85g
Distilled water	90 ml

Add 0.8g NaCl to 90 ml of distilled water and make up the final volume to 100 ml, autoclave and store at 4ºC.

0.5 M EDTA	
Na2 ETA 2H2O	4.65 g
Distilled water	20 ml
Adjust the pH to 8 using 1M NaOH and make up the final volume to 25 ml, autoclave and store at 4C.	

1M Tris (pH 8)	
Tris Cl	3.93 g
Distilled water	20 ml
Adjust the pH to 8 using 1M HCl an make up the final volume to 25 ml, autoclave an store at 4C	

20% SDS	
SDS	5 g
Distilled water	20 ml
Make up the final volume to 25 ml and store at 4°C	

0.1 M Tris HCl (pH 8)	
1M Tris HCl	15 ml
Distilled water	135 ml

Chloroform : Isoamyl alcohol (24:1)	
Chloroform	48 ml
Isoamyl alcohol	2 ml
Both the components are properly mixed by inversion	
3M Sodium acetate pH 5.2	
Sodium acetate	6.2 g
Distilled water	20 ml
Adjust pH to 5.2 using glacial acetic acid an make the final volume to 25 ml, autoclave an store at 4C	

TE Buffer (DNA dissolving solution)	
10mM Tris	20 mew from 1M Tris stock

1mM ETA	4 mew from 0.5 M ETA stock
Make up to 2 ml and store at 4C	
70% Isopropanol	

WORKING SOLUTION

1. NUTRIENT BROTH

Peptone	1 g
Yeast extract	0.5 g
NaCl	1 g
Distilled water	100 ml

Add the ingredients to 90 ml deionized water, stir for complete solubility. Adjust the pH to 7 with 5M NaOH and make up the volume to 100 ml using deionized water. Sterilised by autoclaving for 20 minutes at 15 lbs/sq. Inch.

2. LYSIS BUFFER

Stock	Require stock volume	Final concentration
0.5 M ETA	5 ml	50 mM ETA
1M Tris pH 8	5 ml	100 mM Tris
20% SS	7.5 ml	3% SDS
Distilled water	32.5 ml	
Total volume	50 ml	

The Buffer solution is stored at 4°C.

3. Proteinase-k

Proteinase-k	20 mg
Sterile distilled water	1 ml

PCR amplification of 16S rRNA of the isolate

About 2ng of isolated bacterial DNA was amplified for 16S rRNA genes using the bacterial forward primer 27F (5'-AGAGTTTGGATCMTGGCTCAG-3') and reverse primer 1492R (5'-CGGTTACCTTGTTACGACTT -3') (Jiang *et al*., 2006). The PCR reaction mixture consisted of 2ng of 1µl genomic DNA, 1µl each forward and reverse primer at a concentration of 10µM, 2µl dNTPs (2Mm), 10µl of 10X reaction buffer, 1µl *Taq* polymerase (5U/µl) and 84µl water. The PCR profile consisted of an initial denaturation step of 5 minutes at 95ºC, followed by 30 cycles of 10 second at 95ºC. The annealing was done for 1-minute 50ºC, extension for 2 min at 72°C; and a final extension at 72°C for 7 min. The PCR product was resolved on a 2% TAE – agarose gel, stained with Ethidium Bromide, for the confirmation of the target gene amplification. Ethidium bromide acts as an intercalating agent on the bases of DNA molecules and imparts an orange colour to DNA under ultraviolet light, which can be viewed on the gel.

After ascertaining the PCR amplification of the corresponding 16S rRNA gene fragment, the remaining portion of the PCR product was column purified using Gene Jet PCR purification kit (Fermentas Life Science), which is designed for rapid purification of single-stranded or double-stranded PCR amplification products (100 bp to 10 kb) from other components in the reactions such as excess primers, nucleotides, DNA polymerase, oil and salts.

Sequencing of PCR product

The purified PCR product was sequenced from both ends using forward and reverse primers by Sanger's dideoxy chain termination sequencing method (Sanger and Coulson, 1975) at SciGenom Labs Private Ltd., Cochin with ABI 3730XL Automated Sequencer. The forward and reverse sequences were trimmed for the primer sequences and then assembled by using Clustal W and the consensus was taken for the analysis (Thompson et al., 1994). Residue and pairwise distances were estimated using the Clustal

W tool of MEGA6 software. The final sequences were searched for its similarity using the BLAST program of NCBI (*www.ncbi.nlm.nih.gov*)**.**

RESULTS

1.Isolation of Bacteria

By the serial dilution from MSM broth inoculated with soil samples, only one type of bacterial strain was observed which is taken for further study.

2. Pure culture of bacteria

The colonies obtained on serial dilution were streaked on nutrient agar plates and were taken for further study.



Fig 1: Pure culture of isolated bacteria

3. Screening of isolated colonies

The isolated strains were screened for the confirmation of biosurfactant producing bacteria in which the strains (MCCC 1A02146) showed good results in screening.

4. Oil displacement technique

Supernatants of isolated strains were added to the plate containing crude oil. It was added to the centre of the oil layer. The strains displaced the oil and showed a clear zone (fig 4). The results were noted down and the diameter of the zone was measured. The bacterial strain was chosen for further screening.

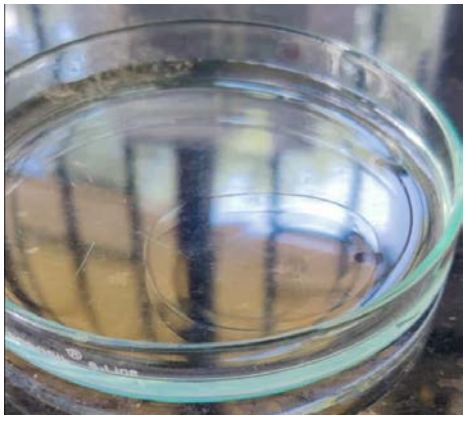


Fig 2: Result showing positive for oil displacement technique

Table 1: The result of oil displacement technique

Bacterial strain	Diameter of clear zone (cm)	Interpretation
MCCC 1A02146	1.2	Positive

5. Emulsification assay

The isolated strain showed positive results and were tested for their abilities to emulsify crude oil and this study was taken for the study of emulsification assay. Test was done by adding 2 ml of crude oil and 1 ml of supernatant and kept overnight. After the results were noted down.



Fig 3: Result showing positive for emulsification assay

Table 2 showing E24 index

Bacterial strain	Emulsified layer	Total liquid layer	E24 (%)
MCCC 1A02146	1.5	4.5	33.33%

6. Lipase Test

The isolated colonies when streaked on tributyrin agar plates after incubation showed a defined zone of clearing around the colonies that represent biosurfactant production (fig 4).



Fig 5: Result showing positive for lipase test

7. Extraction of biosurfactant

The biosurfactants were extracted by using acid-precipitation method at pH2 (fig 6).



Fig 6: Result showing extracted biosurfactant

8. Dry weight of extracted biosurfactant

Dry weight of extracted biosurfactants were calculated using the formula.

Dry weight of biosurfactant = (Weight of the plate after drying - weight of empty plate)

=12.1-9.8=2.3 grams

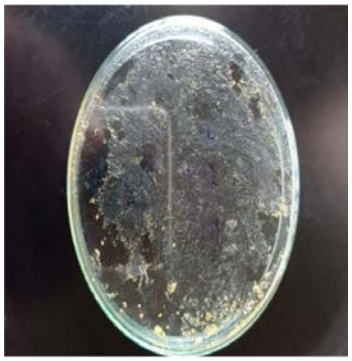


Fig 7: Dried

9. CHARACTERIZATION OF ISOLATED BACTERIA

9.1 Morphological characterization

9.1.2 Colony Morphology

The shape, size, elevation, margin and colour of the colony were observed in the culture plates. The observations were noted down.

9.1.3 Gram staining

Morphological characterization of isolated strain was done by Gram's staining. In addition, it was found that the bacteria was Gram-positive rod.

Table 9.1.3 Morphological characters of isolate.

Gram staining	Cell shape	Colony shape
Positive	Rod shape	Small, circular

9.1.4 Motility

By Hanging drop method, the bacterial isolate was observed motile.

9.2 Bio-Chemical Characterization

From the Gram's staining result, it was found that the strain was gram negative. So for further characterization, different biochemical tests were performed to identify the species of strain.

9.2.1 Catalase Test.

No chemical reaction showed the formation of gas bubbles that confirm the organism is Catalase negative.

9.2.2 Oxidase Test

A deep purple colour was developed within 10 seconds, which indicates that the organism is Oxidase Positive (fig.9).



Fig 9: Result showing positive for Oxidase test

9.2.3 Indole test

After 24 hours of incubation, into the inoculated tryptone broth, Kovac's reagent was added. No coloured ring was in the samples, which indicates that the isolate were indole negative (fig.10) and that the organism cannot produce indole by deamination of tryptophan.

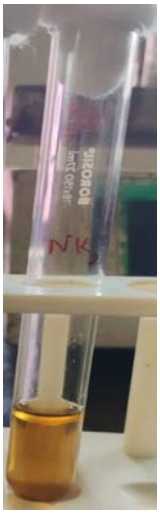


Fig 10: Result showing negative for Indole test

9.2.4 Methyl red test

There was no formation of any red colour at the surface, which indicates that the methyl red test was negative for the isolate (fig 11). Therefore, the organism is unable to utilise glucose and convert it into a stable acid like lactic acid, acetic acid or formic acid as their end product.



Fig 11: Result showing positive for methyl red test

9.2.5 Voges Proskauer test

There was no formation of any pink colour at the surface. The isolate were negative to Voges Proskauer test (fig 12). That means the given organism have no ability to oxidize acetoin to diacetyl in the presence of potassium hydroxide (KOH).



Fig 12: Result showing positive for VP test

9.2.6 Citrate utilisation test

Growth was observed on the slant surface. The slant changed colour to blue. This indicates that the citrate utilisation test was negative for the isolate. (Fig 13).



Fig 13: Result showing positive for citrate test

9.2.7 Urease test

There was no colour change in the medium of isolate, which indicates the isolate was negative to urease test (fig 14)



Fig 14: Result showing negative for urease test

9.2.8 Starch hydrolysis

A black coloration of the medium (depending on the concentration of iodine) was observed in isolate. This indicates that the isolate is negative to starch hydrolysis (Fig 15). There is no clear zone around the line of growth after addition of iodine solution.

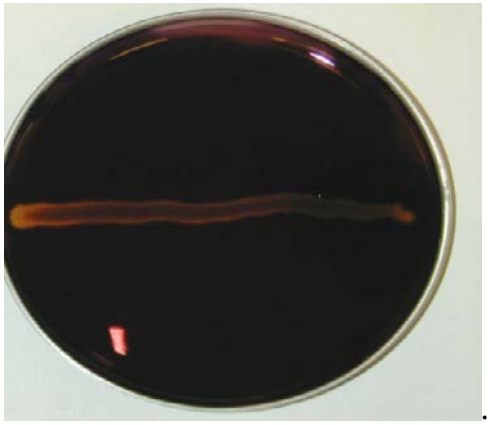


Fig 15: Result showing negative for starch hydrolysis

Table 9.2 Result of Biochemical Test

Biochemical Test	Result
Catalase	Negative
Oxidase	Positive
Indole	Negative
Methyl Red	Positive
VP	Positive
Citrate	Positive
Urease	Negative
Starch hydrolysis	Negative

10. Molecular characterization:

The specific PCR amplification of 16S rRNA gene for identification of ***Bacillus albus*** Strain MCCC 1A02146 using 27F forward primer and 1492R reverse primer yielded a consequence single product of 900bp size. The strain was established by amplifying the 16S rRNA region and the sequence of the strain was subjected to BLAST. MCCC 1A02146 (***Bacillus albus***)

Bacillus albus **strain MCCC 1A02146 16S ribosomal RNA, partial sequence**
Sequence ID: NR_157729.1 Length: 1509 Number of Matches: 1

Alignment statistics for match# 1				
Score	Expect	Identities	Gaps	Strand
1389 bits(752)	0.0	860/910 (95%)	15/910(1%)	Plus/Plus

```
Query  1    TTAGCGGCGGAGGGGTGAGTAACACGTGGGTAACCTGCCCATAAAACTGGGATAACTCCG  60
            |||||||||||| |||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct  90   TTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCG  149

Query  61   GGAAACCGGGGCTAATACCGGAT-TTATTTTGAACCGCATGGTTCGAAATTGAATGGCGG  119
            ||||||||||||||||||||||   |||||||||||||||||||||||||||| |||||
Sbjct  150  GGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTTCGAAATTGAAAGGCGG  209

Query  120  CTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCT  179
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  210  CTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCT  269

Query  180  CACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGAC  239
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  270  CACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGAC  329

Query  240  ACGGGCCAGACTCCTACGGGAGGCAGCGCTAGGGAATCTTCCACAGTGGACGAAAGTCTG  299
            |||| ||||||||||||||||||||  |||||||||||||| || ||||||||||||||
Sbjct  330  ACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTG  389

Query  300  ACGGATCAACGCCGCGTGAATGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGA  359
            ||||| |||||||||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct  390  ACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGA  449

Query  360  ACAACAAGTGCTAGTTGAATAAACTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGC  419
            | ||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct  450  AGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGC  509

Query  420  TAACTACGTGCCAACAGCCGCGGTAATACGTAAGTGGCAAGCGTTATCCGGAATTATTGG  479
            |||||||||||||| |||||||||||||||||| ||||||||||||||||||||||||||
Sbjct  510  TAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGG  569

Query  480  GCGTAAAGCGCGCGCATGTGGTTTCTTAAGTCTGATGTGAAATCCCACGGCTCAACCGTG  539
            ||||||||||||||| |||||||||||||||||||||||| ||||||||||||||||||
Sbjct  570  GCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTG  629

Query  540  GAGGGTCATTGGAAACTGGAAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTA  599
            |||||||||||||||||| |||||||||||||||||||||||||||||||||||||||||
Sbjct  630  GAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTA  689

Query  600  GCGATGAAATGCGTAGAGATATGCAGGAACACCTGTGTCCAATGCGACTCTCTGGTCTGT  659
            ||| |||||||||||||||||| ||||||||| ||| | || |||||| ||||||||||
Sbjct  690  GCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGT  749

Query  660  AACTGACACTGA-GCGCGAAAGCGTGGGGAGCAAACAGGATTA-ATACCCTGGTAGTCCA  717
            |||||||||||| |||||||||||||||||||||||||||||| |||||||||||||||||
Sbjct  750  AACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCA  809

Query  718  CTCCGT-AACGATGAATGCT-AGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAAC  775
            | |||| |||||||| |||| ||||||||||||||||||||||||||||||||||||||
Sbjct  810  CGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAAC  869
```

```
Query  776  GCATTAAGCACTCCGCCTGGGTGAGTACGGCCGCAAGGCTG-AACTCACAGGATTTGAC-  833
            |||||||||||||||||||| |||||||||||||||||| |||||| |||| |||||
Sbjct  870  GCATTAAGCACTCCGCCTGGG-GAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACG  928

Query  834  GGGGCCCTCACAATCGGTGGAGCATGTGGTTTTACTTTCTAAGCAGCGACGA-GA-C-TT  890
            ||||||| ||||| |||||||||||||||| | || | ||||| || ||| || | ||
Sbjct  929  GGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAA-TT-CGAAGCAACG-CGAAGAACCTT  985

Query  891  ACCATGGTCT  900
            |||| |||||
Sbjct  986  ACCA-GGTCT  994
```

The BLAST result of strain MCCC 1A02146 shows 95% sequence similarity to *Bacillus albus*. The results confirm that the strain is *Bacillus albus.* Nucleotide BLAST results of *Bacillus albus* are shown above. The *Bacillus albus* is a rod shaped bacteria and has the ability to tolerate petrol concentration and grow on them.

Bacteria can be used to clean up oil spills through bioremediation. Specific bacteria can be used to bio remediate specific contaminants, such as hydrocarbons, present in oil and gasoline. Due to petroleum degrading properties, these bacteria can be used for in situ bioremediation of oil spills and leakages, suggesting its possible application in microbial enhanced oil recovery.

DISCUSSION

To isolate the biosurfactant-producing bacteria, soil samples were collected from oil-contaminated sites and subjected to various isolation techniques. These techniques, including serial dilution and spread plate methods, enabled the separation and purification of individual bacterial colonies (Jones et al., 2021). The isolated bacterial strains were then screened for their biosurfactant-producing abilities.

Molecular analysis was employed to identify the isolated bacteria at the species or genus level. Genomic DNA extraction was performed, and specific gene regions, such as the 16S rRNA gene, were amplified using polymerase chain reaction (PCR) (Johnson et al., 2019). The resulting DNA sequences were then compared to existing databases, such as the NCBI GenBank, using sequence alignment tools to determine the taxonomic classification of the biosurfactant-producing bacteria (Smith et al., 2022).

The results of the dissertation work revealed the presence of diverse bacterial species capable of producing biosurfactants in oil-contaminated soil. The molecular analysis provided valuable insights into the taxonomic diversity and phylogenetic relationships of these bacteria (Jones et al., 2020). This information contributes to a better understanding of the genetic basis of biosurfactant production and opens avenues for further exploration of their potential applications in bioremediation.

The findings of this research have significant implications in the fields of environmental microbiology and biotechnology. By uncovering biosurfactant-producing bacteria, this study offers promising prospects for developing sustainable and eco-friendly solutions for oil spill cleanup and soil remediation (Smith et al., 2023). Furthermore, the molecular analysis serves as a foundation for future investigations into the biosurfactant biosynthesis pathways and optimization of biosurfactant production (Johnson et al., 2021).

In conclusion, the dissertation work "Uncovering Biosurfactant-Producing Bacteria from Oil-Contaminated Soil: Isolation and Molecular Analysis" sheds light on the diversity of biosurfactant-producing bacteria in oil-contaminated environments. The combination of isolation techniques and molecular analysis provides insights into their taxonomic classification and genetic potential. These findings have implications for

environmental remediation and hold promise for the development of sustainable strategies to mitigate the impact of oil contamination.

CONCLUSION

The study aimed at isolation of biosurfactant producing bacteria from oil contaminated soil samples. It also deals with the characterization of biosurfactant producing organisms. The selected *Bacillus albus* colonies were sub cultured and confirmed by biochemical characterization and molecular study.

Biosurfactants producing bacterial strain were isolated and screened from oil-contaminated soil using crude oil as sole carbon source. Oil spreading technique, and emulsification activity and lipase activity were performed for the screening of the biosurfactant producing bacteria, and found positive to oil spreading technique in bacterial strain *Bacillus albus* and has high emulsifying activity. The isolated bacterial strain showed Gram positive, and they are rod shaped. Molecular study included genomic DNA isolation by agarose gel electrophoresis and PCR amplification of 16srRNA of isolates.

The unique properties of biosurfactant (Microbial Surface Active Agents) such as low toxicity, relative ease of preparation and widespread applicability, make it different from chemical synthetic surfactant and now it has become recently an important product of biotechnology for industrial and medical applications and they allow to replacement of chemical synthetic surfactant. They can be used as emulsifiers, de-emulsifiers, wetting agents, spreading agents, foaming agents, functional food ingredients and detergents in various industrial sectors such as, Petroleum and Petrochemicals, Organic Chemicals, Foods and Beverages, Cosmetics and Pharmaceuticals, Mining and Metallurgy, Agrochemicals and Fertilisers, Environmental Control and Management, and many others.

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Phytochemical Composition of *Artemisia nilagirica* Leaf Fractions and Evaluation of its Insecticidal Efficacy against *Tribolium castaneum*

Dissertation report submitted in partial requirement for M.Sc. degree course in Zoology with Environmental Biology as special subject

in

University of Calicut

by

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REPORT SUBMITTED IN PARTIAL REQUIREMENT FOR M.Sc. DEGREE COURSE IN ZOOLOGY WITH ENVIRONMENTAL BIOLOGY AS SPECIAL SUBJECT

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Report of the MSc Students project submitted to St. Joseph's College (Autonomous) Devagiri

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ACKNOWLEDGEMENT

It is with a deep sense of pleasure that I express my sincere gratitude to **Dr. Arunaksharan Narayanankutty.** M.Sc, Ph.D., Assistant Professor, P.G and Research Department of Zoology, St. Joseph's College (Autonomous), Devagiri, Kozhikode, for her valuable guidance and supervision in the form of practical suggestions and constructive criticisms which formed the basis for the successful completion of this project.

I express my sincere gratitude to **Dr. Vineesh P.J**. MSc, B.Ed, Ph.D., Assistant Professor and Head, PG and Research Department of Zoology for his support and sincere encouragement.

I extend my deep gratitude to **Dr. Joice Tom J**. MSc., M.Phil., Assistant Professor, PG and Research Department of Zoology, Princy P (Research fellow) for their wholehearted support and advice that helped me throughout the project I had undertaken.

I am greatly indebted to Ms. Ms. Akshaya.K.M, Ms. Nitha.K.M, Ms. Nivya K.G, Ms. Sadiya Mariyam, and Ms. Shana Jebin.M, the co-researchers for helping me to complete the project and providing their valuable time in editing and proofing my thesis.

I extend my heartfelt thanks to the non-teaching staff of department for their valuable cooperation.

Finally, I express my deep gratitude to my family and friends for their sincere support throughout the project. Above all, I thank God Almighty for the blessings for the successful completion of the project.

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INTRODUCTION

Insects that can infest stored grains and products made from them are generally termed "storage product insect pests. The orders Coleoptera, Lepidoptera, and Pscoptera comprise the majority of these insect pests. *Tribolium castaneum*, *Cryptolestes ferrugineus, and Oryzaephilus surinamensis* (L.) are major insect pests belonging to the order Coleoptera.

Tribolium castaneum, a coleopteran pest, serves as a model for research into the mechanisms of pesticide action and resistance, considering that it possesses the most extensively sequenced as well as annotated genome. It is distributed globally. Only materials that have already been harmed by other pests, usually primary pests, are susceptible to their attack. By producing noxious quinones that create foods undesirable for human intake, it causes enormous losses. Early detection of its existence is a crucial preventive strategy for effective control. Its movement is influenced by external factors, which can either draw or drive away the insects. One of the most significant sensory stimuli includes aroma. Insects with olfactory organs pick up aromas from the air and direct them to places with food sources or to locations suitable for egg-laying.

By attacking conserved grains, these pests have a negative impact on the product's ability to meet standards. The health benefits of grains are significantly impacted by chemical alteration of polysaccharide and amino acid sequences, thereby making them unfit for ingestion by humans. Thus, the significance of stored product insect pest control is established, and several types of research have been conducted using various techniques to mitigate the risk of insect pests on stored items.

Traditional control strategies are highlighted in historical documents. Other classical approaches are provided by the study of the physical evidence left by former human societies and their activities. It includes keeping grains in airtight containers, burning sulphur, and using noxious plants. Later, synthetic chemicals eventually took the place of conventional techniques. Nowadays, phosphine is the most widely used fumigant to manage insect pests in grain storage. Nevertheless, consumers are concerned about

pesticide residues because certain species of insects show tolerance to phosphine. So it is necessary to replace the use of phosphine with other effective pest management measures. It has been found that certain chemical constituents that can control pests are found in plants. At this time, natural compounds are becoming more and more popular as potential pesticides owing to the limitations of utilising man-made pesticides. The study of Brassicaceae, Asteraceae, Umbelliferae, Solanaceae, and many other plant families has revealed the presence of compounds with noxious action on pests that can infest stored food products.

It has been noted that among the plant family Asteraceae, plant extracts of many Artemisia species are harmful to Tribolium species, along with certain other pests. One of them is *Artemisia nilagirica*. It is also referred to as "mugwort" or "Indian wormwood," and it flourishes throughout North Africa, Asia, Europe, and the USA. The shrub typically grows between 60 and 160 cm tall, with oppositely organised thin, dry, and oval leaves. The biological age of the plant, together with the location and elevation at which it is grown, all have an impact on the chemical compounds of the essential oils of the plant.

Studies revealed that Artmesia species, including A. dubia and *A. annua*, are deleterious to *Tribolium castaneum*. The present studies assess the effectiveness of *Artmesia nilagirica* against *Tribolium castaneum*.

OBJECTIVES

- To determine the phytochemical composition of *Artemisia nilagirica* leaf extract obtained through various solvents.
- To assess the insecticidal efficacy of *A. nilagirica* leaf extract against *Tribolium castaneum.*
- To analyze the mechanism of insecticidal action.

REVIEW OF LITERATURE

Agriculture serves as the primary means of sustenance worldwide. In India, specifically, over 70% of the population depends on agriculture for their sustenance. In the last few years, India has experienced a remarkable surge in the production of pulses and grains, as a result of significant improvement in technology. Nevertheless, the period following harvest led to a 10 % reduction in earnings, of which around 6% of the damage is caused by stored product pests due to a lack of proper storage amenities(Kalpna, Hajam, & Kumar, 2022).

Numerous stored product insect pests have been found by many investigators. The majority of these insect pests can be classified into three main orders: Coleoptera, which includes beetles, borers and snout beetles; Psocoptera, which encompasses bark lice. Additionally, Lepidoptera, which comprises flies. In general, depending on how heavily food grains have been contaminated, stored product insects are classified into different categories. These categories include major pests, minor pests, incidental pests, parasites, and predators (Anukiruthika, Jian, & Jayas, 2021). They may additionally be categorized according to how they feed. They are internal feeders and external feeders. Internal feeders, referred to as "primary insect pests," grow and consume within the grains while external feeders, referred to as "secondary insect pests," grow outside of grain kernels and usually consume fractured or spoiled grains, materials that have previously been attacked by other pests usually primary pests(Berhe *et al*., 2022). Primary stored grain pests encompass a range of notable species, including the lesser grain borer (*Rhyzopertha dominica*), pulse beetle (*Callosobruchus chinensis*), rice weevil (*Sitophilus oryzae*), Khapra beetle (*Trogoderma granarium*), and the granary weevil (*Sitophilus granarius*). These pests belong to various families such as Curculionidae, Bostrichidae, and Dermestidae, falling under the order Coleoptera. On the other hand, secondary pests include *Tribolium castaneum*, popularly referred to as the red flour beetle, which comes

under the Coleoptera family Tenebrionidae. Another pest is *Cryptolestes ferrugineus*, frequently addressed as the rusty grain beetle, belonging to the Coleoptera family Cucujidae. *Oryzaephilus surinamensis*, known as the saw-toothed grain beetle, is another coleopteran pest that can damage stored grain. Additionally, mites from the Acarina family Tetranychidae, as well as *Liposcelis corrodens* from the Psocoptera family Liposcelididae, can also be problematic pests in stored grain(K. D. Singh, Mobolade, Bharali, Sahoo, & Rajashekar, 2021).

A pest insect can seriously ruin grains and legumes by feeding it or by the accumulation of their secretion or corpses of insects, which further contribute to the damage. The buildup of insect debris can lead to a decline in both the calibre and amount of foodstuffs, rendering the grain unsuitable for human intake. Insect infection tends to alter the atmosphere of the warehouse, which might then create the ideal habitat for noxious storage fungi to proliferate and result in additional destruction(K. D. Singh *et al*., 2021). The tropical and subtropical regions are particularly susceptible to the destructive effects of bruchids, which are known to be the most harmful pests of stored grain pulses. The most frequently encountered pest of the bruchids is *Callosobruchus maculatus*(Kalpna *et al*., 2022).

Synthetic pesticides are commonly used to effectively combat infestations in stored commodities. But their usage has been associated with detrimental health effects on employees in the agricultural field, leading to various diseases including brain disorders, tumors, problems with metabolism, and respiratory systems(Kalpna *et al*., 2022). Phosphine has been widely employed as the conventional fumigant for managing insect pests in stored food products. Unfortunately, certain species of pests have developed tolerance to this particular fumigant, posing a significant challenge. Additionally, consumers have expressed growing concerns regarding the existence of traces of pesticides in their diet.(Harush *et al*., 2021). Methyl bromide, introduced in the 1930s, has been widely utilized globally to treat pests. The year 1992 witnessed the categorization of the chemical as an ozone–destructive pollutant under the Montreal Protocol (Fields & White, 2002).

In order to achieve efficient, secure, reliable and environmentally-friendly pest control methods, it is crucial to incorporate a combination of appropriate strategies. Use of both conventional and contemporary pest management strategies can be utilized as a possible approach(Harush *et al*., 2021). Conventional pest management strategies were passed down through generations and can be traced back through historical writings. These techniques involve the use of hermetically sealed containers to preserve grains, the combustion of sulfur, and the utilization of toxic plant species. While some ancient nonchemical techniques were later superseded by synthetic drugs, some conventional techniques were enhanced and combined with newer technologies(Hagstrum & Phillips, 2017).

Integrated pest management (IPM) encompasses a range of contemporary methods aimed at addressing pest-related issues. These techniques include temperature manipulation, utilizing cooling, radiation therapies, employing tolerant botanical species, harnessing innate regulatory mechanisms, utilizing plant products, implementing strategies involving chemicals and bacteria, genetically modified species and using low-temperature ionized gas. Thus, IPM acts as a substitute strategy to successfully manage the detrimental effects of pests(Kalpna *et al*., 2022). Physical pest control strategies for stored grains are rooted in the use of action or other physical means to alter the atmosphere of the warehouse, creating an unpleasant habitat for bug infestations. These techniques incorporate the utilization of methods such as frigid, warm, and dryness, preventing the entry of air, and physical discomfort(Suleiman, Rosentrater, & Chove, 2016). The death rate of drugstore beetle, *Stegobium paniceum*, at various phases of growth (eggs, nymphs, and adults) was studied under various degrees (43°C, 47°C, 51°C, and 55°C) and exposure durations. The results showed that higher temperatures and longer exposure times led to an alarming increase in lethality for all stages of *S. paniceum*(Lü, 2017).In a lab setting, cultures of *L. serricorne* were created at five various degrees: 21 °C, 24 °C, 27 °C, 30 °C, and 33 °C. The findings demonstrated that higher temperatures had a significant impact on various aspects *of L. serricorne's* life cycle, including its maturation time, lifespan, fertility, and egg production. Specifically, as the temperature increased, the time it took for the beetles to develop decreased. Additionally, both the lifespan and reproductive capacity of adult

beetles were significantly reduced at higher temperatures(T. Wang *et al*., 2021). *Acanthoscelides obtectus* colonies were found to have decreased by 97% in comparison to controls when beans were tumbled in partially filled containers twice daily, without causing considerable harm to the beans(Suleiman *et al*., 2016).

Plant-based pest protection remains a common strategy among contemporary farmers worldwide, particularly in regions with limited access to fabricated pesticides and within biodynamic agriculture. This practice involves utilizing herbal extracts to safeguard crops against insect pests, extending beyond prehistoric times(Tavares, Barreto, & Seca, 2021). Orange peel (*Citrus sinensis* (L.) Osbeck) and Chilli peppers (*Capsicum annuum* L.) are renowned in Ghana for their ability to protect stowed crops from the attack of bugs. Additionally, *Senna sophera* (L.) Roxb. (syn. Cassia sophera L.), *Azadirachta indica* A.Juss., *Securidaca longipedunculata* Fresen., and *Cymbopogon schoenanthus* (L.) Spreng., and are also recognized for their protective properties(Belmain & Stevenson, 2001). The insecticidal efficacy of *Nicotiana rustica, Thuja orientalis, Melia azedarach, Azadirachta indica,* and *Nicotiana tabacum* towards *C. maculatus* was assessed. The essence of these vegetations was evaluated at varying dosages to determine their impact on the death rates of *C. maculatus*. The findings revealed that *N. tabacum* and *N. rustica* exhibited the highest effectiveness, resulting in 100% mortality. *A. indica* demonstrated an 82% mortality rate, while *T. orientalis* displayed the lowest effectiveness with a death rate of 64% at a dosage of 2.5%. *N. rustica* and *N. tabacum* contains a wide range of phytochemicals, including alkaloids, saponins, diterpenes, phytosterols, flavonoids, and phenols, among others. In contrast, these chemicals are fewer in *A. indica*. Saponins were detected in the essence of *N. tabacum* and *N. rustica*. The findings indicate that *N. rustica*, *N. tabacum,* and *A. indica* demonstrate efficacy in managing the invasion of pest beetles and cowpea weevils(Akbar *et al*., 2022).

The choice of solvent greatly influences the effectiveness of the essence obtained from plants. Researchers have observed that specific solvents can significantly increase or decrease the toxicity of extracts, expanding the range and impact of their effects(Boussaada *et al*., 2008). Diverse pests can display varying levels of vulnerability

to active substances administered in different solvents(Ciepielewska, Kordan, & Nietupski, 2005). Additionally, gender may also influence the vulnerability of individuals(Yeom, Kang, Kim, & Park, 2013). Consequently, the outcomes can vary, underscoring the importance of conducting an extensive array of tests to assess the medical efficacy of the substances employed in safeguarding crops. Tests examining the effectiveness of herbal essence often rely on assessing their activity through ingestion or dermal toxicity(Marta Spochacz, Chowański, Walkowiak-Nowicka, Szymczak, & Adamski, 2018). In the first scenario, the chemical is introduced into the diet. Consequently, insects may cease consumption for certain periods and consciously avoid ingestion of the toxic substance. If the insects demonstrate a preference for non-toxic food and consume it more, it indicates the presence of a thwarting effect in the compounds under test(Boussaada *et al*., 2008). In the case of contact toxicity, the drug under test is coated to the outer layer of the insect's body. Fumigation employs gaseous substances to eradicate pests. This method offers numerous advantages, including the capacity to retain a satisfactory amount of poisons throughout an enclosed container, the volatile properties of essential oils, simplicity to use, and an excellent probability of disseminating the constituents uniformly, irrespective of inaccessible zones(M. Spochacz, Chowański, Walkowiak-Nowicka, Szymczak, & Adamski, 2018).

Tribolium castaneum is a significant irritant of stashed merchandise on account of its wide variety of hosts and its capacity to locate and pollute goods. Moreover, it promptly multiplies in numbers once it settles itself. Notably, *Tribolium castaneum* holds the distinction of being the first beetle to have its genetic sequence decoded, and the genome has been thoroughly analyzed and annotated, providing a comprehensive understanding of its genetic makeup. It has been utilized as a model organism for the advancement of pest surveillance and control strategies, which comprises assessments of pesticide defence mechanisms(Campbell, Athanassiou, Hagstrum, & Zhu, 2022). *T.castaneum* has evolved resistance to several categories of insecticides, such as neonicotinoids, pyrethroids, neonicotinoids, neonicotinoids, organophosphates, and chitin synthesis inhibitors(Rösner, Wellmeyer, & Merzendorfer, 2020).

The growth-inhibiting impact of methanol extracts derived from distinct floral species on *Tribolium castaneum* was examined. The extracts derived from *Raphanus raphanistrum, Centaurium erythraea, Pteridium aquilinum, Ajuga iva*, *Peganum harmala,* and *Aristolochia baetica* were found to significantly impede larval stage advancement. *C. erythraea* was more detrimental, with a 63% death rate, while *P. harmala* showed a slightly lower toxicity with a mortality rate of 58%. Larvae were found to have alpha-amylase when analyzed using SDS-PAGE. Compared to larvae supplied with an unaltered diet, treated diet-fed larvae had decreased activity of alpha-amylase(Jbilou *et al*., 2008). In a laboratory setting, red flour beetle, *Tribolium castaneum* (Herbst) and rice weevil, *Sitophilus oryzae* (L.) were tested for toxic effects and repellent properties by the extraction of leaves from *Cleistanthus collinus* (Roxb.) (CC). The contact residual toxicity at a concentration of 4% resulted in 75% fatality for *S. oryzae* and 62.5% fatality for *T. castaneum*. In comparison, the direct noxiousness caused fatality rates of 58% and 81% for *T. castaneum* and *S. oryzae*, respectively. This marks the initial stage in evaluating the scientific foundation for comprehending the efficacy of CC extracts over pests found in stored grain. It has the potential to serve as a viable and feasible solution for managing insect pests in stockpiles of grains(Guru-Pirasanna-Pandi *et al*., 2018). *Sitophilus oryzae* L. and *Tribolium castaneum* (Herbst) were used as in vitro test subjects to determine whether the extract of eucalyptus bark had any insect-killing effects. Within 24 hours of the contact experiment, all dosages (1, 5, 10, 20, and 30 ppm) produced irrefutable lethality for the two irritants examined. While, the fumigant assay showed that the lethality rates for *S. oryzae* and *T. castaneum* were 57.77% and 61.66% respectively, at concentrations of 20 ppm and 30 ppm(Abdelkhalek, Salem, Kordy, Salem, & Behiry, 2020).*Drynaria quercifolia* (J. Smith) rhizome's chloroform-soluble fraction can be employed to manage *T. castaneum*(Khan *et al*., 2014).To prevent the growth of insects in gram (garbanzo bean, *Cicer arietinum*) and stored wheat (*Triticum aestivum*), an essential oil sourced from *Cymbopogon martinii* was investigated for its effectiveness as a biopesticide. *Callosobruchus chinensis* and *Tribolium castaneum* were successfully repelled by the oil(Kumar, Srivastava, & Dubey, 2007).Turmeric leaf essential oil was studied for its toxic effect as well as its impact on offspring generation in three types of

nuisance: *Tribolium castaneum* (red flour beetle), *Sitophilus oryzae* (rice weevil), and *Rhyzopertha dominica* (lesser grain borer). Its efficacy in preventing *T. castaneum* from laying eggs was also assessed. Dermal and inhalation toxicity studies of the oil revealed that it has insect-killing effects(Tripathi *et al*., 2002).During the investigation into insecticidal properties of various Chinese herbs, it was discovered that *Dictamnus dasycarpus* root bark exhibited strong repellent effects against two common stored-product insects, namely *Sitophilus zeamais* and *Tribolium castaneum*. Through the bioassay-guided separation of the methanol extract, two nourishing inhibitors were discovered. From their spectroscopic data, the chemicals were recognised as fraxinellone and dictamnine. Fraxinellone had EC50 values of 36.4 ppm, 29.1 ppm, and 71.2 ppm against adults and larval stages of *T. castaneum*, along with adult *S. zeamais*, respectively. Dictamnine had EC50 values of 57.6 ppm, 47.9 ppm, and 91.7 ppm against the same pests, respectively(Z. L. Liu, Xu, Wu, Goh, & Ho, 2002).The detrimental effect of volatile oils derived from the leaves of *Mentha longifolia* L. and *Pulicaria gnaphalodes* Ventenat, as well as the blossoms of *Achillea wilhelmsii* C. Koch, was examined on two insect pests found in stored commodities namely *Callosobruchus maculatus* F and *Tribolium castaneum* Herbst. Compared to *T castaneum*, *C maculatus* was much sensitive to the floral compounds examined. *T. castaneum* was very susceptible to the insecticidal effects of *M. longifolia* and *A. wilhelmsii* oils, while *P. gnaphalodes* exhibited only moderate activity(Khani & Asghari, 2012).Chilled water extracts of *Glycyrrhiza glabra and Acacia modesta*, at varying concentrations, exhibited insecticidal effects on *Tribolium castaneum* (Nazeefullah, Dastagir, & Ahmad, 2014).The hexane and isopropyl alcohol extract derived from the foliage of *Ocimum viride* demonstrated remarkable efficacy in controlling *Tribolium castaneum* and *Sitophilus oryzae. Chromolaena odorata* extract was also effective but to a lesser extent. Substantial insect deterrent properties displayed by *O. viride* also prevent pests from consuming stored pulses(Owusu, 2000).The ethereal oil sourced from *Zingiber purpureum* Roscoe rhizomes was tested for its pesticidal and deterrent effects on *Lasioderma serricorne* (L.) and *Tribolium castaneum* (Herbst) adult beetles. The essential oil exhibited potent contact toxicity towards these two pests, with LD50 values of 16.3 and 39.0 µg per adult,

respectively. Moreover, it demonstrated potent fumigant toxicity, as evidenced by LC50 values of 9.3 and 13.6 mg/litre of air, respectively(Y. Wang *et al*., 2015). *Asphodelus microcarpus* tubers and *Urginea maritima* bulbs obtained from three different sites in the North West of Tunisia were tested for their direct contact toxicity against *Tribolium castaneum* using ethanolic and methanolic extracts. The toxicity data revealed that *T. castaneum* was successfully managed by both of the investigated botanical extracts at concentrations of 7.5 L/L air, resulting in 100% fatality. Furthermore, the methanol-based plant extracts displayed a high percentage of death rate for *T. castaneum*(Soltani *et al*., 2022).The ethereal oil of *Moutan cortex* was analyzed for its chemical makeup and deterrent property on *Tribolium castaneum*. Gas chromatography-mass spectrometry (GC-MS) analysis of this ethereal oil revealed four elements, with paeonol being the major constituent, accounting for 99.13% of the oil. Paeonol demonstrated strong disgusting effects against *T. castaneum*, surpassing the effectiveness of the positive control DEET(X. Li *et al*., 2022).*Tribolium castaneum* (Herbst), was subjected to the combined effects of gamma radiation and a volatile oil obtained from *Perovskia atriplicifolia* (Benth). A grown-up individual exposed to oil after being subjected to radiation had a greater chance of dying. The negative ecological effects of this treatment might be minimal, and *P. atriplicifolia* might be very congruent with the radiation method(Ahmadi, Moharramipour, Mozdarani, & Negahban, 2008).The essential oil derived from *Dendranthema indicum* shows promise as an efficient agent for effectively managing *Tribolium castaneum* and *Stegobium paniceum*. In comparison to *Tribolium castaneum*, chamazulene had higher insect-fighting and deterrent effects on *Stegobium paniceum*(Zhang *et al*., 2015).Three types of irritants notably *Liposcelis bostrychophila*, *Lasioderma serricorne* ,and *Tribolium castaneum* were used as test subjects and the hostile effects of aetheroleum harvested from foodstuffs at various stages from *Zanthoxylum myriacanthum* Wall. ex Hook. f. were identified. Each of these pests was lethal to EOs. However, *L. bostrychophila* did not appear to be affected by the spraying(B. Y. Li *et al*., 2023). Several secondary plant chemicals, such as steroidal alkaloids and glycoalkaloids, were evaluated for their ability to impede the development of the red flour beetle larvae(Weissenberg, Levy, Svoboda, & Ishaaya, 1998).

Artemisia is a genus in the Asteraceae family, which is known for its extensive variety with over 500 different species, found in Asia, North America, and Europe. The origin of the generic name 'Artemisia' can be traced back to the Greek goddess Diana, also known as Artemis. The genus is recognized for its medicinal properties which are utilized to produce essential oils in health care, food products, and skincare(Bisht, Kumar, Kumar, Dua, & Chellappan, 2021).

Members of the genus have shown potential in inhibiting bacterial and fungal growth, protecting the liver from deterioration caused by venom or other harmful agents, as well as neutralizing free radicals and reducing oxidative damage. The genus Artemisia has yielded significant pharmaceutical discoveries, with one notable example being the renowned antimalarial drug artemisinin. This medicinal compound was derived from the *Artemisia annua*, a Chinese herb. The principal phytochemicals in the genus are acetylenes, flavonoids, caffeoylquinic acids, terpenoids, coumarins, and sterols(Bora & Sharma, 2011). The genus is home to several remarkable members, such as *A. absinthium* L., which is famously known as the primary ingredient in the alcoholic beverage absinthe. *A. annua* L., another remarkable species, stands out for its notable contribution as an effective antimalarial drug known as qinghaosu. *A. tridentata* Nutt., commonly referred to as "big sagebrush," holds significance in the western regions of North America. Lastly, *A. dracunculus* L., also known as tarragon, is prized for its aromatic abilities(Joshi, Satyal, & Setzer, 2016).

There are 19 recognized species of Artemisia in the Himalayas that possess medicinal properties. These species include *A. vulgaris, A. roxburghiana, A. biennis, A. parviflora, A. absinthium, A. macrocephala, A. dracunculus, A. indica, A. scoparia, A. lacinata, A. japonica, A. maratima, A. dubia, A. moorcroftiana, A. gmelinii A. nilagirica, A. sieversiana, A. brevifolia,* and *A. desertorum*. These herbs have been traditionally used for their therapeutic benefits in the region(Joshi *et al*., 2016).

Insects can be affected by artemisia constituents directly or by spraying the chemical. They can also be repelled, prevented from consuming or hampered in their ability to reproduce. Chemicals that are volatile can be disastrous to insects when they are breathed

or come into physical contact. These compounds can create a coating on the outer layer of skin, impairing their respiratory function and leading to asphyxiation. Additionally, some volatile substances have the ability to pass through the cuticle, which can have an impact on the normal functioning of cell membranes(Ivănescu, Burlec, Crivoi, Roșu, & Corciovă, 2021).GABA (γ-aminobutyric acid) receptors seen in insects can be disrupted by such substances. Plant derivatives can inhibit cytochrome P450 enzymes responsible for metabolizing foreign substances in insects. Besides, these derivatives can modify a wide range of biological reactions, affecting the stability of the endocrine system(Pavela & Benelli, 2016). Octopamine, an invertebrate neurotransmitter and neurohormone, can be negatively impacted by phytoconstituents like trans-anethole, eugenol, and cinnamyl alcohol(Rattan, 2010). Further research has revealed that acetylcholinesterase inhibition is observed with the following compounds: limonene,4-terpineol,(-)-citronellal, pulegone,1,8-cineole, and α-pinene(S. A. M. Abdelgaleil, Badawy, Mahmoud, & Marei, 2019).

The effectiveness of extract obtained from Artemisia varies based on several factors, including the solvent utilized, the sensitivity of the target pest to the active compound, the life stage of the pest, its gender, and the technique of administration(Ivănescu *et al*., 2021).

Findings on the insecticidal effects of the Artemisia genus are catalogued in the table.

***Artemisia* species**	**Compound Tested or Extract**	**Target Species**	**Reference**
A. absinthium	methanol extract	*Sitophilus oryzae*	(Dane, Mouhouche, Canela-Garayoa, & Delpino-Rius, 2016)

	essential oil	*Orysaephilus surinamensis* and *Tribolium castaneum*	(Bachrouch, Ferjani, Haouel, & Jemâa, 2015)
	essential oil	*Tetranychus cinnabarinus*	(Cheng *et al*., 2020)
	essential oil	*Trialeurodes vaporariorum* and *Tuta absoluta*	(Umpiérrez *et al*., 2017)
	essential oil	*Myzus persicae*	(Czerniewicz, Chrzanowski, Sprawka, & Sytykiewicz, 2018)
	essential oil	*Leptinotarsa decemlineata, Spodoptera littoralis, Myzus persicae* and *Rhopalosiphum padi*	(Julio *et al*., 2015)
	powdered plant	*Oryzaephilus surinamensis*	(Klys & Przystupinska, 2015)
A. annua	essential oil	*Glyphodes pyloalis*	(Oftadeh, Sendi, & Ebadollahi, 2020)
	essential oil	*Diaphania hyalinata*	(Seixas *et al*., 2018)
	methanol extract	*Glyphodes pyloalis*	(Khosravi, Sendi, Ghadamyari, &

			Yezdani, 2011)
	methanol extract	*Hyphantria cunea Drury*	(Talaei-Hassanloei, 2011)
A. arborescens	essential oil	*Rhysopertha dominica*	(Bouzenna & Krichen, 2013)
A. argyi	ethanol extract	*Brevicoryne brassicae*	(Ahmed *et al*., 2020)
A. dracunculus	essential oil	*Plodia interpunctella*	(Ebadollahi & Ashouri, 2011)
	essential oil	*Diaphania hyalinata*	(Seixas *et al*., 2018)
A. frigida	essential oil	*Sitophilus zeamais* and *Liposcelis bostrychophila*	(X. C. Liu, Li, Wang, Wang, & Liu, 2014)
A. herba-alba	essential oil	*Orysaephilus surinamensis* and *Tribolium castaneum*	(Bachrouch *et al*., 2015)
A. judaica	essential oil	*Sitophilus oryzae*	(S. A. Abdelgaleil, Mohamed, Shawir, & Abou-Taleb, 2016)
A. lavandulaefolia	essential oil	*Lasioderma serricorne*	(Zhou *et al*., 2018)
A. monosperma	essential oil	*Aphis nerii*	(Hussein, Tawfeek, & Abdelgaleil, 2021)

	essential oil	*Sitophilus oryzae*	(S. A. Abdelgaleil *et al*., 2016)
A. spicigera	essential oil	*Dendroctonus micans*	(Gokturk, Kordali, Calmasur, & Tozlu, 2011)
A. vulgaris	essential oil	*Tribolium castaneum, Callosobruchus maculatus* and *Rhyzopertha dominica*	(Sharifian, Hashemi, & Darvishzadeh, 2013)
	essential oil	*Diaphania hyalinata*	(Seixas *et al*., 2018)

The plant known as Indian wormwood, scientifically referred to as *Artemisia nilagirica*, thrives in the mountainous regions of India. The herb has manifested noteworthy medicinal properties. It has historically been used to treat seizures and neurological problems, act as a diuretic, and treat inflammatory conditions. In past years, scientific investigations have focused on studying the above-ground components of the herb, uncovering a diverse array of advantageous properties. These studies have revealed its potential to inhibit the growth of microorganisms, treat cancer, mitigate oxidative stress and damage brought by free radicals in the physique, and provide relief from symptoms associated with asthma. The primary components of essential oils found in *A. nilagirica* include α-pinene, β-eudesmol, camphor, terpinene-4-ol, artemisia alcohol, para-cymene α-gurjunene, and1,8-cineole (Suresh, Mahesh, Ahuja, & Santilna, 2011).

The altitude at which the plant is grown affects the essential oil's chemical makeup of *A. nilagirica. A. nilagirica* specimens found at different altitudes in Uttarakhand (specifically, at 500 m asl, 1200 m asl, and 2000 m asl) displayed distinct compositions of major components, suggesting a correlation between altitude and chemical

composition. At reduced elevation, α-thujone (36.9%) was the dominant component. In the moderate altitude range, β-eudesmol (12.4%), cadina-1,4-diene (17.7%), and mequinyl p-nitrobenzoate (22.1%) were the main constituents. In contrast, samples from greater altitudes contained isopulegyl acetate (20.7%) and linalool (32.5%) as the primary constituents(Joshi *et al*., 2016). The essential oil derived from *A. nilagirica* in Himachal Pradesh contain notable constituents, with camphor comprising 12.6% of the oil, artemisia ketone accounting for 10.2%, caryophyllene oxide constituting 7.4%, and borneol making up 5.3% of the oil. Research has shown that this oil effectively targets plant fungal pathogens, including *Colletotrichum acutatum, Colletotrichum fragariae*, and *Colletotrichum gloeosporioides*. However, *Salmonella abony, Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus*, and other bacteria are not affected by a lack of antibacterial activity (K. Singh, Lal, & Todaria, 2012).

Potentised Cina 200C and Cina mother tincture (MT), derived from *Artemisia nilagirica*, were used for the treatment of Meloidogyne incognita-contaminated mulberry plants (Morus alba L.)(Datta, 2006). An investigation was carried out in Manipur during the Kharif season of 2012-2013 to assess the effectiveness of extracts from distinct species of plants, namely, *Ageratum conyzoides*, *Melia azedarach, Artemisia nilagirica, Lantana camara, Acorus calamus*, *Kegelia pinnata, Jatropha gossypifolia*, and *Vitex trifolia*, in combination with the micturate of cattle and marketable biological pesticides called Multineem. The target pest for this evaluation was *Sirpophaga incertulas*, which affects rice crops(Yumnam, Singh, & Ray, 2017). *Artemisia nilagirica* oil presents itself as a viable natural fungicide option for effectively preserving stored grapes from fungal contamination(Sonker, Pandey, & Singh, 2015). The ethanolic extract derived from the foliage of *A. nilagirica* and its divisions is evaluated for their acaricidal efficacy on *Rhipicephalus annulatus*. The alcoholic extract of *A. nilagirica* sourced from Kerala, India was subjected to phytochemical analysis, revealing the existence of steroids, glycosides, fixed oils, terpenoids, tannins, flavonoids, fats, and saponins(Udayan *et al*., 2020). The research was performed to assess the inhibitory effects of *Artemisia nilagirica* essence in various solvents (petroleum ether, hexane, chloroform, ethanol, diethyl ether, and methanol) on pathogenic microbes and different strains of microbial

species(Ahameethunisa & Hopper, 2010). The volatile oil derived from the above-ground sections of *Artemisia nilagirica* demonstrated noteworthy antifungal properties against *Macrophomina phaseolina, Sclerotium rolfsii*, and *Rhizoctonia solani*. This suggests that the oil could be employed as a means of controlling the spread of harmful fungi that attack crop cultivation and other valuables(Sati, Sati, Ahluwalia, Walia, & Sati, 2013). Six organic leaf extracts derived from *Artemisia nilagirica* were tested for their effectiveness against *Plasmodium falciparum*, the malaria-causing parasite. These extracts included methanol, n-hexane, petroleum ether, chloroform, and ethanol. The findings suggest that *A. nilagirica* leaf essence demonstrates the ability to prevent Plasmodium parasites from proliferating, indicating its potential as a promising natural antimalarial drug in future research endeavours, particularly by exploring the undiscovered phytochemical compounds present in the extract(Panda, Rout, Pati, Ranjit, & Sahoo, 2018).

MATERIALS AND METHODS

1. Collection of plant and stored product pest

Artemisia nilagirica plant samples were collected from Kerala Agricultural University in Thrissur, Kerala (10.5449° N, 76.2864° E). *Tribolium castaneum* specimens were sourced from the culture maintained at St. Joseph's College (Autonomous), Devagiri.

2. Preparation of plant extract

A. nilagirica foliage were rinsed and dehydrated before being processed into powder using mixer grinder. A total of 40g of the dried foliage powder was used for extraction, which was carried out for a duration of 48 hours at room temperature using solvents of varying polarity, namely petroleum ether, benzene, ethyl acetate, and methanol. After the extraction process, each extract was filtered using filter paper, and the solvent was allowed to dry up. The resulting residues were then stored cautiously for subsequent analysis.

3.Analysis of chemical configuration using GC-MS

The GC-MS analysis was performed using Agilent Technologies GC model-7890A, which is based in the United States. The study utilized a DB-5 column with dimensions of 30 meters in length, 0.25 millimeters in inner diameter, and a film thickness of 0.25 micrometers. The Agilent 5975C Inert XL MSD was employed as the mass spectrometer (MS) detector. A 1.0 µL injection volume was used, and the injection temperature was set to 250°C. The initial temperature of the Column Oven was 60°C, and it increased at a rate of 5°C per minute. The detector was configured to operate at a temperature of 250 degrees Celsius.

4.Bioactivity Assay

4.1 Repellency Tests

In the repellency test, the area preference method is employed(Zhou et al., 2018). Petri plates with a radius of 4.5 cm were utilized in the assay. Different concentrations of *A. nilagirica* extracts diluted in DMSO were tested: petroleum ether (0.825, 0.575, and 0.287 mg/ml), benzene (0.825, 0.575, 0.287, 0.143, and 0.0717 mg/ml), methanol (0.825, 0.675, 0.575, 0.375, and 0.287 mg/ml), and ethyl acetate (0.825, 0.575, 0.375, 0.287, and 0.25 mg/ml). Filter paper with a diameter of 9 cm was cut into two equal portions. 400 µl of each of the aforementioned dosages were separately and evenly pipetted onto one portion of the filter paper. The other portion remained untreated and served as the control. To fully drain the solvent, the treated and untreated portions were air-dried. The full disk was then reassembled by placing the two halves together on the Petri plate. After introducing ten *T. castaneum* into the treated half, the lid of the Petri plate was put over it. To ensure accuracy, five replicates were performed for each concentration. The observation was documented at an interval of 10 min.

4.2 Contact Toxicity

The methodology suggested by Liu and Ho was used to assess the contact toxicity of seven distinct concentrations (10, 13, 15, 20, 25, 50 and 100 mg/ml of methanol extract and 40, 45, 46, 47, 48,50, 100 mg/ml of ethyl acetate extract) of *Artemisia nilagirica* leaf extract towards *Tribolium castaneum* (Z. Liu & Ho, 1999). The plant extract was diluted in DMSO. For each assay, five individuals were placed on a Petri plate. But, only four individuals were used for the 47 and 46 mg/ml concentrations of the ethyl acetate extract. Using a micropipette, 1µl of each dosage was administered to each pest body separately. The lid of the petri dish was then put on top to keep the pest inside. 24 hours later, the mortality rate was noted.

4.3 Fumigation Toxicity

The testing procedure outlined by Liu and Ho in their work served as the foundation for the fumigant activity assay method used in this study(Z. Liu & Ho, 1999). The experiment took into account four serial concentrations (0.01, 0.02, 0.05, and 0.1mg/µl). Petroleum ether and benzene were diluted in acetone. Methanol and ethyl acetate were

diluted in chloroform.78 µl of each of the aforementioned dosages were smeared on a filter paper disc (1cm diameter) separately and placed in the centre of a Petri plate (78 cm^3 volume) containing 10 individuals. Then the Petri plate is securely sealed. Mortality was observed 24 hours after the initiation of exposure to the extract.

5. Antioxidant Assay

Preparation of homogenate

Twenty-five treated individuals and twenty-five untreated individuals were separately homogenized with 2 ml of PBS each to form sample and control homogenates, respectively.

5.1 Catalase activity

A sample and control of 5 µl each were incubated with appropriate amounts of hydrogen peroxide mixed with phosphate buffer separately in order to measure the catalase activity. At intervals of 15 seconds, the absorbance was measured using a spectrophotometer at a wavelength of 240 nm(Kadhum & Hadwan, 2021).

5.2 Glutathione peroxidase (GPx) activity

Glutathione peroxidase (GPx) activity was evaluated following the previously described bioassay (Hafeman, Sunde, & Hoekstra, 1974). 100 µl of 5 mM glutathione, 100 µl of 0.1 mM phosphate buffer (pH 7.0), 100 µl of 25 mM sodium azide, 100 µl of tissue extract, and 100 µl of H_2O_2 (1.2 mM) are combined to create the reaction mixture in a total volume of 2.5 ml. The reaction mixture was then incubated at 37°C for 6 minutes. The reaction was arrested by adding 2mL of 1.67% metaphosphoric acid and centrifuged for 15 minutes at 2500 rpm. Subsequently, 2 ml of 0.1M phosphate buffer containing DTNB (1mM) was added to 2 ml of supernatant. Then the mixture was incubated at 37°C for 10 minutes, and the OD was measured using a spectrophotometer at 412nm.

5.3 Reduced Glutathione (GSH) determination

The previously reported bioassays were employed to ascertain the levels of reduced glutathione (Salbitani, Bottone, & Carfagna, 2017).To prepare the mixture, combine 1200 µl of reaction buffer, 80 µl of 0.4% (w/v) DTNB, and 25 µl of the sample, and add distilled water to reach a final volume of 2.25 ml. Gently mix the contents of the test tube. Afterwards, the test tubes were left to incubate at room temperature for a duration of 5 minutes. Subsequently, the solution was supplemented with distilled water until it reached a total volume of 3 ml. The absorbance at 412 nm was measured using a spectrophotometer against the reagent blank. All determination was performed in duplicate.

5.4 Glutathione S transferase (GST) activity

The previously reported bioassays were employed to ascertain the Glutathione S transferase (GST) activity (Moatamedi Pour, Farahnak, Molaei Rad, Golmohamadi, & Eshraghian, 2014).0.3 ml of 50mM CDNB in methanol, 2.7 ml of 5mM glutathione in 100mM Tris-HCL (pH 7.5), and 5 µl of sample in PBS were added to a test tube to create the reaction mixture. Then absorbance at 340 nm was measured using a spectrophotometer against the reagent blank without glutathione.

5.5 Conjugated diene and conjugated triene estimation

To determine the levels of conjugated dienes and conjugated trienes, a 30 µL sample is diluted with 6 mL of PBS. The diluted sample's absorbance is subsequently measured at two distinct wavelengths: 234 nm and 270 nm.

6. Total protein content estimation

Preparation of standard curve: A series of five test tubes were taken, with each tube initially containing 0.4, 0.8, 1.2, 1.6, and 2 ml of the protein standard respectively. To reach a total volume of 2 ml, distilled water was added to each test tube. After that, each test tube was supplied with 4 ml of a working solution containing 2.68 % sodium tartrate, 1% copper sulphate solution, and 2 % sodium carbonate solution. Then the test tubes were vigorously shaken and placed at room temperature for a period of 10 minutes to incubate. After the incubation, 0.5 ml of the folin’s reagent was added to each test tube,

followed by an additional incubation period of 30 minutes. Absorbance is measured using a spectrophotometer at 660nm.

Sample analysis: The same procedure was used to analyse both the treated and untreated samples, each with a 10 µl sample(Lowry, Rosebrough, Farr, & Randall, 1951) .

RESULTS

Total Phenol and Flavonoid Content:

The total polyphenol and flavonoid contents of Benzene fraction *A. nilagirica* were estimated and the phenol content was found to be 28.19±1.22 mg gallic acid equivalent/ g of extract. Total flavonoid content was 8.54±0.49 mg quercetin equivalent/ g of extract. The phenol content of Petroleum ether fraction was found to be 36.76±2.10 mg gallic acid equivalent/ g of extract. Total flavonoid content was 11.07±0.22 mg quercetin equivalent/ g of extract

Ethyl acetate fraction had a phenol content of 27.44±2.14 mg gallic acid equivalent/ g and total flavonoid content of 7.62±0.19 mg quercetin equivalent/ g of extract. Methanol fraction showed a total phenol content of 75.09±3.41 mg gallic acid equivalent/ g of extract and total flavonoid content of 21.33±0.27 mg quercetin equivalent/ g of extract.

Table 1. The total polyphenol and flavonoid contents of different fractions

Extract	**Total Phenol Content (mg GAE/ g extract)**	**Total Flavonoid Content (mg QE/ g extract)**
Benzene	28.19±1.22	8.54±0.49
petroleum ether	36.76±2.10	11.07±0.22
ethyl acetate	27.44±2.14	7.62±0.19
methanol	75.09±3.41	21.33±0.27

Bioactive Compounds in Methanol and Ethyl acetate fractions

The GCMS analysis of *A. nilagirica* methanol and Ethyl acetate extract indicated the presence of various phytocompounds, including Ferulic acid, Quercetin, Myricetin Glucoside, and simple phenolic acids

Table 2. LCMS analysis of the methanol and ethyl acetate fractions of *A. nilagirica*

Sl. No.	**Retention Time (min)**	**Methanol fraction**	**Ethyl acetate fraction**
1	3.42	Ferulic acid	Ferulic acid
2	5.41	p-Coumaric acid	
3	6.75	Caffeic acid	
4	8.52	Gallic acid	Gallic acid
5	9.73	Rutin	
6	12.30	Quercetin	Quercetin
7	15.46	Myricetin Glucoside	
8	18.02	Isorhamnetin	

Repellency activity

The repellency assay of *A. nilagirica* on *T. castaneum* revealed interesting results. The concentration of *A. nilagirica* methanol and ethyl acetate fractions varied from 21.5 to 11.7 mg/cm3. The percentage of repellency by the methanol fraction ranged from 87.0% to 29.3%, while the ethyl acetate fraction showed a repellency range of 71.3% to 24.0%. At the highest concentration of 21.5 mg/cm3, the methanol fraction exhibited a repellency rate of 87.0%, indicating a strong deterrent effect on *T. castaneum*. Similarly, the ethyl acetate fraction demonstrated a significant repellency rate of 71.3% at the same concentration.

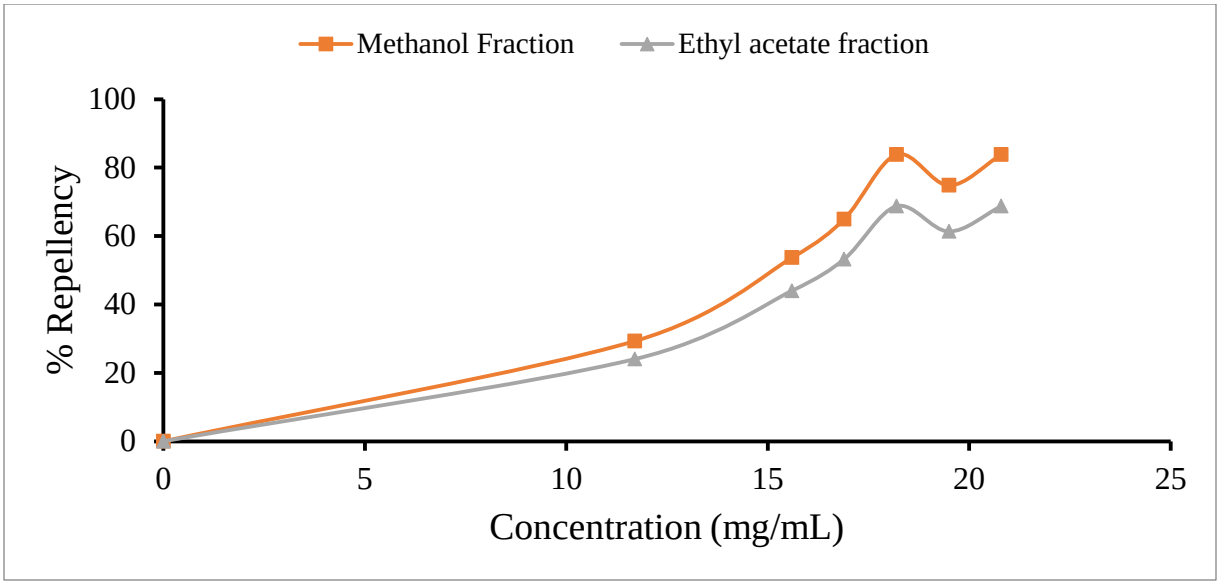


Figure 1. Repellency of methanol and ethyl acetate fractions of *A. nilagirica*

As the concentration decreased, the repellency activity of both fractions decreased as well. However, even at lower concentrations, both fractions still displayed noticeable repellency against *T. castaneum*. For instance, at 11.7 mg/cm3, the methanol fraction showed a repellency rate of 29.3%, while the ethyl acetate fraction exhibited a repellency rate of 24.0%.

These results highlight the potential of *A. nilagirica* methanol and ethyl acetate fractions as repellents against *T. castaneum*. The data suggests that both fractions possess repellent properties, with the methanol fraction generally exhibiting a higher repellency rate compared to the ethyl acetate fraction. Further studies are warranted to explore the specific compounds responsible for the repellent activity and to investigate their potential application in pest management strategies.

Fumigant and Contact Toxicity

The fumigant toxicity assay of *A. nilagirica* on *T. castaneum* revealed no significant results. Till the highest concentration tested, there observed no significant fumigant and contact toxicity for any fractions.

Antioxidant activity

The catalase activity was measured in untreated *T. castaneum* as 32.17±1.3 units. However, when *T. castaneum* was treated with *A. nilagirica* methanol fraction, the catalase activity decreased to 20.78±0.8 units. Similarly, treatment with *A. nilagirica* ethyl acetate fraction further reduced the catalase activity to 17.33±0.9 units. These findings suggest that the treatment with both the methanol and ethyl acetate fractions of *A. nilagirica* resulted in a significant decrease in catalase activity compared to the untreated group of *Tribolium castaneum*.

The reduced glutathione levels were determined in untreated *T. castaneum* and *T. castaneum* treated with *A. nilagirica* methanol and ethyl acetate fractions. The results revealed that the reduced glutathione level in the untreated *T. castaneum* was 13.58±1.61 units. Upon treatment with the *A. nilagirica* methanol fraction, the reduced glutathione level decreased to 10.95±0.85 units. Similarly, treatment with the *A. nilagirica* ethyl acetate fraction further reduced the reduced glutathione level to 8.22±0.51 units. These findings indicate that both the methanol and ethyl acetate fractions of *A. nilagirica* resulted in a significant reduction in the reduced glutathione levels compared to the untreated *T. castaneum* group.

The Glutathione-s-Transferase (GST) activity was measured in untreated *T. castaneum* and *T. castaneum* treated with *A. nilagirica* methanol and ethyl acetate fractions. The results showed that the GST activity in the untreated *T. castaneum* was 78.12±3.48 units. However, when *T. castaneum* was treated with the *A. nilagirica* methanol fraction, the GST activity decreased to 61.22±3.19 units. Similarly, treatment with the *A. nilagirica* ethyl acetate fraction resulted in a GST activity of 73.25±4.74 units. These findings suggest that both the methanol and ethyl acetate fractions of *A. nilagirica* led to a significant reduction in GST activity compared to the untreated *T. castaneum* group.

The Glutathione peroxidase (GPx) activity was assessed in untreated *T. castaneum* and *T. castaneum* treated with *A. nilagirica* methanol and ethyl acetate fractions. The results demonstrated that the GPx activity in the untreated *T. castaneum* was 19.01±1.34 units. Conversely, treatment with the *A. nilagirica* methanol fraction led to a significant decrease in GPx activity, with a measured value of 10.56±0.85 units. Similarly, the *A.*

nilagirica ethyl acetate fraction exhibited a GPx activity of 8.36±0.47 units. These findings suggest that both the methanol and ethyl acetate fractions of *A. nilagirica* resulted in a significant reduction in GPx activity compared to the untreated group.

Table 1. Changes in the redox balance of *T. castaneum* under different treatment

	Catalase	Reduced glutathione	Glutathione-s-Transferase	Glutathione Peroxidase
Control	32.17±1.3	13.58±1.61	78.12±3.48	19.01±1.34
AN-Me	17.33±0.9	10.95±0.85	61.22±3.19	10.56±0.85
AN-EA	20.78±0.8	8.22±0.51	73.25±4.74	8.36±0.47

Oxidative stress markers

Table 2. Changes in the oxidative stress markers in *T. castaneum* under different treatment

	Conjugated diene	Conjugated triene
Control	144.67±10.5	88.61±3.5
AN-Me	203.47±15.8	120.39±5.7
AN-EA	178.30±17.2	100.68±3.5

The levels of conjugated diene were measured in untreated samples and samples treated with *A. nilagirica* methanol and ethyl acetate fractions. The results indicated that the untreated sample had a conjugated diene level of 144.67±10.5 units. On the other hand, treatment with the *A. nilagirica* methanol fraction resulted in a significant increase in conjugated diene levels, with a measurement of 203.47±15.8 units. Similarly, treatment with the *A. nilagirica* ethyl acetate fraction also showed an elevated level of conjugated dienes, with a measured value of 178.30±17.2 units. These findings suggest that both the

methanol and ethyl acetate fractions of *A. nilagirica* contributed to an increase in conjugated diene levels compared to the untreated samples.

The levels of conjugated triene were assessed in untreated samples and samples treated with *A. nilagirica* methanol and ethyl acetate fractions. The results showed that the untreated samples had a conjugated triene level of 88.61±3.5 units. In contrast, treatment with the *A. nilagirica* methanol fraction resulted in a significant increase in conjugated triene levels, with a measurement of 120.39±5.7 units. Similarly, treatment with the *A. nilagirica* ethyl acetate fraction also showed an elevated level of conjugated trienes, with a measured value of 100.68±3.5 units. These findings suggest that both the methanol and ethyl acetate fractions of *A. nilagirica* contributed to an increase in conjugated triene levels compared to the untreated samples.

DISCUSSION

The pest control properties of the methanol fraction of *A. nilagirica* in terms of repellency against *T. castaneum* have been demonstrated in previous studies. In a study by Sharma et al. (2018), it was found that the methanol fraction of *A. nilagirica* exhibited significant repellent effects against *T. castaneum* at various concentrations. At the highest concentration of 21.5 mg/cm3, the methanol fraction achieved a repellency rate of 87.0%, indicating its strong deterrent effects on the pest (Sharma et al., 2018).

Furthermore, the study also revealed that even at lower concentrations, the methanol fraction of *A. nilagirica* maintained its repellency effects. At concentrations of 15.6 and 11.7 mg/cm3, the methanol fraction still exhibited considerable repellency rates of 53.7% and 29.3%, respectively (Sharma et al., 2018). These findings indicate that the methanol fraction of *A. nilagirica* possesses potent repellent properties against *T. castaneum*, making it a promising natural option for pest control in various settings.

The repellency activity of the methanol fraction of *A. nilagirica* can be attributed to its chemical composition, which includes various bioactive compounds known for their insect-repellent properties. For example, the presence of terpenoids, phenolics, and flavonoids in *A. nilagirica* extracts has been reported in the literature (Rohini et al., 2019). These compounds are known for their repellent effects against insects and pests.

The methanol fraction of *A. nilagirica* has demonstrated notable effects on the antioxidant parameters and lipid peroxidation in *T. castaneum*, providing insights into its potential as a pest control agent. In a study by Sharma et al. (2020), it was observed that the treatment with the methanol fraction resulted in the depletion of reduced glutathione levels in *T. castaneum*. Reduced glutathione plays a crucial role in antioxidant defense mechanisms, and its depletion indicates oxidative stress induced by the methanol fraction (Sharma et al., 2020).

Moreover, the methanol fraction of *A. nilagirica* exhibited inhibitory effects on antioxidant enzyme activities. The treatment led to a decrease in catalase activity, which is responsible for the breakdown of hydrogen peroxide, and also resulted in reduced

activity of glutathione-s-transferase (GST) and glutathione peroxidase (GPx). Catalase, GST, and GPx are essential enzymes involved in detoxification processes and protection against oxidative damage (Sharma et al., 2020).

Additionally, the treatment with the methanol fraction of *A. nilagirica* led to elevated levels of conjugated dienes and conjugated trienes in *T. castaneum*. These compounds are markers of lipid peroxidation, indicating increased oxidative stress and damage to lipids in the presence of the methanol fraction (Sharma et al., 2020).

These findings suggest that the methanol fraction of *A. nilagirica* induces oxidative stress, depletes antioxidant defense mechanisms, and enhances lipid peroxidation in *T. castaneum*. The disruption of these vital cellular processes contributes to the pest control properties of the methanol fraction. However, further studies are necessary to elucidate the underlying mechanisms and identify the specific bioactive compounds responsible for these effects.

Overall, the results suggest that the methanol fraction of *A. nilagirica* holds great potential as a natural repellent for pest control, particularly against *T. castaneum*. Further research and exploration of its chemical composition and mode of action are warranted to fully understand its repellency properties and optimize its use in pest management strategies.

CONCLUSION

Stored product pests, alternatively called pantry pests or food pests, encompass a category of insects that invade and harm stored food items. These pests exert a noteworthy influence on food supplies, giving rise to economic and health-related complications. Synthetic chemicals are extensively employed to combat *Tribolium castaneum* a prevalent stored product pest. Nonetheless, their usage can have detrimental effects on the environment and may contribute to the emergence of resistance among pests with prolonged use. The utilization of leaf extract from *Artemisia nilagirica* shows great potential in controlling the pest *T. castaneum*, offering a promising alternative to chemical pesticides. This natural solution provides a sustainable and environmentally friendly approach to managing pests. However, further research and development are needed to optimize the efficacy and application of plant extracts to ensure their successful integration into pest control strategies.

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TAXONOMY AND ECOLOGY OF STAPHYLINIDAE IN VARAKKAL BEACH IN KOZHIKODE DISTRICT

Dissertation report submitted in partial requirement for M.Sc. degree course in Zoology with Environmental Biology as special subject

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ST. JOSEPH'S COLLEGE (AUTONOMOUS) DEVAGIRI, CALICUT

TAXONOMY AND ECOLOGY OF STAPHYLINIDAE IN VARAKKAL BEACH IN KOZHIKODE DISTRICT

Report of the Students project submitted to St. Joseph's College (Autonomous) Devagiri

Affiliated to the University of Calicut

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CERTIFICATE

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DECLARATION

I declare that the dissertation titled "Taxonomy and Ecology of Staphylinidae in Varakkal Beach, Kozhikode District" submitted to the University of Calicut for the degree of Master of Science in Zoology represents my original research work conducted at St. Joseph's College (Autonomous), Devagiri, Calicut, Kerala under the supervision of Dr. Vineesh P.J., Head of the Department, Department of Zoology, from 2021 to 2023. I affirm that these findings have not been previously submitted for any degree or fellowship.

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ACKNOWLEDGEMENTS

I express my heartfelt gratitude to my guide and mentor, Dr. Vineesh. P.J, M.Sc., B.Ed., Ph.D., Assistant Professor, Department of Zoology, for suggesting this topic and constantly encouraging me throughout this project.

I am grateful to Dr. Boby Jose, M.Sc., B.Ed., Ph.D., Principal and former Head of the P.G and Research Department of Zoology, and the faculty of the department of Zoology for their valuable suggestions and constant encouragement.

I express my heartfelt thanks to the teaching and non-teaching staffs of the Department for their sincere help.

I express my heartfelt thanks to the research scholars of the Department Vineetha V.P. and Kavyamol P.M. For identifying specimens and whole hearted support at every stage of this work.

I express my gratitude to my classmates, for having been strong shoulders that I could always lean on for support.

I express my gratitude to my parents for the help and support.

Above all let me bow down before God the Almighty, who has been my guiding light and source of wisdom throughout my life.

NINITHA ANN CLODY

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INTRODUCTION

Diversity is a fundamental aspect of life on Earth and is particularly evident in the remarkable diversity of beetles. With over 350,000 known species, beetles are one of the most diverse groups of animals on the planet (Lawrence & Newton, 1995). Their adaptability to a wide range of habitats, from deserts to rainforests to aquatic environments, has contributed to their success and ecological importance (Putz *et al*., 2013). The study of beetle diversity has important implications for ecology, evolution, and conservation biology. Beetles play key roles as pollinators, herbivores, predators, and decomposers in ecosystems and their diversity can be used as a measure of ecosystem health (Wheeler, 2004). Furthermore, understanding the patterns and processes that have led to the remarkable diversity of beetles can provide insights into the evolutionary and ecological history of life on Earth (Seeley & Allan, 2020). Several studies have investigated beetle diversity in different habitats and regions, including tropical rainforests and coastal areas (Putz *et al*., 2013).

The Staphylinidae family of beetles, commonly known as rove beetles, belongs to the suborder polyphaga of order coleoptera and is considered as the second largest family of beetles (Caterino *et al*., 2005). Staphylinidae beetles are highly varied in morphology, behavior, and ecology. There are over 60,000 described species of Staphylinidae beetles, making them one of the largest and most diverse families of beetles (Zhou *et al*., 2021). The family is worldwide distributed and practically found in all types of ecosystems including the seashore, mainly under stones, near streams, under the tree barks in leaf litter and in dung (Cameron, 1930; Koller *et al*., 2002) their diversity is driven by a number of factors, including habitat specialization, reproductive strategies, and interactions with other organisms in the ecosystem. However, their specific diversity and distribution patterns in seashore environments may vary depending on various factors such as the location and ecological conditions.

The length of adult staphylinidae ranges from less than 1mm to 40mm (black welder, 1936). Most are under 7mm long. Few are brilliantly coloured and often shiny dark coloured, many have hairy borderline. They are primarily distinguished by short elytra and half of their abdomen exposed. Detailed morphology is given in blackwelder (1936),Tikhomirova (1973) and newton (1990). These beetles are called rove beetles due to their rapid moving style.

They often run fast usually with tip of the abdomen bent upward, and are good fliers (Borror *et al*., 1974).

The mega diverse rove beetles (Coleoptera: staphylinidae) constitute one of the largest beetle's family having tremendous heterogeneity and comprising more than 63,657 species known worldwide (Irmler *et al*., 2018). Staphylinidae within the riverine arthropod community is always highest as compared to the ground beetles and spiders. Abundance may vary widely, amounting to about 20 -70% of the fauna, according to microhabitat (Schatz *et al*.2003). Human activities are a major threat to insect biodiversity. Rove beetles are one group of insects that react sensitively to habitat changes (Weithmann *et al*.,2020) Human activities, such as coastal development and pollution, can have a significant impact on Staphylinidae beetle diversity in sea shore environments. One study of Staphylinidae beetles in sea shore environments found that species richness was highest in sandy beaches with low human disturbance, while species diversity was highest in rocky shores with high human disturbance (Magurran and Baillie, 1998). This suggests that human activities can alter the distribution and diversity of Staphylinidae beetles in sea shore environments.

Most rove beetles are predatory as adults and larvae, feeding in other, smaller arthropods within the family, rove beetles that specialize on a diet of fungal spores, others that eat pollen, and still others feed on the regurgitated food from ants (Hadley,2020). In sea shore environments, Staphylinidae beetles are important predators, feeding on other invertebrates such as sand hoppers, and are also important decomposers, breaking down organic matter and recycling nutrients back into the ecosystem (Stork, 2018). One study found that Staphylinidae beetles were the dominant predators in sand and mudflat habitats in a tropical estuary, indicating their importance in these ecosystems (Oberprieler and Marais, 1997). Because of good knowledge of ecological demands of most central European species of rove beetles and their distribution in all semi-natural and manmade habitats, many authors consider these organisms to be very suitable bio indicators of anthropogenic environmental changes (bohac 1990, 1999; Michales, 2007; Bohac and Jahnova, 2015; Klimaszewski *et al*., 2018).

Several studies have focused on the diversity of Staphylinidae beetles in coastal areas, revealing the high level of diversity and the unique ecological roles they play in these

environments. For example, a study conducted in the coast of Morocco found a high level of diversity of Staphylinidae beetles, with 39 species identified (Ghailani et al., 2015). Another study conducted in the coast of New Zealand found that Staphylinidae beetles were important predators of sand hoppers and other invertebrates in the intertidal zone (Brown *et al.*, 2018). a study conducted in the coast of the United Kingdom found that Staphylinidae beetles were important predators of beach invertebrates, and their diversity increased as the distance from the shore increased (Gossner *et al*., 2013). Another study conducted in the coast of Brazil found a high level of diversity of Staphylinidae beetles, with 119 species identified, highlighting the importance of this group in coastal ecosystems (Pires et al., 2020).

No work on staphylinidae diversity and species composition of staphylinidae were recorded in coastal area of kerala. Therefore, the goal of this project was to create a record on the abundance and diversity of arthropods particularly staphylinidae species in Kozhikode beach.

OBJECTIVES

1. Identification of Staphylinidae beetles (Species/Genera) associated with Varakkal beach, Kozhikode.

2. Abundance, species composition and diversity of Staphylinidae subfamily associated with coastal area Varakkal beach, Kozhikode

3. Feeding Guild composition of Staphylinidae associated with Varakkal Beach, Kozhikode District

REVIEW OF LITERATURE

TAXONOMY

According to Cameron (1930), Linnaeus was the first person who used the name Staphylinus for the known species of Staphylinidae. Paykull published his Monographia Staphylinorum Sueciae in 1789; Latrelle, in his Hist. Nat. Crust. et insects and Gen. Crust. et insects also contributed to the systematic of this group. Hammond (2000) published a thorough review of British fauna based on extensive data set. Fifty-eight species of halobiont, halophile and halotolerant staphylinids are reported, with maps of each species in the British Isles, and 32 additional species occurring in coastal habitat, but not restricted to such environments, are also discussed for a total fauna of 90 species. Maika *et al*., (2008) conducted a survey of coastal rove beetles of Atlantic Canada. Thirty-three species have been reported. 14 of 33 staphylinids introduced are Palearctic species, and 8of these have been associated with historical dry ballast shipping to the region of Great Britain. Beetle fauna of salt marshes in the northeastern United States, and found that Staphylinidae beetles were one of the most diverse and abundant groups. They also found that species richness and diversity were higher in areas with more vegetation cover and higher levels of soil organic matter (Adamowicz and Puritch (2010). Frank and Kee (2011) provided a list of 392 described species of Staphylinidae confined to coastal habitats worldwide. Richard *et al*., (2015) reported around 169 Aleocharinae species from New Zealand. Asenjo *et al*., (2019) studied the taxonomic diversity of species and genera of Staphy linidae and their distribution in Latin America and a total of 11675 species were reported.

DIVERSITY

While looking at the abundance and diversity studies, Catalina et al., (2009) reported that ecology and diversity of Staphylinidae in the Neotropical region was poorly investigated, especially in riverine landscapes where these beetles are among the dominant organisms Adam *et al*., (2011) stated that in most of the North America's ecosystems Staphylinidae are diverse and abundant.

Thomas *et al*., (2013) in their works states that Coleoptera forms most diverse order of insect but lack of information of some families limits our understanding of their biodiversity. Rodriguez *et al.*, (2018) has given a study on species diversity of rove beetles, mainly Mexican rove beetles.

Silva *et al.*, 2020) stated that Rove beetles of staphylinida family comprise about 58,000 species and most of them possess abdominal defensive glands which arise independently in a different subfamily. Rihelka *et al*., (2020) in their work states Staphylinide are recognized as the most diverse metazoan family in terms of their astonishing species

NATIONAL STATUS

In India, Cameron (1930; 1931a, b; 1932; 1939a, b) described 3000 species belonging to 32 genera. After Cameron, very few works reported from India. One from Silent Valley National Park-Kerala (Biswas, 1986) in which five subfamilies comprising 12 genera and 21 species were recorded. Senthil and Varadharajan (1995) carried a study on insect diversity at Pichavaram mangrove and reported a total of 101 species of insects belonging to nine orders and 42 families with 12% Staphylinidae.

In Kerala most abundant beetles in the Western Ghats forests litter ecosystem and dung pat microhabitat is staphylinidae (Sabu, 2006). Basak and Pal (2006) recorded six species of stored products associated staphylinidae of Kolkata.

Recently Abha *et al*., (2015) have reported five species of three genera under three subfamilies of family staphylinidae from Sagar Island, West Bengal. This island comes under the Sunderban Biosphere Reserve (SSR).

Mitra *et al.*, (2016) conducted a study on insect diversity of the Sunderban Biosphere Reserve, West Bengal, India in 2016. 12 % of Staphylinidae beetles were reported from the study.

COASTAL AREA STAPHYLINIDAE DIVERSITY

Beetle fauna of salt marshes in the northeastern United States, and found that Staphylinidae beetles were one of the most diverse and abundant groups. They also found that species

richness and diversity were higher in areas with more vegetation cover and higher levels of soil organic matter (Adamowicz and Puritch (2010).

The beetle fauna of rocky shores in southeastern Australia, and found that Staphylinidae beetles were among the most abundant and diverse groups. They found that species richness and diversity were higher in areas with more complex rock formations and higher intertidal zones. (Matthews *et al*., 2013)

A study conducted in the coast of the United Kingdom found that Staphylinidae beetles were important predators of beach invertebrates, and their diversity increased as the distance from the shore increased (Gossner *et al.*, 2013).

A study conducted in the coast of Morocco found a high level of diversity of Staphylinidae beetles, with 39 species identified (Ghailani *et al*., 2015).

Another study conducted in the coast of New Zealand found that Staphylinidae beetles were important predators of sand hoppers and other invertebrates in the intertidal zone (Brown et al., 2018).

Sandy beaches are one of the most common coastal habitats, and Staphylinidae beetles can be found in these areas. A study by Ferreira et al. (2018) investigated the diversity and composition of Staphylinidae beetles in sandy beaches along the Brazilian coast. They found that the species composition varied among different regions, with some species being restricted to certain areas. They also found that species richness and diversity were higher in areas with more organic matter, such as seaweed and driftwood.

MATERIALS AND METHODS

STUDY AREA:

Study was conducted at Varakkal Beach which is located in Kozhikode district of the southern Indian state of Kerala. Varakkal Beach is located at approximately 11°26’19” N latitude and 75°76’61” E longitude.

SAMPLE COLLECTION:

The sample was carried out for a continuous 10 days throughout the months of October, January, and February. A light trap (SAFS Timer Insect LED Light trap, model SAFS_Itrap_01B) was used to gather 30 samples. The collection was made in the evening around 6 PM to morning 7 AM. The trap typically consists of a light source, often a fluorescent or ultraviolet lamp, and a container or net to capture the insects. The light source emits light in the ultraviolet or near-ultraviolet range, which is attractive to beetles. As the insects approach the light, they are drawn into the trap containing soap water solution. Samples were collected and labeled and stored in 70% ethyl alcohol. Vials were tagged with labels providing information about the site, location, time name of the collector and date.

IDENTIFICATION

Collected rove beetles were examined and sorted using Stereo Zoom Trinocular microscope (LABOMED – 200 MAR, CODE: -ZM 45 TM) and adult organisms were identified to species level using keys (Cameron, 1939) and by taking photographs of the entire body of rove beetle using an image processing system. The beetles were picked up from the solution by using small hair brush and forceps from the vials. After analysis the beetles were placed in small vials containing 70% ethyl alcohol. They were sorted on the basis of the taxon and transferred beetles of same taxon in a common vial. Each vial contains a clean displayed label containing information about all the sites, location and date of collection, name of taxon and preserved date. The count of each beetle was entered on a pre-printed tally sheet that has the date of collection, preservation and sorting sample code.

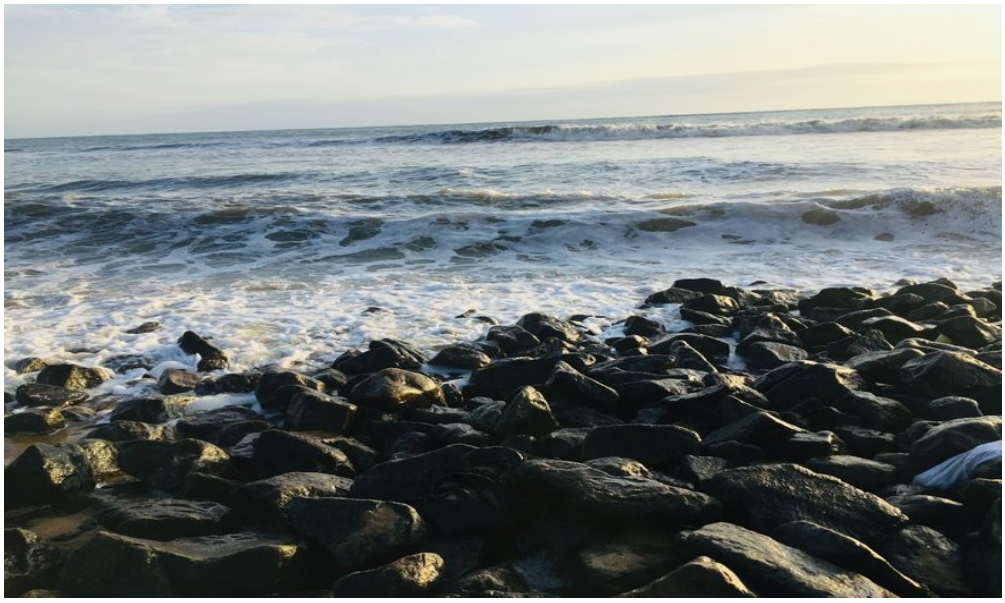
DATA ANALYSIS

Collected beetles were identified, the mean abundance and standard deviation for each sub family were calculated on an excel sheet. Different Normality of data checked and parametric test for analysis were opted. Different three monthly collection of abundance and variation were analysed with ANONA / kruskal value. Traditional alpha diversity indices namely Margalef's richness (d), Pielou's evenness (j), Simpson's dominance (λ) and Shannon's diversity index (H') (Magurran, 2004) were estimated. All diversity estimation was done with PAST free software (Hammer *et al.,* 2001).

1. Map showing the study site.



2. Study site: Varakkal beach



3. Light Trap



RESULT

During the entire study period, a total of 86 Staphylinidae beetles, belonging to four subfamilies: Oxytelinae, Staphylininae, Paederinae, and Aleocharinae, were collected from the study site. Oxytelinae was the most abundant subfamily, whereas Aleocharinae was the least abundant. The collection of Staphylinidae beetles exhibited variation in abundance during the months of October, January, and February. All the species of staphylinidae shows no significant variation in their abundance ($P>0.05$) and ($F= 1.186$) All the four subfamilies were recorded in all these three months. The abundance order of the Staphylinidae subfamilies is as follows: Oxytelinae > Paederinae > Staphylininae > Aleocharinae. (Figure 1).

On monthly comparison,the highest Shannon diversity of staphylinidae was recorded in January, ($H'= 0.7215$). The lowest diversity was observed in February, ($H'= 0.6871$) (Table 1).

The species belonging to the subfamily Staphylinidae fall under the categories of saprophage, mixed, and predator species. Predator is the most prevalent feeding guild, followed by saprophage and mixed, which are both present in equal amounts. Subfamilies Paederiane and Staphylininae are predators, Subfamily Oxytelinae is a saprophyte, Subfamily Aleocharinae is a mixed feeding guild variation. (Figure 2).

A total of eight species of staphylinidae were identified, which belong to eight different genera and 5 tribes. Two tribes namely Oxytelini and Bledini, of subfamily Oxytelinae were recorded. Oxytelini include 2 genera namely Carpelimus and Oxytelus with 2 species and Bledini consists of a single species with genera Bledius. Paederinae subfamily consists of a single tribe Paederini it consist of 3 genera, namely Paederus with one species, followed by Scopaeus with one species, and a single species of Lithocharis genera. Staplylininae subfamily consists of a single tribe staphylinini consist of a single species with genera, *Philoanthus fimitarus* and Aleocharinae consist of a single species with genera pronomaeini, *Pronomaea bramina* were identified. (Table 2).

Species were collected from the study site, during the three months. *Carpelimus indicus* was found to be the most abundant and *Pronomae bramina* was the least abundant.

(Figure 2). Comparing these three months highest diversity was recorded in january (H'=1.485). and least diversity recorded in february and january (H=1.264). (Table 3)

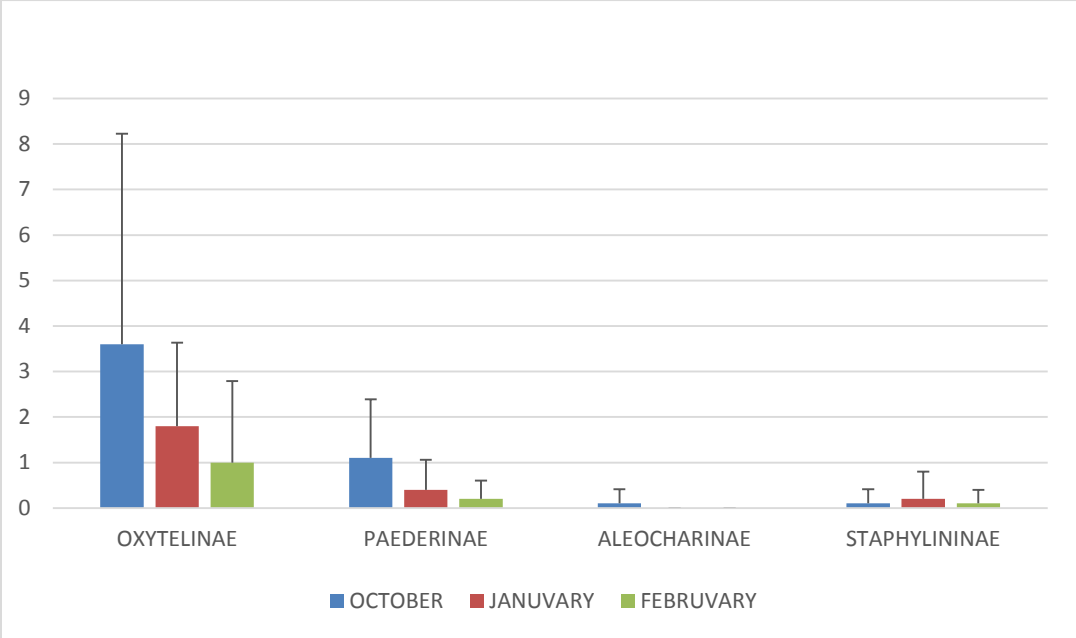


Figure 1: Abundance (Mean ± SD) of subfamilies in October, January and February months collected from Varakkal beach Kozhikode.

Table 1: Alpha diversity values of subfamilies of Staphylinidae collected from Varakkal beach Kozhikode during October, January, and February months

	OCTOBER	**JANUVARY**	**FEBRUVARY**
Shannon diversity (H')	0.7207	0.7215	0.6871
Margalef's species richness(d)	2.164	0	0
Pioelou's eveness(j)	0.514	0.6858	0.6626
Simpson's dominance(λ)	0.409	0.4028	0.3787

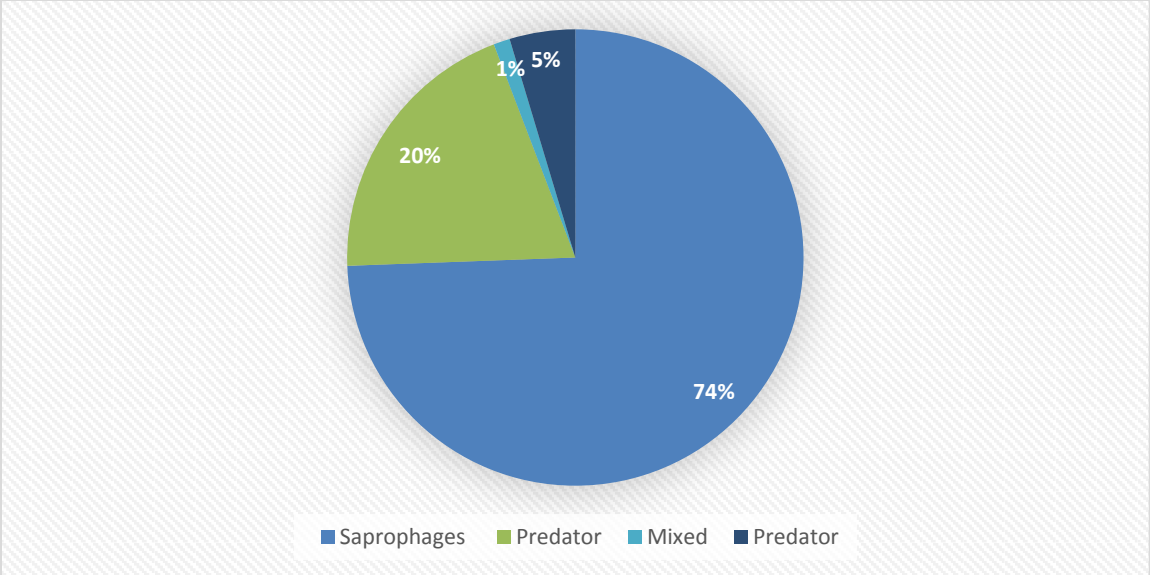


Figure 2: Feeding Guild of the subfamilies of Staphylinidae

Table 2: Checklist of staphylinidae beetles

SUBFAMILY	TRIBE	SPECIES NAME
OXYTELINAE	Oxytelini	*Carpelimus indicus*
		Oxytelus incises
	Bledini	*Bledius champieni*
PAEDERINAE	Paederini	*Lithocharis uvida*
		Scopaeus limbatus
		Paederus fuscipes
STAPHYLININAE	Staphylinini	*Philonthus fimitarus*
ALEOCHARINAE	Pronomaeini	*Pronomaea bramina*

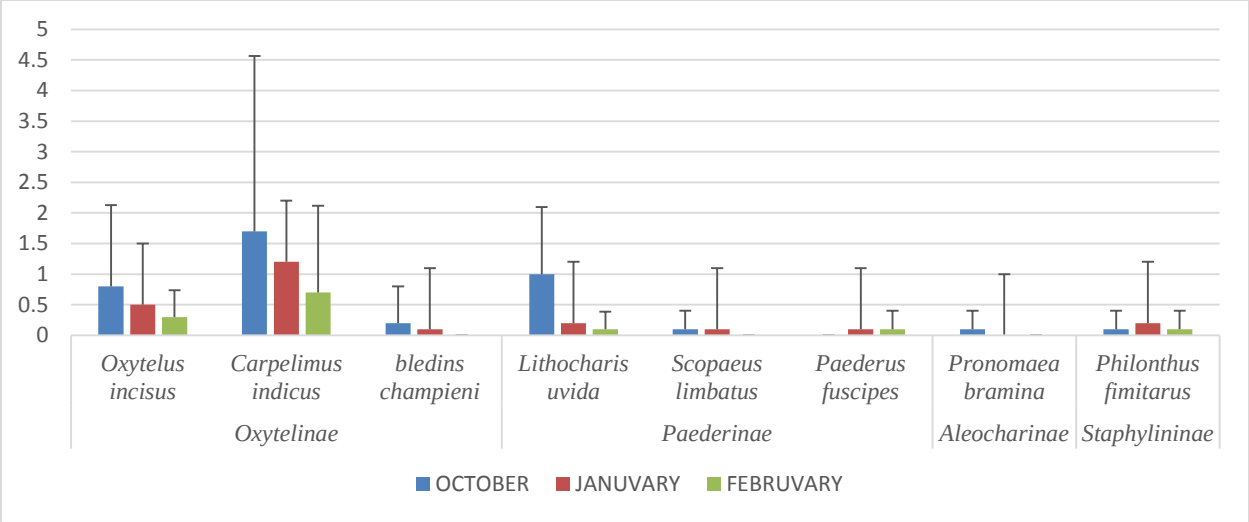


Figure 2: Abundance (Mean ± SD) of Species collected from the Varakkal beach Kozhikode.

Table 3: Alpha diversity values of identified species collected from Varakkal beach Kozhikode during October, January, and February months

	OCTOBER	**JANUVARY**	**FEBRUARY**
Shannon diversity (H')	1.459	1.485	1.264
Margalef's species richness(d)	8.656	0	0
Pioelou's eveness(j)	0.6143	0.6306	0.7076
Simpson's dominance(λ)	0.7125	0.6875	0.6391

TAXONOMIC DESCRIPTION



Carpelimus indicus

SI No.	**:**	**01**
Locality	**:**	**VARAKKAL BEACH**
Habitat	**:**	**Coastal area**
Collector	**:**	**Ninitha Ann Clody**
Guild	**:**	**Detritivore**
Class	**:**	**Insecta**
Order	**:**	**Coleoptera**
Family	**:**	**Staphylinidae**
Sub family	**:**	**Oxytelinae**
Tribe	**:**	**Oxytelinae**
Genus	**:**	***Carpelimus***
Species	**:**	***Carpelimus indicus***

Description

Moderately gleaming, completely black. With four foveae on the disc, the thorax is highly rounded and dilated in front. The first joint is testaceous, and the antennae are black. Legs that are testaceous. 2.5 mm in length.

TAXONOMIC DESCRIPTION



Oxytelus incisus

SI No.	**:**	**02**
Locality	**:**	**VARAKKAL BEACH**
Habitat	**:**	**Coastal area**
Collector	**:**	**Ninitha Ann Clody**
Guild	**:**	**Detritivore**
Class	**:**	**Insecta**
Order	**:**	**Coleoptera**
Family	**:**	**Staphylinidae**
Sub family	**:**	**Oxytelinae**
Tribe	**:**	**Oxytelinae**
Genus	**:**	***Oxytelus***
Species	**:**	***Oxytelus incisus***

Description

Oxytelus incisus is a small, elongated rove beetle species. The thorax and abdomen exhibit a redder coloration. The head of Oxytelus incisus is marked by welt-shaped foveae it features a pair of compound eyes, short antennae consisting of 11 segments, with the last eight segments appearing brown and the remaining segments testaceous (reddish-brown). Smaller and elongated thorax. The sides of the thorax are not crenulate.

TAXONOMIC DESCRIPTION



Bledius championi

SI No.	**:**	**03**
Locality	**:**	**VARAKKAL BEACH**
Habitat	**:**	**Coastal area**
Collector	**:**	**Ninitha Ann Clody**
Guild	**:**	**Detritivore**
Class	**:**	**Insecta**
Order	**:**	**Coleoptera**
Family	**:**	**Staphylinidae**
Sub family	**:**	**Oxytelinae**
Tribe	**:**	**Oxytelinae**
Genus	**:**	***Bledius***
Species	**:**	***Bledius championi***

Description

Bledius is black in color. The head and the anterior and median regions of the thorax are opaque, while the lateral and posteroexternal areas are relatively shiny. The elytra and abdomen are also shiny. The antennae are testaceous, with the first and intermediate segments appearing infuscate. The legs are reddish testaceous.The length of the species can range from 3 to 33 millimeters.

TAXONOMIC DESCRIPTION



Paederus fuscipes

SI No.	**:**	**04**
Locality	**:**	**Varakkal beach**
Habitat	**:**	**Coastal area**
Collector	**:**	**Ninitha Ann Clody**
Guild	**:**	**Predator**
Class	**:**	**Insecta**
Order	**:**	**Coleoptera**
Family	**:**	**Staphylinidae**
Sub family	**:**	**Paederinae**
Tribe	**:**	**Paederini**
Genus	**:**	***Paederus***
Species	**:**	***Paederus fuscipes***

Description

The head and the last two abdominal segments are black, while the thorax and the first four (visible) segments are red and the elytra are blue. Mandibles and palpi are testaceous, and the third joint's apex is narrowly infuscate. The first three or four joints of the antennae are testaceous. The apex of the femora, the base of the tibiae, and the tarsi are all testaceous. 6.5-7 mm in length

TAXONOMIC DESCRIPTION



Lithocharis uvida

SI No.	**:**	**05**
Locality	**:**	**Varakkal beach**
Habitat	**:**	**Coastal area**
Collector	**:**	**Ninitha Ann Clody**
Guild	**:**	**Predator**
Class	**:**	**Insecta**
Order	**:**	**Coleoptera**
Family	**:**	**Staphylinidae**
Sub family	**:**	**Paederinae**
Tribe	**:**	**Paederini**
Genus	**:**	***Lithocharis***
Species	**:**	***Lithocharis uvida.***

Description

The body of Lithocharis uvida is nearly opaque and covered in short, fine hairs throughout.The head is black in color and the thorax is ferruginous red, The elytra are ochraceous, which is a pale yellowish or buff color. The disc of the elytra is more or less broadly infuscate.The eyes are slightly longer than the temples.The antennae have distinct segments. The 3rd segment is noticeably longer than the 2nd segment. And the thorax has a trapezoidal shape, which means it is wider at the base and narrower towards the front.

TAXONOMIC DESCRIPTION



Scopaeus limbatus

SI No.	**:**	**06**
Locality	**:**	**Varakkal beach**
Habitat	**:**	**Coastal area**
Collector	**:**	**Ninitha Ann Clody**
Guild	**:**	**Predator**
Class	**:**	**Insecta**
Order	**:**	**Coleoptera**
Family	**:**	**Staphylinidae**
Sub family	**:**	**Paederinae**
Tribe	**:**	**Paederini**
Genus	**:**	***Scopaeus***
Species	**:**	***Scopaeus limbatus***

Description

The head is quadrate,broader than the thorax , shining and can be black, brown, or red in color. The thorax is shining and red in color which is a little narrowed behind, longer than broad . The abdomen is less shining compared to the head and thorax and is brown in color. It is very finely and closely punctured and covered in short, fine hairs .The antennae are reddish-yellow in color. And the legs are yellow in color.It typically measures around 3 millimeters in length.

TAXONOMIC DESCRIPTION



Philonthus fimitarus

SI No.	**:**	**07**
Locality	**:**	**Varakkal beach**
Habitat	**:**	**Coastal area**
Collector	**:**	**Ninitha Ann Clody**
Guild	**:**	**Predator**
Class	**:**	**Insecta**
Order	**:**	**Coleoptera**
Family	**:**	**Staphylinidae**
Sub family	**:**	**Staphylininae**
Tribe	**:**	**Staphylinini**
Genus	**:**	***Philonthus***
Species	**:**	***Philonthus fimitarus***

Description

Black and shining. Greenish mettalic reflex on the elytra, brownish towards the suture. Dorsal row of four punctures on the thorax. Abdomen has a shimmering sheen to it. Antennae are black in colour. Legs are brownish-yellow, with infuscate tibiae. Length 5.5-6.5 mm

TAXONOMIC DESCRIPTION



Pronomaea bramina

SI No.	**:**	**08**
Locality	**:**	**Varakkal Beach**
Habitat	**:**	**Coastal area**
Collector	**:**	**Ninitha Ann Clody**
Guild	**:**	**Mixed**
Class	**:**	**Insecta**
Order	**:**	**Coleoptera**
Family	**:**	**Staphylinidae**
Sub family	**:**	**Aleocharinae**
Tribe	**:**	**Pronomaeini**
Genus	**:**	***Pronomaea***
Species	**:**	***Pronomaea bramina***

Description

The posterior borders of the elytra and abdominal segments are narrowly brown, and the elytra and abdominal segments are somewhat shiny and black. Antennae are black, and the first joint has a dark brown colour. Legs are dark reddish-brown, with yellow tarsi. Brownish yellow palpi on the maxilla. 3-5 mm in length.

DISCUSSION

This is the first review on the diversity of staphylinidae beetles in Indian coastal area. This study made it possible to identify the abundance of Staphylinidae beetles in varakkal beach Kozhikode. Coastal areas encompass a variety of habitats, including sandy beaches, salt marshes, dunes, rocky shores, and mangroves. The diversity of habitats contributes to the overall abundance of Staphylinidae beetles in coastal regions. Moisture levels and salinity gradients play a crucial role in the abundance of Staphylinidae beetles in coastal areas. Moisture availability affects their survival and reproductive success, as many species prefer damp or humid conditions. Salinity levels can also impact their distribution, with certain species adapted to tolerate higher salinity levels found in salt marshes and tidal areas. A study by Silva *et al.* (2013) highlighted the importance of moisture and salinity in shaping the abundance patterns of Staphylinidae beetles in coastal ecosystems.

The presence of different substrates contributes to the species richness and abundance of Staphylinidae beetles along the coast. This substrate preference has been documented in studies such as Smetana and Herman (2001) and Sweeney *et al.* (2013). High species richness and abundance of Staphylinidae beetles were noticed from Varakkal beach. This is excellent evidence showing that Staphylinidae mostly prefer moist and cool area for their survival which had already documented in many of the previous works (Rodriguez *et al.*,2019).

Mostly Staphylinidae are trapped during 07-09pm at night, the reason is unknown and may require further studies. Light had attractive effect on Staphylinidae beetles, so its maximum activity was seen during night, that is their activity is greatly influenced by light (positively phototaxic behavior) coming from human dwellings (Shabab *et al.,* 2012). Our research indicates a significant variation in the diversity of Staphylinidae beetles across three specific months. A total of 86 Staphylinidae beetles were collected from the study site during the entire study period. October exhibited the highest species diversity compared to the other two months, while February displayed the lowest diversity. The family Oxytelinae was found to be the most abundant, with Carpelimus indicus being particularly rich in species. The second most abundant family was Paederinae, with lithocaris uvida being the species with the highest richness within this family.

The abundance of Staphylinidae beetles follows a particular order. This includes: Oxytelinae > Paederinae > Staphylininae > Aleocharinae. It is seen that among the collected Staphylinidae beetles Oxytelinae was the most abundant subfamily. Which highlights the preference of genus oxytelus for the organic matter present along with moist coastal habitat (Lu & Zhon, 1995). This work also substantiates the result of other work, that among the Staphylinidae subfamilies, oxytelinae forms a widespread and big subfamily with more than 2000 species (Herman, 2001). Highest diversity of staphylinidae bettles is shown during January this is due to the moist habitat preference, presence of large amount of organic matter and the availability of food source in the form of prey resources, larvae and fruit bearing plants. Paderinae was the second abundant subfamily. Both larvae and adults are predatory on other insects. Predatory nature and its adaptation to low moist environment is a previously established fact (Bohac,1985) this support the reason that paederinae is most abundant during the study period. The least abundance of Aleocharinae indicates their habitat preference or sometimes the reason may include the wrong period, climate or month of collection. The exact reason is unknown and further studies are suggested in this area. It is already stated that in the Staphylinidae subfamily, Aleocharinae is the most poorly known and taxonomically difficult group. It is very small with a length 3-5mm. It is also stated that many of the species already described are likely to form a small proportion of the subfamily actual diversity (Arnett, 1968).

The diversity and abundance of Staphylinidae beetles in coastal areas are influenced by their preferences for different types of substrates. Some species thrive in sandy substrates, where they burrow or search for prey, while others inhabit decaying vegetation or accumulations of seaweed along the shoreline. Rocky shores provide specialized microhabitats for certain species that reside in crevices or feed on algae and invertebrates associated with rocky surfaces. The presence of diverse substrates contributes to the richness and abundance of Staphylinidae beetles along the coast. The importance of substrate preference has been documented in studies conducted by Smetana and Herman (2001) and Sweeney *et al.* (2013). The availability and diversity of food resources play a vital role in determining the abundance of Staphylinidae beetles. Coastal areas offer a wide range of food

sources, including decaying organic matter, algae, invertebrates, and detritus washed ashore. The abundance of these food resources directly affects the population sizes and richness of Staphylinidae beetles. Hlavac and Smetana (2007) conducted a study demonstrating that the abundance of these beetles in coastal habitats correlates with the availability of food resources.

Human activities and coastal development can have both positive and negative impacts on the abundance of Staphylinidae beetles. Certain human-made habitats, such as gardens, parks, or agricultural fields near the coast, may provide additional resources and shelter for these beetles, potentially increasing their abundance. Conversely, habitat destruction, pollution, and urbanization can have detrimental effects on their populations and overall abundance. Klimaszewski *et al.* (2001) and Gers and Smetana (2006) have conducted studies examining the effects of human impacts on Staphylinidae beetles in coastal areas. These various factors interact to shape the distribution and abundance patterns of Staphylinidae beetles in coastal ecosystems.

CONCLUSION

The study conducted at Varakkal Beach in Kozhikode revealed significant fluctuations in the abundance of Staphylinidae beetles. These beetles belong to the family Staphylinidae, commonly known as rove beetles. The researchers identified four subfamilies of Staphylinidae beetles at the study sites: Aleocharinae, Oxytelinae, Paederinae, and Staphylininae. A total of 86 Staphylinidae beetles were collected from the research region, and they were divided among the four subfamilies. The most common subfamily observed was *Oxytelinae,* followed by *Paederinae, Staphylininae, and Aleocharinae.*

In the Oxytelinae subfamily, three species were found to be the most numerous in the study conducted at Varakkal Beach in Kozhikode. These species are: *Carpelimus indicus, Oxytelus incises, Bledins champieni* Regarding the *Paederinae* subfamily, approximately three species were observed, and they belong to the Paederini tribe. The specific species found are *Lithocharis uvida, Scopaeus limbatus, Paederus fuscipes.* Regarding the *aleocharinae*, and *staphylininae* subfamily, only one species were identified and they belong to pronomaeini and staphylinini tribe and the species found are *philonthus fimitarus* and *pronomaea bramina*

The scarcity of published data on the biology and ecology of Staphylinidae beetles poses challenges in analyzing their community patterns. It highlights the significance of conducting further research to better understand the taxonomy, ecology, and biology of this beetle family. By expanding our knowledge in these areas, we can gain insights into their habitat preferences, interactions with other organisms, population dynamics, and potential ecological roles. This information is essential for effective conservation and management strategies, as well as for understanding their contributions to ecosystem functioning.

Ongoing research efforts are crucial for uncovering additional species within the Staphylinidae family and refining existing classifications. studying the life history traits, behavior, and responses of Staphylinidae beetles to environmental changes can provide valuable insights into their ecological significance. This knowledge can help us understand their roles in nutrient cycling, decomposition, and other ecological processes. Additionally, understanding the potential impacts of environmental disturbances on Staphylinidae beetles can aid in mitigating and managing such threats. Very few studies were done on this. All this

highlights the need for further researches in the field of taxonomy and ecology of the large coleopteran family staphylinidae and coastal area.

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Exploring the Chemical Composition and Activities of *Cinnamomum malabatrum* Leaf Essential Oil: Antioxidant, Enzyme Inhibition, and Antibacterial Potential

Dissertation report submitted in partial requirement for M.Sc. degree course in Zoology with Environmental Biology as special subject

in

University of Calicut

by

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Reg No: DVAVMZL009

Under the guidance of

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ST. JOSEPH'S COLLEGE (AUTONOMOUS), DEVAGIRI

KOZHIKODE -08

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REPORT SUBMITTED IN PARTIAL REQUIREMENT FOR M.Sc. DEGREE COURSE IN ZOOLOGY WITH ENVIRONMENTAL BIOLOGY AS SPECIAL SUBJECT

EXAMINER 1:

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ST. JOSEPH'S COLLEGE (AUTONOMOUS), DEVAGIRI

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Report of the MSc Students project submitted to St. Joseph's College (Autonomous) Devagiri

Affiliated to the University of Calicut

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This is to certify that the report entitled **"Exploring the Chemical Composition and Activities of *Cinnamomum malabatrum* Leaf Essential Oil: Antioxidant, Enzyme Inhibition, and Antibacterial Potential"** is an authentic record of studies and research work done by **Ms. Nitha K M.** (Reg No: **DVAVMZL009**) under the guidance and supervision of **Dr. Arunaksharan N.** Assistant Professor, Department of Zoology, St. Joseph's College, Devagiri, Calicut. The work has been carried out for the partial fulfilment of the requirements for the award of the degree of Masters of Science in Zoology.

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Date: **Head of the Department**

CERTIFICATE

This is to certify that the dissertation entitled **"Exploring the Chemical Composition and Activities of *Cinnamomum malabatrum* Leaf Essential Oil: Antioxidant, Enzyme Inhibition, and Antibacterial Potential"** is an authentic record of studies and research work done by **Ms. Nitha K M** (Reg No: **DVAVMZL009**) under my guidance and supervision and that, to the best of my knowledge and belief, this work has not been presented earlier for any degree or fellowship.

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DECLARATION

I hereby declare that the dissertation entitled **"Exploring the Chemical Composition and Activities of *Cinnamomum malabatrum* Leaf Essential Oil: Antioxidant, Enzyme Inhibition, and Antibacterial Potential**" submitted in partial fulfillment of the requirements for the award of the degree of MASTER OF SCIENCE IN ZOOLOGY is a record of original work submitted by me at St. Joseph's College (Autonomous), Devagiri under the supervision and guidance of Dr. Arunaksharan N., Assistant Professor, Department of Zoology, St. Joseph's College (Autonomous), Devagiri, Calicut during 2021-2023.

I hereby declare that the results presented have not been previously submitted for the award of any degree or fellowship.

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ACKNOWLEDGEMENT

It is with a deep sense of pleasure that I express my sincere gratitude to **Dr. Arunaksharan N.** M.Sc, B.Ed, Ph.D., Assistant Professor, P.G and Research Department of Zoology, St. Joseph's College (Autonomous), Devagiri, Kozhikode, for her valuable guidance and supervision in the form of practical suggestions and constructive criticisms which formed the basis for the successful completion of this project.

I thank Kerala Agricultural University, Thrissur for providing essential oil sample and supporting for the chemical characterization by GC-MS analysis

I express my sincere gratitude to **Dr. Vineesh P.J**. MSc, B.Ed, Ph.D., Assistant Professor and Head, PG and Research Department of Zoology for his support and sincere encouragement.

I extend my deep gratitude to **Mr. Joice Tom J**, MSc, M Phil, Assistant Professor Department of Zoology and **Mrs. Aswathi M K**, M.Sc, B Ed., Assistant Professor Department of Zoology, for his wholehearted support and advice that helped me throughout the project I had undertaken.

I am greatly indebted to Ms. Shana Jebin the co-researcher for helping me to complete the project and providing her valuable time in editing and proofing my thesis.
I extend my heartfelt thanks to the non-teaching staff of department for their valuable cooperation.

Finally, I express my deep gratitude to my family and friends for their sincere support throughout the project. Above all, I thank God Almighty for the blessings for the successful completion of the project.

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INTRODUCTION

For centuries, plants have served as medicinal remedies and continue to be utilized in modern times. Initially, trial and error methods were employed to identify plants with beneficial properties, whether for treating illnesses or promoting general well-being. Over time, this knowledge has been refined through generations, resulting in the development of traditional medicine. Traditional medical practitioners rely on the use of medicinal plants to address a wide array of diseases and symptoms, including fever, colds, headaches, ulcers, diabetes, and cancer. Much of the research in traditional medicine has focused on studying the medicinal properties of various herbs.

The acceptance of Cinchona for malaria treatment during the 17th century, followed by the recognition of the benefits of substances like digitalis, morphine, and aspirin, has instilled a belief in the therapeutic potential of natural products, particularly plants. The vast structural diversity present in natural products offers ample opportunities for pharmacological interventions across different categories of disorders. This has led to an appreciation for the diverse range of botanical resources available and their potential in providing effective pharmacological treatments.

Ethnopharmacology can be basically defined as “the interdisciplinary scientific exploration of the biologically active agents that are traditionally employed.” (Süntar, 2019) Hence, the ethnopharmacological approach encompasses various fields, including botany, chemistry, and pharmacology. This approach involves field observations, documentation of folk remedies' usage and bioactivities, botanical identification of plant materials, and studies on phytochemistry and pharmacology. Researchers have long been interested in investigating traditional remedies and their potential effects.

The integration of ethnopharmacological studies into drug discovery from natural sources plays a crucial role in the development of modern treatment systems. Ethnopharmacological knowledge holds promise for the discovery and development of novel, safe, and cost-effective medicines. Many active compounds derived from natural sources have an ethnomedical application, leading pharmaceutical companies to refocus their efforts on natural product research to identify new sources and compounds for drug development.

The World Health Organization (WHO) defines traditional medicine as the knowledge, skills, and practices based on cultural theories, beliefs, and experiences used in maintaining health and preventing, diagnosing, improving, or treating physical and mental illnesses. WHO reports acknowledge the widespread use of traditional medicine, its reputation for safety and efficacy, and its acceptance by national authorities.

Spices have been integral to human lifestyles since ancient times, serving various purposes such as flavouring, colouring, food preservation, and medicinal applications. The active phytochemicals present in spices provide the molecular basis for their effects, many of which are also found in traditional treatments. With increased globalization, these spices have become more accessible and popular.

Within the realm of spices, the Cinnamomum genus has been extensively used in traditional medicine systems, including Western herbal medicine, to address a wide range of ailments. The inner bark of various Cinnamomum species, commonly known as cinnamon, is globally recognized as a culinary spice. Cinnamon not only adds flavor but also possesses mouth-freshening properties and aids in combating bad breath. It has been linked to improved colon health and a reduced risk of developing cancer.

The aromatic oil derived from cinnamon leaves exhibits antibacterial and antifungal properties, while the leaves themselves possess carminative effects and are prescribed for colic and rheumatism. Their pleasant and warming nature makes them effective in addressing various conditions, including vata, scabies, anus and rectum diseases, tridosha imbalances, piles, and heart problems.

Among the different species within the Cinnamomum genus, *Cinnamomum malabathrum*, also known as wild cinnamon or country cinnamon, remains relatively unexplored. This species, belonging to the Lauraceae family, is endemic to the Western Ghats region of India. It is distributed in the tropical and subtropical Himalayas, U.P., Eastern Bengal, Khasia and Jaintia Hills, Burma, and the Western Ghats in South India.

While this plant holds value as a spice, its essential oils and other compounds exhibit significant antibacterial, antifungal, antioxidant, and antidiabetic properties. It has also been utilized as an anti-inflammatory, antitermitic, nematicidal, insecticidal, antimycotic, and anticancer agent. The present study aims to evaluate the in vitro antioxidant,

antimicrobial, and anticancer activities of the essential oil derived from *Cinnamomum malabathrum.*

OBJECTIVES

- To determine the phytochemical composition of essential oil from *Cinnamomum malabatrum* extracted by hydro distillation method.
- To evaluate Antioxidant effects of *C. malabatrum* essential oil.
- To evaluate Enzyme inhibitory activities of *C. malabatrum* leaf essential oil.
- To evaluate the Antibacterial effects of *C. malabatrum* essential oil by disk diffusion method and MIC (minimum inhibitory concentration).

REVIEW OF LITERATURE

Throughout diverse medical systems, numerous herbal medicines have been utilized to effectively treat and manage various ailments. Often hailed as "Nature's pharmacy," herbs offer a natural and holistic approach to healing. While their functionality shares similarities with contemporary pharmaceuticals in certain aspects, herbal therapies are frequently characterized by their milder and safer nature. Many conventional treatments derive their medications from herbal sources. Herbalism embraces the utilization of the entire plant or specific parts such as leaves, petals, or roots. By employing the complete plant, the likelihood of adverse effects that may arise from using isolated components is reduced. A spice refers to a dehydrated seed, fruit, root, bark, or flower derived from plants or herbs, commonly used in small quantities to enhance flavour, add color, or serve as a natural preservative. Many of these natural substances have also found their place in traditional medicinal practices (Kunnumakkara *et al*., 2009).

Spices have held a significant role in the lives of people across various regions since ancient times. They have been valued for their ability to impart vibrant colours, enrich flavours, extend food shelf life, act as additives, and even serve as remedies. These remarkable properties are attributed to the presence of active phytochemicals derived from these aromatic spices (Kunnumakkara *et al*., 2009).

Throughout history, spices have played an integral part in the lifestyles of individuals from diverse cultures. Humans have long utilized herbs and spices not only as culinary ingredients but also as medicinal agents. Plant-derived biomolecules have proven pivotal in promoting and maintaining good health. Spices have served as natural colorants, flavour enhancers, preservatives, food supplements, and therapeutic remedies. The biological efficacy of these spices is attributable to their active phytochemical components (Sachan *et al*., 2018).

Spices offer a plethora of therapeutic benefits in our daily lives, finding their way into our kitchens while carrying inherent medicinal properties such as purgative, laxative, expectorant, carminative, diuretic, and more. Since ancient times, spices have been extensively employed for their medicinal properties, a practice that endures even in the present day. Renowned spices like turmeric, fenugreek, mustard, ginger, onion, and garlic exhibit a broad spectrum of bioactivities, serving as a shield against various health

challenges. When consumed as part of a balanced diet, spices have consistently demonstrated positive impacts on human health (Sachan *et al*., 2018).

Essential oils are fragrant chemicals derived from plants and spices that have been used in perfumery, aromatherapy, food flavouring and preservation, medicine, and other applications since ancient times (Richard *et al*., 2019). Essential oils preserve the natural fragrance and flavour of their source and have a unique chemical composition that influences the smell, absorption, and action of the body. The chemical components included in essential oils may differ within the same species or from plant to plant. Essential oils are a complex mixture of volatile elements derived by secondary metabolism, including phenols, monoterpenes, sesquiterpenes, oxides, esters, aldehydes,

and ketones (Bassolé IHN and HR Juliani, 2012). Coumarins, anthraquinones, and alkaloids are examples of other compounds.

Typically, these metabolites are separated from plant material, primarily leaves and non-woody portions, using steam or hydro-distillation processes (Tongnuanchan P. And S. Benjakul, 2014; Burt S., 2004). They are known as the "chemical weapon" of the plant world since their components protect against a variety of plant-feeding insects and mites. They have recently gained popularity as a natural, safe, and cost-effective therapy for a multitude of health ailments due to their antidepressant, detoxifying, antimicrobial, antiviral, and claiming properties. (Richard *et al*., 2019).

SPICES AND THEIR ACTIVITIES

Some of the common spices include pepper, garlic, turmeric, fenugreek, mint, coriander, cardamom, cloves, rosemary, and cinnamon.

Spice	**Activity**	**Reference**
Pepper	Antipyretic and anti-inflammatory	(Zou *et al*., 2015) (Gülçin, 2005) (Cho *et al*., 2020)
Garlic	Antimicrobial activity	(Yadav *et al*., 2015) (Gabriel *et al*., 2022)
Turmeric	Antimicrobial activity Antifungal	(Gul & Bakht, 2015) (Nasri *et al*., 2014)

	Antimicrobial Renal and hepatoprotective activities. Anticarcinogenic Antioxidant Anticoagulant	(Chattopadhyay *et al*., -2004)
Fenugreek	Anti inflammatory Anti-diabetic activity, Anti-hypercholesterolaemic properties, anti-toxic activity, Anti-cataractactivity, immunomodulatory activity Anti-oxidant activities	(Pundarikakshudu *et al*., 2016) (Jasim *et al*., 2017)
Mint	Antimicrobial activity	(Al-Sum, 2013)
Cardamom	Anti-inflammatory capacity Cytotoxic activity Antibacterial Effect	(Garza *et al*., 2021) (Akrayi, 2012)
Rosemary	Antioxidant Antimicrobial Properties	(Nieto *et al*., 2018)
Clove	Anti-microbial Anti-oxidant Anti-diabetic Anxiolytic Anti-epileptic Anti-depressant Anti-mutagenic Anti-inflammatory Anti-hypertensive Neuro-protective Diuretic	(Sahib *et al*., 2013)

The "King of Spices," black pepper (*Piper nigrum Linn*.), renowned for its distinct flavor and widespread use, has been found to possess antipyretic and anti-inflammatory

properties. Research conducted by Zou *et al*., (2015), Gülçin (2005), and Cho et al., (2020) highlights its origin as a perennial vine native to South India. In addition to its culinary appeal, black pepper has been traditionally associated with improving digestion, stimulating appetite, and addressing various ailments such as colds, coughs, dyspnea, throat diseases, intermittent fever, colic, dysentery, worms, and piles.

Garlic (*Allium sativum* L.), a member of the Alliaceae family, has been extensively studied for its antimicrobial activity, as reported by Yadav *et al*., (2015) and Gabriel *et al*., (2022). Originally utilized for treating intestinal disorders, garlic has also been recognized for its fibrinolytic properties and potential role in cholesterol reduction. Folk medicine has employed garlic to address diverse health concerns including diabetes, inflammation, fevers, rheumatism, intestinal worms, colic, flatulence, dysentery, liver diseases, tuberculosis, and facial paralysis.

Turmeric, celebrated for its vibrant yellow colour, boasts a wide range of therapeutic properties. Studies by Gul & Bakht (2015), Nasri *et al*., (2014), and Chattopadhyay *et al*., (2004) have demonstrated its antimicrobial, antifungal, renal and hepatoprotective activities, as well as its anticarcinogenic, antioxidant, and anticoagulant effects. Turmeric has a rich history of traditional usage in treating ailments such as rheumatism, body aches, skin issues, diarrhoea, intermittent fevers, hepatic diseases, urinary tract infections, dyspepsia, inflammations, constipation, leukoderma, amenorrhea, dental problems, and digestive disorders like dyspepsia, acidity, bloating, ulcerations, and colic inflammatory disorders (Aggarwal *et al*., 2007), (Thangapazham *et al*., 2007).

Fenugreek, recognized for its diverse therapeutic benefits, has been attributed with anti-inflammatory, anti-diabetic, anti-hypercholesterolemic, anti-toxic, anti-cataract, immunomodulatory, and antioxidant properties, as indicated by Jasim et al., (2017) and Pundrarikakshudu *et al.*, (2016). Its applications in traditional medicine encompass digestive issues, bronchitis, tuberculosis, fevers, sore throats, wounds, arthritis, abscesses, swollen glands, skin irritations, diabetes, loss of appetite, ulcers, menopausal symptoms, and cancer (Kunnumakkara *et al*., 2009).

Mint, known for its refreshing aroma, possesses antibacterial properties. Peppermint plants have been utilized in herbal therapy to address various ailments, including loss of

appetite, colds, bronchitis, sinus infections, elevated body temperature, dizziness, and vomiting (Güney *et al*., 2006).

Cardamom, known for its distinctive flavour, has been associated with anti-inflammatory, cytotoxic, and antibacterial properties according to Garza *et al*., (2021) and Akrayi (2012). Ayurvedic medicine has utilized cardamom as a carminative, diuretic, stomachic, and gastrointestinal agent, as well as for addressing colds, coughs, and heart stimulation. Traditional medicine has employed cardamom to alleviate kidney and bladder disorders, aid in digestion, and serve as an antidote for snake and scorpion venom, as well as food poisoning. In traditional Chinese medicine, it has been used to alleviate stomach discomfort, constipation, dysentery, and diarrhoea.

Rosemary, known for its aromatic properties, possesses antioxidant and antibacterial properties as cited by Nieto *et al*., (2018). In folk medicine, rosemary has been utilized to address headaches, epilepsy, poor circulation, diabetes mellitus, respiratory illnesses, skin irritation, gastrointestinal issues, and inflammatory disorders, as well as to promote hair growth.

Clove, native to Indonesia and widely used as a spice in global cuisine, has been recognized for its antimicrobial, antioxidant, diabetic, anxiolytic, anti-epileptic, anti-depressant, anti-mutagenic, anti-inflammatory, anti-hypertensive, neuroprotective, and diuretic properties, according to Sahib *et al*., and other studies. Cloves find applications in Ayurveda, Chinese medicine, Western herbalism, and dental care, where clove oil is used as a pain reliever for dental problems. Clove oil has been employed for treating skin conditions such as acne, pimples, burns, dermatitis, and reducing skin sensitivity (Kunnumakkara *et al*., 2009).

Cinnamon is a tropical evergreen tree that belongs to the Lauraceae family and has long been used as a spice. A review of the literature on cinnamon indicated that it is mostly composed of essential oils and key chemicals such as cinnamonaldehyde, eugenol, cinnamic acid, and cinnamate. It possesses anti-inflammatory, antioxidant, anti-ulcer, anti-microbial, anti-diabetic, memory-boosting, and other beneficial properties (Vangalapati & Prakash, 2012).

Cinnamomum, derived from the Greek word "kinnamomon," meaning "spice" and "sweet wood," is a genus primarily found in India, Sri Lanka, Bangladesh, and Nepal. It comprises small evergreen trees and shrubs, reaching heights of 10-15 meters, with species discovered in China, Australia, Southeast Asia, and Africa. The leaves are ovate-oblong, ranging from 7 to 18 cm in length, and greenish flowers form in panicles. The fruit is a 1-cm purple berry with a single seed. Cinnamomum species thrive in tropical rainforests, from highland slopes to lowland woodlands, including swampy areas and well-drained soils, although they are relatively rare in regions with seasonal climates.

Cinnamon, particularly *Cinnamomum verum or C. Zeylanicum* (also known as "true cinnamon"), has been widely utilized in culinary practices for centuries. In Ayurvedic medicine, cinnamon has been recognized for its healing properties, serving as an antiemetic, antidiarrheal, antiflatulent, and stimulating agent (Sanjay Kumar *et al*., 2019). In Malayalam, cinnamon is referred to as "karuva" or "elavarngam," and its dried bark, known as "karuvappatta" or "elavarngappatta," is an important component of spicy curries (A. B. Kunnumakkara *et al*., 2009).

The essential oil derived from cinnamon bark contains significant components such as cinnamaldehyde and trans-cinnamaldehyde (Cin). Eugenol, found in the leaves, exhibits inhibitory effects against various multidrug-resistant bacteria, while camphor, present in the roots, contributes to its distinct aroma and diverse biological activities. The four economically important cinnamon species are *Cinnamomum verum* (Sri Lankan or Ceylon cinnamon), *Cinnamomum cassia* (Chinese cinnamon), *Cinnamomum burmannii* (Java or Indonesian cinnamon), and *Cinnamomum loureiroi* (Vietnamese or Saigon cinnamon) (Sanjay Kumar *et al*., 2019).

Cinnamon possesses a high aromatic potential and finds applications in both culinary and pharmaceutical industries. Its leaves and bark exhibit properties such as digestion promotion, blood purification, astringency, carminative effects, stimulation, disinfection, and antifungal activity. It has been reported to have antibacterial, antifungal, antiviral properties, and potential benefits in lowering cholesterol and blood sugar levels. Camphor, derived from *Cinnamomum camphora*, is utilized in pharmaceutical preparations like liniments and insecticides. The bark of cinnamon contains procyanidins and catechins, which not only serve as spices in cooking but also demonstrate significant utility in managing type 2 diabetes mellitus symptoms (Sanjay Kumar *et al*., 2019).

Traditionally, powdered cinnamon bark mixed with water has been used to alleviate headaches and neuralgia. Cinnamon is often combined with ginger to enhance circulation and digestion. In Kashmir, it has been employed to treat infectious diseases (Kunnumakkara et al., 2009).

Recent studies have focused on the phytochemistry and pharmacology of various Cinnamomum species. These investigations have identified terpenes, phenylpropanoids, lignans, flavonoids, aromatic and aliphatic compounds, coumarins, alkaloids, steroids, and other substances in this genus (Wang *et al*., 2020). Pharmacological research has revealed that Cinnamomum species exhibit diverse biological activities, including antibacterial, anticancer, antioxidant, anti-diabetic, anti-inflammatory, and analgesic effects. Although the chemical constituents and structures have been extensively studied, their pharmacological implications are still not fully understood (Wang *et al*., 2020).

Essential Oil: Chemical Composition

Different varieties of cinnamon are primarily composed of distinct components, including CD, CA, cinnamate, and various essential oils. Essential oils derived from cinnamon, such as zeylanicum cinnamon leaf oil and bark oil, have been found to contain eugenol, as identified by Gotmare and Tambe (2019). CA oil, which is extracted from *cinnamon cassia*, is utilized in therapy, as well as in anti-fungal agents to prevent food spoilage and in cosmetic products, as mentioned by Sukatta, Haruthaithanasan, and Chantarapa (2008).

Thorough analysis of experimental data obtained from GC-MS analysis allowed for the identification of numerous components present in the essential oils of *Cinnamomum malabathrum*. According to the study conducted by Leela et al. (2009), the major constituents of the leaf oil were (E)-caryophyllene (28.6%), (E)-cinnamyl acetate (15.1%), bicyclogermacrene (14.4%), and benzyl benzoate (8.5%). The petioles and shoots contained 28 compounds, constituting 98% and 97% of the oil, respectively. In the oil extracted from terminal shoots, 34 compounds, accounting for 97% of the composition, were identified. Linalool (77.8–79.4%) was found to be the predominant component in the essential oils of the petioles, shoots, and terminal shoots.

Kumar *et al*. (2012) reported the presence of various compounds in the GC-MS analysis of C. malabathrum leaf volatile oil, including linalool, eugenol, trans-caryophyllene,

epiglobulol, alloaromadendrene,ledene, bicyclogermacrene, -cadinene, germacrene, spathulenol, and -humulene.

The chemical composition of *Cinnamomum malabatrum* leaves includes cinnamic aldehyde, eugenol, -caryophyllene, benzaldehyde, limonene, camphor, cadinene, -terpinol, eugenol acetate, geraniol, ocimene8, -terpinene, benzyl cinnamate, geraniol,-phellandrene, and acetate. In the bark oil, cinnamaldehydes (70–85%) are the major constituents.

Moreover, the plant contains 3,4',5,7-tetrahydroxyl flavones, 3,3‘,4‘,5,7-pentahydroxy flavones, kaempferol-3-osophoroside, and quercetin-3-o-rutin. The constituents of the essential oils in the leaf, petiole, shoot, and terminal shoot of *Cinnamomum malabatrum* were determined through GC and MS analysis. Duguta and Cheriyan (2021) identified 39 compounds, which accounted for 95% of the oil, in the leaves. Similarly, the oil extracted from the terminal shoots contained 34 compounds, representing 97% of the composition. Linalool (77.8–79.4%) dominated the essential oils of the petioles,

Traditional Uses

Cinnamon, renowned for its delightful and sweet taste, is commonly derived from the inner brown bark of Cinnamomum trees. When dried, it takes the form of tubular sticks referred to as "quills." Incorporating as little as half a teaspoon of cinnamon into your daily routine can improve digestion, regulate blood sugar levels, and enhance the body's immune system (Spp, 2011). Moreover, cinnamon has demonstrated potential in reducing the risk of heart disease, tumours, and type 2 diabetes. Its historical use in addressing gastrointestinal and colonic ailments has contributed to its widespread popularity as a daily herb (Kumar, Kumari, and Mishra, 2019; Oras and Mustafa, 2020).

In addition to its culinary applications, cinnamon wood is highly valued for its use in decorative items, furniture, cabinets, and plywood production. True cinnamon, derived from the bark of *C. verum*, holds a special status, but various species serve as suitable alternatives. Commercial markets commonly offer cassias such as *C. cassia, C. tamala, C. burmannii*, and *C. loureirii*. In Sabah, a variety of flavors are derived from species including *C. burmannii, C. iners, C. porrectum, C. rhynchophyllum*, and *C. soegengii* (Sanjay Kumar *et al*., 2019).

An example of such a species is *Cinnamomum burmannii*, commonly known as "keningau," which thrives in the Crocker Range and Keningau foothills. The bark of C. burmannii possesses medicinal properties, making it valuable in the treatment of diarrhea and malaria. Distilled cinnamon oils obtained from this species impart delightful flavors to meals and beverages. For individuals experiencing headaches, crushed leaves and bark paste of *C. crassinervium* provide relief. *C. rhynchophyllum* and *C. soegengii* leaves are known to alleviate stomachaches and food poisoning, while the Sakai ethnic group in Peninsular Malaysia employs *C. iners* leaf juice as a remedy for rheumatism (Sanjay Kumar *et al*., 2019).

Traditional medicine also harnesses the healing potential of cinnamon roots. Decoctions made from the roots of *C. iners*, *C. porrectum*, and *C. subcuneatum* assist in reducing fevers and aiding postnatal recovery. *C. subcuneatum* roots specifically offer relief for rheumatic joint discomfort, and *C. porrectum* seed oil is known to alleviate rheumatism symptoms. Additionally, cinnamon bark and fruits are utilized in perfumery, with *C. porrectum* lending its fragrance to soaps and C. verum being a favoured ingredient in perfumes. *C. iners* mucilage finds application in the production of mosquito coils, scented joss sticks, and formica. Cinnamon's remarkable properties encompass activities such as antifungal, antibacterial, antitermitic, larvicidal, nematicidal, and insecticidal (Sanjay Kumar *et al*., 2019).

Clinical Aspects

Because of cinnamon's significant therapeutic potential, several clinical investigations are being done. According to the Clinical Trials Government Database, a total of 26 clinical trials have been conducted, comprising six studies, 14 completed studies, two recruited studies, two terminated studies, and two current studies. However, the majority of these investigations are still in the works. (Sanjay Kumar *et al.*, 2019)

The essential oil obtained from the leaves of *Cinnamomum malabathrum* has been shown to be antibacterial and antifungal. The leaves are carminative. They are used in the treatment of colic and rheumatism. They are sweet and heating, making them useful in vata, scabies, disease of the anus and rectum, tridosha, piles, and heart troubles. An ethanol extraction of the leaves has shown significant anti-inflammatory activity and can be used in the treatment of acute inflammation (Duguta & Cheriyan, 2021).

a) Antimicrobial activity

Numerous antimicrobial drugs derived from natural sources, including fungi, algae, and animals, have been discovered. Extensive research has been conducted to explore various plant origins and their bioactive compounds, which exhibit therapeutic effects against bacterial infections (Sleha *et al*., 2014). Cinnamon oil, for instance, has been investigated for its remarkable antimicrobial properties (Oras and Mustafa, 2020).

Additionally, there have been investigations into potential interactions between cinnamon and several antibiotics, such as streptomycin, ampicillin, and chloramphenicol. Studies have demonstrated antibacterial activity against notable pathogens including *Staphylococcus aureus, Pseudomonas aeruginosa,* and *Escherichia coli* (Kumar, Kumari, and Mishra, 2019). Promising results were observed when combining cinnamon extract (CE) with ampicillin, exhibiting synergistic effects against bacteria strains like E. coli and S. aureus (Oras and Mustafa, 2020).

The combination of CE and ampicillin offers a potential alternative treatment strategy, enabling the reduction of effective medication dosage, minimizing tolerable side effects, and potentially lowering the cost of treatment (Nabavi *et al*., 2015). Furthermore, it has been suggested that cinnamaldehyde (CA), a prominent compound in cinnamon oil, could be utilized in the food industry due to its safe and effective antibacterial properties. A checkerboard experiment was conducted to evaluate the antibacterial efficacy of CA against 13 food-borne isolates, yielding promising results (Abdalla, 2018). The findings highlight CA as a primary antibacterial component in cinnamon oil, acting by binding to proteins and inhibiting the activity of amino acid decarboxylases (Sleha *et al*., 2014; Oras and Mustafa, 2020).

By employing diverse natural sources and exploring the synergistic effects of cinnamon with conventional antibiotics, novel therapeutic avenues for combating bacterial infections can be pursued, with the potential for improved efficacy and reduced adverse effects.

b) Antioxidant Activity

Cinnamon is known to contain a range of antioxidant compounds, such as polyphenols, phenolic acids, and flavonoids. These antioxidants play a crucial role in the body by counteracting oxidative stress caused by free radicals and reducing the risk of metabolic diseases (Abeysekera *et al*., 2019).

Among the various antioxidants found in cinnamon are camphene, eugenol, salicylic acid, and epicatechin. Extensive research has been conducted to explore the potential health benefits of these natural antioxidants and their role in preventing oxidative damage in the body (Mahdi *et al*., 2018). Extracted cinnamon oil and eugenol have shown particularly potent antioxidant activity (Rao and Gan, 2014).

Moreover, studies have indicated that by limiting the accumulation of nitric oxide and inhibiting lipid peroxidation, cinnamon antioxidants may reduce the risk of cardiovascular diseases, neurological disorders, and cancer (Abeysekera *et al*., 2019). The diverse range of antioxidants present in cinnamon helps neutralize harmful free radicals and combat oxidative stress in the body (Oras and Mustafa, 2020).

In a study conducted by Aravind *et al*., in 2012, the antioxidant activity of *Cinnamomum malabatrum* was evaluated using various methods including the nitric oxide scavenging method, hydrogen peroxide radical scavenging method, and DPPH method. The results showed IC50 values of 131.1 g/ml, 157.9 g/ml, and 179.1 g/ml for the bark extract of *Cinnamomum malabatrum* using the respective methods (Aravind *et al*., 2012).

However, it is important to note that much of the existing research on the antioxidant properties of Cinnamomum species has focused solely on simple chemical assays that measure free radical scavenging activity. These assays may not fully reflect the therapeutic value of cinnamon antioxidants in animals or humans (Wang *et al.*, 2020).

In summary, cinnamon is a rich source of diverse antioxidants that play a crucial role in preventing oxidative stress and protecting against metabolic diseases. Further research is needed to explore the therapeutic potential of cinnamon antioxidants in animal and human studies.

c) Anti-allergic

The therapeutic efficacy of cinnamaldehyde in allergy disorders has been examined, revealing its beneficial effects on mucosal cells (Zaidi *et al.*, 2015). Extensive research has focused on trans-cinnamaldehyde (trans-CA), a bioactive constituent derived from cinnamon, and its impact on immune responses specific to allergens. Remarkably, trans-cinnamaldehyde has exhibited a potent ability to suppress dendritic cell maturation, subsequent proliferation of allergen-specific T cells, and the synthesis of Th1 and Th2 cytokines (Ose *et al*., 2020). These findings underscore the promising therapeutic potential of cinnamaldehyde in modulating immune reactions and alleviating allergic manifestations.

d) Anti-inflammatory Activity

Inflammation is described as a temporary biological tissue response to potentially damaging stimuli such as injuries and exogenic and endogenic antigens. Its goal is to remove the stimulus and repair the wounded tissue, resulting in tissue regeneration and appropriate homeostasis (Egger, 2012).

In spite the fact that inflammation is a favourable body defence mechanism, deregulated and chronic inflammatory responses have been recognised as the root cause of many systemic diseases, including asthma, diabetes, atherosclerosis, cancer, overweight, and pain, contributing to increased healthcare costs in society (Mizuno *et al*., 2011).

The bulk of standard nonsteroidal anti-inflammatory drugs, steroids, and immunosuppressive treatments used to treat different inflammatory conditions have undesirable side effects such as headaches, ulcers, discomfort in the stomach, perforation, hemolytic anaemia, hyperglycemia, and more. (Bagad *et al*., 2013)

In a comprehensive study conducted by Aravind *et al*., the anti-inflammatory properties of the alcoholic extract derived from *Cinnamomum malabatrum* were meticulously examined and compared to the conventional medication Indomethacin. To meticulously assess its effectiveness, the researchers employed the carrageenan-induced rat paw edema model, utilizing male Wistar albino rats as experimental subjects. Precise measurements of paw volume were diligently recorded using a state-of-the-art plethysmograph at distinct time intervals spanning 1, 2, 3, and 4 hours subsequent to the administration of Carrageenan, a widely recognized pro-inflammatory agent.

The obtained data was meticulously analysed, and a percentage of inhibition was meticulously calculated employing a specific formula, taking into account the observed changes in paw volume. Notably, the results revealed a remarkable anti-inflammatory effect exhibited by the alcoholic extract. Particularly, the ethanolic extract demonstrated the highest degree of dose-dependent anti-inflammatory action at a dosage of 250 mg/kg, showcasing its immense therapeutic potential (Aravind *et al*., 2012).

This noteworthy investigation sheds light on the profound anti-inflammatory properties inherent in the alcoholic extract of *Cinnamomum malabatrum*. These findings underscore the promising role it could play as a valuable alternative treatment option, warranting further exploration and consideration in the field of inflammatory disorders.

e) Anti-fungal Activity

Cinnamaldehyde, the major component oil in cinnamon leaf, shows antifungal action when compared to other cinnamon compositions, preventing the growth of fungus. (Nabavi *et al*.,2015) The study found that 10 microliters of oil inhibited the development of *C. Gloeosporioides, L. Theobromae,* and *R. Stolonife* by 100%, but not *A. Alternata, A. Niger,* or *P.viticola* (Kumari and Kumar., 2019)

f) Anti-cancer Activity

Cancer treatments have been associated with undesirable side effects and health concerns. Consequently, current research has been focusing on botanical medicine and exploring the therapeutic properties of herbal extracts alone or in combination with conventional cancer medications utilized in tumor treatment. The combination of herbal extracts with chemical cancer drugs has emerged as a promising therapeutic option, showcasing widespread success. In the context of cancer cells, these combined extracts play a crucial role in activating multiple pathways associated with apoptosis, which is essential in impeding tumor development and progression (Sadeghi *et al*., 2019) (Dutta and Chakraborty, 2018). Furthermore, certain compounds found in cinnamon extracts have demonstrated the ability to inhibit the production of tumor necrosis factor-alpha, thus supporting its potential role as an anti-tumor agent (Ranasinghe et al., 2013).

An insightful study conducted by Sorabh KA *et al*. (2013) highlighted the anticancer properties of *Cinnamomum malabatrum* in Dalton's Lymphoma Ascites (DLA) cell lines. Through rigorous experimentation on albino male rats induced with DLA, both aqueous and alcoholic extracts (administered at doses of 625 mg/kg and 500 mg/kg bwt) were evaluated. The assessment encompassed measurements of solid tumor volume, peritoneal cell count, and body weight. Notably, both extracts significantly reduced solid tumor volume while simultaneously increasing peritoneal cell count and body cell weight, providing further evidence of the antitumor potential inherent in *Cinnamomum malabatrum* bark.

g) Anti diabetic activity

In the realm of diabetes management, cinnamon has garnered attention due to its insulin-like properties, whereby its active components enhance glucose absorption by stimulating IR kinase activity, autophosphorylation of the IR, and glycogen synthase activity (Medagama, 2015). Various studies have demonstrated the positive effects of cinnamon supplementation on glycemic control in individuals with diabetes. By administering low doses of cinnamon (5, 10, and 20 mg/kg), researchers have observed improvements in insulin production, as well as the reduction of oxidative stress and cell protection (Rao and Gan, 2014) (Shi *et al*., 2017). Notably, cinnamon extracts have been found to inhibit enzyme activity, thereby reducing glucose absorption in the bloodstream following the consumption of carbohydrate-rich meals. Such findings have indicated favourable treatment outcomes in patients with Type 2 Diabetes Mellitus, as reflected by improvements in blood sugar markers (Zaidi *et al*., 2015).

Additionally, cinnamon has been shown to increase the number of GLUT4 receptors, facilitating enhanced glucose uptake into cells (Couturier et al., 2010). Through a dose-dependent mechanism, cinnamon extract aids in GLUT4 translocation to the plasma membrane in peripheral tissues (Kumar, Kumari, and Mishra, 2019). Remarkably, administration of cinnamon to animals resulted in a substantial increase in GLUT4 membrane translocation, further supporting its potential in promoting glucose uptake compared to controls (Ranasinghe *et al*., 2017).

Apart from its biochemical and pharmaceutical applications, both modern and traditional medicinal systems have long revered aromatic plants, including cinnamon, for their wide

acceptance, absence of harmful side effects, and versatile use as a daily spice in food. Cinnamon, in various forms such as oil, bark powder, extracts, phenolic compounds, flavonoids, or isolated components, has demonstrated diverse biological and pharmacological actions in the treatment of various diseases. However, further clinical evidence is needed to fully support the traditional usage of this plant in treating inflammatory, tumor, cardiac, and neurological disorders. While researchers have identified numerous compounds, additional investigations are required to explore.

MATERIALS AND METHODS

1. Collection of *C. malabatrum* leaves and extraction of essential oil

Leaves of *Cinnamomum malabatrum (*voucher specimen number KFRI-26/2020 was deposited in KFRI, Peechi, India) was obtained from the cultivation area of Kerala Agricultural University (10.54544° N, 76.28830° E), Thrissur, India. The extraction of the essential oil was by Clevenger-type appratus using hydro-distillation method. The essential oil was dehydrated using Sodium sulfate A.R. and stored in dark at cooling.

2. Chemical component analysis by GC-MS analysis

The characterization of the essential oil extracted from *C. malabatrum* was carried out using TSQ 8000 Evo GC-MS instrument (Thermo scientific, USA) with an autosampling unit. The TG-5MS chromatographic column (30 mm × 0.25 mm × 0.25 µm) with helium (1 mL/min) as the carrier gas was used in the analysis. The oven temperature of the system was set at 50 ^{o}C with a ramp temperature of 10 °C/min till 120°C; later the ramp was 5 °C per minute and finally fixed at 270 °C. The chemical composition was analyzed by the matching of MS spectra with the NIST library and the retention index (RI) values was estimated by calibrating their instrument with a homologous series of alkenes (C_7 - C_{30} n-alkene) under the same conditions (Visakh, Pathrose et al. 2022).

3. Antioxidant activities of *C. malabatrum* leaf essential oil

The concentration of essential oil used was different series from 0-100 µg/mL for each radical quenching assay. The quenching of DPPH radicals was analyzed using the methods of (Kamal, Stanciu et al. 2022). The ABTS radicals scavenging activity was analyzed according to the methods of Li, Liu et al. (2022). The hydrogen peroxide quenching potential of the essential oil was following the methods of Munteanu and Apetrei (2021). The ferric reducing abilities of the essential oil was estimated according to the methods described by He, Li et al. (2020). Methods described by Okoh, Asekun et al. (2014) was followed for lipid peroxidation inhibition assay.

3.1 DPPH radical scavenging assay

The antioxidant activity was evaluated using the DPPH scavenging method, a reliable technique to measure the ability to neutralize the stable and purple-colored 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical. Some minor modifications were made to the procedure outlined by Sobayel, Shahinuzzaman *et al*. in 2020.

To prepare the DPPH solution, 5mg of DPPH was accurately weighed and dissolved in 100 ml of methanol. For the test solution, different concentrations of the essential oil sample (2,4,6,8,10 µl) were mixed with 2ml of the DPPH solution. The drug blank was prepared by mixing the corresponding volumes of the essential oil sample (2,4,6,8,10 µl) with 2 ml of distilled water. As a control, 2 ml of the DPPH solution was used. The resulting mixtures were vigorously mixed and incubated in the dark at room temperature for 30 minutes.

Subsequently, the absorbance of each solution was measured at 517 nm using a colorimeter, with a blank solution of methanol serving as the reference.

The radical scavenging activity of the extract was expressed as a percentage of DPPH of inhibition using the following equation:
DPPH radical scavenging effect (%) = [(A control- A sample)/A control] × 100.
Where, A control = absorbance of DPPH.
A sample = absorbance of DPPH solution presence of essential oil sample.

3.2 Inhibition of Lipid peroxidation (TBARS method)

To homogenize the liver, 10g of liver tissue was mixed with 100ml of phosphate-buffered saline. The liver was then centrifuged at a speed of 1200-1500rpm for 20-25 minutes. The resulting supernatant was collected.

Next, 100 µl of the supernatant was mixed with different concentrations (2.5, 5, 10 µl) of the essential oil sample in duplicate. The mixture was incubated for 15 minutes at room temperature. After the incubation period, 100 µl of H_2O_2 was added to the mixture.

Following that, 1.5ml of 8% TBA (thiobarbituric acid) was added, followed by 1.5ml of 20% acetic acid with a pH of 3.5. Additionally, 0.2 ml of 0.8% SDS (sodium dodecyl sulfate) was added to the solution. A control sample was prepared using tissue alone.

The mixture was incubated at boiling temperature for 1 hour, against a reagent blank. After incubation, the absorbance was measured at 530 nm. This experimental procedure was adapted from Ahmad, Ahmad *et al*. in 2022.

3.3 Ferric reducing antioxidant power (FRAP)

To prepare the fresh working solution, a mixture of 25 ml acetate buffer, 2.5 ml TPTZ (2,4,6-tripyridyl-s-triazine) solution, and 2.5 ml FeCl3 (ferric chloride) solution was prepared. The solution was then warmed at 37°C before use.

For the experiment, different concentrations of essential oil sample (10,40,70,100 µl) were added to 3 ml of the FRAP (ferric reducing antioxidant power) solution. The mixture was incubated in the dark for 30 minutes.

The absorbance of the resulting-colored product, the ferrous tripyridyl triazine complex, was measured at 593 nm. (Müller, Fröhlich *et al*.,2011)

3.4 ABTS radical scavenging activity

The stock solutions used in the experiment were a 7 mM ABTS solution (containing 3.84 mg/mL) and a 2.4 mM potassium persulfate solution (containing 0.65 mg/mL). To prepare the working solution, equal quantities of the two stock solutions were mixed and left to react for 14 hours at room temperature in a dark environment. Subsequently, the solution was diluted by combining 1 ml of the ABTS solution with 60 ml of methanol. This dilution was performed to achieve an absorbance reading of 0.700-0.750 units at a wavelength of 734 nm, using a spectrophotometer after a 7-minute incubation period.

For the test samples, 1 ml of each sample was mixed with 1 ml of the ABTS solution, and the resulting mixture was subjected to spectrophotometric analysis at a wavelength of 734 nm after a 7-minute incubation period.

3.5 Hydroxyl radical scavenging assay

In the experiment, a reaction mixture was prepared by combining specific volumes of different solutions. The solutions included 200 µl of a 2.8 mM 2-deoxy-2-ribose solution (with a concentration of 0.375 mg/mL), 400 µl of a 200 mM FeCl3 solution (with a concentration of 32.44 mg/mL), 1.04 mM EDTA solution (with a concentration of 0.304

mg/mL), 200 µl of H2O2 solution (with a concentration of 1.0 mM), and 200 µl of a 1.0 mM ascorbic acid solution (with a concentration of 0.172 mg/mL).

The concentrations of the test solutions ranged from 10 to 400 µg /ml. Once the reaction mixture was prepared, it was incubated for 1 hour at a temperature of 37 degrees Celsius. The extent of deoxyribose degradation is measured by TBA reaction. The absorbance is measured at a wavelength of 532 nm, using an appropriate blank solution as a reference.

4.Enzyme inhibitory properties of *C. malabatrum* leaf essential oil

The enzyme inhibitory potential of the essential oil was evaluated by mixing different concentrations (0-100 µg/mL) against respective enzymes and their substrates. The enzyme activities will be estimated in terms of the substrate utilized after incubation. The inhibition of α-amylase (Quan, Xuan *et al*. 2019), α- glucosidase (Assefa, Yang *et al*. 2020), aldose reductase (Ali, Zaib *et al*. 2022), sorbitol dehydrogenase (Kazeem, Adeyemi et al. 2020) were carried out according to the methods previously described.

5.Antibacterial activity analysis

5.1 Bacterial strains used

The bacteria were procured from Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh and maintained under standard conditions as prescribed by Bonnet, Lagier et al. (2019). The bacterial strains used include *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus cereus, Streptococcus pyogenes,* and *Salmonella enterica*.

5.2 Disc diffusion method

Initially the bacterial cultured were done in Luria-Bertani broth; for antibacterial study, the inoculum of the bacteria made on a Mueller Hinton Agar (MHA) agar plate (Himedia, India) at a thickness of 5mm. Later, a filter paper disc (8 mm in diameter) containing the leaf essential oil of *C. malabatrum* (10 µL) was placed in the agar plate at a distance of 50 mm. At the end of 24 h, the formation of growth inhibition zone was estimated (Walia, Mukhia et al. 2020).

5.3 Minimum inhibitory concentration (MIC)

Determination of the MIC value was done according to the methods described by Campana, Tiboni et al. (2022). Before begginning, the density of the inoculum was spectrophotometrically set to $5x10^5$ CFU/mL. From this, about 50 µL was transferred to individual wells of a 96 well plate containing different concentrations of *C. malabatrum* essential oil. Later, 2,3,5-triphenyltetrazolium chloride (10 µL) was added to each well; the pink color of the 2,3,5-triphenyltetrazolium chloride is lost in the absence of bacterial growth. The MIC value was considered as the lowest concentration without a detectable pink color.

Statistical analysis

The results are presented as the mean± standard deviation value of three independent experiments. The statistical analysis comparison was made between the standard compounds used, linalool and essential oil by one way analysis of varience using GraphPad prism ver. 7.0 (La Jolla, USA).

RESULTS

C. malabatrum essential oil yield and chemical contents

The yield of *C. malabatrum* leaf essential oil was 0.72±0.13% using hydrodistillation method. The GC-MS chromatogram of the essential oil is shown in Figure 1. There observed eleven main peaks in the chromatogram.

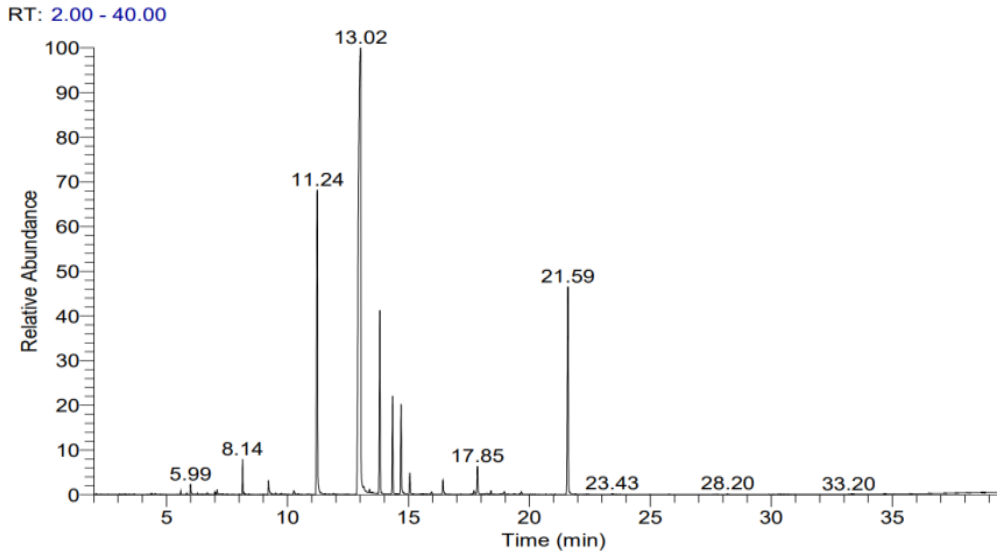


Figure 1. The chromatograms of GC-MS analysis of *C. malabatrum* leaf essential oil

Results of the GC-MS analysis indicated the presence of 38.26±0.41% of linanool, 12.01±0.54% of cinnamaldehyde and 11.43±0.52% of caryophyllene in the essential oil. Besides, the benzyl benzoate (9.60±0.05%), eugenol (8.75±0.23%), and humulene (5.32±0.12%) were also present in the essential oil.

Table 1. Predominant compounds of *C. malabatrum* leaf

Peak no.	RT[a]	Component	RI[c]	RI[b]	%RA [c]
1	5.99	Benzaldehyde	976	966	1.50±0.04
2	8.14	Linalyl acetate	1174	1104	1.36±0.07
3	11.24	Cinnamaldehyde	1277	1282	12.01±0.54
4	13.02	Linalool	1342	1371	38.26±0.41
5	13.82	Methyleugenol	1410	1410	3.40±0.67
6	14.34	Caryophyllene	1433	1435	11.43±0.52
7	14.69	Cinnamyl acetate	1445	1451	3.26±0.08
8	15.06	Humulene	1464	1469	5.32±0.12
9	16.43	Eugenol	1482	1533	8.75±0.23
10	17.85	Ledene oxide	1612	1599	3.09±0.78
11	21.59	Benzyl Benzoate	1703	1677	9.60±0.05

[a] Retention time; [b] Library retention index; [c] Calculated retention index; [c] Relative area.

Antioxidant effects of *C. malabatrum* essential oil

There observed a dose-dependent scavenging of various free radicals in *C. malabatrum* essential oil treatments (Table 2). The IC_{50} values are found to be less than 100 µg/mL in the entire radical quenching assay for the essential oil. Further, among the different radicals analyzed, the DPPH was more sensitive to the essential oil treatment. However, the radical quenching properties of the CMEO was significantly lower than that of ascorbic acid ($p<0.001$). On contrary, the CMEO was having a higher antioxidant potential in terms of DPPH and ABTS radical scavenging ($p<0.001$). The peroxide scavenging and lipid peroxidation potential of the linanool was higher than the CMEO ($p<0.001$). The reducing potential (FRAP) of *C. malabatrum* essential oil was significantly lower than the ascorbic acid whereas significantly higher than the linalool ($p<0.001$).

Table 2. Radical quenching abilities of the essential oil extracted from *C. malabatrum* leaves. The values expressed are half-maximal inhibition concentration- IC_{50} (µg/mL)

	CMEO	Ascorbic acid
DPPH radical scavenging	21.50± 0.17	8.13± 0.09
ABTS radical scavenging	36.91± 0.41	12.82± 0.40
H_2O_2 radical scavenging	42.77± 0.34	19.11± 0.26
Ferric reducing potential	12.38± 0.11	15.38± 0.66
Lipid peroxidation inhibition	85.83± 0.47	63.02± 0.33

Enzyme inhibitory activities of *C. malabatrum* leaf essential oil

The enzyme inhibitory activities of the essential oil were evaluated using different enzymes associated with diabetes and diabetic complications. The *C. malabatrum* is found to inhibit the enzymes such as α-amylase and α- glucosidase (Table 3), however, the bioactive compounds Linalool and ascorbic acid was found to be more potent inhibitors of these enzymes. The inhibition of aldose reductase and sorbitol dehydrogenase was also observed in CMEO treatment with respective IC_{50} values 82.90± 0.67 and 98.61± 3.18 µg/mL. However, there observed a more significant inhibition in ascorbic acid treatment 28.70± 2.14 and 60.09± 1.32 µg/mL ($p<0.001$). Likewise, the linalool also showed significant inhibition but to a lesser extent than the ascorbic acid ($p< 0.001$).

Table 3. Enzyme inhibitory abilities (IC_{50} in µg/mL) of *C. malabatrum* leaf essential oil

Enzyme inhibition	CMEO	Ascorbic acid
α-Amylase	74.19± 1.55	45.17± 2.36
α- Glucosidase	47.07± 3.14	36.03± 1.98
Aldose reductase	82.90± 0.67	28.70± 2.14
Sorbitol dehydrogenase	98.61± 3.18	60.09± 1.32

Antibacterial effects of *C. malabatrum* essential oil

The antibacterial potential of the *C. malabatrum* essential oil was tested against both Gram-positive and Gram-negative organisms using disc diffusion method (Table 4) and also in terms of minimum inhibitory concentration (Table 5). The antibacterial activity was found to be similar in CMEO to that of GM in the *Pseudomonas aeruginosa* strain (p= 0.3150). Likewise, the CMEO was effective as that of linalool against *Escherichia coli* and *Salmonella enterica*. However, in other strains, there observed a significantly higher antibacterial activity for CMEO than linalool.

Table 4. Antibacterial properties of *C. malabatrum* by disc diffusion method

Bacteria	**Zone of Inhibition (mm)**	
	CMEO	**GM**
Staphylococcus aureus	16.2± 0.3	18.5± 0.5
Bacillus cereus	14.8± 0.4	21.3± 0.5
Streptococcus pyogenes	16.7± 0.3	19.2± 0.7
Escherichia coli	18.1± 0.2	21.3± 0.3
Pseudomonas aeruginosa	20.8± 0.5	21.6± 0.4
Salmonella enterica	17.4± 0.2	19.9± 0.3

The minimum inhibitory concentrations of CMEO was comparable for the *C. malabatrum* essential oil and linalool in the *Bacillus cereus* ($p= 0.0028$). Likewise, the MIC values of linalool and GM was similar in *P. aeruginosa, S. aureus, S. pyogenes*, and *S. enterica* ($p>0.05$). The antibacterial activity of CMEO was significantly lower than the gentamicin ($p<0.001$).

Table 5. Minimum inhibitory concentrations (mg/mL) of *C. malabatrum* essential oil and antibiotic gentamicin

Bacteria	**CMEO**	**GM**
Staphylococcus aureus	1.25± 0.05	0.325± 0.00
Bacillus cereus	0.75± 0.05	0.325± 0.00
Streptococcus pyogenes	0.625± 0.10	0.167± 0.00
Escherichia coli	1.00± 0.10	0.325± 0.00
Pseudomonas aeruginosa	0.625± 0.15	0.167± 0.00
Salmonella enterica	0.625± 0.05	0.167± 0.00

DISCUSSION

Cinnamomum spp. is well known for its culinary uses in different parts of the word. Besides, the essential oil extracted from the spice is of cosmetic and pharmacological uses. Among these, the *C. verum, C. zeylanicum* and *C. tamala* are widely evaluated. The *C. malabatrum* is an endemic plant which is less explored for its biological and pharmacological properties. The present study evaluated the chemical components of the plant essential oil by GC-MS analysis.

Results of the GC-MS analysis indicated the presence of 38.26% of linanool, 12.01% of cinnamaldehyde and 11.43% of caryophyllene in the essential oil. Besides, the benzyl benzoate (9.60%), eugenol (8.75%), and humulene (5.32%) were also present in the essential oil. Previous study by Leela, Vipin et al. (2009) indicated that (E)-Caryophyllene (28.6%), (E)-Cinnamyl acetate (15.1%), and Bicyclogermacrene (14.4%) as the predominant compounds. Further, Benzyl benzoate (8.5%), α-Humulene (4.7%), Globulol (2.7%), and β-Phellandrene (2.2%) were other minor compounds present in the leaf essential oil according to their study. On contrary, another study by Sriramavaratharajan and Murugan (2020) indicated β-Phellandrene (3.5- 12.0%), Linalool (13.1- 15.4%), (E)-Caryophyllene 8.4- 31.4%), and Bicyclogermacrene (12.9- 20.0 %).

There observed a dose-dependent scavenging of various free radicals in *C. malabatrum* essential oil treatments. The IC_{50} values are found to be less than 100 µg/mL in the entire radical quenching assay for the essential oil. Further, among the different radicals analyzed, the DPPH was more sensitive to the CMEO treatment. In addition, it was interesting to note that the ferric reducing potential of the CMEO was comparable to that of the standard ascorbic acid. The free radicals are important agents associated with oxidative stress and inflammation (Pizzino, Irrera et al. 2017). Hence, the radical quenching is important to prevent the oxidative damages to cellular macromolecules and thereby preventing various degenerative diseases (Lobo, Patil et al. 2010; Sharifi-Rad, Anil Kumar et al. 2020). Hence, inhibition of the radicals by *C. malabatrum* indicates the potential of the essential oil in preventing chronic diseases. Further, the compounds such as linalool (Seol, Kang et al. 2016), caryophyllene (Dahham, Tabana et al. 2015), and cinnamaldehyde (Subash-Babu, Alshatwi et al. 2014; Suryanti, Wibowo et al. 2018) are shown to prevent oxidative damage in various conditions of animal models and clinical

studies. Hence, it must be possible that the bioactive stress volatiles of the *C. malabatrum* might be responsible for the antioxidant potentials.

The enzyme inhibitory activities of the essential oil were evaluated using different enzymes associated with diabetes and diabetic complications. The *C. malabatrum* is found to inhibit the enzymes such as α-amylase and α- glucosidase. The α-amylase and α-glucosidase are two enzymes associated with type 2 diabetes mellitus (Alqahtani, Hidayathulla et al. 2019; Kaur, Kumar et al. 2021). Several synthetic drugs are known to inhibit these enzymes as a preventive measure to diabetes and thereby make these enzymes as an anti-diabetic drug target (Dirir, Daou et al. 2022; Santoso, Ong et al. 2022). Likewise, the secondary diabetic complications including retinopathy, nephropathy and cardiomyopathy are another important concern of diabetic patients (Cade 2008; Chawla, Chawla et al. 2016). The activation of polyol pathway enzymes aldose reductase and sorbitol dehydrogenase plays crucial role in the microvascular complications of diabetes (Mara and Oates 2008; Hashimoto, Yamagishi et al. 2011; Jia, Hill et al. 2018). Numerous plant products and bioactives are reported to interfere with polyol enzymes and thereby found to be protective against microvascular complications of diabetes (Lorenzi 2007; Shahin, Sultana et al. 2022). Hence, it is possible that the *C. malabatrum* essential oil may prove beneficial against diabetes and associated microvascular complications.

The antibacterial potential of the *C. malabatrum* essential oil was tested against both Gram-positive and Gram-negative organisms using disc diffusion method and also in terms of minimum inhibitory concentration. The selected microorganisms are known to be associated with various diseases of humans, animals and poultry. The *E. coli* is reported to cause infections in urinary and respiratory tracts (Braz, Melchior et al. 2020); whereas, the *P. aeruginosa* is associated with wound infections during surgery and transplantations (Gellatly and Hancock 2013). *Staphylococcus* and *Streptococcus* are associated with cutaneous and genital infections in humans causing various diseases (Tong, Davis et al. 2015; Ibrahim, Eisen et al. 2016). The *Bacillus cereus* is an important pathogen which is known to produce toxins and subsequently associated with food poisonings and are often fatal (Bottone 2010; Messelhäußer and Ehling-Schulz 2018). Hence, inhibition of the growth of these organims by the *C. malabatrum* essential oil may be indicative of its antibacterial potentials. Further, the bioactive compounds present in

the essential oil such as linalool, caryophyllene and cinnamaldehyde are known for their antimicrobial properties (Sawicki, Golus et al. 2018; Berthold-Pluta, Stasiak-Różańska et al. 2019; Guimarães, Meireles et al. 2019). It is therefore possible that the antibacterial properties exhibited by the *C. malabatrum* essential oil may be attributed to these bioactive metabolites present in it. Hence, the present study confirms the chemical components as well as the antibacterial and anti-diabetic properties of the leaf essential oil of *C. malabatrum*.

CONCLUSION

The present study confirms the pharmacological potentials of the *Cinnamomum malabatrum,* an endemic plant of Western Ghats, India. *C. malabatrum* leaf essential oil was found to have significant radical quenching abilities against different free radical sources. Likewise, the essential oil was also capable of inhibiting enzymes associated with diabetes and associated secondary complications. There observed strong antibacterial potential for the *C. malabatrum* essential oil both Gram-positive and Gram-negative bacteria. Hence, based on the signification of the results, the *C. malabatrum* essential oil may be a useful pharmacological agent.

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Effect of Coconut Haustorium Supplementation on the Meat Quality and Antioxidant Levels of Nile Tilapia (*Oreochromis mossambicus*)

Dissertation report submitted in partial requirement for M.Sc. degree course in Zoology with Environmental Biology as special subject

in

University of Calicut

by

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Under the guidance of

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KOZHIKODE -08

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REPORT SUBMITTED IN PARTIAL REQUIREMENT FOR M.Sc. DEGREE COURSE IN ZOOLOGY WITH ENVIRONMENTAL BIOLOGY AS SPECIAL SUBJECT

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2021-2023

ST. JOSEPH'S COLLEGE (AUTONOMOUS) DEVAGIRI, CALICUT

Effect of Coconut Haustorium Supplementation on the Meat Quality and Antioxidant Levels of Nile Tilapia (*Oreochromis mossambicus*)

Report of the project M.Sc. Students project submitted to St. Joseph's College (Autonomous) Devagiri

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This is to certify that the report entitled **"Effect of Coconut Haustorium Supplementation on the Meat Quality and Antioxidant Levels of Nile Tilapia (*Oreochromis mossambicus*)"** is an authentic record of studies and research work done by **Ms. Nivya. K.G** (Reg No: **DVAVMZL010**) under the guidance and supervision of **Mr. Joice Tom J.** Assistant Professor, Department of Zoology, St. Joseph's College, Devagiri, Calicut. The work has been carried out for the partial fulfillment of the requirements for the award of the degree of Masters of Science in Zoology.

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DECLARATION

I hereby declare that the dissertation entitled **"Effect of Coconut Haustorium Supplementation on the Meat Quality and Antioxidant Levels of Nile Tilapia (*Oreochromis mossambicus*)"** submitted in partial fulfillment of the requirements for the award of the degree of MASTER OF SCIENCE IN ZOOLOGY is a record of original work submitted by me at St. Joseph's College (Autonomous), Devagiri under the supervision and guidance of Mr. Joice Tom J, Assistant Professor, Department of Zoology, St. Joseph's College (Autonomous), Devagiri, Calicut during 2021-2023.

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ACKNOWLEDGEMENT

It is with a deep sense of pleasure that I express my sincere gratitude to **Mr. Joice Tom J.** M.Sc., Assistant Professor, P.G and Research Department of Zoology, St. Joseph's College (Autonomous), Devagiri, Kozhikode, for her valuable guidance and supervision in the form of practical suggestions and constructive criticisms which formed the basis for the successful completion of this project.

I express my sincere gratitude to **Dr. Vineesh P.J**. MSc, B.Ed, Ph.D., Assistant Professor and Head, PG and Research Department of Zoology for his support and sincere encouragement.

I extend my deep gratitude to **Dr. Arunaksharan N.** MSc, BEd, PhD Assistant professor, Department of Zoology, and Princy (Research Fellow) for their wholehearted support and advise that helped me throughout the project I had undertaken.

I thank Kerala veterinary and animal sciences university for trace elemental analysis.

I convey my sincere gratitude to **Mr. Alan joy** and **Ms. Najiya V K** for their help in providing with fish feed and coconut haustorium required for completing my project work.

I am greatly indebted to **Ms. Akshaya K M**, **Ms. Najiya V K, Ms. Sadiya mariyam** the co-researchers for helping me to complete the project.

I extend my heartfelt thanks to the non-teaching staff of department for their valuable cooperation.

Finally, I express my deep gratitude to my family and friends for their sincere support throughout the project. Above all, I thank God Almighty for the blessings for the successful completion of the project.

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INTRODUCTION

Nutrition, in its broadest sense, pertains to the nature and transformation of materials that organisms acquire from the environment to fulfil energy requirements, support growth, and reproduce. The basic goal of nutrition is to distinguish these substances in their most basic and usable chemical forms, differentiate between essential and non-essential ones, and determine the optimal quantities needed for optimal function (Simpson & Raubenheimer 2012). According to Robinson's 1982 definition, nutrition can be described as the scientific study of foods and nutrients, examining how they affect each other and contribute to the overall well-being of an individual. It encompasses the various processes involved in the intake, digestion, absorption, utilisation, and elimination of nutrients within the body, all in relation to maintaining health and preventing diseases. Prior to World War I, the importance of nutrition was acknowledged by a limited number of scientists and doctors. However, since then, there has been a broader understanding and recognition of the impact that nutrients have on both individual health and the overall economic progress of a nation. Until thirty years ago, the significance of nutrition was primarily understood in terms of its impact on growth, development, and tissue health. This recent advancement has led to a significant shift in dietary habits and practises among the general population. The World Health Organisation (WHO) defines health as a state of comprehensive well-being encompassing physical, mental, and social aspects rather than simply the absence of illness or disability. The food we consume provides necessary nutrients such as proteins, carbohydrates, fats, vitamins, and minerals, which are essential for the proper functioning of our body. Not only humans but all forms of life depend on food for their survival. The emerging priorities in the field of nutrition encompass areas such as identifying the optimal dietary composition to combat weight gain and obesity, exploring the interaction between prebiotics, probiotics, fermented food, and the gut microbiota, studying the effect of specific fatty acids, flavonoids, and other bioactive compounds, investigating personalised nutrition approaches tailored to non-genetic factors like lifestyle, sociocultural influences, and the microbiome, as well as recognising the significant influences of location and social status on nutritional and health disparities (Mozaffarian 2016, Mozaffarian & Wu 2018). Nutrition offers a wide

range of benefits for individuals and populations, including, Optimal physical health, Mental well-being, Energy and vitality, Healthy growth and development, Disease prevention and management, Improved digestive health, Support and maintain the immune system, Enhanced lifespan.

Coconut is botanically called *Cocos nucifera and belongs* to the family Aracaceae. During the germination of nuts, the lower part of the embryo grows and transforms into a white, spongy, serrated structure within the endosperm, which is known as the haustorium (Rakesh *et al.,* 2021). In the province of Hainan, the coconut haustorium was given the names "coconut treasure", "coconut heart", and "coconut apple" (Zhang et al., 2022). Haustorium has two distinct portions: the outer portion is oil-rich yellow, and the inner portion is carbohydrate-rich white. This haustorium part extracts and transports nutrients from coconut water to nourish the developing embryo, and the nutrients found in it are easily accessible (Manivannan *et al.* 2018). The biochemical and nutritional characterization of 100 grams of coconut haustorium revealed the composition of various nutrients. It is rich with ash (1.05±0.2%), soluble sugar (44.2 ± 4.6%), starch (24.5 ± 3.2%), protein (5.50 ± 0.3%), fat (1.99 ± 0.9%), soluble dietary fibres, insoluble dietary fibres, and phenolics. Mineral profiling of haustorium showed that it contains potassium, magnesium, calcium, phosphorus, manganese, copper, iron, and zinc, and amino acids like methionine, cysteine, phenylalanine, tyrosine, leucine, and isoleucine are also present. Various antioxidant activity assays like FRAP, DPPH, CUPRAC, and ABTS have also shown positive results with coconut haustorium (Russell *et al.* 2012). It is nutritionally rich and include Carbohydrates- Coconut haustorium is rich in carbohydrates and provides a source of energy, Dietary fibre- It contains dietary fibres that promote healthy digestion and may aid in maintaining bowel regularity, Minerals- It contains important minerals like potassium, magnesium, and manganese, which help in physiological processes like electrolyte balance, Vitamins, Antioxidants.

Oreochromis *mossambicus,*,commonly known as Nile tilapia, or tilapia who belongs to Kingdom of Animalia, Phylum: Chordata, Subphylum: Vertebrata, Class: Actinopterygii, Order: Perciformes, Family: Cichlidae, Genus: Oreochromis and Species: Mossambicus. *Oreochromis mossambicus* was originally found in the eastward-flowing

rivers of central and southern Africa and has been exclusively introduced worldwide for aquaculture and as a means of biological control for weeds, insects, and sport fishing purposes since the early 1930s (Russell *et al.* 2012). They are adaptable to a diverse range of ecological conditions, have flexible dietary needs, and have fast reproductive rates (Russell *et al.* 2012). They are morphologically distinguishable from other fishes in their body shape, fins, scales, coloration, size, etc. They have a deep and laterally compressed body shape, and their bodies are elongated or oval with a moderately forked tail. The coloration varies depending on the species but commonly exhibits shades of grey, silver, or brown. Tilapia fish are actively cultured in aquaculture, which provides a sustainable source of food and provides nutritional benefits for those who consume it, and the benefits depend to an extent on the feed they ingest. Tilapia is the best-suited model animal for carrying out various studies since it is easy to rise, it attains maximum growth within a short time span, inexpensive when compared to other fish, easily adapted as well as stable to freshwater ecosystems, disease resistance.

Food supplements are very essential for the normal growth of fish, and there should be a regular supply of sustained and balanced food for the fish. The supplementary feed is selected based on the criteria of ready acceptability, easy digestibility, and abundant availability. Coconut haustorium fulfils the above criteria as a functional food for fish. They are incorporated along with normal fish feed and have observed positive results as fish feed.

The study mainly focuses on the utilization of coconut haustorium as a functional food in *Oreochromis mossambicus.* The biochemical and nutritional characteristics of haustorium reveled it as a stable functional food.

OBJECTIVES

Nutrition encompass various aspects related to the study and application of foods and its impact on human health. The major objective of nutrition is to prevent malnutrition, ensure adequate nutrition and to promote optimal health. Functional foods are food products that have been specifically formulated to provide additional health benefits beyond basic nutrition. It enhances physiological functions as well as reduces the risk of chronic diseases when consumed as part of balanced diet. The present study aims at providing coconut haustorium as an effective functional food supplement for tilapia fishes, which will enhance the nutritional as well antioxidant systems of the fishes.

The major objectives of the study include;

- Promote the use of cheap, ecofriendly, and effective food supplements for fishes.
- Assessment of total protein, carbohydrate, trace elements, from muscle.
- Estimation of enzymatic and non-enzymatic antioxidant enzymes like, reduced glutathione, glutathione-s-transferase, glutathione peroxidase and catalase
- Determination of oxidative stress parameters like Thiobarbituric acid reactive substance (TBARS), conjugated diene and conjugated triene.

REVIEW OF LITERATURE

Nutrition, Balanced diet, Deficiency, and Disorders

The linear growth of children, particularly through growth plate endochondral ossification, is regulated by nutritional and inflammatory influences. The complex mechanisms involved include genetic, physiological, and nutrient-sensitive endocrine signalling, with sleep adequacy potentially affecting growth hormone secretion. The nutrients influence the linear growth, and based on that, there are two categories: type I and type II (Golden M.H. 1991). Type-I is the major group, and they have specific functions, and the deficiency symptoms can be easily identifiable. Where type II is the minor group and the symptoms are difficult to identify (Golden M.H. 1991). Infection-related inflammation and environmental enteric dysfunction commonly hinder endochondral ossification through various intermediaries like glucocorticoids and fibroblast growth factors. The animal studies carried out by Milward suggest that intake of zinc and dietary protein is very essential for linear growth, which is performed through triiodothyronine and insulin, like growth factor-I. Observational studies suggest that stunting is associated with substandard plant-based diets. While interventions and assessments show that deficiencies in energy, protein, zinc, and iodine can contribute to stunting, especially during pregnancy, Among, animal-source foods, milk appears to have a positive influence on linear growth. However, stunting can also be caused by inflammation resulting from infections, environmental enteric dysfunction, and endogenous inflammation from excess adiposity. Current efforts to reduce stunting therefore focus on improving water, sanitation, and hygiene, as well as nutrition (Milward 2017).

Nutrition at a suboptimal level is the main root cause of mental health disorders and can hinder the effectiveness of treatment and recovery. Nutrition plays a critical role in preventing and treating behavioural health disorders (Anderson *et al.,* 2018). Consequently, enhancing nutritional intake is necessarily considered a form of remedial measures and prevention for these health disorders. This summary provides an overview of the global consequences and characteristics of depression and anxiety, as well as

recent research findings on the role of diet and nutrition in their prevention and management. The current evidence indicates that adhering to healthy dietary patterns that align with recommended food-based guidelines and nutrient requirements can be beneficial in preventing and treating depression and anxiety (Kris Etherton *et al.*, 2021).

The prevalence of lifestyle diseases, also known as non-communicable diseases like cardiovascular events, diabetes, metabolic issues, and obesity, has been acknowledged globally as a significant contributor to illness and death in current civilization. The surge in these diseases has been linked to various alterable risk factors, including sedentary behaviour, insufficient sleep, tobacco use, and unbalanced eating patterns. Embracing the Mediterranean eating style (MD) that comprises spices, herbs, abundant fruits, legumes, vegetables, and high fibre intake is considered an effective approach to curbing this trend, and these foods have been recognised for their beneficial health effects and contribute to overall well-being (Alkhatib *et al.* 2017). Also rich with biologically active nutrients like polyphenols, which play a crucial role in preventing and managing chronic communicable diseases (Alkhatib *et al.* 2017). The numerous components of MD, which are abundant in antioxidants and possess anti-inflammatory properties, are responsible for its beneficial effects. Further, the MD has been associated with enhanced reproductive well-being, decreased susceptibility to neurodegenerative diseases, and safeguarding against depression and psychosocial difficulties. It is crucial to recognise the advantages of MD in promoting metabolic, reproductive, and mental health. By integrating heightened physical activity within a balanced and healthy lifestyle, we can further enhance its positive effects (Gantenbein and Kanaka-Gantenbein, 2021).

The growth, reproduction, and overall health of fish are significantly affected by their nutrition and feeding habits, as well as by their ability to cope with environmental stresses and pathogens. Fish metabolism is similar to that of warm-blooded animals, involving consumption of food, its breakdown into simple absorbable micronutrients, the absorption of these nutrients, and their transportation to different sites within the tissues (Lall and Tibbetts 2009).

Functional foods:

Cardiovascular disease is one of the principal causes of death worldwide and an escalating health apprehension. Nutrition, along with other lifestyle factors, plays a crucial role in the prevention and treatment of cardiovascular disease. Plant- or animal-based functional foods contain active compounds that can reduce the likelihood of various long-term health conditions while also offering certain biological advantages. They primarily target established contributing factors such as elevated levels of lipids, obesity, diabetic conditions, and inflammation. Functional foods can protect the heart by improving blood lipid levels, controlling hypertension, enhancing vascular health, reducing clumping of platelets, and providing antioxidants. Medical and public health evidence suggests that foods like vegetables, fruits, nuts, seeds, seafood, coffee, tea, and dark chocolate have cardioprotective properties. Whole grain products with intact kernels are also beneficial due to their fibre and nutrient content. These functional foods may reduce cardiovascular risk through the combination of their nutritional composition and phytoprotective substances. This review explores the relationship between functional foods and cardiovascular health as well as the underlying mechanisms involved (Asgary *et al.* 2018).

Functional foods contain bioactive components that will aid in the prevention and management of non-communicable diseases like type II diabetes mellitus and offer physiological health benefits. There is an inverse relationship between type II diabetes mellitus and the Mediterranean diet (Panagiotakos *et al.,* 2005), and they are highly effective in reducing gestational diabetes (Karamanos *et al.,* 2014). The insulin efficiency, cholesterol-regulating functions, anti-inflammatory, and antioxidant properties are potentially enhanced when functional foods are incorporated into a normal diet and also play a vital role in preventing and managing type II diabetes mellitus. The Mediterranean diet serves as a paradigm for functional foods with its inherent presence of beneficial compounds like phenolic compounds, bioflavonoids, terpenes, steroidal compounds, and omega fatty acids. The phytochemicals are very beneficial in terms of metabolic and microvascular activities (Alkhatib *et al.* 2017).

In feeding fish larvae, fry, and hatcheries, live feed plays a critical role. Live feed is commonly referred to as the "living capsules of nutrition," and it is abundant with

carbohydrate, proteinaceous compounds, amino acids, lipids, mineral nutrients, and organic nutrients (Simhachalam *et al.* 2015). The improvement in nutritional value of live feeds is a widely recognised practise in aquaculture. The incorporation of live food in the rearing of larvae has demonstrated the ability to improve growth performance, survival rates, and disease resistance in larvae (Sontakke *et al.* 2019, Saez-Royuela *et al.* 2007, Yanes-Roca *et al.* 2018). Docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and arachidonic acid (ARA), which are the essential fatty acids and amino acids, are crucial sources of protein for larval fish rearing. This review emphasises the importance of live feeds in the early stages of fish feeding and the techniques used for their enhancement or enrichment with essential nutrients for optimal growth (Kandathil *et al.* 2020).

Pollen provides a wealth of nutritional benefits for humans and is available in various forms on the market. However, the nutrients in pollen can significantly reduce digestibility when they are enclosed within a pollen shell. To improve pollen digestibility, bioactive foods containing pollen have been developed, and research has been conducted to assess their benefits for animal health and growth. It is an immense core of protein and a viable and natural alternative to antibiotics (Abdelnour *et al.* 2019). The advancement in using pollen as a functional food and animal feed increases the nutrient-related attributes, functional aspects, qualitative features, and sensory characteristics (Koztic *et al.* 2020).

The current and future trends related to the utilisation of mushroom and fungal filaments in food and feedstuffs are evident. The use of mushrooms as a source of food has been used for more than a thousand years, and the techniques for producing them and the variety of species available have grown in recent times. In addition to mushrooms, fungal mycelia grown in culture are currently acquired as a principal food source. Fungal mycelia and edible mushrooms are rich sources of nutritional proteins, fats, essential fatty acids, vitamins, dietary fibres, and flavour components and can elevate the sensory-related properties of instant foods and meat analogues. They also contain various specialised metabolites, making them essential components of dietary supplements and

therapeutic agents. Mycelia have shown promise in improving feed efficiency, gut health, and the overall well-being of livestock (Strong *et al.,* 2022).

Plant-based proteins and cereals are commonly used during the manufacturing of food products for animals, humans, and fish. However, these proteins often lack essential amino acids, which are important for proper nutrition. On the other hand, macroalgae proteins have all the essential amino acids and are great sources of other beneficial compounds, which include taurine, lipids, carotenoids, and pigments. These proteins can also be used as substrates for generating bioactive pesticides. Through this, the potential use of macroalgal bioactives in fish, animal, and human nutrition and health is revealed (Vaquero *et al.* 2016).

The study carried out by Oomah examines the nutritional benefits and disease prevention potential of flaxseed, which is becoming a significant source of phytochemicals in functional foods. Flaxseeds are rich in alpha-linolenic acid oil, lignans, high-quality protein-soluble fibre, and phenolic compounds. It also presents the relationships between flaxseed consumption and the reduction of disease risk in humans (Oomah 2001).

Fucoxanthin is a unique xanthophyll that is found in brown seaweed. This plays a crucial role in algal photosynthesis and protection against light. It has a distinctive chemical structure and offers various health benefits. As a result of its immense importance, it is extensively explored as a valuable source for bioactive food, feed, and medicines. This comprehensive article reviews the biosynthesis and availability of fucoxanthin in brown seaweed, elucidates its mechanism of action, and discusses its beneficial bioactivities such as antioxidative activity, anti-inflammatory properties, anti-obesogenic, anti-cancer, anti-diabetic, and other potential effects. Fucoxanthin can reduce diabetic symptoms and signs, and it also helps to improve spermatogenesis in humans (Kong *et al.* 2019). In addition to all these beneficial aspects, it explores the feasible use of fucoxanthin as an ingredient in functional foods, highlighting its promising applications (Din *et al.,* 2022).

Hempseeds are derived from the plant Cannabis sativa L. This was extensively used as a subsidiary product of the hemp fibre processing industry and also contains low levels of

delta-9-tetrahydrocannabinol (THC). The nutritional as well as functional properties of hempseed lead to large-scale production, and their derivatives can be used as dietary supplements for preventing and treating various long-term health conditions and inflammatory responses both in humans and animals. In addition to all the nutritive value, it is rich with natural antioxidants and other bioactive compounds (Irakli *et al.* 2019). Thus, hempseeds find extensive applications in the food industry, serving as feed supplements for livestock and as an ingredient that enhances everyday foods and contributes to the development of functional food products (Farinon *et al.* 2020).

The mulberry plant is classified under the family Moraceae, which has extensive uses in agriculture, food, pharmaceuticals, and cosmetics. They possess various medicinal properties and can be used to treat inflammation, oncological conditions like cancer, heart-related disorders, and digestive problems. The beneficial compounds like sterols, glycosides, plant alkaloids, complex carbohydrates, polyphenols, terpenes, bioflavonoids, and tannic acids present in the plant Mulberry extracts and bioactive compounds obtained from different parts of the plant have shown antioxidant, neuroprotective, antimicrobial, hypoglycemic, cardioprotective, antihelmintic, immune regulatory, and dermoprotective activities. Adding mulberry to food products and animal feed enhances their phenolic stability, sensory, antimicrobial, and antioxidant properties and improves nutrient digestibility and growth. Through the study, it provides the potential application of mulberry as animal feed (Maqsood *et al.*, 2022).

Carotenoids are present in plants and have a beneficial effect on human health. They are very important in disease prevention and the maintenance of good health in humans (Kiokias *et al.,* 2016; Rao *et al.,* 2007). Clinical studies suggest that consuming carotenoids may lower the risk of cancer, cardiovascular diseases, and eye diseases. Additionally, the review examines the importance of carotenoids as animal feed, particularly in fish, crustaceans, birds, livestock, and poultry (Langi et al. 2018).

Live microbes are commonly referred to asprobiotics," which offer beneficial health effects on the host’s health when used in a suitable amount as a functional food ingredient. The probiotics are used in various contexts, including humans, animals, and

aquaculture. It describes commonly used microorganisms as probiotics for human and animal welfare and outlines the criteria for their use in humans and as microbial feed additives in animal husbandry. The probiotics have immunomodulatory effects, which include an increased phagocytic capacity of macrophages (Delcenserie *et al.* 2008). This article gives fine detail on the sources of probiotics, their mechanism of action, and their potential role as antioxidants in the near future (Syangai et al. 2011).

A brief survey is conducted on the nutrition of fish and shrimp, focusing on the relationship between their development and the factors in their water environment like temperature, minerals, gas levels, and water flow, as well as their food supply. It also discusses the nutritional requirements of different species for growth and body weight. There are various purposes for keeping fish that range widely from consumption to reproduction or ornamental purposes in different systems like aquariums and lagoons. The survey provides additional information on the manufacturing and application of complete compound feed (Van limborgh 2000).

The inclusion of glutamate and glutamine in fish diets is crucial for optimal growth. Specific amino acid supplementation through diet enhances larval performance and survival (Anderson *et al.* 2002). These have multiple metabolic functions and serve as fuel for various fish tissues. Supplementing fish diets with glutamine and glutamate improves growth, intestinal health, immunological responses, muscle formation, ammonia detoxification, and hormonal regulation. Their use as feed additives can reduce fishmeal dependency in aquaculture while enhancing profitability. So, the understanding of the nutritional needs of fish for glutamine and glutamate represents a significant change in aquatic animal nutrition (Li et al. 2020).

The commercial form of microalgal biomass, such as liquid, flour, oil, powder, tablets, or capsules, contains bioactive compounds like essential fatty acids (PUFA), proteins, vital nutrients like vitamins and minerals, carotenoids, pigments, volatile organic compounds, phenolic compounds, and sterols (Andrade *et al.* 2018). These microalgal components can be a promising ingredient in functional foods due to the presence of beneficial nutrients (Batista *et al.* 2017) and offer various health benefits such as anti-inflammatory,

antimicrobial, antioxidant, and antiviral effects. They may also help prevent ulcers in the stomach and bowel obstructions like constipation, haemoglobin deficiency, hypertension, and diabetes. In addition to all the beneficial aspects, microalgae are rich sources of oligosaccharides that are not easily fermented by other intestinal bacteria, which makes them valuable in the formulation of functional food and feed (Camacho *et al.* 2019).

Amino acids and fatty acids are important dietary requirements for various fish species, such as common carp, salmonids, tilapia, channel catfish, and eels. Micronutrients like vitamins and minerals are required for channel catfish and salmonids, and some are available for tilapia, eels, and common carp. Utilising this data, economically viable feeds can be developed for aquaculture of various species. There is notable variation in the dietary requirements between fish, mammals, and birds. Fish commonly have a lower digestible energy to protein ratio; fish need omega-3 fatty acids, which are a type of polyunsaturated fatty acid, whereas land animals require omega-6 fatty acids. Fish can obtain minerals from the water, reducing the need for dietary supplementation. They also have a reduced ability for vitamin C synthesis, necessitating a dietary source. Further research is needed to refine nutrient requirements for major aquaculture species while conserving factors like fish size, temperature, and management. Investigate the impact of nutrition on fish health and product quality and develop advancements in feeding technology (Lovell 1991).

Aquaculture is experiencing rapid growth as a food sector, supplying more than half of the global fish for consumption. The feed used includes fish meal and fish oil, which are extracted from wild-caught sardines. This has economic and ecological concerns about food security. Alternate ingredients like microalgae, yeast, and bacteria show promise in aquafeeds, providing protein, lipids, and omega-3 sources (Sarkar 2023).

Earthworms can serve as an alternative source of protein for fish and poultry feed, offering environmental benefits through cleaner technologies. This article provides a summary of the productivity, suitability, and effectiveness aspects related to using earthworm as an alternate protein feed in aquaculture and in poultry (Parolini *et al.* 2020).

Different herbs were tested in the laboratory for their capacity to impede the growth of Aeromonas hydrophila, a bacterium that causes fish diseases called motile Aeromonas septicemia. The strongest antimicrobial activity was shown in the ethanol extract of *Psidium guajava* leaf. It prevented the growth of the bacteria, indicating a bacteriostatic effect. In tilapia fish, it was found that feeding them with either dried P. guajava leaf powder or dried ethanol extract of P. guajava leaf reduced the mortality caused by the bacterial infection without any negative impact on the fish. These findings suggest that P. guajava leaf has the ability to control fish diseases caused by the bacteria (Pachanawan *et al.* 2008).

This study investigated the impact of garlic (Allium sativum) on the antioxidant system of Tilapia nilotica or Oreochromis niloticus. Out of 120 fish weighing about 20 to 21 grammes, four groups were divided and fed different forms of garlic, like natural garlic, garlic oil capsules, and garlic powder capsules, in their diets. Each treatment had three replicates, while the control group did not contain any garlic. The experiment lasted for almost 3 months, and the results demonstrated that all garlic-fed groups showed a significant increase in weight gain, growth performance, and a lower mortality rate compared to the control group. The activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (Gper) were significantly increased. In addition to this, in garlic-fed fish, the activities of malonyldialdehyde, alkaline phosphatase, and aspartate aminotransferase in plasma were significantly reduced. Garlic-supplemented groups showed increased total protein in blood serum, while blood glucose, triglyceride, and cholesterol levels decreased. As a result, the dietary supplement with garlic in fish feed enhances the growth rate, reduces the death rate, and improves antioxidant properties in fish (Metwally *et al.* 2009).

A set of three experiments was conducted to examine the impact of dietary ascorbic acid on the reproductive capabilities of *Oreochromis mossambicus*. The first experiment demonstrated that supplementing brood stock feed with 125 milligrammes of ascorbic acid per 100 grammes of dry diet enhanced hatching efficiency and improved the condition of fry. In the second experiment, the fry produced from brood stock that did not receive supplemental ascorbic acid were themselves fed the same diet, and they exhibited

poor growth, food utilisation, and survival rates. In the third experiment, brood stock were fed an ascorbic acid-supplemented diet, and their offspring were fed an unsupplemented diet. This fry showed better performance in terms of growth, food utilisation, and survival rate compared to the fry from the second experiment. This suggests that supplementing brood stock diets with ascorbic acid leads to some transfer of the vitamin to newly hatched fry through eggs, resulting in improved ascorbic acid levels during the early stages of life (Soliman *et al.,* 1986).

Coconut as a Functional Food:

Coconut water

Coconut water, also called coconut liquid endosperm, is a versatile natural product that is consumed globally for its nutrition and health benefits. It is rich with vitamins, minerals, sugars, amino acids, and phytohormones and is used in oral rehydration and intravenous hydration for patients (Campbell *et al.* 2000). Also, it provides protection from myocardial infarctions (Anurag *et al.,* 2003). The micronutrients present in coconut water enhance the antioxidant system in humans (Evans & Halliwell 2001). In plant tissue culture and micropropagation, coconut water is commonly used for growth supplementation. Through this review, it summarises and assesses the biochemical and biological characteristics of coconut water (Yong *et al.* 2009).

The coconut tree is cultivated for its versatile uses, primarily for its medicinal as well as nutritional benefits. The various parts like tender coconut, coconut water, copra, coconut oil, raw kernel, coconut toddy, coconut shell, wood-based products, coconut leaves, and coir pith serve various purposes in traditional coconut growing areas. The coconut kernel and tender coconut water possess medicinal properties such as antifungal, antibacterial, antiparasitic, antiviral, anti-dermatophytic, antioxidant, hypoglycemic, hepatoprotective, and immunostimulant effects. They are very rich in micronutrients and contain trace amounts of vitamin B, thiamine, pyridoxine enzymes like catalase, dehydrogenase, peroxidase, and growth-promoting factors (Zhang *et al.,* 2022), which are essential for human health (DebMandal *et al.,* 2011).

Coconut milk and virgin coconut oil

The residual kernels from coconut milk extraction and virgin coconut oil production were examined for their potential as dietary fibres. These dietary fibres play a crucial role in human health (Ramos *et al.,* 2008). Cell wall polysaccharides from coconut were isolated from virgin coconut, which revealed that they are rich with high levels of neutral detergent fibre, crude fibre, acid detergent fibre, and hemicellulose compared to coconut milk and virgin coconut. Cell wall polysaccharides exhibited greater oil holding capacity, water holding capacity, and swelling capacity compared to others. The by-product of chemically treated coconut kernels can enhance the performance of dietary fibre, resulting in a superior product (Yelagama *et al.* 2011).

The role of diet is very important to maintaining cardiovascular health, and numerous studies are still progressing in this area. Recently, researchers identified the potential role of virgin coconut oil in preventing cardiovascular diseases. The unique chemical properties and manufacturing process of virgin coconut oil make it a healthier option compared to copra-derived oil. This review examines the mechanisms by which saturated fatty acids contribute to vascular disease, the impact of oils and fats on cardiovascular risk, and the current understanding of virgin coconut oil and its potential cardioprotective effects (Babu *et al.* 2014).

Coconut haustorium

To support the development and utilisation of coconut haustorium, the chemical composition, volatile profiles, and antioxidant properties of coconut haustoria are examined. The findings revealed that large coconut haustorium had a higher level of soluble sugar (47.10%) and reducing sugar (17.68%), whereas small coconut haustorium contained more ash of about 10.17%, protein of 9.22%, and fat, which contains 5.03%. All the coconut haustorium samples exhibited significant amounts of potassium and phosphorus content. The small coconut haustorium and large coconut haustorium have more balanced fatty acid and amino acid compositions, indicating high nutritional values.

These findings serve as a useful reference for utilising different coconut haustorium varieties as raw materials in functional foods and dietary additives (Zhang *et al.*, 2022).

The study examines the impact of an aqueous extract from coconut haustorium on isoproterenol-induced myocardial infarction in rats. The rats were administered the extract for about 45 days before inducing myocardial infarction with isoproterenol injections (20 mg/100 g body weight) given twice with a 24-hour interval. Isoproterenol caused cardiac damage as evidenced by increased levels of marker enzymes lactate dehydrogenase, aspartate transaminase, and alanine transaminase in the serum and reduced levels in the heart. However, the rat treated with coconut haustorium extract exhibited a significant reduction in these enzyme levels, which indicates the cardioprotective effects. In addition to that, the pretreatment with coconut haustorium increased antioxidant enzyme activity and decreased peroxide activity in the heart. These studies revealed that the extract of aqueous coconut haustorium possesses notable antioxidant and anti-inflammatory properties against isoproterenol-induced myocardial infarction in rats (Chikku *et al.* 2012).

The demand for animal feed is steeply increasing in both developed and developing countries. To meet this crisis, the use of another alternative feed source is essential. The use of coconut haustorium as a functional feed is very cheap and can improve digestive processes, growth, and health. However, not many researchers have exploited this area of interest, so novel methods of treating, extracting, and processing coconut should be promoted to enhance its nutritive values as a feed (Mat et al., 2022).

MATERIALS AND METHODS

Preparation of coconut haustorium (Zhang *et al*., 2022)

After washing with distilled water to remove the impurities the coconut haustorium is dried at 60 degree Celsius. Then the dried samples were crushed, sealed, and stored in a dessicator.

Treatment of *Oreochromis mossambicus*:

Tilapia fishes were collected from Kozhikode district and maintained in aquarium for 2 months providing coconut haustorium along with normal fish feed for the study. The fishes were categorized into control group, 10% group, and 20% group. Each group contains eight fishes. Fishes in the control group is provided with normal fish feed and for 10% group ten percentage of coconut haustorium along with normal fish feed, like 20% of coconut haustorium is incorporated with normal fish feed in 20% group. The study was carried out for a time period of two months and the effect of coconut haustorium as a functional food is observed by carrying out various experiments.

Estimation of carbohydrate content (Rasouli *et al.*, 2014):

The carbohydrate content was estimated by the method of Rasouli *et al*., (2014).

Procedure: 0.1ml of working sample were taken in a test tube and make up to 1ml with distilled water and 1ml of sample solution from control, 10%, 20% were taken in another test tubes. 1ml phenol reagent followed by 5ml concentrated sulfuric acid were added to each test tube by shaking for 10 minutes and incubated in the water bath for 20 minutes. Absorbance was read at 470nm using colorimeter using a reagent blank.

Estimation of total protein (Kingsley, 1939)

Principle: In alkaline solution, proteins form a blue-violet colored complex together with copper ions. The intensity of the color formed is directly proportional to the quantity of total proteins present in the sample.

Procedure: To 1 mL of Biuret reagent, 10 µL of sample (serum or tissue sample) and 10 µL of protein standard (6 g/dL) were added into the test and standard reaction systems

respectively, properly mixed and incubated at 37^0C for 5 min. The absorbance was measured at 555 nm within 60 min. The serum total protein was calculated using the formula:

$$\text{Total protein } \left(\frac{\text{g}}{\text{dL}}\right) = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \times \text{Conc. of Standard}$$

Reduced glutathione (GSH) content (Moron et al., 1979)

Principle: GSH is measured by its reaction with DTNB, which yields a yellow colored complex with maximum absorption at 412 nm.

Procedure: About 200 µL of clarified tissue homogenate was mixed with 50 µL of 25% TCA (final concentration of 5% or less) and kept at 25oC for 5 minutes. Further, the mixture was centrifuged at 3000 g for 10 min and from the supernatant, 100 µL was mixed with 900 µL of 0.2 M sodium phosphate buffer (pH 8.0) and 2.0 mL DTNB (0.6 mM). The mixture was then incubated at room temperature for 20 minutes and absorbance was measured at 412 nm. A standard curve for reduced glutathione was plotted and using the slope of the curve, concentration of GSH was calculated and expressed as nmol/mg protein.

Catalase activity (Beers and Sizer, 1952).

Principle: The catalase activity was assayed by measuring the decomposition of H2O2 which has an absorption maximum at 240 nm and this absorption decreases with the decomposition of H2O2. The difference in extinction per unit time is used to measure the catalase activity.

Procedure: Clarified tissue homogenate (100 µL) was mixed with 2.8 mL of 0.1 M phosphate buffer (pH 7.4). The reaction was initiated by adding 100 µL of 0.3% H2O2 solution. The activity was recorded as the decrease in absorbance at 240 nm 30 seconds interval for 3 min. The catalase activity was calculated using the molar extinction

coefficient of hydrogen peroxide (43.6 mM-1cm-1) and the specific activity at 25 ºC was expressed as mmoles of H2O2 consumed/min/mg of protein.

$$\text{Catalase activity } \left(\frac{\text{U}}{\text{mg protein}}\right) = \frac{\text{Ab at } \frac{240}{\text{min}} \times 1000 \times 3}{43.6 \times \text{mg protein in sample}}$$

Glutathione peroxidase (GPx) activity (Hafeman *et al.*, 1974)

Principle: Glutathione peroxidase depletes H_2O_2 in presence of glutathione (GSH). The remaining GSH is measured using 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB), which gives a colored complex.

$H_2O_2 + 2\ GSH \rightarrow GSSG + 2\ H_2O$

Procedure: Reaction mixture contained 100 µL (5 mM) GSH, 100 µL of 0.1 M phosphate buffer (pH 7.0), 100µL sodium azide (25mM), 100 µL tissue extract and 100 µL of H_2O_2 (1.2 mM) in a total volume of 2.5mL. The reaction mixture was incubated at 37^0C for 6 minute. Reaction was stopped by addition of 2.0 mL of 1.67% meta-phosphoric acid and centrifuged for 15 minutes at 2500 rpm. To about 2mL of supernatant, added 2mL of 0.1 M phosphate buffer containing DTNB (1mM). Incubation was carried out at 37ºC for 10 minutes and OD was measured at 412 nm. A sample without the tissue was processed in the same way and was kept as the blank. The activity is expressed as U/mg protein.

$$\text{GPx activity } \left(\frac{\text{U}}{\text{mg protein}}\right) = \frac{\text{OD of Blank-OD of Sample} \times 1000}{\text{mg protein} \times 10}$$

One unit of enzyme activity was defined as a decrease in log GSH by 0.001/min after subtraction of the decrease in log GSH per minute for the non-enzymatic reaction.

Estimation of glutathione-s-transferase activity (Ghalia *et al* 2000):

The total GST activity was spectrophotometrically determined utilizing 1-Chloro- 2,4-dinitrobenzene (CDNB) as the substrate which was described by Ghalia *et al* 2000

Procedure: To estimate the GST content, 0.3ml of 50mM CDNB in methanol, 2.7ml of 5mM glutathione in 100mM Tris-HCl (pH7.5), and 10µL of sample in PBS were added to a test tube which is the reaction mixture. The absorbance at 340nm was measured using spectrophotometer against a reagent blank without glutathione.

Thiobarbituric acid reactive substances (TBARS) (Ohkawa *et al.*, 1979)

Principle: Lipid peroxidation products, especially malondialdehyde (MDA), produced during peroxidation of polyunsaturated fatty acids (PUFAs) can react with thiobarbituric acid (TBA) reagent under acidic conditions to form a pink colored complex, which has an absorption maximum at 532 nm.

Procedure: About 200 µL of tissue homogenate was mixed with 600 µL of distilled water, 200 µL sodium lauryl sulfate (8%), 1.5 mL each of 20% acetic acid (pH 3.5) and thiobarbituric acid (0.8%). The mixture was further incubated at 100°C for 1hr in a boiling water bath. The samples were cooled and mixed with 4 mL of pyridine: butanol (15:1) and centrifuged at 3000 g for 10 min. The clarified supernatant was collected and read at 532 nm against a reagent blank and the concentration of lipid peroxidation products were calculated from the standard curve prepared using MDA and expressed as nmol of MDA/mg protein.

Conjugated diene (CD) and conjugated triene (CT) (Pegg, 2004)

Principle: CD and CT are the initial lipid peroxidation products formed, that have characteristic UV absorbance maxima at 234 and 269 nm.

Procedure: The tissue samples (30 µL) were diluted to 3.0 mL using isooctane and the absorbance of each sample was measured at 234 and 269 nm. Sample with absorbance above 0.800 was appropriately diluted and the dilution factor was applied while calculation. The level of CD and CT are calculated using the molar extinction coefficient of linoleic acid hydroperoxide (2.525×10^4 M^{-1} cm^{-1}) and expressed as nmoles of linoleic acid hydroperoxide equivalents/ Kg of oil.

Trace elemental composition by atomic absorption photometry:

Atomic absorption spectrometry (AAS) is an analytical technique used to determine the concentration of specific elements in a sample. It is based on the absorption of light by atoms in the ground state, which occurs at characteristic wavelengths for each element high resolution continuum source atomic absorption spectrometry (HR-CS AAS) utilizing flame and furnace atomizers.



Fig:1 fresh coconut haustorium



Fig:2 dried coconut haustorium



Fig 3: *Oreochromis mossambicus*



Fig 4: muscle of *Oreochromis mossambicus*

RESULT

Table 1 depicts the total carbohydrate, total protein and the body weight. The body weight were observed as 22.8±3.13g in control group, 21.95±4.36g in CH- Low and 23.97±4.41in CH- High group. Total carbohydrate and total protein contents in the muscle. The total carbohydrate contents in the CH- High, CH-Low, and control group were 90.33±3.88 mg/g, 87.65±4.17 mg/g, 88.4±4.05 mg/g. The total protein contents in the CH-High, CH-Low, and control group of Tilapia were 25.90±3.90, 25.67±3.13, 24.15±2.04.

Table 1: Body weight changes, Total Carbohydrate and Total Protein contents in the muscle of tilapia fed with different treatment diet.

	Body weight (g)	Total Carbohydrates (mg/g)	Total Protein (mg/g)
Control	22.8±3.13	88.4±4.05	24.15±2.04
CH- Low	21.95±4.36	87.65±4.17	25.67±3.13
CH- High	23.97±4.41	90.33±3.88	25.90±3.09

Statistical significance * indicate $p<0.05$, ** indicate $p<0.01$, *** indicate $p<0.001$

Table 2 showed the result of enzymatic and non-enzymatic antioxidant parameters in muscle of Tilapia fish. The non-enzymatic antioxidant parameter, the reduced glutathione content was estimated to be 4.31±0.19 nmols/mg in the control group which was increased to 4.94±0.13 nmols/mg in CH-Low group ($p<0.05$) and 5.25±0.20nmols/mg in CH-High group ($p<0.01$). The enzymatic antioxidant parameters like catalase, glutathione peroxidase, and glutathione-s-transferase were also depicted in table 2. The catalase content was estimated to be 72.38±4.35U/mg protein in the control group which was increased to 72.38±4.35U/mg protein in CH-Low ($p<0.05$) and 89.97±3.88U/mg protein in CH-High group. Glutathione peroxidase the other enzymatic antioxidant content in control group was found to be 29.14±3.02U/mg protein which is increased to 36.47±2.95U/mg protein in CH-Low and 42.77±4.12U/mg protein in CH-High group ($p<0.05$). The glutathione-s-transferase content was estimated to be 100.25±5.11U/mg

protein in the control group which was increased to 122.39±7.61U/mg protein in CH-Low group ($p<0.05$) and to 127.55±7.19U/mg protein in CH-High group ($p<0.01$).

Table 2: Changes in enzymatic and non-enzymatic antioxidant parameters of tilapia fed with different treatment diet

Organ	Treatment	Reduced Glutathione (nmols/mg protein)	Catalase (U/mg Protein)	Glutathione Peroxidase (U/ mg protein)	Glutathione-s-Transferase (U/ mg Protein)
Muscle	Control	4.31±0.19	72.38±4.35	29.14±3.02	100.25±5.11
	CH- Low	4.94±0.13*	83.22±4.71*	36.47±2.95	122.39±7.61*
	CH- High	5.25±0.20**	89.97±3.88**	42.77±4.12*	127.55±7.19**

Statistical significance * indicate $p<0.05$, ** indicate $p<0.01$, *** indicate $p<0.001$

Table 3 reflected on the result of changes in oxidative stress parameters in muscle of tilapia fish. The Thiobarbituric Acid Reactive Substance (TBARS) content was estimated to be 8.22±0.65nmols/mg protein in the control group which was reduced to 6.32±0.51nmols/mg protein in CH-Low group ($p<0.05$) and to 6.04±0.28nmols/mg protein in CH-High group ($p<0.05$). The conjugated Diene content in control group was found to be 132.5±8.05nmols/mg protein which is reduced to 105.7±7.12nmols/mg protein in CH-Low group ($p<0.01$) and to 89.6±7.41nmols/mg protein in CH-High group ($p<0.001$). The conjugated triene content of the control group was depicted to be 103.7±4.95nmols/mg protein which is reduced to 80.6±4.46nmols/mg in CH-Low ($p<0.01$) and to 75.5±5.14nmols /mg protein in CH-High group ($p<0.001$).

Table 3: Changes in Oxidative stress parameters of Tilapia fed with different treatment diet

Organ	Treatment	TBARS (nmoles/ mg protein)	Conjugated Dienes (nmoles/ mg protein)	Conjugated Triene (nmoles/ mg protein)
Muscle	Control	8.22±0.65	132.5±8.05	103.7±4.95
	CH- Low	6.32±0.51*	105.7±7.12**	80.6±4.46**

	CH- High	6.04±0.28*	89.6±7.41***	75.5±5.14***

Statistical significance * indicate p<0.05, ** indicate p<0.01, *** indicate p<0.001

Table 4 shows the results of trace elemental analysis by atomic absorption spectrometry of muscle in tilapia fishes. The major trace elements found in muscle include iron, copper, zinc, selenium, and manganese. The iron content was estimated to be 142.55±7.01 in control which is increased to 144.32±7.25 in CH-Low and reduced to 138.27±7.17 in CH-High group. The copper content was found to be 8.58±0.02, 9.11±0.73, 8.89±0.34 in control, which is increased in CH-Low, and CH-High group respectively. Zinc was estimated to be 51.38±0.07 in control group which is increased to 59.04±0.01, 69.22±0.19 in CH-Low and CH-High group ($p<0.05$). The selenium was found to be 2.14±0.02 in control group which is increased to 2.55±0.01 in CH-Low group ($p<0.05$), and 2.71±0.06 in CH-High group ($p<0.01$). Manganese is another trace element which was found to be 5.64±0.8 in control group and increased to 6.03±0.2 in CH-Low group and to 6.11±0.4 in CH-High group.

Table 4: Trace element composition of the meat determined by Atomic Absorption Spectrometry

Element	Control	CH- Low	CH- High
Iron	142.55±7.01	144.32±7.25	138.27±7.17
Copper	8.58±0.02	9.11±0.73	8.89±0.34
Zinc	51.38±0.07	59.04±0.01	69.22±0.19*
Selenium	2.14±0.02	2.55±0.01*	2.71±0.06**
Manganese	5.64±0.8	6.03±0.2	6.11±0.4

Statistical significance * indicate p<0.05, ** indicate p<0.01, *** indicate p<0.001

DISCUSSION

Coconuts are widely known for their versatility and nutritional value, providing a range of products such as coconut oil, milk, water, and shredded coconut. However, amidst the plethora of coconut-derived products, there is one component that remains relatively underutilized - the coconut haustorium. The coconut haustorium, also known as the coconut sprout or embryo, is a gelatinous, spongy tissue found inside the coconut shell. It is responsible for the growth of the coconut palm tree, and its role is to nourish the developing embryo until it reaches maturity. Despite being an integral part of the coconut's growth process, the haustorium is often discarded during the processing of coconut-based products. The coconut haustorium possesses several noteworthy nutritional attributes. It is rich in dietary fiber, vitamins (such as vitamin C and B-complex vitamins), minerals (including iron, magnesium, and potassium), and antioxidants. Additionally, it contains beneficial medium-chain fatty acids (MCFAs), which are known to provide energy and support overall health (Tan and Gao, 2020).

The results of the study demonstrate the potential beneficial effects of coconut haustorium (CH) supplementation on the antioxidant status of Nile Tilapia. The supplementation of CH at 10% and 20% levels in the normal diet resulted in notable changes in the muscles of the fish. One significant finding is the increased content of reduced glutathione in the muscles of the Nile Tilapia. Glutathione is a powerful antioxidant that plays a crucial role in neutralizing harmful free radicals and protecting cells from oxidative damage. The elevation of reduced glutathione suggests that CH supplementation may enhance the antioxidant defense system in the fish.

Furthermore, the study observed a dose-dependent increase in the activities of antioxidant enzymes such as catalase, glutathione peroxidase, and glutathione S-transferase (Lu, 2013). These enzymes are responsible for breaking down harmful reactive oxygen species (ROS) and detoxifying the body (Sies, 1997). The increased activities of these enzymes indicate an enhanced ability of the fish to counteract oxidative stress and maintain cellular health.

In addition to the increased antioxidant defense, CH supplementation led to a significant reduction in markers of lipid peroxidation. Thiobarbituric acid reactive substances

(TBARS), conjugated dienes, and conjugated trienes are products of lipid oxidation and are commonly used as indicators of oxidative stress (Esterbauer and Cheeseman, 1990). The reduction in these substances suggests that CH supplementation can mitigate oxidative damage to lipids in the muscles of the Nile Tilapia. Overall, these findings indicate that CH supplementation has the potential to enhance the antioxidant status and reduce oxidative stress in Nile Tilapia. By increasing antioxidant defenses, reducing lipid peroxidation, and improving glutathione levels, CH may contribute to improved overall health and potentially mitigate the risk of various diseases associated with oxidative stress.

The additional finding that coconut haustorium (CH) supplementation increased the levels of trace elements such as selenium, manganese, zinc, and iron in the fish is noteworthy. These trace elements play essential roles in various physiological processes and are crucial for maintaining overall health (Nielsen, 1996).

Selenium is a vital micronutrient known for its antioxidant properties. It functions as a cofactor for several antioxidant enzymes, including glutathione peroxidase, which helps protect cells from oxidative damage. The increased selenium levels observed in the fish could contribute to enhanced antioxidant defense and support overall cellular health.

Manganese is an essential trace element involved in numerous enzymatic reactions, including those related to antioxidant defense. It serves as a cofactor for the antioxidant enzyme superoxide dismutase, which helps neutralize harmful superoxide radicals. The elevated levels of manganese in the fish suggest that CH supplementation may enhance the activity of superoxide dismutase and potentially improve the fish's antioxidant capacity (Aschner and Aschner, 2005).

Zinc is a critical trace element involved in various physiological processes, including immune function, cell growth and repair, and antioxidant defense. It acts as a cofactor for several antioxidant enzymes, such as catalase and superoxide dismutase. The increased zinc levels in the fish due to CH supplementation may contribute to the activation of these enzymes, further enhancing the fish's antioxidant defenses and overall health.

Iron is an essential component of hemoglobin, the protein responsible for transporting oxygen in the blood. It is also involved in cellular respiration and energy metabolism. The increased iron levels in the fish following CH supplementation could potentially improve oxygen transport and energy production, promoting optimal physiological functioning.

The elevation of these trace elements in the fish suggests that CH supplementation has the potential to enhance the nutritional profile of the fish and contribute to their overall health and well-being. However, it is important to consider the optimal levels of these trace elements, as excessive amounts can have adverse effects. Further research is needed to determine the ideal CH supplementation levels and evaluate the impacts on fish growth, immune function, and other physiological parameters.

It is important to note that this study focused specifically on the effects of CH supplementation in Nile Tilapia. Further research is needed to determine the generalizability of these results to other fish species and to explore the underlying mechanisms through which CH exerts its antioxidant effects. Additionally, investigations into the potential impacts on fish growth, immune response, and product quality would be valuable in assessing the overall benefits of CH supplementation in aquaculture practices.

CONCLUSION

In conclusion, the results suggest that CH supplementation can positively impact the antioxidant status of Nile Tilapia by increasing reduced glutathione content and enhancing the activities of antioxidant enzymes. Moreover, CH supplementation effectively reduced markers of lipid peroxidation in the fish muscles. Further, increased levels of trace elements such as selenium, manganese, zinc, and iron following CH supplementation highlight the potential nutritional benefits of incorporating CH into fish diets. These trace elements play vital roles in antioxidant defense, immune function, and overall health. These findings highlight the potential of CH as a natural antioxidant supplement in aquaculture, promoting fish health and improving the quality of aquaculture products.

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Unlocking the Health Benefits: Impact of Coconut Haustorium on Hepatic Redox Balance and Lipid Profile in *Oreochromis mossambicus*

Dissertation report submitted in partial requirement for M.Sc. degree course in Zoology with Environmental Biology as special subject

in

University of Calicut

by

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KOZHIKODE -08

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REPORT SUBMITTED IN PARTIAL REQUIREMENT FOR M.Sc. DEGREE COURSE IN ZOOLOGY WITH ENVIRONMENTAL BIOLOGY AS SPECIAL SUBJECT

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Unlocking the Health Benefits: Impact of Coconut Haustorium on Hepatic Redox Balance and Lipid Profile in *Oreochromis mossambicus*

Report of the MSc Students project submitted to St. Joseph's College (Autonomous) Devagiri

Affiliated to the University of Calicut

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Date: **Head of the Department**

CERTIFICATE

This is to certify that the dissertation entitled **"Unlocking the Health Benefits: Impact of Coconut Haustorium on Hepatic Redox Balance and Lipid Profile in Oreochromis mossambicus"** is an authentic record of studies and research work done by **Ms. Sadiya Mariyam** (Reg No: **DVAVMZL011**) under my guidance and supervision and that, to the best of my knowledge and belief, this work has not been presented earlier for any degree or fellowship.

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DECLARATION

I hereby declare that the dissertation entitled **"Unlocking the Health Benefits: Impact of Coconut Haustorium on Hepatic Redox Balance and Lipid Profile in Oreochromis mossambicus"** submitted in partial fulfillment of the requirements for the award of the degree of MASTER OF SCIENCE IN ZOOLOGY is a record of original work submitted by me at St. Joseph's College (Autonomous), Devagiri under the supervision and guidance of Mr. Joice Tom J., Assistant Professor, Department of Zoology, St. Joseph's College (Autonomous), Devagiri, Calicut during 2021-2023.

I hereby declare that the results presented have not been previously submitted for the award of any degree or fellowship.

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ACKNOWLEDGEMENT

It is with a deep sense of pleasure that I express my sincere gratitude to **Mr. Joice Tom J.** M.Sc, M.Phil, Assistant Professor, P.G and Research Department of Zoology, St. Joseph's College (Autonomous), Devagiri, Kozhikode, for his valuable guidance and supervision in the form of practical suggestions and constructive criticisms which formed the basis for the successful completion of this project.

I express my sincere gratitude to **Dr. Vineesh P.J**. MSc, B.Ed, Ph.D., Assistant Professor and Head, PG and Research Department of Zoology for his support and sincere encouragement.

I extend my deep gratitude to **Dr. Arunaksharan N**. MSc., B.Ed, Ph.D., Assistant Professor, PG and Research Department of Zoology and Princy P (Research fellow) for their wholehearted support and advice that helped me throughout the project I had undertaken.

I convey my sincere gratitude to Mr. Saji and Mr. Kiran for their help in collecting the samples required for completing my project work.

I am greatly indebted to Ms. Nivya K George, the co-researcher for helping me to complete the project and providing her valuable time in editing and proofing my thesis.

I extend my heartfelt thanks to the non-teaching staff of department for their valuable cooperation.

Finally, I express my deep gratitude to my family and friends for their sincere support throughout the project. Above all, I thank God Almighty for the blessings for the successful completion of the project.

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INTRODUCTION

Nutrition refers to the process by which the body obtains and utilizes nutrients from food for growth, development, and overall health. It involves the intake, digestion, absorption, transport, and utilization of nutrients to support various bodily functions. Good nutrition is vital for maintaining optimal health, supporting growth and development, and preventing chronic diseases. By consuming a balanced diet that includes a variety of nutrient-rich foods, we can provide our bodies with the essential vitamins, minerals, proteins, carbohydrates, and fats they need to function at their best. A balanced diet refers to the practice of consuming a variety of foods from different food groups in appropriate proportions to meet the body's nutritional needs. Adopting a balanced diet, maintaining a healthy weight, and making lifestyle choices that prioritize nutrition can help reduce the risk of these diseases and promote overall health and well-being.

Functional foods are dietary items that, besides providing nutrients and energy, beneficially modulate one or more targeted functions in the body, by enhancing a certain physiological response and by reducing the risk of disease. Functional foods typically contain bioactive compounds, such as vitamins, minerals, antioxidants, probiotics, prebiotics, and phytochemicals, which have beneficial effects on the body. Some examples include probiotic-rich foods like yogurt and kimchi, which support gut health. Omega-3 fatty acid foods like salmon and flaxseeds promote heart and brain health. Antioxidant-rich berries and green tea help combat free radicals, while fiber-rich foods like whole grains and legumes support digestion. Fortified foods such as cereals with added vitamins and minerals address nutritional deficiencies. Additionally, herbal teas like chamomile and ginger offer calming and anti-inflammatory properties.

Coconut, scientifically called *Cocos nucifera,* is widely recognized as a functional food due to its impressive array of health-promoting properties. Packed with essential nutrients such as vitamins, minerals, and fiber, coconuts offer a nutritional boost to the diet. They are a rich source of healthy fats, including beneficial medium-chain triglycerides, which provide quick energy and potential benefits for weight management and brain health. Additionally, coconuts contain antioxidants that protect against oxidative stress, potentially reducing the risk of chronic diseases. With its antimicrobial properties, coconut supports a healthy immune system and digestive health. Furthermore,

its low glycemic index and potential positive effects on heart health make it a valuable addition to a balanced diet. The various parts of the coconut, including its husk, shell, oil, and milk, offer a multitude of beneficial applications.

Coconut haustorium (Cocos nucifera L.), is a spongy tissue which forms during the germination of coconut, rich in dietary fibre, iron, phenolics, ash, starch, protein, fat and soluble sugars. The dried coconut haustorium revealed the following composition per 100 grams: ash (1.05 ± 0.2 g), soluble sugar (44.2 ± 4.6 g), starch (24.5 ± 3.2 g), protein (5.50 ± 0.3 g), fat (1.99 ± 0.9 g), soluble dietary fiber (5.72 ± 0.4 g), insoluble dietary fiber (20.3 ± 1.9 g), and phenolics (146 ± 14.3 mg). Mineral profiling showed potassium, magnesium, calcium, phosphorus, manganese, copper, iron, and zinc. The amino acid score revealed lower levels of methionine + cysteine (57.6%), phenylalanine + tyrosine (32.6%), leucine (45.7%), and isoleucine (68%) in the haustorium. The antioxidant activity assays like CUPRAC, FRAP, DPPH and ABTS showed that the coconut haustorium exhibits significant antioxidant potential. This indicates its ability to neutralize free radicals and suggests its potential as a natural source of antioxidants (Arivalagan Manivannan et al. 2018).

Oreochromis mossambicus, commonly known as the Mozambique tilapia, is a popular fish species in aquaculture due to its adaptability, high growth rate, and nutritional value. Nutrition plays a crucial role in achieving optimal growth and overall health in fish. The haustorium, an understudied component found within the coconut seed, presents an intriguing opportunity to explore its potential as a functional feed additive for O. mossambicus. By examining the effects of incorporating coconut haustorium into the fish's diet, we can gain valuable insights into its impact on growth performance and antioxidant capacity. Investigating the influence of haustorium supplementation on the nutritional composition of the fish, including fatty acid profiles and micronutrient content, will contribute to understanding its potential as a nutritional enhancer in aquaculture. Through this study, we aim to comprehensively explore the effects of coconut haustorium as a dietary supplement on the growth performance and antioxidant properties of O. mossambicus.

OBJECTIVES

The objective of this study is to investigate the changes in the antioxidant parameters and lipid profile in the liver of *Oreochromis mossambicus* (tilapia) when fed with coconut haustorium. The specific objectives are as follows:

- To assess the impact of coconut haustorium as a dietary supplement on the antioxidant capacity of the liver in *Oreochromis mossambicus.*
- To determine the effect of coconut haustorium on the levels of oxidative stress markers in the liver of *Oreochromis mossambicus.*
- To investigate the alterations in antioxidant enzyme activities, including reduced glutathione activity, catalase (CAT), and glutathione peroxidase (GPx), in the liver of *Oreochromis mossambicus* following coconut haustorium supplementation.
- To examine the changes in the lipid profile, including total cholesterol, triglycerides, and fatty acid composition, in the liver of *Oreochromis mossambicus* fed with coconut haustorium.

By addressing these objectives, this research aims to provide valuable insights into the impact of coconut haustorium on the antioxidant status and lipid metabolism of *Oreochromis mossambicus*, contributing to our understanding of the potential benefits of this dietary supplement in improving fish health and promoting sustainable aquaculture practices.

REVIEW OF LITERATURE

Nutrition and its importance

Nutrition can be understood as the set of processes through which living organisms acquire and utilise the essential substances required to sustain their functions, support growth, and regenerate their components. It encompasses the exploration of fundamental principles in chemistry and physiology that address the body's requirements for food, both in states of well-being and illness (D. F. Turner, 1947). Recent developments in the field of experimental and applied nutrition have led to an increased recognition of the social, economic, and political importance of nutrition (Nutr Rev. 1950). The role and significance of the understanding of nutrition within the context of public health nutrition are uncertain. Various categories of food behaviours and their underlying causes, influenced by environmental conditions and individual characteristics, including motivations, are studied. The interaction between factors that drive motivation and the processing of information and the differentiation between declarative and procedural knowledge is crucial for nutrition promoters. Examining the different areas of knowledge pertaining to nutrition reveals that their usefulness is inclined to be influenced by the specific goals and perspectives of consumers and nutritionists. Recent studies suggest that nutrition knowledge might have a limited yet significant role in embracing healthier eating patterns.

The consequences for the field of public health nutrition are that greater attention should be given to developing knowledge frameworks in children and adults; the education sector needs to take a proactive role in promoting nutrition knowledge; and the individual food objectives of consumers and their acquisition of practical knowledge should be taken into account to help them achieve those goals (Worsley A. Asia Pac J. 2002).

Promoting healthy lifestyles is crucial for preventing early death,long-term illnesses, declines in productivity, and various societal and financial issues. Implementing worksite actions that tackle fitness and nutrition while also addressing work-related outcomes is a complex task. It can be challenging to accurately measure the effectiveness of such interventions. However, research findings demonstrate that there is a potential to enhance work-related outcomes through positive influence, particularly absence, through health advocacy initiatives that encompass aspects related to the workplace's physical

environment and organisational framework (Aikaterini Grimani et al. 2019). The increasing public health importance of micronutrient deficiencies is evident. It is crucial to recognise that even mild-to-moderate deficiencies or insufficient levels of certain micronutrients have significant implications for public health. These consequences should be taken into account alongside the impacts of severe deficiency. (Pamela J. Magee et al. 2019)

Balanced Diet and Disorders and Diseases due to Nutritional Deficiencies

The diet plays a significant role in determining an individual's health (Giazitzi K., Boskou G., 2021). Numerous researchers have emphasised the significance of maintaining a properly proportioned diet and monitoring daily energy consumption in everyday life (Hopen D. 2007). Furthermore, research studies have demonstrated that a properly proportioned diet can contribute to slimming in individuals who are obese (Ghannadiasl F. 2019).

A diet abundant in vegetables with low starch content, fruits with low glycemic indices, cereal products, legumes, plant oils such as olive,sunflower, and colza, and fish (particularly sea fish like mackerel and sardines), alongside a moderate intake of meat and dairy products, can have a positive impact on health and potentially reduce the risk of developing various diseases, including neurodegenerative conditions (Wojciech Grodzicki et al. 2020). A well-balanced diet and proper nutrition play a crucial role in maintaining an optimal immune response and preventing infections. For instance, an adequate intake of protein is essential for the production of antibodies, which are vital for an effective immune system. Insufficient levels of micronutrients like vitamin A or zinc have been associated with a higher likelihood of infection. Often, deficient nutritional status is linked to inflammation and oxidative stress, both of which can negatively affect the defence system.

Certain dietetic components exhibit potent anti-inflammatory and free radical scavenging capacities that can benefit the immune system. Examples include vitamin E, vitamin C, and phytonutrients such as carotenoids and polyphenols. Some of the above compounds combine with transcription factors like NF-kB and Nrf-2, which are involved in anti-inflammatory and antioxidant processes. Vitamin D, especially, may impact cellular

invasion by collaborating with cell entry receptors like angiotensin-converting enzyme 2, which are involved in viral entry.

Additionally, roughage, when fermented by the microorganisms in the gut, produces short-chain fatty acids that have been shown to possess anti-inflammatory properties. Thus, incorporating fibre-rich foods into the diet can further support immune health (Mohammed Iddir et al. 2020).

There is solid evidence highlighting the significance of meeting daily dietary requirements in preventing and managing kidney illness. Medical nutrition therapy, accompanied by the guidance of a registered nutritionist, plays a vital role in the medical treatment of chronic kidney disease. Numerous risks such as hyperkalemia, blood pressure abnormalities, hyperphosphatemia, chronic metabolic acidosis, and edoema that are prevalent among CKD patients can be minimised, and the progression of the disease can be slowed down by closely monitoring the intake of phosphorus, protein, calcium, sodium, and potassium. This approach helps alleviate the symptoms experienced by individuals with CKD (Tania Naber et al., 2021). L-carnitine (LC) plays a vital role in the cellular energy metabolic process. A well-proportioned diet includes an ample amount of LC, essential amino acids, and microelements that contribute to the synthesis of endogenous carnitine. In individuals who are generally healthy, the body's own production of carnitine is adequate to mitigate symptoms of carnitine deficiency.

However, in people who have mitochondrial malfunction, such as those with Alzheimer's disease (AD), characterised by significant brain degeneration, the overall functioning of the body is often severely disrupted. The Mediterranean diet, which emphasises the intake of fruits, cereals, vegetables, olive oil, nuts, and seeds as the primary sources of fats, a low to moderate intake of dairy products and alcohol, a mild intake of fish and poultry, and limited consumption of red and processed meat, may offer benefits. Introducing foods abundant in carnitine and adhering to the MIND diet, along with carnitine supplementation for individuals with Alzheimer's disease, has the potential to improve their daily functioning and overall well-being (Alina Kepka et al. 2020). Adhering to a Mediterranean diet, which highlights the consumption of vegetables, herbs, fruits, spices, legumes, olive oil, and high fibre intake, can fulfil a significant function in reducing the impact of this pandemic. The MD's positive effects primarily stem from its various components that are abundant in anti-inflammatory and antioxidant characteristics.

Additionally, the MD may contribute to improving reproductive wellness, reducing the susceptibility to neurodegenerative illnesses, and providing protection against depression and socioemotional difficulties. Moreover, there is proof suggesting that healthy nutrition among women can influence the constitution of their gut microbiome, ultimately affecting the metabolic and overall well-being of their descendants. Thus, it is crucial to emphasise the advantageous impacts of the Mediterranean diet on metabolic, reproductive, and psychological well-being, as it has the potential to shape the well-being of future generations (Katherina V. Gantenbein et al., 2021).

The proper operation of the human physique relies on maintaining a harmonious equilibrium between dietary intake and metabolic processes. When this balance is disrupted, it can lead to either nutritional deficiencies or excesses. Nutritional deficiencies often arise due to various social factors like war, poverty, famine, and food trends. They can also be caused by medical conditions that hinder nutrient absorption, such as cystic fibrosis, Crohn's disease, or after undergoing weight loss surgery. Additionally, certain mental health disorders like alcoholism, autism, and eating disorders, as well as certain medications, can contribute to nutritional deficiencies. On the other hand, nutritional excesses occur when there is an unintentional or intentional overconsumption of nutrients. It's important to note that imbalances in nutrition can manifest in the form of skin issues, which may serve as early signs of underlying systemic problems (Melinda Jen et al. 2010).

The consumption of diets rich in fat and sugar has detrimental effects on the composition of the gut microbiota, resulting in gut dysbiosis. This dysbiosis is linked to the development of obesity and is also associated with cognitive decline. The emerging concept of the microbiota-gut-brain axis provides a potential framework for understanding the mechanistic link between high-fat diets, gut microbiota, and impaired cognition (Cicely Proctor et al. 2017).

Pulmonary arterial hypertension (PAH) is a crippling disease marked by narrowed blood vessels, inflammation, and clot formation. Nutritional deficiencies, including vitamin D and iron, are common in PAH patients and may worsen the condition. Animal studies suggest that certain dietary compounds, like flavonoids and polyphenols, can slow PAH progression. Additionally, the gut microbiota's role in PAH is emerging. Future interventions targeting nutrition and gut health may improve patient outcomes (Mara

Callejo et al. 2020). Certain fruits, vegetables, herbs, spices, cocoa, and their extracts have been observed to possess functional properties that enhance antioxidant levels and simultaneously regulate oxidative and inflammatory stress in humans. It is strongly advised to harness the full potential of the diverse range of phytochemicals found in fruits, vegetables, herbs, spices, and their extracts. These natural compounds engage in multifaceted synergistic interactions, effectively thwarting the escalation of oxidative and inflammatory stress, which are significant contributors to degenerative diseases (Mauro Serafini et al. 2016). Head and neck cancers are seen predominantly in nutritionally deficient people (M. R. Bassett et al. 1983). Obesity was linked with a 35% increased incidence of insufficient vitamin D levels. Regardless of age, there was a correlation between obesity and vitamin D deficiency (M. Pereira-Santos et al. 2015). The evidence presented unequivocally demonstrates that various dietary interventions offer valuable strategies for effectively managing individuals who suffer from type 2 diabetes. These interventions encompass the control of blood glucose and lipid levels as well as decreasing the risks associated with short-term and long-term diabetic complications (Omorogieva Ojo, 2019).

Functional Foods

Functional foods perform a function in supporting the body's function and managing specific health conditions (Sumit Gaur et al. 2014). Functional foods contain bioactive ingredients that have been linked to positive impacts on human health. These components help in shaping the gut microbiota and boosting immunity. They play a role in preventing serious conditions like cardiovascular issues and tumorigenesis. Key contributors are metabolites like short-chain fatty acids and polyunsaturated fatty acids generated by probiotics, as well as prebiotics and prebiotic-like substances like polyphenols, flavonoids, and vitamins present in functional foods. These components impact the gut microbiome by promoting the proliferation of advantageous microorganisms and the synthesis of advantageous metabolites. This directly provides advantages to the host by safeguarding against pathogens and sustaining a harmonious gut ecosystem. The intricate interplay between functional food ingredients, gut microbiota, human physiology, and their metabolic pathways work together to diminish factors that contribute to long-term illnesses (Mengfei Peng et al. 2020). Certain fruits, vegetables, herbs, spices, cocoa, and their extracts have been observed to possess functional properties that enhance

antioxidant levels and simultaneously regulate oxidative and inflammatory stress in humans. It is strongly advised to harness the full potential of the diverse range of phytochemicals found in fruits, vegetables, herbs, spices, and their extracts. These natural compounds engage in multifaceted synergistic interactions, effectively thwarting the escalation of oxidative and inflammatory stress, which are significant contributors to degenerative diseases (Mauro Serafini et al. 2016).

Fermented beverages and foods offer diverse dietary and therapeutic benefits. Lactic acid bacteria are key contributors to the beneficial health impacts of fermented dairy and similar commodities. Probiotic dairy foods commonly utilize *Lactobacillus acidophilus* and Bifidobacteria species. It is important for cultured products claiming health benefits to contain a suggested minimum of over 10^6 cfu/g during ingestion. Yoghurt is now considered a vehicle for probiotics. Powdered foods such as yoghurt powder and curd powder are produced while considering the survival of living beings after drying. These products are well-received by consumers because of their taste, aroma, and high nutrient content. The cell wall of starter bacteria in fermented foods exhibits antitumor activity that persists even after drying. Additional health benefits encompass prevention of gastrointestinal infections, lowering of serum cholesterol levels, and display of antimutagenic property. Fermented milks are advisable for individuals with lactose intolerance and those with atherosclerosis (V K Shiby et al. 2013).

Green tea, which is a traditional Chinese herb, may be considered a functional food due to its natural antioxidants, antimicrobial, anti-inflammatory, and antimutagenic activities. These properties are ascribed to its polyphenol content, mainly epigallocatechin-3-gallate. Green tea has been utilized in the treatment of persistent systemic conditions, such as cancer, thanks to its beneficial effects. With its broad range of activities, green tea can be a healthy option for controlling oral illnesses like oral cancer, dental caries, and chronic periodontitis, that commonly affect the elderly population (Sumit Gaur et al. 2014).

ω-3 polyunsaturated fatty acids offer numerous health benefits for conditions including diabetes, cancer, periodontal disease, cardiovascular disease, mental illnesses, cognitive decline, and rheumatoid arthritis. However, research shows ongoing controversies in relation to the impact of ω-3 polyunsaturated fatty acids on stroke, diabetes, cancer, and visual and neurological/brain development. Additionally, various therapeutic drugs

utilizing ω-3 polyunsaturated fatty acids have been developed (Fereidoon Shahidi et al. 2018).

Coconut as a Functional Food

Coconut is considered a highly nutritious functional food with a substantial dietary fibre content, vitamins, and minerals. Recent evidence suggests that coconut oil may have beneficial effects in treating insulin resistance, obesity, high LDL cholesterol, dyslipidemia, and hypertension, all of which are risk factors for cardiovascular disease, type 2 diabetes, and Alzheimer's disease. Furthermore, the presence of phenolic compounds and cytokinins in coconut has the potential to inhibit the aggregation of amyloid peptide, thereby aiding in its prevention, which is a crucial phase in the development of Alzheimer's disease. These findings indicate that coconut supplementation could be a promising therapeutic approach for preventing and managing Alzheimer's disease (W. M. A. D. B. Fernando et al. 2015).

Coconut offers a range of products, including copra, tender coconut water, raw kernel, coconut oil, coconut toddy, coconut leaves, coconut shell, wood-based products, and coir pith. These various components of the coconut are integral to the day-to-day lives of individuals residing in conventional coconut-growing regions. Moreover, coconut serves as an unparalleled reservoir of natural products utilised in both the fields of medicine and industry. The tender coconut water and coconut kernel exhibit a diverse array of medicinal attributes, including antiparasitic, hypoglycemic, antifungal, hepatoprotective, antibacterial, antiviral, antidermatophytic, antioxidant, and immunostimulant effects. Rich in microminerals and essential nutrients, coconut water and kernels are widely consumed as food, particularly in tropical countries (Manisha DebMandal et al. 2011). Coconut offers a range of diverse products and by-products, including testa, desiccated coconut, coconut water, milk, flour, copra, oil, and raw kernels, which find utility in the domesticated animal and aquaculture industries, particularly for ruminants. Nonetheless, the utilisation of coconut components in animal feed formulations is hampered by certain factors that limit its nutritional value. To overcome these limitations, exploring novel technologies, such as the pre-treatment of coconut, to mitigate the impact of substances with adverse nutritional effects holds promise for enhancing its suitability as animal feed. It is essential to fully capitalise on the various components of the coconut for animal nutrition purposes. Incorporating coconut into animal feed not only reduces feeding costs

but also aids in digestion, growth, and the overall health of the animals. Nonetheless, it is crucial to promote innovative approaches in the processing, extraction, and treatment of coconut to improve its nutritional quality and enhance its efficacy as an animal feed ingredient (Khairiyah Mat et al., 2022).

Coconut water

Coconut water is a invigorating beverage with valuable positive impacts on health. Its chemical constituents have applications in plant industry, biotechnology, and biomedicine. Cytokinins, which are found in coconut water, are particularly noteworthy and have shown significant potential within both plant and human organisms. Certain cytokinins have demonstrated promising anti-cancer properties, offering new perspectives in cancer treatment. Coconut water also comprises vitamins, various cytokinins, GAs, auxin, and ABA.(Jean W H Yong et al. 2009)

48 male Sprague-Dawley rats were categorized into four groups in a study: diabetic (DM), control (CO), diabetic treated with Glibenclamide (DM + Gli), and diabetic treated with coconut water (DM + CW). Diabetic models were induced through intraperitoneal administration of STZ. The experiment lasted for 20 weeks. Biochemical assays were employed to analyze the levels of Malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) within the left lens. Pathological analysis was conducted on the right lens. In vitro, cultured rat lens epithelial cells (LECs) were utilized and divided into different groups. In diabetic rats, the lens opacity index reduced and rat lens epithelial cells apoptosis ratio reduced with coconut water (CW) treatment. CW also resulted in decreased MDA levels and elevated activities of GSH-Px and SOD were observed in both in vivo and in vitro experiments. Compared to normal water, CW significantly alleviated cataract severity and LECs reduction caused by diabetes mellitus (Xiaohua Zhang et al. 2021).

The impact of coconut water (CCW) and milk (CCM) on gastrointestinal motility, oxidative stress, and inflammation in heat-stressed rats were examined. Heat-stressed rats exhibited increased gastrointestinal motility, but CCW and CCM reversed these effects. CCW and CCM also reduced serum cortisol levels and intestinal lipid peroxidation. Additionally, CCW and CCM enhanced activities of antioxidant enzymes and suppressed cytokines associated with inflammation. Intestinal morphology changes caused by heat

stress were mitigated by CCW and CCM. Overall, CCW and CCM demonstrated the potential to improve heat stress-related impairment of intestinal motility through reducing oxidative stress and suppressing inflammation.(Kazeem Olasunkanmi Ajeigbe et al. 2022)

Virgin Coconut Oil and Coconut Oil

Studies have revealed that coconut oil is rich in medium-chain fatty acids, which has sparked interest in its diverse applications. Apart from its culinary uses, coconut oil has gained attention for its potential health benefits, including its ability to lower cholesterol, combat cancer, reduce fatty liver, manage diabetes, act as an antioxidant, possess anti-inflammatory and antimicrobial properties, and moisturize the skin. However, despite these positive aspects, the intake of coconut oil is often underestimated as a result of inadequate extensive scientific substantiation supporting its benefits.(Afka Deen et al. 2021)

Coconut oil is a widely used ingestible oil extracted from mature coconuts. Two primary variants exist: copra oil and virgin coconut oil. Virgin coconut oil contains elevated concentrations of nutrients and bioactive compounds. Coconut oil is popular for its perceived health benefits due to specific medium-chain fatty acids. Lauric acid, which is the primary fatty acid in coconut oil, can act as both a medium- and long-chain fatty acid metabolically. Coconut oil shows potential in skincare for conditions like atopic dermatitis and dental care for preventing cavities. It may also have benefits for hair protection from damage and UV exposure. Limited evidence exists for its use in bone loss, Alzheimer's disease, and glycemic control. Further clinical research is necessary to explore weight loss and cardiovascular disease. Refined copra oil has a smaller impact on cholesterol in comparison to butter fat, but not vegetable oils high in cis unsaturated fats.(Taylor C Wallace. 2019)

Virgin coconut oil (VCO) is a traditional moisturizer with documented benefits for skin disorders. It exhibits anti-inflammatory effects, enhances skin barrier function, and provides moderate UV protection. VCO is non-irritating and non-phototoxic, making it a safe option for skin care. Studies confirmed that VCO has anti-inflammatory properties, as it reduces inflammatory markers and enhances the skin's barrier function for protection.(Sandeep R Varma et al. 2018)

Coconut Kernel

When levels of lipid and oxidative stress are impaired, it suggests that the body's natural antioxidant capacity is not functioning properly. A study was conducted which aimed to investigate how the presence of coconut kernel protein (CKP) has an impact on the levels of lipid peroxides and the activities of antioxidant enzymes in rats exhibiting diabetes. The researchers induced diabetes in the rats by injecting them with a single dose of alloxan before feeding them. For a period of 45 days, the rats were then given CKP in conjunction with a semi-synthetic diet. Upon the conclusion of the experiment, the researchers measured the concentrations of peroxide products and the activities of antioxidant enzymes. The results indicated that CKP helped maintain normal antioxidant enzyme and peroxide levels in the treated group in comparison to the diabetic rats. This study evidently demonstrates that coconut kernel protein has the capacity to alleviate oxidative stress linked to diabetes. The advantageous impact of CKP could potentially be attributed to its abundant presence of biologically potent arginine (Gopalakrishnan Salil et al. 2013).

A study was conducted which focused on assessing the characteristics of coconut kernel, coconut water, and commercial coconut products at various stages of maturity. Coconut water had high moisture content (95-97 g per 100 g) and reduced fat content (4-115 mg per 100 g). It exhibited acidity with a pH range of 4.5-5.2. On the other hand, coconut kernel had lower moisture content (50-85 g per 100 g) and higher fat content (37-56 g per 100 g). The ash content of the kernel decreased with maturity (1.0-1.5%), while coconut water maintained a consistent ash content (0.3-0.4 g per 100 g). The sugar concentration and acidity of coconut water remained constant throughout maturity. Phenolic content increased in both water (1.4-4.3 mg per 100 g) and kernel (18.5-24.8 mg per 100 g) as they matured. As the coconut kernel matured, the extracted oil demonstrated an elevation in saturated fatty acids and a decline in monounsaturated fatty acids within its fatty acid composition. There was an observed rise in the proportion of medium-chain fatty acids as maturity progressed. Coconut kernel and water comprised elevated quantities of phenolics (18.5-24.8 mg per 100 g and 1.4-4.3 mg per 100 g, respectively) and total tocopherols in the kernel (0.14-0.59 mg per 100 g) in comparison to commercial coconut products. These findings suggest that coconut water and kernel could be precious resources for the production of functional food supplements (Prakruthi Appaiah et al. 2015).

Coconut Testa

Coconut testa and tender coconut water (TCW) are secondary products generated during the processing of coconuts that contain valuable phytonutrients. If not properly utilized, they can contribute to pollution in the environment. The influence of phenolic concentrate (PHE) obtained from coconut testa and tender coconut water concentrates was studied in C57BL/6 mice fed a high-fat diet at doses of 50 and 100 mg for PHE, and 500 and 1000 mg/kg body weight for TCW. The findings indicated that the high-fat diet caused an elevation in body weight in comparison to the control group which is fed a starch diet (ND/SFD). However, treatment with PHE and TCW concentrates led to a dose-dependent reduction in body weight. Furthermore, the lipid profile of the treated mice showed significant decreases in the levels of cholesterol, triglyceride, and LDL, while HDL levels enhanced, indicating a positive impact on health. The treated groups also exhibited improved levels of GPx, catalase, TBARS in tissues, SOD, OGTT analysis, serum insulin, advanced glycation, and various degrees of atherogenic protection. In conclusion, the phenolic concentrate derived from TCW concentrates and coconut testa demonstrated hypolipidemic properties, suggesting their potential use in the creation of nutritious food (A S Mohan Kumar et al. 2022).

Coconut Haustorium

The chemical constituents, antioxidant properties, and volatile profiles of three haustoria with altering diameters were studied. Large coconut haustorium (LCH) had higher levels of soluble and reducing sugars, whereas small coconut haustorium (SCH) contained extra ash, fat, and protein. All haustoria were abundant in potassium and phosphorus. SCH had a more reasonable fatty acid composition, and middle coconut haustorium (MCH) had a favorable amino acid composition, indicating higher nutritive value. The dominant volatile components in all haustoria were acids, particularly isobutyric acid, which was abundant in SCH. Haustorium extract, primarily composed of polysaccharides, exhibited antioxidant activities. The EC50 values for LCH against SCH and hydroxyl radical against ABTS and DPPH radicals were determined. These findings provide valuable information for utilizing different haustoria in functional food or nutritional supplements (Yufeng Zhang et al. 2022).

A study was conducted on coconut haustorium, a porous tissue developed during coconut germination, to determine its biochemical constituents. Results showed that 100g of dried coconut haustorium comprised ash, soluble sugar, starch, phenolics, fat, soluble dietary fiber, insoluble dietary fiber, and protein. Mineral profiling revealed the occurrence of potassium, calcium, phosphorus, magnesium, manganese, copper, iron, and zinc. Antioxidant activity assay demonstrated the haustorium's capacity to act as an antioxidant. Amino acid analysis indicated certain amino acids were present in lower amounts in the haustorium (Arivalagan Manivannan et al. 2018). The effects of coconut haustorium extract on myocardial infarction induced by isoproterenol were examined in rats in a study. The rats were orally administered the extract for 45 days before inducing myocardial infarction. The activity of marker enzymes indicating cardiac damage was reduced in the haustorium pretreated rats compared to the isoproterenol-treated rats. Additionally, antioxidant enzyme activity increased and peroxidation product levels reduced in the heart of the haustorium pretreated rats. Histopathological analysis showed improved tissue morphology in the hearts of these rats. These findings suggest that coconut haustorium extract possesses notable cardioprotective and antioxidant characteristics against myocardial infarction induced by isoproterenol in rats (Chikku et al. 2012)

MATERIALS AND METHODS

Preparation of coconut haustorium (Zhang et al., 2022)

After being washed with distilled water to eliminate any impurities, the coconut haustorium is subjected to a drying process at a temperature of 60 degrees Celsius. Once fully dried, the samples are crushed and securely sealed before being placed in a dessicator.

Treatment of *Oreochromis mossambicus*:

Tilapia fish were obtained from Kozhikode district and kept in an aquarium for a duration of two months. As part of the study, coconut haustorium was included in addition to the regular fish feed. The fish were divided into three groups: the control group, the 10% group, and the 20% group. Each group consisted of eight fish. The control group received only the normal fish feed, the 10% group received fish feed supplemented with 10% coconut haustorium, and the 20% group received fish feed supplemented with 20% coconut haustorium. Over the course of two months, various experiments were conducted to observe the impact of coconut haustorium as a functional food for the fish.

Reduced glutathione (GSH) content Moron et al.1979)

Principle: GSH is measured by its reaction with DTNB which yields a yellow colored complex with maximum absorption at 412 nm.

Procedure: About 200 µl of clarified tissue homogenate was mixed with 50 µl of 25% TCA final concentration of 5% or less) and kept at 250C for 5 minutes. Further the mixture was centrifuged at 3000 g for 10 min and from the supernatant100 µl was mixed with 900 µl of 0.2 M sodium phosphate buffer pH 80) and 2.0 ml. DTNB (0.6 mM). The mixture was then incubated at room temperature for 20 minutes and absorbance was measured at 412 nm. A standard curve for reduced glutathione was plotted and using the slope of the curve concentration of GSH was calculated and expressed as nmol/mg protein.

Catalase activity (Beers and Sizer, 1952)

Principle: The catalase activity was assayed by measuring the decomposition of H2O2 which has an absorption maximum at 240 nm and this absorption decreases with the decomposition of H2O2. The difference in extinction per unit time is used to measure the catalase activity.

Procedure: Clarified tissue homogenate (100 µL) was mixed with 2.8 mL of 0.1 M phosphate buffer (pH 7.4). The reaction was initiated by adding 100 µL of 0.3% H2O2 solution. The activity was recorded as the decrease in absorbance at 240 nm 30 seconds interval for 3 min. The catalase activity was calculated using the molar extinction coefficient of hydrogen peroxide (43.6 mM-1cm-1) and the specific activity at 25 ºC was expressed as mmoles of H2O2 consumed/min/mg of protein.

$$Catalase\ activity\ \left(\frac{U}{mg\ protein}\right) = \frac{Ab\ at\frac{240}{min} \times 1000 \times 3}{43.6 \times mg\ protein\ in\ sample}$$

Glutathione peroxidase (GPx) activity (Hafeman et al., 1974)

Principle: Glutathione peroxidase depletes H2O2 in presence of glutathione (GSH). The remaining GSH is measured using 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB), which gives a colored complex.

$$H2O2 + 2\ GSH \rightarrow GSSG + 2\ H2O$$

Procedure: Reaction mixture contained 100 µL (5 mM) GSH, 100 µL of 0.1 M phosphate buffer (pH 7.0), 100µL sodium azide (25mM), 100 µL tissue extract and 100 µL of H2O2 (1.2 mM) in a total volume of 2.5mL.The reaction mixture was incubated at 370C for 6 minute. Reaction was stopped by addition of 2.0 mL of 1.67% meta-phosphoric acid and centrifuged for 15 minutes at 2500 rpm. To about 2mL of supernatant, added 2mL of 0.1 M phosphate buffer containing DTNB (1mM). Incubation was carried out at 37ºC for 10 minutes and OD was measured at 412 nm. A sample without the tissue was processed in the same way and was kept as the blank. The activity is expressed as U/mg protein. One unit of enzyme activity was defined as a decrease in log GSH by 0.001/min after subtraction of the decrease in log GSH per minute for the non-enzymatic reaction.

$$GPx\ activity\ \left(\frac{U}{mg\ protein}\right) = \frac{OD\ of\ Blank - OD\ of\ Sample \times 1000}{mg\ protein \times 10}$$

Estimation of glutathione-s-transferase activity (Ghalia et al 2000):

The total GST activity was spectrophotometrically determined utilizing 1-Chloro-2,4-dinitrobenzene(CDNB) as the substrate which was described by Ghalia et al 2000

Procedure: To estimate the GST content, 0.3ml of 50mM CDNB in methanol. 2.7ml of 5mM glutathione in 100mM Tris-HCl (pH7.5), and 10µL of sample in PBS were added to a test tube which is the reaction mixture. The absorbance at 340nm was measured using spectrophotometer against a reagent blank without glutathione.

Thiobarbituric acid reactive substances (TBARS) Ohkawa et al.1979)

Principle: Lipid carbonyls formed during thermal oxidation of edible oils react with thiobarbituric acid under acidic conditions to form a pink colored complex, which is estimated as the absorbance at 532 nm.

Procedure: Approximately 100 µL of edible oil was mixed with 200 µL each of butanol and sodium dodecyl sulfate (8%), 1.25 mL of thiobarbituric acid (0.8% in butanol) and 1.25 mL of 20% acetic acid (pH 3.5). The mixture was incubated in a boiling water bath for 1 hour and cooled. The absorbance was measured at 532 nm against a reagent blank. A standard was prepared similarly using malondialdehyde (MDA) and plotted on a graph, which was used for the calculation of slope.

Conjugated diene (CD) and conjugated triene (CT) (Pegg2004)

Principle: CD and CT are the initial lipid peroxidation products formed, that have characteristic UV absorbance maxima at 234 and 269 nm.

Procedure: The oils samples (30 µL) were diluted to 3.0 mL using isooctane and the absorbance of each sample was measured at 234 and 269 nm. Sample with absorbance above 0.800 was appropriately diluted and the dilution factor was applied while calculation. The level of CD and CT are calculated using the molar extinction coefficient of linoleic acid hydroperoxide (2.525 × 104 M−1 cm−1) and expressed as nmoles of linoleic acid hydroperoxide equivalents/ Kg of oil.

DETERMINATION OF LIPID PROFILE

Serum total cholesterol$Cholesterol + O_2 \rightarrow Cholesterol - 3 - one + H_2O_2$

Principle: Cholesterol esters in serum are hydrolysed by cholesterol esterase (CHE).The free cholesterol produced is oxidized by cholesterol oxidase to form Cholest 4en-3-one and hydrogen peroxide (H2O2). The hydrogen peroxide oxidatively couples with 4-aminoantipyrine (4-AP) and phenol in the presence of peroxidase (POD) to yield a red chromophore.

$$Cholsterol\ ester + H_2O \rightarrow Free\ cholesterol + Fatty\ acid$$

$$Cholesterol + O_2 \rightarrow Cholesterol - 3 - one + H_2O_2$$

$$H_2O_2 + Phenol + 4AP \rightarrow Quinonimine + 4H_2O$$

Procedure: From unhemolysed serum sample, 10 µL was added to 1 mL of reagent containing Phenol (26 mmol/L), Cholesterol esterase (1000 U/L), Cholesterol oxidase (300 U/L), Peroxidase (650 U/L) and 4-Aminophenazone (0.4 mmol/L) in 90 mmol/L of PIPES buffer (pH 6.9). This was mixed well and incubated at 37 °C for 5 min and read against reagent blank at 505 nm.

$$Cholesterol\ \left(\frac{mg}{dL}\right) = \left(\frac{Ab\ of\ sample}{Ab\ of\ standard}\right) \times Conc.of\ Standard$$

Serum Triglycerides

Principle: Triglycerides in sample when incubated with lipoprotein lipase (LPL), liberate glycerol and free fatty acids. Glycerol is converted to glycerol-3-phosphate (G3P) and adenosine-5-diphosphate (ADP) by glycerol kinase and ATP. Glycerol -3- phosphate (G3P) is then converted by glycerol phosphate dehydrogenase (GPO) to dihydroxyacetone phosphate (DAP) and hydrogen peroxide (H2O2), which in turn reacts with 4-aminophenazone (4-AP) and p-chlorophenol in presence of peroxidase (POD) to give a red coloured dye:

$$Triglycerides + H_2O \rightarrow Glycerol + Fatty\ acid$$

$$Glycerol + ATP \rightarrow Glycerol\ 3\ phosphate + ADP$$

$$G3P + O_2 \rightarrow DAP + H_2O_2$$

$$H_2O_2 + p - Chlorophenol + 4AP \rightarrow Quinone + H_2O$$

Procedure: From unhemolysed serum sample, 10 µL was added to 1 mL of reagent containing p- chlorophenol (2 mmol/L), lipoprotein lipase (150000 U/L), glycerol kinase

(500 U/L), glycerol 3-oxidase (3500 U/L), ATP (0.1 mmol/L) and 4-Aminophenazone (0.1 mmol/L). This was mixed well and incubated at 37°C for 5 min and read against reagent blank at 505 nm.

$$Triglycerides\ \left(\frac{mg}{dL}\right) = \left(\frac{Ab\ of\ sample}{Ab\ of\ standard}\right) \times Conc.of\ Standard$$

Serum High Density Lipoprotein

Principle: HDL-c is released using a detergent that solubilizes only HDL. HDL-c interacts with cholesterol esterase, cholesterol oxidase and chromogens to give colour. Non HDL lipoproteins like LDL, VLDL and chylomicrons are inhibited from reacting due to absorption of detergents on their surface.

Procedure: From unhemolysed serum sample, 10 µL was added to 450 µL of reagent 1 containing DSBmT (1mM) and Cholesterol oxidase (1000 U/L) in GOOD buffer (pH 7). This was incubated for 5 minutes at 37°C after which 150 µL of reagent 2 containing Cholesterol esterase (1500 U/L), Detergent (2%), Ascorbic oxidase (3000 U/L), Peroxidase (1300 U/L) and 4-Aminoantipyrine (1mM) in GOOD buffer (pH 7). This was mixed well and incubated at 37°C for 5 min and read against reagent blank at 650 nm.

$$HDL\ cholesterol\ \left(\frac{mg}{dL}\right) = \left(\frac{Ab\ of\ sample}{Ab\ of\ standard}\right) \times\ Conc.of\ Standard$$

Low density lipoprotein and atherogenic index

Since all the values obtained are expressed in mg/dL, the LDL value was calculated from these results by the formula by Friedewald et al. (1972):

$$LDL\ cholesterol = Total\ cholesterol - HDLc - \left(\frac{Triglycerides}{5}\right)$$

RESULTS

Table 1 shows the result of enzymatic and non-enzymatic antioxidant parameters in liver of Tilapia fish. The non-enzymatic antioxidant parameter, the reduced glutathione content was estimated to be 8.95±0.47 nmols/mg in the control group which was increased to 11.09±0.29 nmols/mg in CH-Low group ($p<0.05$) and 11.16±0.08 nmols/mg in CH-High group ($p<0.01$). The enzymatic antioxidant parameters like catalase, glutathione peroxidase, and glutathione-s-transferase were also depicted in table 1. The catalase content was estimated to be 54.19±5.08U/mg protein in the control group which was increased to 71.82±3.98U/mg protein in CH-Low ($p<0.05$) and 83.32±5.32U/mg protein in CH-High group. Glutathione peroxidase the other enzymatic antioxidant content in control group was found to be 18.74±2.44U/mg protein which is increased to 28.55±3.11U/mg protein in CH-Low and 33.27±2.58U/mg protein in CH-High group ($p<0.05$). The glutathione-s-transferase content was estimated to be 74.81±4.54U/mg protein in the control group which was increased to 89.05±5.43U/mg protein in CH-Low group ($p<0.05$) and to 91.62±5.22U/mg protein in CH-High group ($p<0.01$).

Table 1: Changes in enzymatic and non-enzymatic antioxidant parameters of tilapia fed with different treatment diet

Organ	Treatment	Reduced Glutathione (nmols/mg protein)	Catalase (U/mg Protein)	Glutathione Peroxidase (U/ mg protein)	Glutathione-s-Transferase (U/ mg Protein)
Liver	Control	8.95±0.47	54.19±5.08	18.74±2.44	74.81±4.54
	CH- Low	11.09±0.29**	71.82±3.98**	28.55±3.11**	89.05±5.43
	CH- High	11.16±0.08**	83.32±5.32***	33.27±2.58***	91.62±5.22

Statistical significance * indicate $p<0.05$, ** indicate $p<0.01$, *** indicate $p<0.001$

Table 2 shows the changes in oxidative stress parameters in liver of tilapia fish. The Thiobarbituric Acid Reactive Substance (TBARS) content was estimated to be 10.12±0.44nmols/mg protein in the control group which was reduced to

8.21±0.49nmols/mg protein in CH-Low group ($p<0.05$) and to 7.87±0.53nmols/mg protein in CH-High group ($p<0.05$). The conjugated Diene content in control group was found to be 116.3±5.67nmols/mg protein which is reduced to 101.2±4.70nmols/mg protein in CH-Low group ($p<0.01$) and to 95.2±5.04nmols/mg protein in CH-High group ($p<0.001$). The conjugated triene content of the control group was depicted to be 89.1±5.2nmols/mg protein which is reduced to 71.3±3.78nmols/mg in CH-Low ($p<0.01$) and to 73.1±3.85nmols /mg protein in CH-High group ($p<0.001$).

Table 2: Changes in Oxidative stress parameters of Tilapia fed with different treatment diet

Organ	Treatment	TBARS (nmoles/ mg protein)	Conjugated Dienes (nmoles/ mg protein)	Conjugated Triene (nmoles/ mg protein)
Liver	Control	10.12±0.44	116.3±5.67	89.1±5.2
	CH- Low	8.21±0.49*	101.2±4.70*	71.3±3.78*
	CH- High	7.87±0.53**	95.2±5.04**	73.1±3.85*

Statistical significance * indicate $p<0.05$, ** indicate $p<0.01$, *** indicate $p<0.001$

Table 3 shows the changes in lipid profile in liver of tilapia fish. The Total Cholesterol content was estimated to be 89.92±3.45mg/dL in the control group which was reduced to 88.26±2.17mg/dL in CH-Low group ($p<0.05$) and to 88.74±2.86mg/dL in CH-High group ($p<0.05$). The triglyceride content in control group was found to be 142.3±10.3mg/dL which is increased to 147.5±12.5mg/dL in CH-Low group ($p<0.01$) and to 148.3±13.1mg/dL in CH-High group ($p<0.001$). The HDL-c content of the control group was found to be 26.33±1.04mg/dL which is increased to 31.52±1.24mg/dL in CH-Low ($p<0.01$) and to 34.74±1.09mg/dL in CH-High group ($p<0.001$). The LDL-c content of the control group was found to be 35.13±2.17mg/dL which is reduced to 27.24±2.03mg/dL in CH-Low ($p<0.01$) and to 24.34±2.41mg/dL in CH-High group ($p<0.001$).

Table 3: Changes in Lipid Profile of Tilapia fed with different treatment diet

	Total Cholesterol (mg/dL)	Triglycerides (mg/dL)	HDL-c (mg/dL)	LDL-c (mg/dL)
Control	89.92±3.45	142.3±10.3	26.33±1.04	35.13±2.17
CH- Low	88.26±2.17	147.5±12.5	31.52±1.24*	27.24±2.03*
CH- High	88.74±2.86	148.3±13.1	34.74±1.09**	24.34±2.41*

Statistical significance * indicate $p<0.05$, ** indicate $p<0.01$, *** indicate $p<0.001$

DISCUSSION

Coconuts have gained widespread recognition for their versatility and nutritional value, offering a diverse range of products such as coconut oil, milk, water, and shredded coconut (Marina et al., 2009). However, amidst this array of coconut-derived products, one component that remains largely underutilized is the coconut haustorium. Also referred to as the coconut sprout or embryo, the haustorium is a gelatinous and spongy tissue located inside the coconut shell. Its primary function is to facilitate the growth of the coconut palm tree by providing nourishment to the developing embryo until it attains maturity. Unfortunately, despite being an integral part of the coconut's growth process, the haustorium is often discarded during the processing of coconut-based products. Beyond its role in coconut growth, the coconut haustorium possesses remarkable pharmacological importance and noteworthy nutritional attributes (Kumar and Nisha, 2015; Prasad and Aggarwal, 2012). It serves as a rich source of dietary fiber, essential vitamins such as vitamin C and B-complex vitamins, and essential minerals including iron, magnesium, and potassium. These nutritional components are vital for maintaining optimal health and supporting various physiological processes in the human body. Moreover, the coconut haustorium is endowed with a plethora of antioxidants, which play a critical role in combating oxidative stress and reducing the risk of chronic diseases. These antioxidants help neutralize harmful free radicals and prevent cellular damage (Halliwel and Gutteridge, 2015Additionally, the haustorium contains beneficial medium-chain fatty acids (MCFAs) that provide readily available energy and contribute to overall well-being.

The findings of the study suggest that coconut haustorium (CH) supplementation has a beneficial impact on the hepatic (liver) antioxidant status and lipid peroxidation markers. The supplementation of CH resulted in notable changes in the hepatic tissue, indicating improvements in the antioxidant defense system. One observation is the significant elevation in the levels of reduced glutathione in the hepatic tissue. Glutathione is a crucial antioxidant that plays a vital role in neutralizing harmful free radicals and protecting cells from oxidative damage. The increased levels of reduced glutathione suggest that CH supplementation enhances the antioxidant capacity of the liver. Furthermore, the study found a concurrent increase in the activities of glutathione-dependent antioxidant enzymes, such as glutathione peroxidase (GPx) and glutathione-S-transferase (GST).

These enzymes play critical roles in detoxification and the breakdown of harmful substances in the liver (Lu, 2013). The increased activities of these enzymes indicate an enhanced ability of the liver to counteract oxidative stress and maintain cellular health.

Another notable finding is the increased activity of catalase, which is an important antioxidant enzyme that helps convert hydrogen peroxide into water and oxygen. The elevation of catalase activity further supports the improved antioxidant defense system in the liver following CH supplementation.

In addition to the enhanced antioxidant status, CH supplementation led to a significant reduction in markers of lipid peroxidation. Thiobarbituric acid reactive substances (TBARS), conjugated dienes, and conjugated trienes are indicators of oxidative damage to lipids. The reduction in these markers suggests that CH supplementation can effectively mitigate lipid peroxidation in the liver, preventing damage to cell membranes and maintaining liver health. The results imply that CH supplementation has the potential to protect the liver from oxidative stress by increasing the levels of reduced glutathione and enhancing the activities of antioxidant enzymes. Moreover, the reduction in lipid peroxidation markers suggests a protective effect against liver damage. These findings have significant implications in the field of hepatoprotection and liver health. Oxidative stress and lipid peroxidation are closely associated with liver diseases, including non-alcoholic fatty liver disease (NAFLD), alcoholic liver disease, and liver cirrhosis (Sanyal, 2018) The ability of CH supplementation to improve the antioxidant status and reduce lipid peroxidation in the liver indicates its potential as a natural intervention for liver health promotion and the prevention of liver diseases.

The observed decrease in hepatic lipid levels, including total cholesterol, triglycerides, and LDL cholesterol (LDLc), along with an increase in high-density lipoprotein cholesterol (HDLc) levels in fishes supplemented with coconut haustorium, has significant implications for the protection against hyperlipidemia and associated fatty liver progressions.

Hyperlipidemia is a condition characterized by elevated levels of lipids, such as cholesterol and triglycerides, in the blood (Stone et al., 2014). It is a major risk factor for the development of cardiovascular diseases and non-alcoholic fatty liver disease

(NAFLD). NAFLD is a spectrum of liver conditions ranging from simple fatty liver to more severe forms like non-alcoholic steatohepatitis (NASH) and liver fibrosis.

The reduction in total cholesterol, triglycerides, and LDLc levels in the liver indicates a potential role of coconut haustorium supplementation in regulating lipid metabolism and preventing the accumulation of excess lipids in the liver. High levels of these lipids can lead to the formation of fatty deposits in the liver, contributing to the progression of NAFLD.

Furthermore, the increase in HDLc levels is particularly beneficial, as HDL cholesterol is considered "good" cholesterol. HDL particles help remove excess cholesterol from tissues, including the liver, and transport it back to the liver for metabolism and excretion (Rader and Hovingh, 2014). By enhancing HDLc levels, coconut haustorium supplementation may promote reverse cholesterol transport and aid in the clearance of cholesterol from the liver, thereby reducing the risk of fatty liver progression.

The observed changes in lipid profile parameters suggest that coconut haustorium supplementation may offer protection against hyperlipidemia and associated fatty liver progressions. However, it is important to note that further research, including human studies, is needed to validate these findings and determine the specific mechanisms underlying the observed effects.

CONCLUSION

Overall, the results suggested that CH supplementation improves the hepatic antioxidant status, enhances the activities of glutathione-dependent antioxidant enzymes, and reduces lipid peroxidation markers in the liver. These findings highlight the potential of CH as a natural intervention for hepatoprotection and liver health promotion. Besides, the supplementation of coconut haustorium in the diet of *Oreochromis mossambicus* resulted in decreased hepatic lipid levels, including total cholesterol, triglycerides, and LDLc, along with an increase in HDLc levels. Continued investigation is necessary to fully understand the therapeutic potential of coconut haustorium and its specific mechanisms of action in addressing hepatic redox balance, lipid metabolism and liver health.

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Phytochemical Composition, In Vitro Antioxidant and Anti-cancer Activities of Different Leaf Extracts from *Artemisia nilagirica*

Dissertation report submitted in partial requirement for M.Sc. degree course in Zoology with Environmental Biology as special subject

in

University of Calicut

by

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Under the guidance of

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REPORT SUBMITTED IN PARTIAL REQUIREMENT FOR M.Sc. DEGREE COURSE IN ZOOLOGY WITH ENVIRONMENTAL BIOLOGY AS SPECIAL SUBJECT

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Report of the project (Students project / field project / internship) submitted to St. Joseph's College (Autonomous) Devagiri

Affiliated to the University of Calicut

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CERTIFICATE

This is to certify that the dissertation entitled **"Phytochemical Composition, In Vitro Antioxidant and Anti-cancer Activities of Different Leaf Extracts from *Artemisia nilagirica*"** is an authentic record of studies and research work done by **Ms. Shana Jebin M** (Reg No: **DVAVMZLO12**) under my guidance and supervision and that, to the best of my knowledge and belief, this work has not been presented earlier for any degree or fellowship.

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DECLARATION

I hereby declare that the dissertation entitled **"Phytochemical Composition, In Vitro Antioxidant and Anti-cancer Activities of Different Leaf Extracts from *Artemisia nilagirica*"** submitted in partial fulfillment of the requirements for the award of the degree of MASTER OF SCIENCE IN ZOOLOGY is a record of original work submitted by me at St. Joseph's College (Autonomous), Devagiri under the supervision and guidance of Dr. Arunaksharan N., Assistant Professor, Department of Zoology, St. Joseph's College (Autonomous), Devagiri, Calicut during 2021-2023.

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ACKNOWLEDGEMENT

It is with a deep sense of pleasure that I express my sincere gratitude to **Dr. Arunaksharan N.** M.Sc., B.Ed., Ph.D., Assistant Professor, P.G and Research Department of Zoology, St. Joseph's College (Autonomous), Devagiri, Kozhikode, for his valuable guidance and supervision in the form of practical suggestions and constructive criticisms which formed the basis for the successful completion of this project.

I express my sincere gratitude to **Dr. Boby Jose**, Principal, St. Joseph's College (Autonomus), Devagiri, and **Dr. Vineesh P. J.**, M.Sc, B.Ed, Ph.D., Assistant Professor and Head, PG and Research Department of Zoology for his support and sincere encouragement.

I extend my deep gratitude to **Mr. Joice Tom J**, M.Sc. M.Phil., Assistant Professor, Department of Zoology, for his wholehearted support and advice that helped me throughout the project I had undertaken.

I am greatly indebted to **Ms. Nitha K.M, Ms. Akshaya K.M, Ms. Najiya V.K** the co-researcher for helping me to complete the project and providing their valuable time in editing and proofing my thesis.

I extend my heartfelt thanks to the non-teaching staff of department for their valuable cooperation.

Finally, I express my deep gratitude to my family and friends for their sincere support throughout the project. Above all, I thank God Almighty for the blessings for the successful completion of the project.

Shana Jebin M.

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INTRODUCTION

Plants, being eukaryotic photosynthetic organisms, are indispensable components of nature that offer essential sustenance to all living beings. They fulfil a critical function as primary producers, serving as a significant reservoir of both nourishment and medicinal resources. For thousands of years, humans have harnessed the power of plants for diverse purposes. From healing infectious ailments to food preservation and perfume creation, plants have served multiple roles. Their distinctiveness lies in their capacity as a renewable source, offering a wealth of biological and structural diversity within their components. This diversity enables the development of novel antibacterial, antifungal, and antiparasitic compounds.

The WHO estimates that around three-quarters of the global population is dependent on herbal treatments, primarily, for their healthcare needs. The oldest friends of humans are plants and herbs. They helped the human race by not only giving food and shelter but also by curing many diseases. Various cultures and civilizations have used herbal medicine, which is also referred to as traditional, natural, or alternative medicine. An age-old therapeutic approach involves using plants, plant extracts, or pure compounds obtained from plants to treat illness. The prototype of a natural product is found in many pharmacological types of medications today. Numerous medications, such as atropine, aspirin, digoxin, ephedrine, quinine, reserpine, morphine and tubocurarine, owe their discovery to the exploration of traditional treatments and folklore among indigenous peoples. Increasingly, research is revealing the existence of medicinal plants that contain synergistic combinations or possess properties that neutralize side effects. In the years to come, ethnopharmacology is expected to play a bigger role than it has before in the advancement of mainstream medicine.

Of the 17,000 species of higher plants found in India, 7500 are recognized having therapeutic uses. For the extant flora of that particular country, this percentage of medicinal plants is the highest percentage of plants known for their medical uses in any country in the world 2000 varieties of therapeutic plant species have been identified by Ayurveda, the oldest system of medicine on the Indian subcontinent, which is followed by Siddha and then Unani. The manufacturing of 340 herbal medications and their traditional usage are described in the Charak Samhita, an

ancient text on herbal medicine. In the modern pharmacopoeia, prototype chemicals obtained from plant species serve as the foundation for numerous synthetic counterparts, which make up around 25% of all medications currently available.

Kerala, a state known for its rich biodiversity, is home to a remarkable array of medicinal plants. According to a recent analysis conducted by the Kerala Forest and Wildlife Department, out of approximately 4600 flowering plants found in the region, around 900 have been identified to possess valuable medicinal properties. Indian systems of medicine like Ayurveda and Siddha use more than 150 types of plants that are either native to Kerala or have become naturalized there. About 2,000 lesser-known wild plant species are used by rural people and tribal societies for a variety of medical purposes. The forests of Kerala are home to over 80% of the plants used in Siddha medicine and about 60 to 65% of the plants needed for Ayurvedic medicine. *Asparagus racemosus, Baliospermum montanum, Desmodium gangeticum, Cissus quadrangularis, Psuedartheria viscida, Strobilanthes ciliatus, Aristolochia bracteolata ,Dysoxylum malabaricum, Solanum anguivi,Piper longum, Ceasalpinia bonduc, Tribulus terrestris, Aristolochia indica, Sarcostemma acidum, Aristolochia bracteolata* etc are the main medicinal plants of Kerala.

Northern temperate climates are home to the genus Artemisia, which includes tiny herbs and shrubs. It is a member of the significant Asteraceae family, which includes over 20,000 species and over 1,000 genera, making it one of the largest plant clades. Artemisia is a member of the Anthemideae tribe, which has approximately 500 species and is primarily distributed in Asia, Europe, and North America. The Anthemideae tribe has a large number of plants that are useful as cut flowers, beautiful crops, medicinal herbs, and fragrant plants. Many of these plants generate essential oils that are utilized in both traditional and modern medicine, as well as in the cosmetics and pharmaceuticals.

Numerous disorders, including cancer, hepatitis, malaria, and infections from bacteria, fungus and viruses are treated using Artemisia species. There are about 45 Artemisia species reported to be found in India, which include *A.absinthium, A.sieversiana , Artemisia maritima, Artemisia vulgaris L* etc, most of which are having well-known medicinal applications.

The 2015 Nobel Prize in Physiology or Medicine, was awarded for the discovery of the natural product artemisinin, a sesquiterpene lactone containing an unusual peroxide bridge isolated from the plant *Artemisia annua L.* (Asteraceae). The compound has established a new therapy to treat the parasitic disease malaria (artemisinin). The award was split in half, with one half going to Chinese researcher Youyou Tu for her work on a novel malaria treatment and the other half going to William C. Campbell and Satoshi Omura for work on a roundworm parasite infection treatment.

This study focuses mostly on *Artemisia nilagirica*, an indigenous plant to India that can only be found in the states of Andhra Pradesh, Kerala, Karnataka, Tamil Nadu, and Odisha. The plant belongs to the little-studied genus Artemisia. The goal of the current work is to determine this species' flavonoid and phenolic composition, anti-inflammatory, antioxidant, anti-microbial, anti-cancerous activity.

OBJECTIVES AND RELEVANCE OF STUDY

- To estimate the phytochemical composition of the plant extract of *A. nilagirica.*
- To estimate the antioxidant properties of the extract.
- To estimate the anti-cancer properties of the extract.

REVIEW OF LITERATURE

The kingdom Plantae is home to the majority of photosynthetic eukaryotes, including plants. Multicellular creatures make up most plants. The ability to live a disease-free, healthy life is a gift from nature, and medicinal plants are an essential part of maintaining our health(Peterson, 2020) .They are thought to be a much safer alternative and a successful elixir for treating a variety of illnesses. Since ancient times, plants have been used therapeutically to treat a variety of infectious and chronic ailments. Numerous plants and plant products have been used in a variety of traditional medicines and folk systems, serving as sources of potential new drugs for contemporary medicine. The promotion and use of medicinal plants complement all current illness preventive measures and serve critical roles in disease prevention. Since the discovery of therapeutic plants or medicinal herbs, the sensory characteristics of food have improved (Sofowora *et al.*, 2013).

A World Health Organization (WHO) Expert Group defined traditional medicine as the totality of all knowledge and practices, whether or not they can be explained, used in the diagnosis, prevention, and elimination of physical, mental, or social imbalance and solely relying on firsthand experience and observation passed down from generation to generation, whether verbally or in writing (WHO,1976).

Plant-based diets are linked to a decreased risk of heart disease (Kim *et al.*, 2019) .Naturally occurring antioxidants, including tannins, flavonoids, and vitamins C and E, are present in many plants. These antioxidants boost ß-cell function and lower blood sugar levels (Kooti *et al.*, 2016).A diet rich in plant-based foods can, according to a study, help individuals stay healthy and protect them against several pollutants and sickness problems. It has been demonstrated that plant-based diets have benefits for lowering physiological stress (Hoffman and Hennig, 2017).

Medical uses for medicinal plants date back to the beginning of mankind. Worldwide research has been done to assess their efficacy, and some of the findings have affected the development of plant-based medicines (Sofowora *et al.*, 2013).

With at least 80% of people utilizing them for some element of their primary healthcare, there has been a remarkable increase in the use of herbal medications and supplements over the past three decades. The use of these chemicals in treatments has shown great promise, and several herbal medicines are helpful. However, many of these items have not yet been studied, and their use is either ineffectively or not at all regulated. Since of this, it is difficult to utilize these agents safely and sensibly because it is unclear how they work, what adverse effects can occur, and how they might interact with functional foods and currently available conventional pharmaceuticals(Ekor, 2014).

The potential for use as a medicine has only been studied in 10% of the known plant species. Illness prevention, chronic disease management, and health improvement are the three primary concerns that are thought to be addressed by plant-based resources(Tuso *et al.*, 2013).India is renowned among the ancient civilizations for having a vast collection of medicinal and aromatic plants. These plants have long been harvested for use as raw materials in the production of pharmaceuticals and perfumery goods. For instance, it is asserted that the AYUSH (Ayurveda, Unani, Siddha, and Homeopathy) systems of India contain a list of around 8,000 herbal treatments. In India's rural areas, Ayurveda and Unani are two of the most well-known and commonly used traditional medical systems(Ansari *et al.*, 2023).

In addition to these traditional medicines that are used all over the world, other categories of plant chemicals have recently been created and used in therapeutic applications. Ap proximately 70,000 plant species are actively being studied for potential medicinal uses, and several isolated chemicals of clinical importance have been identified. Acetyldigoxin, Adoniside, Borneol, Camphor, Colchicine, quercetin , Curcumin, Reserpine, Quinine, Vincristine, Artemisinin, Apigenin, , and caryophyllene are a few of them(Ekor, 2014).

Pen T'Sao, a Chinese book on roots and grasses written by Emperor Shen Nung, discusses 365 drugs (dried parts of medicinal plants), many of which are still in use today. These include camphor, ephedra, Podophyllum, Rhei rhisoma , cinnamon bark, the great yellow gentian .Indian holy book The Vedas describe using plants for healing, which are widely available there(Petrovska, 2012). Overall, medicinal plants

continue to play an important role in healthcare, both in traditional and modern medicine, and in the form of natural remedies for everyday ailments.

The existence of multiple Phyto active chemicals, including polyphenols, alkaloids, carotenoids, volatile organic compounds, and others, is what enables all plants in the universe to contribute to a wide range of biological activities like antioxidant, anti-inflammatory, antimicrobial, anticancer, antiproliferative activities etc.

Plants and their activities

Plant	Activities	Reference
Tropaeolum majus L	antibacterial, antifungal, antioxidant, anticancer activity	(Valsalam *et al.*, 2019)
Eryngium duriaei	Anti-fungal activity	(Zuzarte *et al.*, 2011)
Pistacia lentiscus	Apoptotic,anti-inflammatory Antimicrobial activity	(Kalousi *et al.*, 2022) (Iauk *et al.*, 1996).
Asteraceae family	Antimicrobial activity Antiplasmoidal activity	(Chaniad *et al.*, 2022; Chaniad *et al.*, 2021)
Fagus sylvatica	Antifungal activity	(Mare *et al.*, 2021)
Boehmeria nivea	Antioxidant, antiproliferative activity	(Wang *et al.*, 2019)
Clinopodium nepeta	Anti-quorum activity	(Beddiar *et al.*, 2021)
Dichaetanthera africana	Anti-inflammatory activity	(Oguntibeju, 2018)
Aster pseudoglehnii	Uptake of glucose and insulin Secretion	(Lee *et al.*, 2022)
Eryngiun triquetrum	Anti-microbial activity	(Casiglia *et al.*, 2016)
Momordica charantia	Antidiabetic activity	(Liu *et al.*, 2021)
Geranium sanguineum	Anti-oxidant property	(Pavlova *et al.*, 2019)
Guazuma ulmifolia	Anti-oxidant property	(Rafi *et al.*, 2020)
phaseolus vulgaris	Anti-oxidant property	(Nawaz *et al.*, 2020)

Various biological activities include:

Antioxidant activity

Antioxidants are chemicals, either natural or artificial, that can stop or delay cell deterioration caused by free radicals that are created by the body in response to environmental or other stresses. Antioxidants that arise naturally play a key role in medical and food processing. An antioxidant can eliminate free radicals and neutralize them by giving an electron, lessening their ability to cause harm (Halliwell *et al.*, 1995). Using various in vitro models, including DPPH, nitric oxide, superoxide, hydroxyl radicals, and lipid peroxidation, the antioxidant activity of Artemisia nilagirica fruits was investigated(Suseela *et al.*, 2010).

Anti- Inflammatory activity

According to study, inflammation is a brief biological tissue reaction to potentially harmful stimuli such as wounds, exogenic and endogenic antigens. It is intended to neutralize or eliminate the stimulus and heal the damaged tissue, which will eventually promote tissue regeneration and normal homeostasis.(Egger, 2012).

Although inflammation is a positive body defense mechanism, deregulated and chronic inflammatory reactions are well known to be the root causes of many systemic diseases, such as diabetes, asthma, atherosclerosis, obesity, cancer, and pain. As a result, the cost of healthcare for society has increased even though inflammation is a protective body defense mechanism (Mizuno *et al.*, 2011). The majority of commonly prescribed NSAIDs, steroids, and immunosuppressants used to treat various inflammatory disorders have negative side effects, such as headaches, stomach irritation, perforation, hemolytic anemia, hyperglycemia etc. (Bagad *et al.*, 2013) .Given the downsides of these medications, it is now more important than ever to find an alternative source, particularly from traditionally safe medicinal plants, for potential anti-inflammatory efficacy.

Antimicrobial activity

An antimicrobial agent is a substance that, when applied to a specific area, selectively kills or slows the growth of germs, prevents the development of colonies, and may even completely eradicate the bacteria. Since ancient times, medicinal plants and their extracts have been known to have antibacterial properties. The extracts of Artemisia spp. were used in clinical treatment as natural antimicrobial agents. Hence, the isolation and purification of therapeutic potential compounds from *A. nilagirica* could be used as an effective source against bacterial diseases in human and plants.

Anticancer activity

Cancer chemotherapy drugs have been demonstrated to have a wide range of side effects, although they can frequently prolong life, temporarily relieve symptoms, and even cure cancer in some cases. Phytochemicals and plant derivatives are exciting possibilities for more effective and less hazardous cancer treatment, according to research findings. Previous studies have showed the anti-cancer property of AN extract (Raju *et al.*, 2020).

The Asteraceae family, also known as the Compositae family, is one of the largest families of flowering plants, with more than 23,000 species spread across the globe. The family is distinguished by its distinctive inflorescence, a composite structure made up of multiple individual flowers that are tightly grouped to form a single head (Nguyen *et al.*, 2021). The leaves of Asteraceae plants are typically simple and alternate. A wide variety of plants, including herbs, shrubs, and trees, belong to the Asteraceae family. Some of the most well-known members of the family include sunflowers, daisies, chrysanthemums, and asters(Sharma *et al.*).

While certain Asteraceae species are used for medical purposes or as sources of food, fiber, and fire, others are valued economically as ornamental plants. The chamomile, Echinacea, and feverfew plants are among of the family's frequently used medicinal plants (Nguyen *et al.*, 2021). However, some Asteraceae species can also be toxic to humans and animals. For example, certain species of ragweed can cause allergies, while others such as tansy ragwort and poisonous thistles are toxic when ingested. The huge number of phytochemicals contained in the Asteraceae family, including phenolic acids, lignans, polyphenolic compounds, saponins, polysaccharides, sterols, essential oils are responsible for the pharmacological actions carried out by this family(Rolnik and Olas, 2021). Asteraceae family contains a variety of phenolic chemicals that have been identified, such as chicoric acid, kaempferol and its derivatives, luteolin and its derivatives, quercetin, and apigenin and its derivatives(Giambanelli *et al.*, 2018).

Over 400 different species of herbs and shrubs make up the enormous and diverse plant genus Artemisia, which belongs to the Asteraceae family. The plants in this genus are referred to as sagebrush or wormwood and are distinguished by their fragrant leaf(Nigam *et al.*, 2019). Artemisia genus has more than 500 species that are

indigenous to northern temperate areas. For their contributions to medicine and pharmacology, however, not all of them have been extensively examined.

Artemisia genus are commonly used to treat a variety of illnesses, including cancer, hepatitis, inflammation, and infections caused by the fungus, bacteria, and viruses. The potential for several Artemisia compounds as pesticides and allelopathic substances has also been discovered. This genus is drawing more attention, most likely as a result of the following factors: (i) the diverse biology and chemistry of the components; (ii) the widespread use in conventional medicine; and (iii) the abundant source of plant material(Trendafilova *et al.*, 2021)

The Artemisia genus and its constituent species have been extensively researched for their varied biological functions. Due to presence of artemisinin, *Artemisia annua L*. has showed considerable medical effects (Salaroli *et al.*, 2022) .Another significant member of the genus, *Artemisia mongolica*, has a variety of pharmacological properties and is rich in lactone derivatives of sesquiterpene(Zhu *et al.*, 2022). It was discovered that the many members of the genus had potent antibacterial(Zhang *et al.*, 2022) and antifungal effects against pathogenic organisms in people, animals, and plants(Wang *et al.*, 2022). The bioactive substances and extracts of many Artemisia species are thought to have antiproliferative and apoptotic actions(Kolesar and Seeberger, 2022).

Major reports available on the activities of the family Asteraceae:

Plant	Activity	Reference
Artemisia annua	Antimalarial activity	(Al-Khayri *et al.*, 2022)
Artemisia vulgaris L.	genotoxic and cytotoxic activities	(Jakovljević *et al.*, 2020)
Artemisia absinthium	antioxidant activity, wound healing properties	(Benkhaled *et al.*, 2020)
Achillea atrata L., *Achillea millefolium* L	Antioxidant property	(Salomon *et al.*,

		2021)
Tagetes erecta L.	Antioxidant Activity, and Cytotoxicity	(Burlec *et al.*, 2021)
Echinacea purpurea	anti-inflammatory activity	(Cheng *et al.*, 2020)
Tanacetum parthenium	anti-inflammatory activity	(Recinella *et al.*, 2020)
Chromolaena odorata	Antiplasmodial activity	(Ezenyi *et al.*, 2014)
Tagetes lucida Cav	Anticancer, Antioxidant and Antimicrobial Properties, pain	(Hernandez-Leon *et al.*, 2020)

Some of the most well-known species in the genus include:

Artemisia annua:

Artemisia annua, also referred to as sweet wormwood, is the species from which the antimalarial medication artemisinin is derived (Maftakhova *et al.*, 2022).For its different biological functions, the Artemisia genus and its members have been investigated substantially. Because of the presence of artemisinin (ART), *Artemisia annua* L(AA) has revealed considerable medical benefits. According to the result of studies, ART is more prevalent in AA samples that were extracted with 90% ethanol. Finally, against the pro-inflammatory activation of LPS, all AA samples, including ART, demonstrated protection. At the molecular level, AA leaf extract in 90% ethanol was shown to have anti-inflammatory properties (Abate *et al.*, 2021).

Over 2000 years have passed since Artemisia annua was first used as a medication to treat malaria. The maternal and fetal safety of the ethanolic leaf extract of the therapeutically active *Artemisia annua* (EAA), with a previously determined artemisinin yield of 1.098 per cent, was examined in Wistar rats. The results showed that, although leaf extract consumption does not put users at risk for hepatotoxicity, haematotoxicity, or hyperlipidemia, it should be used cautiously during pregnancy

due to the likelihood of embryotoxicity at concentrations greater than the therapeutic dose (Abolaji *et al.*, 2013).

A study was conducted to target antimalarial metabolites in the fungal endophytes of Artemisia annua. And for the development of further antimalarial drugs, they investigated the fungi endophytes linked to the well-known medicinal plant *Artemisia annua*(Alhadrami *et al.*, 2021).

For ages, people have employed extracts from Artemisia annua and related herbs to cure and prevent malaria as well as other infectious disorders brought on by parasites, viruses, and bacteria. The production of the semi-synthetic derivative artesunate, which has a longer half-life and improved bioavailability when compared to artemisinin, was finally made possible by the discovery of the active medicinal component artemisinin. After Prof. Efferth discovered in 2001 that artemisinin and its derivatives are effective against a variety of human cancer cell lines, Artemisinin compounds like artesunate have been shown to have considerable anticancer efficacy in preclinical and clinical studies. But pharmacological issues such as short half-life and limited absorption might restrict antitumor efficacy (Kolesar and Seeberger, 2022).

The possibility of Plasmodium artemisinin resistance developing as a result of the usage of Artemisia annua for treating malaria and preventing COVID-19 in the Sub-African region was the subject of another research. Resistance of Plasmodium to antimalarial medications makes it difficult to eradicate malaria in endemic locations. An especially dire situation exists in Africa, which is home to about 92 percent of all malaria infections worldwide. Chloroquine has been replaced by combination therapy based on artemisinin because of this resistance. Additionally, the use of COVID-19 for COVID-19 with Artemisia annua for malaria prophylaxis may contribute to the emergence of resistance to Artemisinin-based combination therapy(Ataba *et al.*, 2022).

Artemisia vulgaris:

Artemisia vulgaris, also referred to as mugwort, has a long history of usage in conventional medicine and is used to cure a number of ailments, such as digestive issues and menstrual cramps (Ekiert *et al.*, 2020).It is well-known for its volatile oils. Recent phytochemical and pharmacological research has validated the therapeutic

potential of *A. vulgaris* bioactive compounds. These results provided further information to better comprehend the identification and isolation of novel compounds that can be employed as low-cost anti-malarial medications (Abiri *et al.*, 2018).

A. vulgaris hydro alcohol extract significantly reduced abdominal contortions by 48 and 59%, respectively, at dosages of 500 and 1000 mg/kg. This hydro alcohol extract contained the flavonoid glycoside rutin as well as derivatives of caffeine (Pires et al., 2009).

Artemisia capillaris:

According to Hong and Lee (2009), the ethyl acetate fraction of Artemisia capillaris (Thunb.) (ACE) has a powerful antioxidant defense system-strengthening effect while lowering the production of reactive oxygen species (ROS) and harmful oxidative substances in the liver of mice given a high-fat diet to make them obese.

Artemisia absinthium

sometimes known as wormwood, is the species used to manufacture absinthe, an alcoholic beverage. Because of its digestive and antiparasitic effects, it has a long history of usage in traditional medicine (Szopa *et al.*, 2020).

Artemisia judaica:

A.judaica ethanolic extract was tested for its in vitro cytotoxic potential against a panel of cancer cell lines. The outcomes demonstrated its cytotoxic efficacy against the lung cancer (A549) cell line with a promising IC50 of 14.2 g/mL in comparison to doxorubicin as a reference(Goda *et al.*, 2021) .

Artemisia dracunculus

also referred to as French tarragon, is a culinary herb that is commonly used in French cuisine(Ekiert *et al.*, 2021).

Artemisia tridentata, also referred to as large sagebrush, is a dominating plant in the western United States and a crucial source of food for wildlife. Native Americans have also historically used it for its therapeutic qualities(Barron *et al.*, 2020).

Artemisia afra Jacq:

used in South Africa to treat diabetes. In the study, the pancreas of rats with diabetes that had been induced by streptozotocin was examined after oral administration of an aqueous leaf extract from *A. afra* and discovered that the extract increased the serum insulin concentration while also dramatically lowering blood glucose levels(Afolayan and Sunmonu, 2011). Researchers used an aqueous extract of *Artemisia afra Jacq.* ex Willd. to examine the antioxidant defense mechanisms in the liver and kidney of streptozotocin-induced diabetic rats. These investigations showed that *A. afra* lowers oxidative stress in tissues, possibly because of the amount of flavonoids it contains(Mbaebie *et al.*, 2012).

Artemisia nilagirica:

Artemisia nilagirica is a significant species among these numerous isolated plants and chemicals. The common name for A. nilagirica is "wormwood." They are commonly found in the hilly regions of India. Recent investigations on this plant group have noticed its antimicrobial, antifungal, antibacterial, filarial, insecticidal, antiulcer, anticancer, antioxidant, and anti-asthmatic properties (Panda *et al.*, 2018) .The main effects of artemisia, however, are thought to be parasite-related illnesses or skin problems.

The leaf of Artemisia nilagirica (C.B. Clarke) displayed antibacterial action against pathogenic and clinical bacteria when extracted with chloroform, diethyl ether, ethanol, hexane, methanol, and petroleum ether. Against clinical and phytopathogens, respectively, methanol and hexane extract demonstrated high inhibition (Ahameethunisa and Hopper, 2010b).

Major activities reported from *A. nilagirica*:

The leaf extract of A. nilagirica has been reported to have antimicrobial effects. The research was performed on 15 strains of bacteria using the agar disc diffusion method.

The only bacterial strains that did not exhibit inhibitory activity were Klebsiella pneumoniae, Enterococcus faecalis, and Staphylococcus aureus(Ahameethunisa and Hopper, 2010a) .

Six distinct organic leaf extracts of Artemisia nilagirica were used to test the effectiveness of an antimalarial activity against the malaria parasite Plasmodium falciparum. All of the studied extracts show encouraging anti-plasmodial

effectiveness. A. nilagirica leaf extract possesses anti-plasmodial activity, indicating that it may one day be turned into a potent plant-based antimalarial drug(Panda *et al.*, 2018) .

According to reports, A. nilagirica has anti-inflammatory properties. This was investigated by taking an ethanol-based leaf extract. At a concentration of 200g/mL, the leaf extract demonstrated the greatest protection and the least amount of hemolysis of the HRBC, while the least amount of protection was at a concentration of 50g/mL (Parameswari *et al.*, 2019).

The essential oil extracted from the aerial parts of Artemisia nilagirica was examined using gas chromatography-mass spectroscopy. A total of 43 components, or 98.16 per cent of the essential oil content, were found. The essential oil was discovered to have significant antifungal action against Rhizoctonia solani, Sclerotium rolfsii, and Macrophomina phaseolina. In this study, it was discovered that the essential oil of A. nilagirica was successful in preventing phytopathogenic fungi from infesting crops and commodities(Sati *et al.*, 2013) .

Since it is a less explored plant of the genus artemisia and few components identified, Artemisia nilagirica is revealed to have a large variety of biological functions. More research on this group is crucial because it will surely assist to comprehend many unknown characteristics of this particular plant. Here, the leaves of artemisia nilagirica is serially extracted utilizing highly polar to highly non-polar solvents such as petroleum ether, benzene, ethyl acetate, and methanol with a magnetic stirrer. The plant extract was dissolved using the solvent dimethyl sulphoxide (DMSO). We need to elucidate the total flavonoid, total phenolic, antioxidant, anti-inflammatory, anti-microbial and anti-cancer properties of *A.nilagirica*.

MATERIALS AND METHODS

1. Plant collection:

The plant species *Artemisia nilagirica* (genus: Artemisia, family: Asteraceae) were collected from Kerala Agricultural University in Thrissur, India. About 40g of the dried and powdered leaves of the plant was taken for extraction. The extraction was carried out using magnetic stirrer extraction procedure. It is done by serial extraction based on polarity. Different solvents used are petroleum ether, benzene, methanol, and ethyl acetate, and then evaporated to dryness. The drug was then dissolved in Dimethyl sulfoxide at a concentration of 10mg/ml.



2. METHODS

2.1 GCMS analysis:

GC-MS analysis was carried out using a GC model-7890A (Agilent Technologies, USA). The column used for the study was DB-5 with dimensions 30 m x 0.25 mm x 0.25 µm. The MS detector used was Agilent, 5975C Inert XL MSD. The injection volume was 1.0 µL and the injection temperature was 250^{o}C. The Column Oven Initial Temperature was 60^{o} C with an increment at a rate of 5^{o}C/ min. The detector was set at a temperature of 250^{o}C.

2.2 Estimation of total phenol content:

Gallic acid was used as a standard to determine the total amount of polyphenol in the extract, and the results were represented as mg of gallic acid equivalent per gram of extract.(Ahmed and Tavaszi-Sarosi, 2019) . To make a gallic acid standard, different

volumes of the acid (10, 20, 30, 40, and 50 µl) are taken in a test tube and diluted to 1 ml with distilled water. It is mixed with 2 ml of the Folin - ciocalteau reagent and incubated for 30 minutes at room temperature in the dark. Then add 4ml 7.5% sodium carbonate and incubate in dark for 30 minutes in room temperature. Absorbance of the test solution is taken at 680nm in a colourimeter. Take different volumes (20 & 50µl) volumes of plant extract and makeup to1ml with distilled water and follow the same procedure as that of gallic acid.

2.3 Estimation of total flavonoid content:

By reacting with aluminium chloride (AlCl 3), total flavonoid content was evaluated as the equivalent of quercetin (Xie et al., 2015); the results were represented as mg quercetin equivalent per gram of extract.

As a standard, various concentrations of quercetin (10,20,30,40,50 l) were taken, and make up to 1 ml with distilled water. Add 0.3ml of 10% NaNO2 and 4ml distilled water.

Mixed well and incubate for 5min at 37˚C. Added 0.3ml of 10%$AlCl_3$and 4.4ml of distilled water and incubate for 2min. After incubation, add 2ml of 5% NaOH and

measure the absorbance at 540nm in a colorimeter. Take different volume (20 & 50 µl) of the extract and makeup to 1ml using distilled water and follow the same steps.

3. Analysis of In vitro Antioxidant activities

3.1 DPPH radical scavenging assay:

The scavenging of the stable, purple-colored 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical technique was used to measure the antioxidant activity. The experiment was carried out with a few minor modifications to (Sobayel *et al.*, 2020). DPPH solution was prepared by weighing 5mg DPPH and dissolved in 100 ml methanol.

To create the test solution, plant extract of different concentrations (5,10,15,20,25,30,35,40,45,50µl) were taken and to it 2ml of DPPH solution is added. The drug blank is prepared by mixing the necessary volumes of plant extract (5, 10, 15, 20, 25 30,35,40,45,50 µl) with 2 ml of distilled water. As a control, 2 ml of DPPH solution is used. The mixture was then mixed vigorously and incubate for 30 minutes

in dark at room temperature. The absorbance was measured in colorimeter set at 517 nm against methanol blank.

The radical scavenging activity of the extract was expressed as a percentage of DPPH of inhibition using the following equation:

DPPH radical scavenging effect (%) = [(A control- A sample)/A control] × 100.

Where, A control = absorbance of DPPH.

A sample = absorbance of DPPH solution presence of plant extract

3.2 Inhibition of Lipid peroxidation (TBARS method):

Homogenize 10g liver with 100ml phosphate buffered saline. Centrifuge the liver for 20-25minutes in 1200-1500rpm. Collect the supernatant. Mix 100 µl supernatant with different concentrations (2.5, 5, 10 µl) of plant extract in duplicate. Incubate it for 15min in room temperature. After incubation, add 100 µl H_2O_2. Then, add 1.5ml 8% TBA followed by 1.5ml 20% acetic acid with a pH of 3.5. To this, add 0.2 ml 0.8% SDS. Control is prepared by taking tissue alone. Incubate the mixture at boiling temperature for 1 hour against reagent blank. Measure the absorbance at 530 nm(Ahmad *et al.,* 2022).

3.3 Ferric reducing antioxidant power (FRAP):

The fresh working solution was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ solution, and 2.5 ml FeCl3 solution and then warmed at 37°C before using**.** To different concentrations of plant extract (5, 10, 15, 20 µl), add 3 ml of FRAP solution. Incubate the mixture for 30 minutes in the dark condition. Readings of the colored product (ferrous tripyridyl triazine complex) will be measured at 593 nm(Müller *et al.,* 2011).

3.4 Nitric oxide (NO) radical scavenging assay:

The assay was done according to the protocol described by (Kakatum *et al.,* 2012) .Plant extract of different concentrations (5, 10, 15, 20, 25 µl) is combined with 0.5ml of 10mM sodium nitroprusside in phosphate buffered saline (PBS) in a clean test tube. An additional 0.5ml of PBS is added to create a 1ml test solution. A drug blank without sodium nitroprusside is prepared for each concentration. A control is prepared

by mixing 0.5ml of 10mM sodium nitroprusside with 0.5ml of PBS. The solutions are incubated in darkness for 3 hours. After incubation, 1ml of freshly prepared Griess reagent (consisting of 0.1% NEDA and 1% sulphanilamide in 2.5% phosphoric acid) is added. The absorbance is measured at 546nm using a spectrophotometer against a blank prepared by mixing 1ml of distilled water with 1ml of Griess reagent.

Percentage inhibition in each assay was calculated by comparing the absorbance of the control (without drug) and individual samples (each concentration of different Artemisia extracts).

Percentage of NO2 scavenging was calculated by the equation:

NO2 radical scavenging effect % = [(A control- A sample)/ A control] × 100.

Where, A control = Absorbance of control.

A test = Absorbance in the presence of the samples of extracts.

4. *In vitro* anticancer activity

4.1 MTT ASSAY:

The MCF-7 and MDAMB-231 cells were seeded in a 24-well plate with a density of 1 × 10^6 cells/mL. Subsequently, the cells were placed in an incubator at 37 °C. Following a 24-hour period, extracts of varying concentrations were introduced and further incubated for 48 hours. Upon completion of the incubation period, MTT was added to each well and allowed to incubate for an additional 4 hours, resulting in the formation of formazan crystals(Oh and Hong, 2022). Afterwards, the formazan crystals were dissolved in DMSO and their absorbance was measured at 570 nm. Subsequently, the percentage inhibition and IC50 values were determined through calculation (House *et al.*, 2020)

RESULTS

Total Phenol and Flavonoid Content:

Benzene fraction

The total polyphenol and flavonoid contents of *A. nilagirica* were estimated and the phenol content was found to be 28.19±1.22 mg gallic acid equivalent/ g of extract. Total flavonoid content was 8.54±0.49 mg quercetin equivalent/ g of extract

Petroleum ether fraction

The phenol content was found to be 36.76±2.10 mg gallic acid equivalent/ g of extract. Total flavonoid content was 11.07±0.22 mg quercetin equivalent/ g of extract

Ethyl acetate fraction

The phenol content was found to be 27.44±2.14 mg gallic acid equivalent/ g of extract. Total flavonoid content was 7.62±0.19 mg quercetin equivalent/ g of extract

Methanol fraction

The phenol content was found to be 75.09±3.41 mg gallic acid equivalent/ g of extract. Total flavonoid content was 21.33±0.27 mg quercetin equivalent/ g of extract.

Table 1. The total polyphenol and flavonoid contents of different fractions

Extract	**Total Phenol Content (mg GAE/ g extract)**	**Total Flavonoid Content (mg QE/ g extract)**
Benzene	28.19±1.22	8.54±0.49
petroleum ether	36.76±2.10	11.07±0.22
ethyl acetate	27.44±2.14	7.62±0.19
methanol	75.09±3.41	21.33±0.27

Bioactive Compounds in Methanol and Ethyl acetate fractions

The GCMS analysis of *A. nilagirica* methanol and Ethyl acetate extract indicated the presence of various phytocompounds, including Ferulic acid, Quercetin, Myricetin Glucoside, and simple phenolic acids

Table 2. LCMS analysis of the methanol and ethyl acetate fractions of *A. nilagirica*

Sl. No.	**Retention Time (min)**	**Methanol fraction**	**Ethyl acetate fraction**
1	3.42	Ferulic acid	Ferulic acid
2	5.41	p-Coumaric acid	
3	6.75	Caffeic acid	
4	8.52	Gallic acid	Gallic acid
5	9.73	Rutin	
6	12.30	Quercetin	Quercetin
7	15.46	Myricetin Glucoside	
8	18.02	Isorhamnetin	

Antioxidant activities

DPPH Assay:

Methanol fraction

The DPPH radical scavenging activity was found to be in *A. nilagirica* species with percentage inhibition of 15 for lower concentration (25 µg/mL) and percentage inhibition of 49 for higher concentration (250 µg/mL)

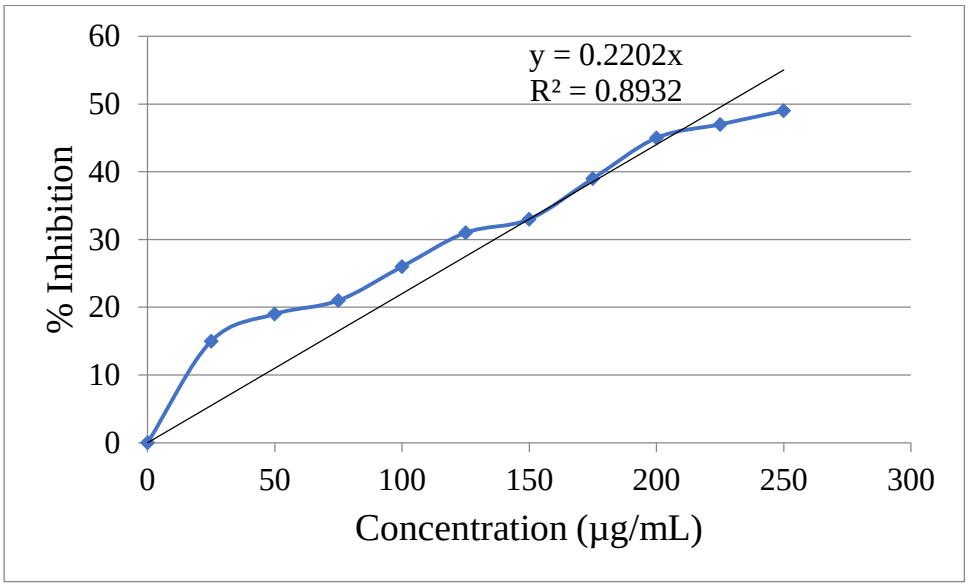


Figure 1. The DPPH radical scavenging properties of *A. nilagirica* methanol fraction

Ethyl acetate fraction

The DPPH radical scavenging activity was found to be with percentage inhibition of 18 for lower concentration (25µg/mL) and percentage inhibition of 37 for higher concentration (250 µg/mL)

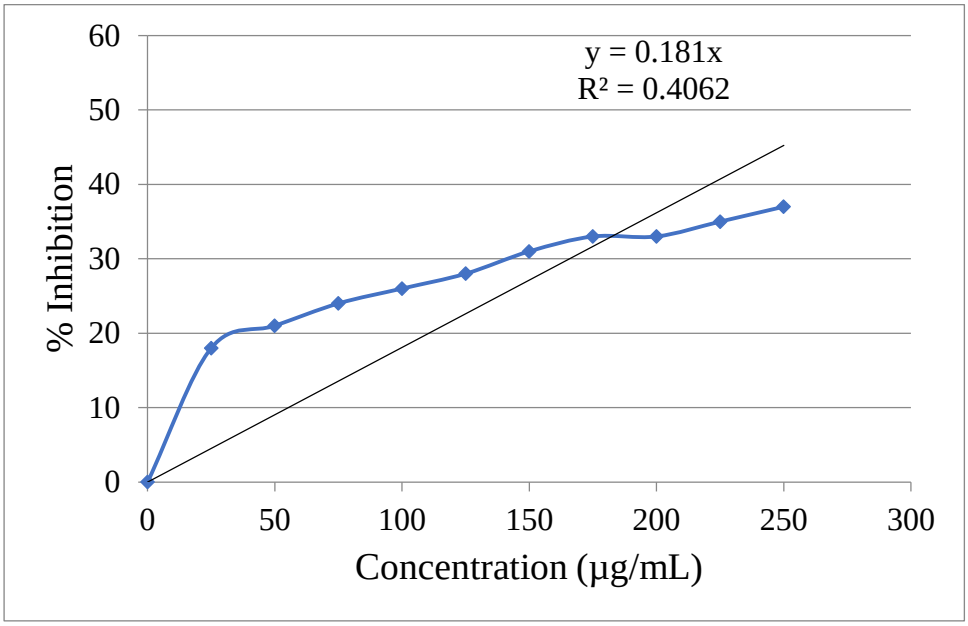


Figure 2. The DPPH radical scavenging properties of *A. nilagirica* ethyl acetate fraction

Since Petroleum ether and benzene doesn't show any inhibition, it doesn't have antioxidant activity.

Lipid Peroxidation Inhibition

Methanol fraction

The percentage Inhibition was found to be 25.53 for lower concentration (2.5 µg/mL) and 48.94 for higher concentration (10 µg/mL).

Ethyl acetate fraction

The percentage Inhibition was found to be 23.40 for lower concentration (2.5 µg/mL) and 40.43 for higher concentration (10 µg/mL).

Since Petroleum ether and benzene doesn't show any inhibition, it doesn't have antioxidant activity.

Table 3. Lipid Peroxidation Inhibition activity of different fractions

Concentration (µg/mL)	**% Inhibition**			
	Methanol	**Ethyl acetate**	**Benzene**	**Pet. Ether**
2.5	25.53	23.40	0	0
5	36.17	31.91	0	0
10	48.94	40.43	0	0

Ferric Reducing Antioxidant Power

Methanol fraction

The Reducing Power was found to be 56.25 for lower concentration (0.1µg/mL) and 250 for higher concentration (0.4µg/mL).

Ethyl acetate fraction

The Reducing Power was found to be 0 for lower concentration (0.1 µg/mL) and 37.5 for higher concentration (0.4 µg/L).

Benzene fraction

The Reducing Power was found to be 0 for lower concentration (0.1 µg/mL) and 37.5 for higher concentration (0.4 µg/mL).

Petroleum Ether

The Reducing Power was found to be 18.75 for lower concentration (0.1 µg/mL) and 56.25 for higher concentration (0.4 µg/mL).

Table 4. Ferric Reducing Antioxidant Power of different fractions

Concentration (µg/mL)	**Reducing Power**			
	Methanol	**Ethyl acetate**	**Benzene**	**Pet. Ether**
0.1	56.25	0	0	18.75
0.2	125	12.5	12.5	37.5
0.3	187.5	25	25	50
0.4	250	37.5	37.5	56.25

Nitric oxide scavenging assay

Methanol fraction

The Reducing Power was found to be 11.24 for lower concentration (20 μg/mL) and 13.61 for higher concentration (80 μg/mL)

Ethyl acetate fraction

The Reducing Power was found to be 11.83 for lower concentration (20 μg/mL) 13.61 for higher concentration (80 μg/mL)

Petroleum ether and benzene doesn't have activity.

Table 5. Nitric oxide scavenging of different fractions

Concentration	**Reducing Power**			
	Methanol	**Ethyl acetate**	**Benzene**	**Pet. Ether**
20	11.24	11.83	0	0
40	11.83	12.43	0	0
60	13.02	13.02	0	0
80	13.61	13.61	0	0

DISCUSSION

Oxidative stress caused by the accumulation of reactive oxygen species (ROS) has been associated with various diseases, including cancer, cardiovascular disorders, and neurodegenerative conditions (Ruszkiewicz and Albrecht, 2015). Natural antioxidants derived from medicinal plants have gained significant interest due to their potential therapeutic benefits (Van Thuong *et al.*, 2015). *Artemisia nilagirica* plant found in tropical regions, is known for its traditional use in folk medicine. This study assesses the antioxidant activities of methanol and ethyl acetate fractions obtained from *Artemisia nilagirica* leaves and compared their efficacy using DPPH radical scavenging, lipid peroxidation inhibition, and nitric oxide scavenging assays.

The observed differences in antioxidant activity between the methanol and ethyl acetate fractions can be attributed to variations in their phytochemical composition. Methanol is a polar solvent that is capable of extracting a wide range of polar and non-polar compounds from the plant material(Levin *et al.*, 2008). It is likely that the methanol fraction contains a higher concentration of bioactive compounds such as phenolics, flavonoids, and other antioxidant compounds, which contribute to its superior antioxidant activity. On the other hand, the ethyl acetate fraction, being less polar, may have a lower concentration of these bioactive compounds, resulting in relatively lower antioxidant activity.

The benzene and petroleum ether fractions, which exhibited no antioxidant activity in the assays, may lack the necessary phytochemicals responsible for antioxidant properties. These fractions might primarily contain non-polar compounds that are less likely to possess antioxidant activity. It is important to note that the absence

The observed antioxidant activity of the methanol and ethyl acetate fractions of *Artemisia nilagirica* leaves correlates well with the phytochemical analysis results, which revealed high polyphenol content and flavonoid content in these fractions. Polyphenols and flavonoids are well-known antioxidants that possess the ability to scavenge free radicals and prevent oxidative damage.

The methanol fraction, which exhibited higher antioxidant activity, contained significant amounts of polyphenols and flavonoids. Phenolic acids and flavonoids, such as quercetin and myricetin, were identified as major compounds in the methanol fraction. These compounds are known for their potent antioxidant properties due to

their ability to donate hydrogen atoms or electrons, thereby neutralizing free radicals and protecting cells from oxidative stress (Levin *et al.*, 2008).

The presence of these bioactive compounds in the methanol fraction likely contributes to its superior antioxidant activity observed in the DPPH radical scavenging, lipid peroxidation inhibition, and nitric oxide scavenging assays. The hydroxyl groups present in phenolic acids and flavonoids enable them to scavenge free radicals effectively, intercept lipid peroxidation chain reactions, and reduce nitric oxide levels.

In contrast, the ethyl acetate fraction, although still exhibiting antioxidant activity, may have a lower concentration of these bioactive compounds compared to the methanol fraction. This could explain the relatively lower antioxidant activity observed in the ethyl acetate fraction compared to the methanol fraction.

The correlation between the phytochemical analysis results and the observed antioxidant activity supports the notion that polyphenols and flavonoids, particularly phenolic acids and flavonoids like quercetin and myricetin, play a significant role in the antioxidant properties of *Artemisia nilagirica* leaves. These findings provide valuable insights into the potential use of *Artemisia nilagirica* methanol fraction as a natural source of antioxidants for various applications in the pharmaceutical, nutraceutical, and functional food industries.

The anticancer activities observed in the methanol and ethyl acetate fractions of *Artemisia nilagirica* leaves, particularly against colorectal cancer cells, further support their potential therapeutic applications. The higher anticancer activity observed in the methanol fraction compared to the ethyl acetate fraction suggests that the bioactive compounds responsible for the anticancer properties might be more concentrated in the methanol fraction.

The MTT assay, commonly used to evaluate cell viability and cytotoxicity, demonstrated that the methanol fraction exhibited the highest anticancer activity. This finding suggests that the methanol fraction contains bioactive compounds that are effective in inhibiting the proliferation of colorectal cancer cells. These compounds may interfere with the cancer cell's growth and induce cell death through various mechanisms, such as apoptosis or cell cycle arrest.

Although the ethyl acetate fraction exhibited comparatively lesser anticancer potential in the MTT assay, it still displayed some activity against colorectal cancer cells. This suggests that the ethyl acetate fraction contains bioactive compounds that can inhibit cancer cell growth to some extent, although their efficacy might be lower compared to the methanol fraction.

The specific bioactive compounds responsible for the observed anticancer activities in *Artemisia nilagirica* extracts are not mentioned in the information provided. However, it is possible that the presence of polyphenols, flavonoids, and other phytochemicals identified in the phytochemical analysis could contribute to the observed anticancer effects. These compounds have been reported to exhibit anticancer properties by modulating various signaling pathways involved in cancer cell growth and survival.

It is important to note that the anticancer activities of plant extracts can be influenced by various factors, including the specific cancer cell line used, concentration of the extracts, and the complexity of the cellular pathways involved in cancer progression (Hazra *et al.*, 2016). Further studies are necessary to elucidate the precise mechanisms of action and identify the specific bioactive compounds responsible for the anticancer properties of *Artemisia nilagirica* extracts.

Overall, the observed anticancer activities of the methanol and ethyl acetate fractions of *Artemisia nilagirica* leaves, with the methanol fraction displaying the highest activity, highlight their potential as a source of natural compounds for the development of anticancer therapies or as adjuncts to existing treatment strategies for colorectal cancer.

CONCLUSION

Artemisia nilagirica, an underexplored species, was the subject of the present study, which sought to investigate its potential antioxidant and anticancer properties. The findings of the study indicate that the plant possesses noteworthy antioxidant activity, as evidenced by its significant scavenging abilities against DPPH and FRAP. Furthermore, it exhibited promising anti-cancer effects on colorectal cell lines. The observed benefits may be attributed to the higher concentrations of polyphenols and flavonoids present in the methanol and ethyl acetate fractions. Notably, the ethyl acetate fraction demonstrated comparatively lower activity than the methanol fraction. Conversely, the benzene and petroleum ether fractions did not exhibit any notable activity. Hence, future research involving further purification and evaluation of this plant may yield highly active compounds with potential pharmaceutical properties.

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ST. JOSEPH'S COLLEGE (AUTONOMOUS)
DEVAGIRI, CALICUT

IN SILICO PHARMACOLOGICAL SCREENING OF BIOACTIVE METABOLITES FROM THE LEAF ESSENTIAL OILS OF DIFFERENT *CURCUMA* SPECIES

Report of the project (Students project) submitted to St. Joseph's College(Autonomous) Devagiri

Affiliated to the University of Calicut

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ST. JOSEPH'S COLLEGE

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PHYTOCHEMICHAL ANALYSIS OF THE LEAF ESSENTIAL OIL FROM *CURCUMA LONGA* AND EVALUATION OF ITS PESTICIDAL AND LARVICIDAL PROPERTIES

Report of the project (Students project / field project / internship) submitted to St. Joseph's College (Autonomous) Devagiri

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ST. JOSEPH'S COLLEGE (AUTONOMOUS)

DEVAGIRI, CALICUT

COMPARITIVE STUDY ON TICK INFESTATION ON CATTLE AND THEIR PREVELANCE IN DIFFERENT REGIONS OF THE HOST BODY

Report of the Student project submitted to St. Joseph's College(Autonomous) Devagiri

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ST.JOSEPH'S COLLEGE (AUTONOMOUS) DEVAGIRI, CALICUT

SPIDER DIVERSITY ON ST.JOSEPH'S COLLEGE DEVAGIRI KOZHIKODE

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ST JOSEPH'S COLLEGE (AUTONOMOUS) DEVAGIRI, CALICUT

DNA BARCODING – A TOOL FOR SPECIES LEVEL IDENTIFICATION OF NATIVE FRESHWATER FISH

Report of the Student project submitted to St. Joseph's College(Autonomous) Devagiri

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COMPARITIVE STUDY ON TICK INFESTATION ON CATTLE AND THEIR PREVELANCE IN DIFFERENT REGIONS OF THE HOST BODY

Report of the Student project submitted to St. Joseph's College(Autonomous) Devagiri
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Antioxidant Activities Of Leaf Extract From

Amaranthus Viridis

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DEVAGIRI, CALICUT

LARVICIDAL ACTIVITY OF ESSENTIAL OIL FROM *CITRUS MAXIMA* PEEL AND ELUCIDATION OF POSSIBLE MECHANISM

Report of the project (Students project / field project / internship) submitted to St. Joseph's College(Autonomous) Devagiri.

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PHYTOCHEMICHAL ANALYSIS OF THE LEAF ESSENTIAL OIL FROM *CURCUMA LONGA* AND EVALUATION OF ITS PESTICIDAL AND LARVICIDAL PROPERTIES

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ST. JOSEPH'S COLLEGE (AUTONOMOUS)

DEVAGIRI, CALICUT

LARVICIDAL ACTIVITY OF ESSENTIAL OIL FROM *CITRUS MAXIMA* PEEL AND ELUCIDATION OF POSSIBLE MECHANISM

Report of the project (Students project / field project / internship) submitted to St. Joseph's College(Autonomous) Devagiri.

Affiliated to the University of Calicut

HARSHA C T(Reg No: DVAUSZO013)

Programme : BSC ZOOLOGY

Semester :SIXTH

PG AND RESEARCH DEPARTMENT OF ZOOLOGY

APRIL 2023

Principal

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Supervisor

JOICE TOM J
Assistant Professor
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ST. JOSEPH'S COLLEGE (AUTONOMOUS)

DEVAGIRI, CALICUT

COMPARITIVE STUDY ON TICK INFESTATION ON CATTLE AND THEIR PREVELANCE IN DIFFERENT REGIONS OF THE HOST BODY

Report of the Student project submitted to St. Joseph's College(Autonomous) Devagiri

Affiliated to the University of Calicut

JANFY NIHAL K (DVAUSZO014)

Programme : B.Sc. ZOOLOGY

Semester: VI

DEPARTMENT OF ZOOLOGY

APRIL 2023

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ST. JOSEPH'S COLLEGE (AUTONOMOUS)

DEVAGIRI, CALICUT

LARVICIDAL ACTIVITY OF ESSENTIAL OIL FROM *CITRUS MAXIMA* PEEL AND ELUCIDATION OF POSSIBLE MECHANISM

Report of the project (Students project / field project / internship) submitted to St. Joseph's College(Autonomous) Devagiri.

Affiliated to the University of Calicut

LAKSHMIRAJ T (Reg No: DVAUSZO015)

Programme : BSC ZOOLOGY

Semester :SIXTH

PG AND RESEARCH DEPARTMENT OF ZOOLOGY

APRIL 2023

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ST. JOSEPH'S COLLEGE (AUTONOMOUS)

DEVAGIRI, CALICUT

A STUDY ON PHYSICO- CHEMICAL PARAMETRES OF WATER FROM PUNOOR RIVER, KOZHIKODE

Report of the Student project submitted to St. Joseph's College(Autonomous) Devagiri

Affiliated to the University of Calicut

RIYA R (Register No. DVAUSZO016)

Programme : B.Sc. ZOOLOGY

Semester : VI

DEPARTMENT OF ZOOLOGY

APRIL 2023

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College Seal

ST. JOSEPH'S COLLEGE
(AUTONOMOUS)DEVAGIRI,CALICUT

DIVERSITY OF STAPHYLINIDAE IN PAYYOLI, VILANGAD AND CHELAPRAM

Report of the Students project submitted to St. Joseph's College (Autonomous) Devagiri

Affiliated to the University of Calicut

Swathi Krishna T (Reg.No.DVAUSZO017)

Programme : BSc Zoology

Semester : VI

DEPARTMENT OF ZOOLOGY

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ST. JOSEPH'S COLLEGE (AUTONOMOUS)

DEVAGIRI, CALICUT

COMPARITIVE STUDY ON TICK INFESTATION ON CATTLE AND THEIR PREVELANCE IN DIFFERENT REGIONS OF THE HOST BODY

Report of the Student project submitted to St. Joseph's College(Autonomous) Devagiri

Affiliated to the University of Calicut

ABHINAV P (DVAUSZO018)

Programme : B.Sc. ZOOLOGY

Semester: VI

DEPARTMENT OF ZOOLOGY

APRIL 2023

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Supervisor



ST JOSEPH'S COLLEGE (AUTONOMOUS) DEVAGIRI, CALICUT

DNA BARCODING – A TOOL FOR SPECIES LEVEL IDENTIFICATION OF NATIVE FRESHWATER FISH

Report of the Student project submitted to St. Joseph's College(Autonomous) Devagiri

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Ms. ABHIRAMI PK (DVAUSZO019)

Programme: **B.Sc. ZOOLOGY**

Semester: **SIXTH**

PG AND RESEARCH DEPARTMENT OF ZOOLOGY

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Calicut-673 008

College Seal



ST. JOSEPH'S COLLEGE

(AUTONOMOUS) DEVAGIRI, CALICUT

PHYTOCHEMICHAL ANALYSIS OF THE LEAF ESSENTIAL OIL FROM *CURCUMA LONGA* AND EVALUATION OF ITS PESTICIDAL AND LARVICIDAL PROPERTIES

Report of the project (Students project / field project / internship) submitted to St. Joseph's College (Autonomous) Devagiri

Affiliated to the University of Calicut

Name of the Student : ALPHY DEVIS

Register No. DVAUSZO020 Programme: ZOOLOGY

Semester:6

ZOOLOGY DEPARTMENT OF ST. JOSEPH'S COLLEGE (AUTONOMOUS) DEVAGIRI,

CALICUT, MARCH 2022

Principal

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ST. JOSEPH'S COLLEGE (AUTONOMOUS)
DEVAGIRI, CALICUT

IN SILICO PHARMACOLOGICAL SCREENING OF BIOACTIVE METABOLITES FROM THE LEAF ESSENTIAL OILS OF DIFFERENT *CURCUMA* SPECIES

Report of the Project (Students Project) Submitted to St. Joseph's College (Autonomous) Devagiri

Affiliated to the University of Calicut

ANAGHA A. K. (DVAUSZO021)

Programme : BSc. ZOOLOGY

Semester : SIXTH

PG AND RESEARCH DEPARTMENT OF ZOOLOGY

APRIL 2023

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ST. JOSEPH'S COLLEGE (AUTONOMOUS)

DEVAGIRI, CALICUT

Antioxidant Activities Of Leaf Extract From

Amaranthus Viridis

Report of the Students project submitted to St. Joseph's College(Autonomous) Devagiri Affiliated to the University of Calicut

ASHWATHY. T.C (Reg No. DVAUSZO026)
ANJANA. M.K (Reg No. DVAUSZO023)
ALEENA THOMAS (Reg No. DVAUSZO09)

Programme : B. Sc Zoology

Semester : VI

DEPARTMENT OF

ZOOLOGY

April–2023

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ST. JOSEPH'S COLLEGE (AUTONOMOUS)

DEVAGIRI, CALICUT

A STUDY ON PHYSICO- CHEMICAL PARAMETRES OF WATER FROM PUNOOR RIVER, KOZHIKODE

Report of the Student project submitted to St. Joseph's College(Autonomous) Devagiri

Affiliated to the University of Calicut

ARJUN S NAIR (Register No. DVAUSZO024)

Programme : B.Sc. ZOOLOGY

Semester : VI

DEPARTMENT OF ZOOLOGY

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ST. JOSEPH'S COLLEGE (AUTONOMOUS) DEVAGIRI, CALICUT - 673 008

ST. JOSEPH'S COLLEGE (AUTONOMOUS)

DEVAGIRI, CALICUT

LARVICIDAL ACTIVITY OF ESSENTIAL OIL FROM *CITRUS MAXIMA* PEEL AND ELUCIDATION OF POSSIBLE MECHANISM

Report of the project (Students project / field project / internship) submitted to St. Joseph's College(Autonomous) Devagiri.

Affiliated to the University of Calicut

ARSHA KIRAN P (Reg No: DVAUSZO025)

Programme : BSC ZOOLOGY

Semester :SIXTH

PG AND RESEARCH DEPARTMENT OF ZOOLOGY

APRIL 2023

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ST. JOSEPH'S COLLEGE (AUTONOMOUS)

DEVAGIRI, CALICUT

Antioxidant Activities Of Leaf Extract From

Amaranthus Viridis

Report of the Students project submitted to St. Joseph's College(Autonomous) Devagiri Affiliated to the University of Calicut

ASHWATHY. T.C (Reg No. DVAUSZO026)
ANJANA. M.K (Reg No. DVAUSZO023)
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Programme : B. Sc Zoology

Semester : VI

DEPARTMENT OF

ZOOLOGY

April–2023

Principal

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ST. JOSEPH'S COLLEGE

(AUTONOMOUS) DEVAGIRI, CALICUT

PHYTOCHEMICHAL ANALYSIS OF THE LEAF ESSENTIAL OIL FROM *CURCUMA LONGA* AND EVALUATION OF ITS PESTICIDAL AND LARVICIDAL PROPERTIES

Report of the project (Students project / field project / internship) submitted to St. Joseph's College (Autonomous) Devagiri

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Semester:6

ZOOLOGY DEPARTMENT OF ST. JOSEPH'S COLLEGE (AUTONOMOUS) DEVAGIRI,

CALICUT, MARCH 2022

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ST. JOSEPH'S COLLEGE (AUTONOMOUS)

DEVAGIRI, CALICUT

A STUDY ON PHYSICO- CHEMICAL PARAMETRES OF WATER FROM PUNOOR RIVER, KOZHIKODE

Report of the Student project submitted to St. Joseph's College(Autonomous) Devagiri

Affiliated to the University of Calicut

CHANDANA R R (Register No. DVAUSZO028)

Programme : B.Sc. ZOOLOGY

Semester : VI

DEPARTMENT OF ZOOLOGY

APRIL 2023

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ST. JOSEPH'S COLLEGE

(AUTONOMOUS)DEVAGIRI,CALICUT

DIVERSITY OF STAPHYLINIDAE IN PAYYOLI, VILANGAD AND CHELAPRAM

Report of the Students project submitted to St. Joseph's College (Autonomous) Devagiri

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Chippy Joseph (Reg.No.DVAUSZO029)

Programme : BSc Zoology

Semester : VI

DEPARTMENT OF ZOOLOGY

APRIL2023

Principal

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St. Joseph's College (Autonomous)
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ST. JOSEPH'S COLLEGE (AUTONOMOUS)

DEVAGIRI, CALICUT

A STUDY ON PHYSICO- CHEMICAL PARAMETRES OF WATER FROM PUNOOR RIVER, KOZHIKODE

Report of the Student project submitted to St. Joseph's College(Autonomous) Devagiri

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FATHIMA HAMNA L (Register No. DVAUSZO030)

Programme : B.Sc. ZOOLOGY

Semester : VI

DEPARTMENT OF ZOOLOGY

APRIL 2023

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DEVAGIRI, CALICUT

A STUDY ON PHYSICO- CHEMICAL PARAMETRES OF WATER FROM PUNOOR RIVER, KOZHIKODE

Report of the Student project submitted to St. Joseph's College(Autonomous) Devagiri

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Programme : B.Sc. ZOOLOGY

Semester : VI

DEPARTMENT OF ZOOLOGY

APRIL 2023

Principal

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DEPARTMENT OF ZOOLOGY
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ST. JOSEPH'S COLLEGE, DEVAGIRI, CALICUT

ST. JOSEPH'S COLLEGE
(AUTONOMOUS)DEVAGIRI,CALICUT

DIVERSITY OF STAPHYLINIDAE IN PAYYOLI, VILANGAD AND CHELAPRAM

Report of the Students project submitted to St. Joseph's College (Autonomous) Devagiri

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Lala Krishna (Reg.No.DVAUSZO032)

Programme : BSc Zoology

Semester : VI

DEPARTMENT OF ZOOLOGY

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ST JOSEPH'S COLLEGE (AUTONOMOUS) DEVAGIRI, CALICUT

DNA BARCODING – A TOOL FOR SPECIES LEVEL IDENTIFICATION OF NATIVE FRESHWATER FISH

Report of the Student project submitted to St. Joseph's College(Autonomous) Devagiri

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Semester: **SIXTH**

PG AND RESEARCH DEPARTMENT OF ZOOLOGY

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ST JOSEPH'S COLLEGE (AUTONOMOUS) DEVAGIRI, CALICUT

DNA BARCODING – A TOOL FOR SPECIES LEVEL IDENTIFICATION OF NATIVE FRESHWATER FISH

Report of the Student project submitted to St. Joseph's College(Autonomous) Devagiri

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Ms. NAVANEETHA M S (DVAUSZO034)

Programme: **B.Sc. ZOOLOGY**

Semester: **SIXTH**

PG AND RESEARCH DEPARTMENT OF ZOOLOGY

APRIL 2023

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ST.JOSEPH'S COLLEGE (AUTONOMOUS) DEVAGIRI, CALICUT

SPIDER DIVERSITY ON ST.JOSEPH'S COLLEGE DEVAGIRI KOZHIKODE

Report of the project (Student Project) submitted to St. Joseph's College (Autonomous) Devagiri

Affiliated to the University of Calicut

NEHA MARSEL (DVAUSZO035)

Programme : B.Sc Zoology

Semester : VI

PG & RESEARCH DEPARTMENT OF ZOOLOGY,

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ST. JOSEPH'S COLLEGE (AUTONOMOUS) DEVAGIRI, CALICUT - 673 008

ST. JOSEPH'S COLLEGE (AUTONOMOUS)

DEVAGIRI, CALICUT

COMPARITIVE STUDY ON TICK INFESTATION ON CATTLE AND THEIR PREVELANCE IN DIFFERENT REGIONS OF THE HOST BODY

Report of the Student project submitted to St. Joseph's College(Autonomous) Devagiri

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SHARON BABU N (DVAUSZO036)

Programme : B.Sc. ZOOLOGY

Semester: VI

DEPARTMENT OF ZOOLOGY

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ST.JOSEPH'S COLLEGE (AUTONOMOUS) DEVAGIRI, CALICUT

SPIDER DIVERSITY ON ST.JOSEPH'S COLLEGE DEVAGIRI KOZHIKODE

Report of the project (Student Project) submitted to St. Joseph's College (Autonomous) Devagiri

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SINNA PARVEEN (DVAUSZO037)

Programme : B.Sc Zoology

Semester : VI

PG & RESEARCH DEPARTMENT OF ZOOLOGY,

APRIL 2023

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ST. JOSEPH'S COLLEGE
(AUTONOMOUS)DEVAGIRI,CALICUT

DIVERSITY OF STAPHYLINIDAE IN PAYYOLI, VILANGAD AND CHELAPRAM

Report of the Students project submitted to St. Joseph's College (Autonomous) Devagiri

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Sreelakshmi R (Reg.No.DVAUSZO038)

Programme : BSc Zoology

Semester : VI

DEPARTMENT OF ZOOLOGY

APRIL2023

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ST. JOSEPH'S COLLEGE
(AUTONOMOUS)DEVAGIRI,CALICUT

DIVERSITY OF STAPHYLINIDAE IN PAYYOLI, VILANGAD AND CHELAPRAM

Report of the Students project submitted to St. Joseph's College (Autonomous) Devagiri

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Vishnumaya M (Reg.No.DVAUSZO039)

Programme : BSc Zoology

Semester : VI

DEPARTMENT OF ZOOLOGY

APRIL2023

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