# A STUDY ON PHARAMACOLOGICAL ACTIVITIES OF *MOMORDICA CYMBALARIA* UNRIPE FRUITS

A PROJECT SUBMITTED TO

**ST.MARY'S COLLEGE (AUTONOMOUS), THOOTHUKUDI**

**Affiliated by Manonmaniam Sundaranar University**

In partial fulfillment of the requirements for the award of the degree of

**MASTER OF SCIENCE IN MICROBIOLOGY**

SUBMITTED BY

**K.AFFRIN NISHA**

**(REG. NO. 20SPMB01)**

**Under the Guidance of**

**Dr.Joys Selva Mary Albert M.Sc., M.Phil., Ph.D.**

**DEPARTMENT OF MICROBIOLOGY**

**ST.MARY'S COLLEGE (AUTONOMOUS),**

**THOOTHUKUDI – 628 001.**

**May – 2022**

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# CERTIFICATE

This is to certify that the project work entitled **"A study on Pharmacological activities of *Momordica cymbalaria unripe fruits*"** submitted to **St Mary's College (Autonomous), Thoothukudi** affiliated to **Manonmaniam Sundaranar University, Tirunelveli** for the partial fulfillment for the award of Master of Science in Microbiology is a bonafide research carried out by **Ms. AFFRIN NISHA. K** under the guidance and supervision of Dr. Joys Selva Mary Albert M.Sc., M. Phil., Ph.D, Head and Assistant Professor of Microbiology St Mary's College (Autonomous), Thoothukudi, for academic year 2022.

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# DECLARATION

I hereby declare that the project work entitled **"A Study on Pharmacological activities of *Momordica cymbalaria* unripe fruits"** is a bonafide record of the work completed by me during the academic year 2022 in St. Mary's College (Autonomous), Thoothukudi and submitted as a partial fulfillment of requirements for the award of the Degree of Master of Science in Microbiology prescribed by the Manonmanimam Sundharanar University. I also affirm that this is an original work done by me under the supervision of Dr. **Joys Selva Mary Albert M.Sc., M.Phil., Ph.D.,** Head and Assistant Professor of Department of Microbiology, St. Mary's College (Autonomous), Thoothukudi.

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**DATE** : 25-5-2022

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**K. AFFRIN NISHA**

# CONTENTS

## ABBREVIATIONS

| | |
|---|---|
| % | Percentage |
| µg | Microgram |
| µl | Microlitre |
| + | Present |
| - | Absent |
| ANOVA | Analysis of variance |
| cm | Centimeter |
| DMSO | Dimethyl sulfoxide |
| DPPH | Diphenyl-1-picrylhydrazyl |
| g | gram |
| GCMS | Gas Chromatography Mass Spectrometry |
| hr | Hours |
| ha | Hectares |
| in | inches |
| IL-1β | interleukin-1β |
| Kg | Kilogram |
| L | litre |
| m | meter |
| mcg | microgram |

| | |
|---|---|
| MHA | Muller Hinton Agar |
| Min | Minutes |
| ml | Milliliter |
| mm | Millimeter |
| mM | Millimole |
| M.e | Methanolic extract |
| *MTCC* | Microbial Type Culture Collection and Gene Bank |
| NS | No sensitivity |
| NSAID | *Non-steroidal anti-inflammatory drugs* |
| OS | oral administration |
| ˚C | Degree Celsius |
| PDA | Potato Dextrose Agar |
| $PGE_2$ | prostaglandin $E_2$ |
| S.D | Standard deviation |
| Sec | Seconds |
| S.E.M | Standard Error of the mean |
| sp | Species |
| Vol. | Volume |
| Wt. | Weight. |

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# CHAPTER 1

## INTRODUCTION

Natural products gain more attention nowadays because of their compatibility with mankind because it's safe, economic and feasible. For aeons, Plant extracts were a source of therapy in local communities. These are still valuable today as a primary healthcare strategy for about 85 percent of the planet's population (Pei *et al.*, 2015) and act as a reservoir for drug research, with 80 % of all synthetic compounds emanating from it (Bauer and Brönstrup,*et al.*, 2014). Simultaneously, there has been a sudden spike with in adoption, evolution, and refinement of herbal drugs over last few centuries (Mohammed Bourhia *et al.*, 2020). Globally more than 75-85% of the people and folk medicine are known to use medicinal plants predominantly in Asia and Africa. Herbal plants were indigenous and slowly eroded by the modernization to allopathic drugs. Due to the scarcity in knowledge about growth, population, species variety, distribution management methods of treatment and conversion in useful medicine has declined (K.Arora 2018).

The inventoried plant species in the current work are frequently used for the treatment of various illnesses and to ensure the medication safety of people. Synthetic drugs, also called "designer" drugs, are created in laboratories. Rogue chemists have since taken these chemicals, modified them slightly so they're technically legal, and sold them as "research chemicals." The synthetic drugs stimulate the central nervous system by inhibiting the reuptake of nor epinephrine and dopamine leading to severe CNS adverse effects or even death (Kaliyaperumal *et al.*, 2012).

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels. Deficient insulin action results from inadequate insulin secretion and or diminished tissue responses to insulin in the complex pathways of hormone action. Impairment of insulin secretion and defects in insulin.According to WHO currently 382 million people are affected globally and diabetes will emerge as 7$^{th}$ leading cause of death in 2030. Currently the medication used for diabetes includes sulfonylurea, metformin, glucosidase inhibitors, trogliatzone, etc. The drugs are used as immunotherapy or in combination to achieve glycemic control but the side effects are unavoidable. So an alternative medicine with few to no side effects is needed to gradually decrease the infectious ability of the disease. Organic macromolecules, enzymatic and

chemical approaches can be used as antidiabetic medicines to stimulate insulin production, lower blood glucose, and suppress specific kinds of diabetes while minimising side effects (Jaison Jeevanandam *et al*., 2022).

Medicinal plants posses stronger antimicrobial qualities compared to other antifungal and antibacterial compounds with narrow range of specificity. These not only help temporarily but aid in curing totally and disseminate diseases and ailments away. Compared to allopathic and other chemical and synthetic antimicrobials the plant extracts are holistic in their approach. The microbe inhibiting activity of plants correlates with antagonistic endophytes which harbor the medicinal plants due to their bio active components .

This manifold assertion in plant drug medical compounds which is growing at 7-15% rate despite the advances in modern medicine (Deepak Kumar Jha *et al* .,2018). The forest in India is a habitat to wide range of diverse plants which are used as raw material for various perfumes and medicines. About 8000 herbal remedies have been confirmed in ayush systems in India.Ayurveda, Unani, Siddha, Homeoapthy utilize indigenous variety of plants with therapeutic aspects and for development of medicine in the respective field. In Indian systems of medicine, most practitioners formulate and concoct their own formulations. The World Health Organization (WHO) has listed 21,000 species of plants that are used medicinally around the world. Of these 2500 species found in India, 150 of them are used commercially on a fairly large scale. India is the largest producer of medicinal herbs and is known as the world's botanical garden (Seth S.D 2004).

Anti microbial therapy is the mainstay line of treatment to treat these infection but recently microbial resistance had been well documented. This lead to use of natural medicinal herb for the treatment of ulcer, cancer, diabetes, cardiac diseases, wounds, foetal health issues ,etc. Major obstacle in amalgamation of plant based medicine in scientific medical techniques is scarce of clinical knowledge in their potential and safety. So there prevails a necessity for carrying out research and investigating the herbal drugs by means of developing simple bioassays, standardization of the compounds, pharmacological and toxicological evaluation on laboratory models, and usage of animal models for safety consumption. It is important to quantify and qualify the active component from these plant extracts (Manisha Modak *et al*.,2007). Antimicrobial activity of a single compound is referred as the intrinsic capacity of substances obtained from it like drugs or toxins to change chemical or physiological functions of a cell, tissue, organ or organism (Jackson *et al*.,2007). Along with this activity, its concentration and duration of cellular exposure to substance is also determined. Secondary metabolite success depends on the presence of high diversity and

wide spectrum of biological activities of the phytochemicals present in them (T Shasthree *et al*., 2021). Environment and humans are closely interconnected. Scientists seek evidence, which spot lime light on the major dominion aspect of microbial ecosystems in with holding the material and non materialistic balance between nature and human health (McCann, 2000). Man consumes vegetables that contain varied minor and major nutrients like carbohydrates, peptides, antioxidants, lipids, and glucosinolates. These also contain biologically active compounds that serve as antioxidants in the metabolism of humans and prevent skin damage, cell necrosis, and cancer cell formation (Rossner U *et al*., 2009).

In spite of the fact that restorative plants have been generally utilized for diabetes treatment all through the world, few of them have been approved by logical criteria. Crude and plant based natural products have been used broadly for their nature of anti diabetic activity .Most of the compounds function as direct or indirect enzyme inhibitors. It uses extensive mechanisms like alpha glycosidase and aldose reductase and inhibition by formation of glycation ,and reduction of plasma glucose level or by alternating the activity of hexo kinases and synthesizing insulin with stimulation of GLUT-4 and decreasing the activity of G6P, and dissolving or reducing the skeletal hexo kinases. The medicinal plants that have such properties include *Abelmoschus moschatus, Alangium salvifolium, Azardirchta indica ,Bidens pilosa, Boerhaavia diffusa, Capsicum frutescens, Cassia alata, Eclipta alba, Embellica officinalis, Ficuscaria, Gentianaoliver, Ipomoea aquatic, Mangifera indica,Hordeum vulgare, Ocimum sanctum, Zingiber officinale, Momordica chantaria, Juniperes communis, Punica granatum*. All of them posses compounds like ferulic acid, oleuropeoside, serquiterpine, alkaloids, limonene that increase the efficiency of anti diabetic drug composition (Fiaz Alam *et al*., 2018).

Modern dietary habits have great influence and broadly changed lifestyles which contribute to the increasing numbers of chronic diseases. Imbalanced oxidative stress and low-grade inflammation lay the base for non-communicable diseases such as metabolic syndrome, cardiovascular, neurodegenerative, and chronic lung diseases, as well as cancer (Checa and Aran, 2020; Ranneh *et al*., 2017).

The plant *Veronica cinera* less (*Asteraceae*) is a common edible weed distributed in Asia, India, China, Nepal, and Africa. It is a predominant component in Chinese medicine and used in treatment of malaria, cancer, diabetes, gastrointestinal disorders and various inflammation. (NourHamidAzhari *et al*.,2018). The extract of the plant can inhibit cytochrome P450 2A6(CYP2A6) and associated with smoking cessation therapy (Prasopthum *et al*.,2015). The

plant is also known to decrease the glucose level and improve the quality of sperm *in vivo* rat models (Ponmjunya *et al*., 2017).

The polysaccharides extracted from *Suillellus luridus* retained the anti diabetic property and when applied as treatment pathological morphology of kidney, liver and pathogenecity was normal on observation. The polysaccharide consists of arabinose, xylose, mannose and galactose. The increase of arabinose and galactose may conduce to the activities of the polysaccharide (Hejen WU *et al*., 2018).

Like most microorganisms, Plasmodium parasites develop resistance with drug therapy over duration. Resistance to chloroquine and, more recently, Arteminisins has really been reported in parasites. Threshold parameters for biological activity and preferential activity have been specified in recent studies. The *in vitro* anti-plasmodial action of hydroethanolic extracts from medicinal plants *Baphia nitida, Cinnamomum zeylanicum, Lippia multiflora, Morinda lucida, Parkia clappertoniana, Tabernaemontana crassa, Terminalia ivorensis, and Treculia Africana* was investigated, along with their antioxidant properties. *Terminalia ivorensis, Parkia clappertoniana,* and *Lippia multiflora* extracts showed substantial anti-plasmodial activity and great selectivity. The remarkable anti-plasmodial potential of *Terminalia ivorensis* may be attributable to its strong phenolic content; nevertheless, more research is required to isolate and identify the active principles in bioactive plant extracts. Chemotherapy is perhaps the most viable ailment apart from treatment with quinine drugs (Regina Appiah-Opong *et al*., 2022).

Pastoralists from Cameroon analyzed the capabilities of various extracts of *B.senegalensis* and *T.dodoneifolius*. Plant extracts have been shown to be non-toxic. These compounds were shown to be extremely toxic to some of the microorganisms examined. Traditional uses of *B. senegalensis* and *T.dodoneifolius* for animal disease prevention may be observed as *B.senegalensis* showed efficacy against methicillin-resistant *S. aureus*. Tannins, saponins, and alkaloids, chemicals with antibacterial effects are found in plant components (Prashant *et al*., 2011). The antimicrobial activities of the various extracts were tested against three bacterial reference strains, including Gram-positive (*Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli)* and (*Salmonella typhi*). The hexane extract of *B. senegalensis* and the methanolic extract of *B.senegalensis* both had a sensitive inhibitory action against S. aureus (Vougat Ngom *et al*., 2022).

Thymus is one of the most well-known *Lamiaceae* genera, with over 215 species. In this family, it is a polymorphic genus. As a result, changes in chemical characteristics between polymorphs could lead to variation in biological functions. The chemical makeup of thymus pubescens essential oil was examined in order to measure antioxidant activity and define active components.TLC-bioautography assays with (ABTS) reagent were used to detect and recognize antioxidant components. The presence of 39 components, accounting for 96.64 percent of total contents, was shown by GC analyses, with thymol (38.67 percent), -terpinene (7.46 percent), and p-cymene (5.54 percent) being the most prominent. The primary antioxidant compound identified using TLC-bioautography of oil was thymol. The antioxidant properties of essential oils were shown to be quite powerful and substantial, and they linked well with ascending dose invitro (Nickavar, Bahman *et al*., 2022).

The total phenolic and flavonoid compositions in methanol extracts of *Olea ferruginea Royle*, *Olea europaea L*., and *Tilia europaea L*., which grow naturally in the north-west Indian Himalaya, were investigated. The highest concentrations of phenolics and flavonoids were detected in methanol extracts of *O. ferruginea* stem bark and leaf (9.28 mg GAE/g few and 14.73 mg QE/g fw, respectively. Leaf and stem bark had the highest ferric reducing antioxidant strength and DPPH radical scavenging activity. These species of tree were discovered to be high in natural chemicals and to have antioxidant properties. As a result, pharmaceutical and local applications for health benefits are recommended (Rajesh Kumar Sharma *et al*., 2022).

Using the solvent extraction method with petroleum ether, benzene, chloroform, ethyl acetate, methanol, and aqueous extracts, this study aims to investigate the antioxidant activity of the leaf, stem, latex, and bark of *Euphorbia neriifolia (EN)* Linn. Tannic acid, quillaja, quercetin, gallic acid, and rutin, respectively, were used to study total tannin content, total saponin content, total flavonoid content, total phenol content, and total flavonol content were also studied individually .The leaf, stem, latex, and bark show significant antioxidant capacity, according to EN's *in vitro* antioxidant assay. It can be inferred that EN leaf has a higher mean content of phytochemicals than the plant's stem, latex, or bark, which may have contributed to its high antioxidant qualities. As a result, medicines with antioxidant activities can be derived from this species. However, *in vitro* and *in vivo* research is still needed to

establish the antioxidant property, as well as toxicological and anticarcinogenic investigations of crude extract against various disorders (Chaudhary P *et al.*, 2022).

Chemical compounds with possible therapeutic benefits can be found in medicinal plants. Twelve phenolic compounds were discovered after extracting phenolic pools from jujube (*Ziziphus jujuba Mill.)* and moringa (*Moringa oleifera Lam.*) leaves using MeOH. Jujube leaf extract is higher in phenolic compounds than moringa leaf extract. Jujube leaf extract was more effective than Moringa leaf extract at fighting free radicals, chelating/reducing iron, and reducing cupric forms. Furthermore, statistic clustering of marked findings on the inhibition of -amylase and -glucosidase enzymes revealed a significant role of phenolic and flavonoid pools in anti-diabetic action (Hanen Wasli *et al.*, 2021).

Patiwala (*Lantana camara*), one of the plants used in Indonesian traditional medicine, is traditionally used to cure itching, wounds, ulcers, swelling, eczema, tetanus, malaria, tumors, rheumatism, and headaches. The patiwala leaf has the ability to scavenge free radicals.. Resveratrol dimer, iso-humolones, oleuropein glucoside, quercetin-3-O-glycoside, myricetin, oleuropein, 12-deoxy-16-hydroxy-phorbol, aloeresin A, humulones, ursolic acid, viniferin, Epicatechin, Epicatechin, oleanolic acid, 5-hydroxy-3′,4′,7-trimerth were some of the compounds isolated from them.The methanolic extract of *L. camara* leaves has the potential to be developed as a natural source of antioxidants. *Lantana camara* extracts and fractions demonstrated extremely high antioxidant activity( Ruslin *et al.*,2022).

Salvia species are found all over the world and include numerous well-known plants that are significant in the pharmaceutical and food sectors due to their acknowledged medicinal and preservation characteristics. *S. verticillata*, like *S. officinalis* and *S. fruticosa*, was discovered to have anti-acetylcholinesterase action, whereas S. glutinosa was found to have the potential to inhibit -glucosidase. Some of them, such as *Salvia officinalis* L. (Dalmatian sage, common sage), *Salvia lavandulifolia Vahl* (Spanish sage), *Salvia sclarea* L. (clary sage), *Salvia fruticosa Mill.* (Greek sage*), Salvia miltiorrhiza Bunge* (danshen), and *Salvia hispanica L.* (chia), are planted globally for Other phenolic acids found in sage leaves which include caffeic, chlorogenic, p-coumaric, and ferulic acid. Sage extracts contain luteolin, apigenin, quercetin, and their glycosidic derivatives, which can be used as nutraceuticals and food preservatives for oxidative stress-related chronic diseases such as diabetic, cardiovascular, and neurological diseases ( Mervić, M *et al.*,2022).

Antimicrobial and antioxidant properties of *Ramalina roesleri Nyl* solvent extracts were tested. *Staphylococcus aureus* and *Streptococcus mutans* were both sensitive to hexane extract. The hexane extract was used to isolate atranorin, protolichesterinic acid, usnic acid, 2-hydroxy-4-methoxy-6-propyl benzoic acid, homosekikaic acid, sekikaic acid, benzoic acid, 2,4-dihydroxy-6-propyl, and 2,4-dihydroxy-3,6-dimethyl benzoate ( R. Sisodia *et al*.,2013).

The total phenolic content (TPC) yield, purity, antioxidant activity, and antibacterial activity of four samples of Arcea nut were determined and compared using conventional liquid–solid extraction and ultrasonic-microwave synergistic extraction . Antibacterial and antioxidant activity against *Streptococcus mutans, Staphylococcus aureus, Escherichia coli,* and *Pseudomonas aeruginosa* were measured. With proper investigation, a possible antibacterial agent and antioxidant can be extracted commercially from areca nut ( Jiang *et al*., 2022).

Plant-based therapeutic and curative medications are becoming increasingly popular in today's society. Ginseng's (*Panax species*) relevance as a functional nutraceutical has kindled interest among these phytomedicines in recent years. Phenolic substances such as gallic acid, chlorogenic acid, and vanillic acid were discovered in both Panax species.

*P. pseudo ginseng* has high levels of palmitic, oleic, and linoleic acid, as well as greater poly-unsaturates. Mineral and trace element analysis revealed that Panax species have high levels of phosphorus, potassium, magnesium, and selenium. This could be utilised as a starting point for enhancing herbal raw materials for pharmaceutical and nutraceutical formulations ( Riadh Ksouri *et al*., 2022).

*Chenopodium botrys* (CB) is an anti-inflammatory, antioxidant, antidiabetic, antibacterial, antifungal, antiparasitic, and antiviral plant that is native to Iran. The effects of CB essential oil on cutaneous wound healing markers in Sprague-Dawley male rats. At 10 days post-injury (DPI), the animals in each group were euthanized. In addition, CB therapy reduced wound surface area, lymphocyte and neutrophil numbers, and increased the number of blood vessels. CB has been shown to have wound-healing properties on the skin, suggesting that it could be used as a medicinal supplement (Taymour Sayyedrostami *et al* .,2018) .

*Acanthus* is a genus of flowering plants in the *Acanthaceae* family. With 2500-3000 species in 250 genera, the Acanthus family is quite vast. Antifungal, cytotoxic, anti-inflammatory, and antipyretic, antioxidant, antiviral, insecticidal, hepatoprotective, immunomodulatory, and

antiplatelet actions are all found in the *Acanthaceae* family. The wound healing capacity of the crude extract of *A. polystachyus* leaves was evaluated using 80 percent methanol in a scientific study. Excision, infection, and incision wound models in Swiss albino mice were used to test the crude extract ointment for wound healing efficacy. In comparison to the negative control group, it considerably decreased the time it took for epithelialization and boosted wound contraction rate and tensile strength (Seyfe Asrade *et al.*, 2018) .

Secondary metabolites isolated from Tupistra plants have been reviewed in this research. There are about 200 phytochemicals, which are divided into different chemical classes of bioactive molecules. One of the most important goals in pharmacological research is to use Tupistra plant extracts and isolated chemicals in biological experiments. Tupistra components have shown antioxidative, antibacterial, antidiabetic, and antihepatic characteristics in the field of pharmacology, although their cytotoxic and anti-inflammatory effects are the most notable. The investigated Tupistra samples' cancer-related effects *in vivo* were largely dependent on apoptosis. Spirostanol sapogenins, as well as spirostanol and furostanol saponins, are the main steroids. In both phytochemical and pharmacological investigations, the various sections of *T. chinensis* have been used the most. Saponins from Tupistra (Thuy Linh NT *et al*.,2022).

Polysaccharides from marine microalgae and seaweeds have immunomodulatory effects on humans and animals. Antiviral, anticancer, hypoglycemic, anticoagulant, and antioxidant effects may also be present in these polysaccharides. Immunosuppressive molecules can be used as therapeutic agents in inflammatory disorders including autoimmune diseases and sepsis. Similarly, immune activation molecules can trigger immune responses that fight cancer and infectious illnesses. The chemical makeup of algal polysaccharides such as alginate, fucoidan, ascophyllan, and porphyran will be discussed. We also go through how they can be used to treat cancer, infectious disease, and inflammation Finally, these uses of marine algal polysaccharides could provide innovative therapeutic options for a variety of disorders (Dhananjay Yadav *et al*.,2021).

*S. latissima,* like many other brown seaweed species, includes a variety of useful and bioactive substances such as polysaccharides, phenolic compounds, dietary fibre, and vitamins (Holdt and Kraan, 2011; Pealver *et al*., 2020). Antioxidant chemicals can help to scavenge excessive oxidative stress and rebalance the intricate inflammatory signalling

system in the context of disease prevention (Liu *et al.*, 2018). Natural antioxidants generated from algae have already been proven to reduce protein and lipid oxidation (Barros-Velázquez *et al.*, 2016; Jacobsen *et al.*, 2019; Jónsdóttir *et al.*, 2016).Phenolic substances, such as phenolic acids or flavonoids, are another source of antioxidant action in seaweeds. Phlorotannins are the most common phenolic chemicals found in brown seaweed (Cotas *et al.*, 2020).

Aqueous methanolic or ethanolic solutions are often used to extract polyphenols and antioxidants from red and browm alage (Aslan *et al.*, 2019; Machu *et al.*, 2015).

Several algae-derived chemicals, such as phlorotannins and fucoidans, have been shown to have anti-allergic properties by interfering with allergy-triggering pathways (Barbosa *et al.*, 2018; Vo 2020).

Seung-Hong Lee *et al.*,2013 examined anti-diabetic impacts of brown, green growth or brown algae determined phlorotannins, marine polyphenols through different components . The marine green algae has phlorotannins, marine polyphenols. The larger part of the examinations on phlorotannins determined from brown green alage have shown their different anti-diabetic components such as α-glucosidase and α-amylase inhibitory impact, glucose take-up impact in skeletal muscle, protein tyrosine phosphates protein hindrance, against diabetes complication.

In the shallow waters of the Arctic region's oceans, *Fucus vesiculosus* is one of the most common brown algae. *F. vesiculosus* could be employed safely in food and medication research as a source of active biochemical substances and dietary ingredients to meet human daily nutritional needs. Researchers are paying more attention to brown algae, and *F. vesiculosus* is utilised in cosmetics, biofertilizers, and animal feed. Fucoidans, polyphenols, fucoxanthin, and vital minerals are abundant in F. vesiculosus, making it a good source of health-promoting substances.. Fucoidans have been shown to have a variety of pharmacological actions, including antioxidant, anti-obesity, antidiabetic, anti-aging, antibacterial, anticancer, anticoagulant, and anti-inflammatory. It could be used safely in food and medication research, as a source of active biochemical components and dietary ingredients, to meet the daily nutritional requirements of humans (Obluchinskaya *et al.*,2022).

Bioactive natural products research has primarily concentrated on marine species such micro- and macro algae and marine invertebrates, as well as terrestrial glycophyte plants. Natural

salt-tolerant plants, known as halophytes, make up roughly 2% of terrestrial plant species and are found in half of the higher plant families. They have a wide range of plant forms and are now regarded as a significant source of bioactive compounds with a variety of biotechnological applications, ranging from food to cosmetic elements. Sea rocket (*Cakile maritima Scop.*) is an edible halophyte plant with a variety of ethno medicinal uses.
The edible halophytic succulent annual herb *Cakile maritima Scop.* (sea rocket) belongs to the *Brassicaceae* (mustard) family. This species is also used as an antiscorbutic, digestive, diuretic, and anti-dandruff agent in ethno medicine. Sea rocket generates phenolic acids, ascorbic acid, hydrocarbons, sterols, and flavonoids ( Joao Rodrigues *et al*., 2020).

Cyanobacteria are blue–green microalgae that generate antibiotics, cytotoxic agents, and antimicrobial compounds as secondary metabolites. *Oscillatoria sp.* was discovered in an isolated colony and was cultured in BG 11 media for bulk cultivation. Fatty acid, triazine derivatives, pyridine derivatives, and acridine derivatives were identified by GCMS analysis of *Oscillatoria sp*. Because the methanolic extract reduced the growth of S. aureus for gram-positive bacteria and *P. aeruginosa* for gram-negative bacteria, *Oscillatoria s*p. serve as antibacterial agents. In the pharmaceutical and health-care industries, cyanobacteria extract should be chosen and considered a novel natural medicine or antibiotic. Antimicrobial, anticancer, antioxidant, and antitumor activities have all been demonstrated in marine cyanobacteria (Oscillatoria sp.). Instead of using chemical drugs or therapies, the extract from cyanobacteria can be used to cure a variety of ailments ( Maniam, G.P. *et al* .,2020).

Phytochemical screening and antibacterial activity of several solvent extracts of marine algae such as *Sargassum swartzii* against some harmful bacteria found in humans and fish. *Sargassum swartzii*, a brown seaweed, was collected in Kudankulam, Tirunelveli district, Tamil Nadu, India. Antibacterial activity against human pathogens such as *E. coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*, and fish pathogens such as *Aeromonas hydrophila* and *Vibrio vulnificus* bacteria. The phenolic content of the ethanol extract was substantially higher, whereas the flavonoides content of the methanol extract was significantly higher ( Sujatha, R *et al.*, 2019).

Non-steroidal anti-inflammatory medications (NSAIDs) are routinely used for pain relief and inflammation reduction. As a result, using natural anti-inflammatory substances with antioxidant capabilities as adjuvant therapy for many chronic conditions could be

advantageous. *P. oceanica* (L.) Delile is a marine angiosperm and the only *Posidoniaceae* species indigenous to the Mediterranean Sea, where it forms underwater meadows of significant ecological value (Vacchi *et al*., 2017). *P. oceanica* leaves have long been utilised by peasants along Western Anatolia's coast as a traditional natural medicine for diabetes and hypertension. Human cancer cell migration is inhibited by *P. oceanica* (Barletta *et al*., 2015; Leri *et al*., 2018).

## HISTORY OF *MOMORDICA CYMBALARIA*:

Athulakkai (*Momordica cymbalaria*) has a long history as a medicinal plant with antimicrobial resistance and also analyze the inhibitory activity of the whole fruit. Momordica cymbalaria is slender, scandent, branched, striate stem. The leaves are orbicular, reniform in outline deeply cordate at the base, sparsely hairy. The root of the plant is woody, tuberous and perennial. The plant is originating in tropical regions of India and South East Asia .Momordica is a monecious trailing plant with large turnip shaped tuberous rootstock. The plant has also been named *Luffa tuberosa* (Roxb.) or *Momordica tuberosa* (Roxb)Locally the fruit is known as Karisikayi in kanada ,athalakkai in Tamil, kattupaval in Malayalam and in marathi known as kuduhunchi.

The plant is mostly cultivate along the banks of water courses and in ledges *Momordiaca cymbalaria* is a plant of indigenous origin from the family *Cucurbitae* grows abundantly in tropical regions of the world. It has a bitter taste and has many medicinal qualities (Walters TW *et al.*, 1988) it grows on many parts of India, Pakistan, Bangladesh, and also extends from Himalayas to Ceylon. It has been known to grow at a height of 1500 in Assam garo hills of Meghalaya (Ram *et al*.,2002) the Western Ghats hold a wide variety of diverse treasure of the momordica genus which are *M.dioca var muricata, M.charantia var charantia, M.sahyadrica* (Joseph and Antony 2008). It is a perineal climber found in India . It is found in Karnataka and Andhra Pradesh, Madhya Pradesh, Maharashtra and Tamilnadu and is predominant only in the monsoon season.

## MORPHOLOGICAL CHARACTERISTICS AND NOMENCLATURE :

*Momordica* is a genus of about 60 species of annual or perennial climbers, herbaceous or rarely small shrubs, and native to tropical and subtropical Africa, Asia, and Australia, and belonging to the *Cucurbitaceae f*amily (Schaefer and Renner,*et al*.,2010). Notably,

*Cucurbitaceae* family is considered to be one of the valuable medicinal families of plants with potent hypoglycemic properties. Recently, this plant has been reported for the presence of bioactive metabolites through GC-MS analysis, potent compound from *Momordica cymbalaria* can be used as an agonist for PPAR-γ (E. Abbirami *et al.*, 2018) . Cucurbitane type triterpenoids, phenolics, glycosides, and a variety of peptides, including *Momordica* anti-HIV protein . Several kinds of cucurbitane type triterpenoids have been discovered in the leaves, stems, and fruits of Momordica species, and they have a variety of pharmacological activities. The common species include *Momordica dioica, Momordica charantia Momordica balsamina, Momordica foetida, Momordica cochinchinensis, Momordica enneaphylla*

The plant has hepatoprotective , cardioprotective, antiulcer , neuroprotective ,anticancer , antidiarhoeal, anti implantation potential (Deepak *et al.*, 2019). It has been also used for skin disease, rheumatism and owing to anthropogenic activities like habiat destruction, grazing, urbanization, poor seed viability and germination. The tuber of the plant is an aborficient and used in treatment of diabetes and is also reported to have anti hyperglycemic activities and cardioprotectiv effect with addition of antioxidant enzymes and lipid profile. (Raju *et al.*, 2008) *M. cymbalaria* has shown significant prophylactic and therapeutic effects on diabetic neuropathy in that it improves myelination and restores neuronal integrity .Further research is needed to demonstrate its efficacy in humans (SM Simi *et al.*, 2014). The anti diabetic plant may also posses' neuroprotective effects additionally. The calcium content of athalakkai is three times higher when compared to bitter gourd and is required for bone and teeth health along with maintenance of normal heart rhythm, blood coagulation, muscle contraction and nerve responses. And it has ascorbic acid content and presence of carotene in negligible amounts (Parvathi and Kumar 2002).

*Momordica cymbalaria* can be successfully used for the synthesis of AgNPs that exhibit effective anti-bacterial as well as cytotoxic potential. Prepared AgNPs were tested for anti-bacterial activity against pathogenic organisms and they have shown higher bactericidal effect against *Staphylococcus aureus*, and *E.coli* in comparison to *Streptococcus pyogenes* and *Pseudomonas aeruginosa* (Shantakani *et al.*, 2021). *M.cymbalaria* seed shows more significant antiallergic activity .The antiallergic activity may be due as polar constituents and the extract also possesses analgesic activity (Amol N Khedkar 2012).

**TAXONOMIC HIERACHY OF *MOMORDICA CYMBALARIA*:**

Kingdom: Plantae

Clade: *Tracheophytes*

Clade: *Angiosperms*

Clade: *Eudicots*

Clade: *Rosids*

Order: *Cucurbitales*

Family: *Cucurbitaceae*

Genus: *Momordica*

Species: *M. cymbalaria*

Binomial name: *Momordica cymbalaria Hook.*

Synonyms: *Luffa tuberosa* (Roxb.)*Momordica tuberosa* (Roxb.)

Figure 1.1 *Momordica cymbalaria* plant parts

Figure 1.2 *Momordica cymbalaria* whole fruits

# CHAPTER 2

## AIM AND OBJECTIVES

- To collect and extract *Momordica cymbalaria* whole fruits using double distilled water and absolute ethanol as solvents.
- To determine qualitatively the phytochemicals from the *M.cymbalaria* fruit extracts.
- To analyse the Gas Chromatography Mass Spectrometric activity of aqueous extract of *M.cymbalaria* fruit.
- To evaluate the toxicity level of the *M.cymbalaria* aqueous and ethanolic extracts
- To determine the antioxidant activity of aqueous and ethanolic extract.
- To determine the anti inflammatory activity of the whole fruit extracts of *M.cymbalaria*
- To determine the anti-diabetic activity of *Momordica cymbalaria* aqueous and ethanolic extract
- To determine the antibacterial activity of *M.cymbalaria* fruit extracts against selected bacterial isolates from diabetic wound.
- To analyse the wound healing activity of the aqueous *M.cymbalaria* extract and ethanolic extract of the same.

# CHAPTER 3

# EXTRACTION AND QUALITATIVE ANALYSIS OF PHYTOCHEMICALS

## 3.1 SAMPLE COLLECTION:

The plant species namely *Momordica cymbalaria* belonging to the *Momordiaceae* family is known for many medicinal properties. The whole fruits were collected from the local market name VOC market ,Thoothukudi ,Tamil nadu.

**Figure 3.1 Sample collection of *Momordica cymabalaria* whole fruits**

## 3.2 PREPARATION OF FRUIT POWDER:

The fruits were washed thoroughly with water for 2-3 times to avoid unwanted debris. The it was air dried in shade away from sunlight for 14-20 days. It was the ground to fine powder using electric mixer and stored in air tight container until further usage.

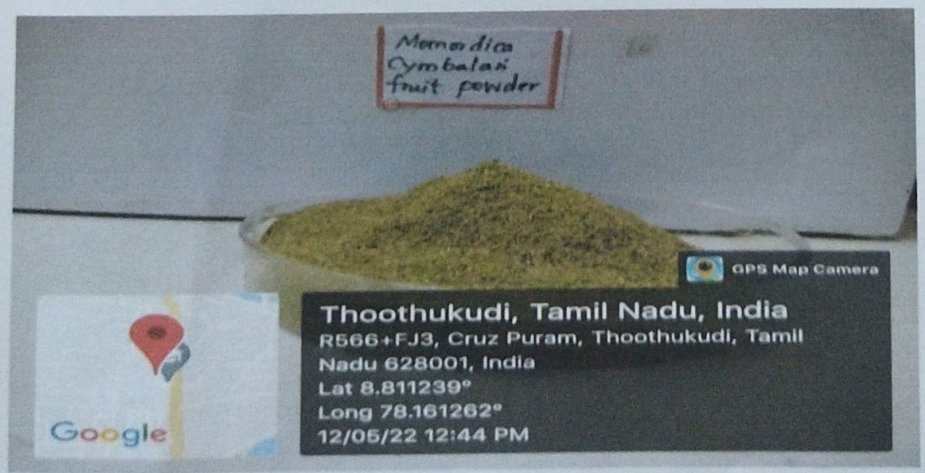



**Plate 3.1 *Momordica cymbalaria* whole fruit powder**

### 3.3 EXTRACT PREPARATION:

Dried powder of the whole fruit of *Momordica cymbalaria* was weighed and it was subjected to soxhelet extraction with double distilled water and ethanol(99.9%) as solvent for up to 8 hrs the extraction process was continued. To 500 ml of the solvent 50 gm of dried powder was added to obtain 10% concentration of the extract. The solution after 8hrs with mixture of *M.cymbalaria* fruit powder and solvent was evaporated at room temperature in a flat tray for 24-48hrs .After evaporation the crude extract was collected and stored at 4˚C for further analysis.

**Figure 3.2Soxhelet extraction of the extract**

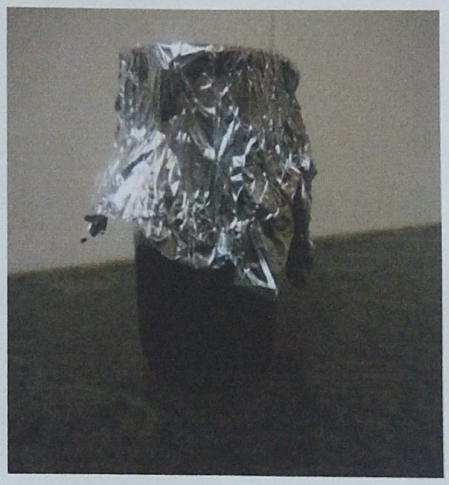


**Figure 3.3 Solution after extraction**

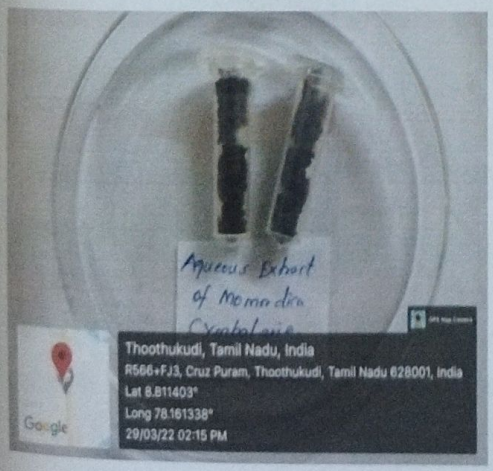



**Figure 3.4 Aqueous extract**


**Figure 3.5 Ethanolic extract**

## 3.4. MATERIALS AND METHODS:

The aqueous and ethanolic extract of *M.cymbalaria* were subjected to various qualitative test for identification of the plant and chemical constituents predent in the species and to analyse the its nature respectively .The concentration of the aqueous and ethanol extracts used here is 10% .If the solution extract is too much concentrated it can be diluted with the respective solvent prior to the test to avoid false results in the tests.

### 3.4.1 QUALITATIVE ANALYSIS OF PHYTOCHEMICALS:

1. **Test for Alkaloids**

To 1ml of the plant extract was taken to this 0.1 ml of saturated solution of picric acid was added. Yellow color appears in presence of alkaloids

2. **Test for Carbohydrates:**

To 2 ml of the plant extract ,1ml of Molish reagent and few drops of conc.$H_2SO_4$ were added. Presence of purple or reddish brown colour indicate the presence of carbohydrates.

3. **Test for Saponins:**

To 2ml of plant extract ,2ml of distilled water was added and gently shaken for 10 mins. length wise. Formation of 1cm layer of foam indicate the presence of saponins.

4. **Test for Flavonoids:**

To 2ml of plant extract 1ml of 2N sodium hydroxide was added. Presence of yellow colour indicate the presence of flavonoids.

5. **Test for Tannins:**

To 1ml of plant extract ,2ml of $Fecl_2$ was added .Formation of dark blue or greenish black color indicate presence of tannins.

6. **Test for Quinone:**

To 1ml of plant extract 1ml of concentrated $H_2SO_4$ was added. Formation of red colour indicate presence of quinone.

7. **Test for Terpenoids:**

To 0.5 ml of extract ,2ml chloroform was added along with concentrated sulphuric acid carefully. Formation of red brown colour at the interface indicate presence of terpenoids.

8. **Test for Sterols:**

5ml of extract was mixed with 2ml of chloroform and 3ml of conc$H_2SO_4$ was carefully added to form a layer. A reddish brown coloration is observed at the interface in presence of sterols.

9. **Test for Phenol:**

To 1ml of extract 2ml of distilled water and few drops of 10% $Fecl_2$ was added. Formation of blue or green colour indicate the presence of phenols.

10. **Test for Coumarins:**

To 1ml of extract 1 ml of 10% NaOH was added. Formation of yellow colour indicate the presence of coumarins.

11. **Test for Cardiac Glycosides:**

To 0.5ml of the extract 2ml of glacial acetic acid and few drops of $Fecl_2$ was added .It was layered with 1ml conc. $H_2SO_4$ . Formation of brown color indicate the presence of cardiac glycoside.

12. **Test for Anthraquinones:**

To 1ml of plant extract ,few drops of 10% ammonia solution were added. Appearance of pink colour precipitate indicate the presence of anthraquninone.

**3.5 RESULTS AND DISCUSSION:**

The qualitative chemical test for the aqueous and ethanol extracts of M.cymbalaria was performed to analyse the phytochemical constituents .The investigation showed the presence of alkaloids, flavonoids, saponins, anthraquninones, terpenoids, cardiac glycoxide, coumarin, etc as shown in the table 3.1 . Many alkaloids are valuable medicinal agents that can be utilized to treat various diseases including malaria, diabetics, cancer, cardiac dysfunction etc. flavonoids have beneficial anti-inflammatory effects and they protect your cells from oxidative damage that can lead to disease. These dietary antioxidants can prevent the development of cardiovascular disease, diabetes, cancer, and cognitive diseases like Alzheimer's and dementia. terpenes to create the flavors and scents of many everyday products, such as perfumes, body products, and even foods. Coumarins are commonly used in the treatment of prostate cancer, renal cell carcinoma and leukemia, and counteract the side effects caused by radiotherapy. Anthraquinone utilized as colorants, anthraquinone derivatives have been used since centuries for medical applications, for example, as laxatives and antimicrobial and antiinflammatory agents. Current therapeutic indications include constipation, arthritis, multiple sclerosis, and cancer. Phenol is used as a disinfectant and antiseptic, and in medicinal preparations such as mouthwash and sore throat lozenges.

Cardiac glycosides are medicines for treating heart failure and certain irregular heartbeats. They are one of several classes of drugs used to treat the heart and related conditions

**Table 3.1 Presence of phyochemicals in aqueous and ethanolic extracts of *Momordica cymbalaria***

| S.NO | PHYTOCHEMICAL TEST | AQUEOUS EXTRACT OF *MOMORDICA CYMBALARIA* | ETHANOLIC EXTRACT OF *MOMORDICA CYMBALARIA* |
|---|---|---|---|
| 1 | ALKALOIDS | + | - |
| 2 | CARBOHYDRATE | - | + |
| 3 | FLAVINOIDS | + | + |
| 4 | SAPONINS | + | - |
| 5 | TANNIN | - | - |
| 6 | QUINONE | + | - |
| 7 | TERPENOIDS | + | - |
| 8 | STEROLS | + | - |
| 9 | PHENOLS | - | - |
| 10 | COUMARIN | + | + |
| 11 | CARDIAC GLYCOSIDE | - | + |
| 12 | ANTHRAQUNINONE | - | - |

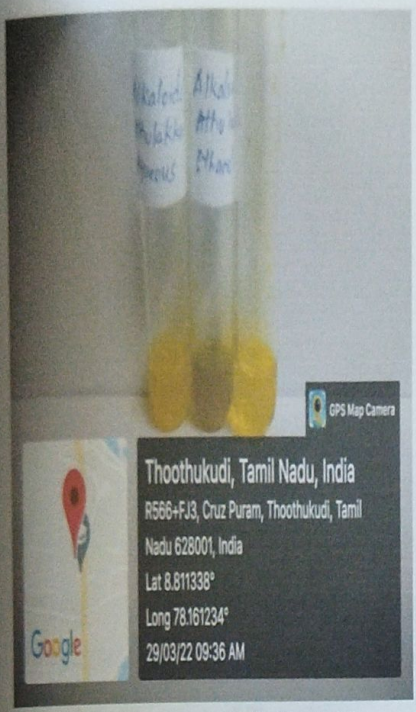



**Plate 3.2 Presence of alkaloids**


**Plate 3.3 Presence of carbohydrate in ethanolic extract**

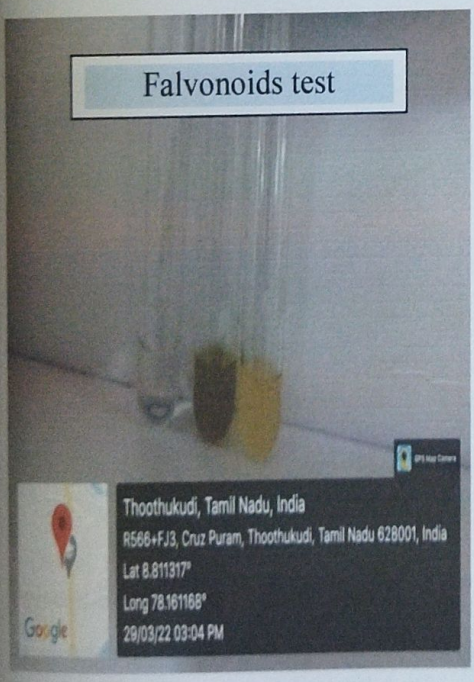



**Plate 3.4 Presence of Flavonoids**

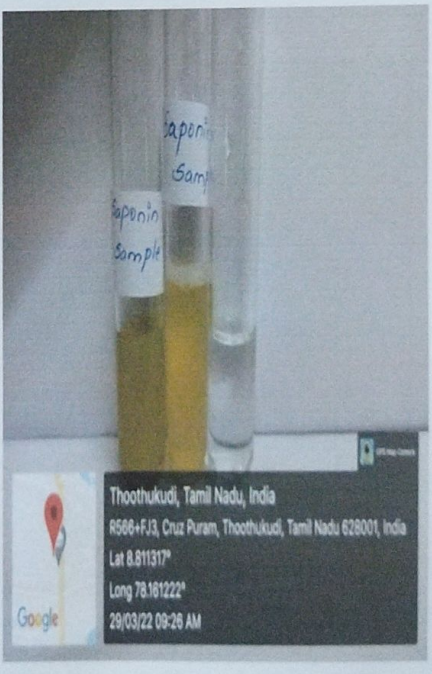



**Plate 3.5 Presence of Saponin in Aqueous extract**

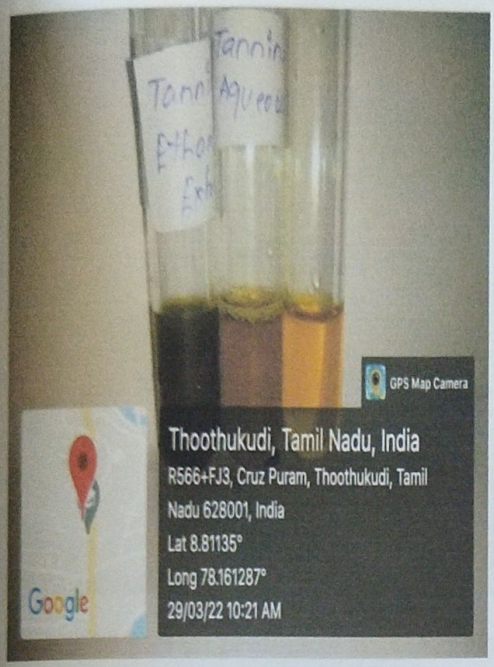



**Plate 3.6 Absence of Tannins**

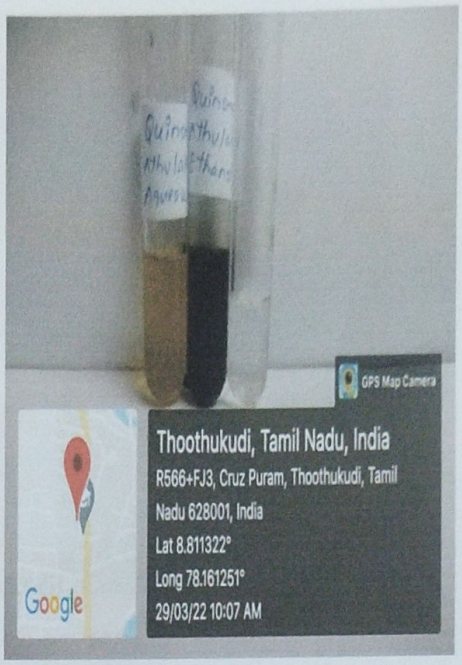



**Plate 3.7 Presence of Quinone in Aqueous extract**

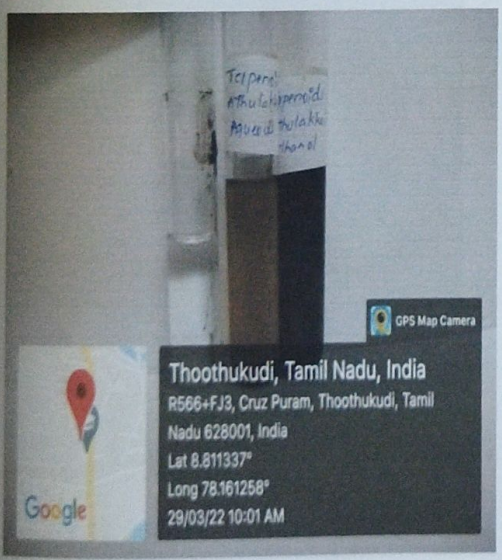



**Plate 3.8 Terpenoids Present in Aqueous extract**

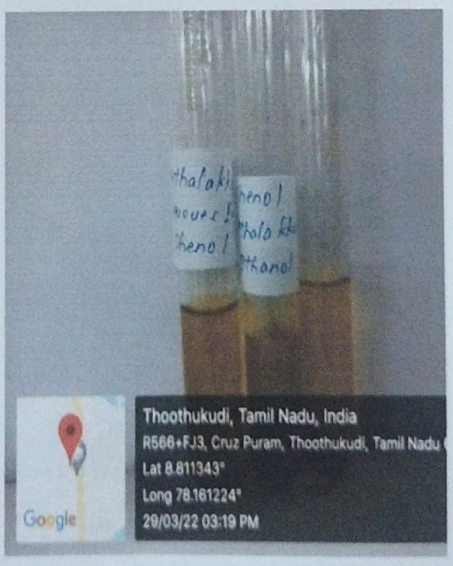



**Plate 3.9 Absence of Phenol**

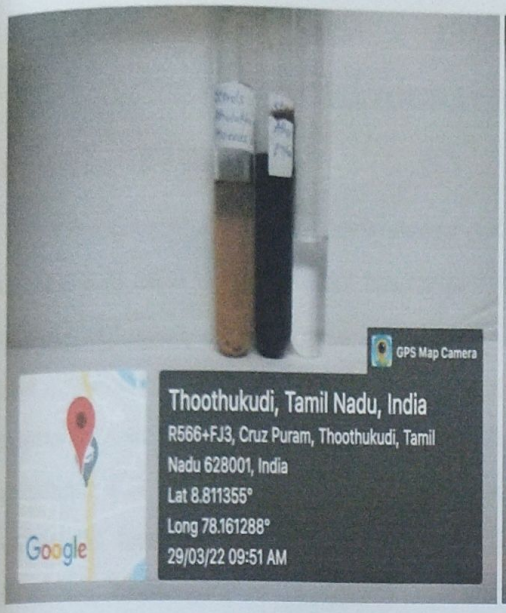



Plate 3.10 Sterols present in Aqueous extract

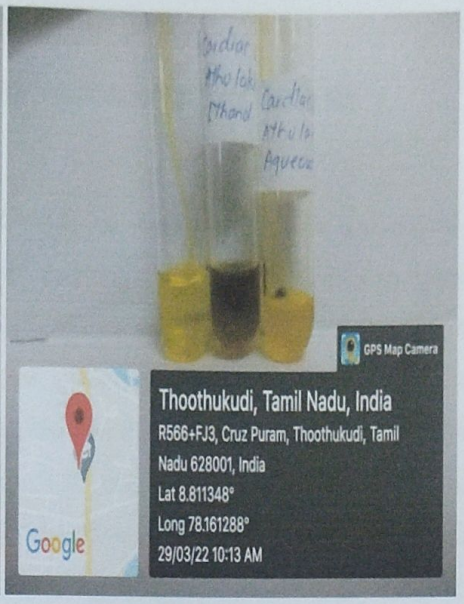



Plate 3.11Cardiac glycoside present in Ethanolic extract

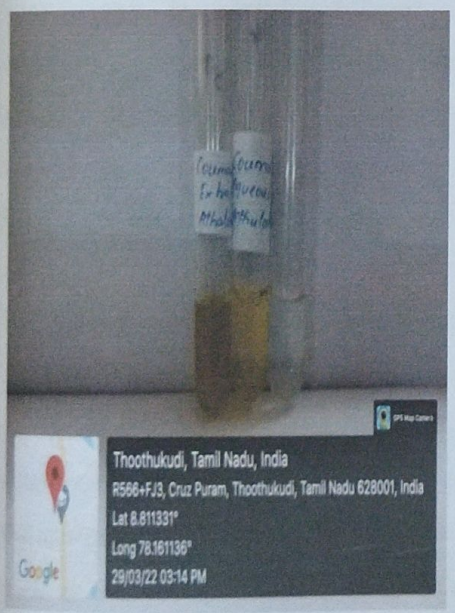



Plate 3.12 Presence of Coumarin


Plate 3.13 Absence of Anthraquninone

# CHAPTER 4:

# GAS CHROMATOGRAPHY -MASS SPECTROMETRY

## 4.1 INTRODUCTION :

Gas chromatography–mass spectrometry (GC–MS) has the potential to be a powerful tool in routine analytical procedure that has rarely been met in practice . The GC–MS analysis serves to critically review current approaches practical compositions in addressing their advantages and disadvantages to meet particular application and medicinal needs. The five main current approaches to fast GC–MS are, which involve the use of: (1) short, microbore capillary GC columns; (2) fast temperature programming; (3) low-pressure GC–MS; (4) supersonic molecular beam for MS at high GC carrier gas flow; and (5) pressure-tunable GC–GC (Kateřina Maštovská *et al.*, 2003). Furthermore, applications GC–MS is used for direct analysis of components in herbal medicine and also for non polar components and volatile fatty acids(Jie MSF *et al.*,1991).Gas chromatography–mass spectrometry (GC-MS) is an analytical method that combines the features of gas-chromatography and mass spectrometry to identify different substances within a test sample(Penton Z *et al.*,2011). Applications of GC-MS include drug detection, fire investigation, environmental analysis, explosives investigation, and identification of unknown samples, including that of material samples obtained from planet Mars during probe missions as early as the 1970s. Like liquid chromatography–mass spectrometry, it allows analysis and detection even of tiny amounts of a substance.

GC-MS has been regarded as a "gold standard" for forensic substance identification because it is used to perform a 100% specific test, which positively identifies the presence of a particular substance. A nonspecific test merely indicates that any of several in a category of substances is present. Although a nonspecific test could statistically suggest the identity of the substance, this could lead to false positive identification. However, the high temperatures used in the GC-MS injection port can result in thermal degradation of injected molecules (Jones M *et al.*,2019) thus resulting in the measurement of degradation products instead of the actual molecules of interest.

Dozens of congenital metabolic diseases also known as inborn errors of metabolism are now detectable by newborn screening tests, especially the testing using gas chromatography–mass spectrometry. GC-MS can determine compounds in urine even in minor concentration. These compounds are normally not present but appear in individuals suffering with metabolic disorders. In combination with isotopic labeling of metabolic compounds, the GC-MS is used

for determining metabolic activity. Because of its simplicity ,sensitivity and effectiveness in separating of components of mixtures GCMS is widely used of qualitative and quantitative analysis of mixtures and purification of compounds (Alessandra Braca *et al*., 2008).

## 4.2.GAS CHROMATOGRAPHY -MASS SPECTROMETRIC INSTRUMENTATION

The Trace GC Ultra and DSQII model MS from Thermo Fisher Scientific Limited, were engaged for analysis. The instrument was set as follows, Injector port temperature set to 250°C, Interface temperature set as 250°C, source kept at 200°C. The oven temperature programmed as a variable, 70°C for 2 mins, 150°C @ 8°C/min, up to 260°C @ 10°C/min. Split ratio set as 1:50 and the injector used was splitless mode. The DB-35 MS Nonpolar column was used whose dimensions were 0.25 mm OD x 0.25 µm ID x 30 meters length procured from Agilent Co., USA. Helium was used as the carrier gas at 1 ml/min. The MS was set to scan from 50 to 650 Da. The source was maintained at 200 °C and <40 mtorr vacuum pressure. The ionization energy was -70eV. The MS was also having inbuilt pre-filter which reduced the neutral particles.

The data system has two inbuilt libraries for searching and matching the spectrum. NIST4 and WILEY9 each contain more than five million references. Only those compounds with spectral fit values equal to or greater than 700 were considered positive identification.

### 4.2.1 IDENTIFICATION OF COMPOUNDS :

Interpretation of mass spectrum of GC – MS was done using the database of National Institute Standard and Technology (NIST) and WILEY (Dool and Kratz,1963). The spectrum of the known component was compared with the spectrum of the known components stored in the inbuilt library.

## 4.3 RESULT AND DISCUSSION:

The studies on the principle analysis in the aqueous extract of Momordica cymbalaria by GCMS analysis distinctly showed 7 compounds. Their active precepts with retention time (RT),Molecular formula ,Molecular weight ,Concentration peak area (%) and are represented. The total number of compound identified were with the retention time of individual compounds. The results revealed the presence of 2-Furancarboxyladehyde (5-Hydroxy methyl) (8.69) was reported to have antimicrobial activity along with uterotonic activity which is also known as ecbolic and are pharmacological agents used to induce contraction or greater tonicity of the uterus. Uterotonics are used both to induce labor and to reduce postpartum hemorrhage. Hexadeconic acid CAS (22.68) and 9,12-Octadeca-dienoic acid

(26.02) were generally reported to have anti inflammatory response ,antioxidant activity ,antibacterial activity and also serve as nematicides (less commonly known as nematocides) are chemical agents used to control parasitic worms such as roundworms and thread worms. Benzoic acid (31.67) which is the simplest benzene based aromatic carboxylic acid is known for its antibacterial activity and used in softeners ,perfumes and cosmetics. The other compound analyzed were found to be 1-hepatotriacho tanol (37.23) which may possess antimicrobial activity. But 2-amino-9-hydroxymethyl tetra hydro furan 2YL -3 hydro-pure(12.01) & 1,6,6-Trimethyl-7-3oxobutenyl3,8-dioxatricyclo Octan-5-one(17.08) which the nature of the compound and activity is not specifically known and necessitate further research.

Chaitanya Gopu *et al.*, 2021 reported the bioactive compound analysis of root methanolic extract of *Momordica cymbalaria* revealed 37 chemical constituents consisting of several antiviral ,anti convulsant , analgesic ,antibacterial, anti inflammatory (Subavathy *et al.*,2016).

**Table 4.1: Compounds present in the aqueous extract of Momodica *cymbalaria*.**

| S. No. | RT | Name | Molecular Weight | Molecular Formula | Nature of the compound | Activity |
|---|---|---|---|---|---|---|
| 1. | 8.69 | 2-Furancarboxaldehyde 5 (Hydroxymethyl) | 126 | $C_6H_6O_3$ | Furanaldehyde | Antimicrobial Preservative and Uterotonic activity |
| 2. | 12.01 | 2-Amino-9-(3,4 – dihydroxy – 5 – hydroxymeThyl – Tetra hydrofuran – 2 YL) – 3 9 dihydro – Pure | 283 | $C_{10}H_{13}N_5O_5$ | - | - |
| 3. | 17.08 | 1,6, 6 – Trimethyl – 7 –(3-oxobut –tenyl 3,8 dioxatricyclo [ 5,1,0 0 (2,4)] Octan – 5 – one | 236 | $C_{13}H_{16}O_4$ | - | - |
| 4. | 22.68 | Hexadecanoic acid (CAS) | 256 | $C_{16}H_{32}O_2$ | Palmitic acid, ester | Pesticide, Nematicide, Antioxidant, Anti-inflammatory, |
| 5. | 26.02 | 9, 12 – Octadeca-dienoic acid( ZC) – (CAS) | 280 | $C_{18}H_{32}O_2$ | Fatty acid, Linolenic acid | Nematicide, Antioxidant, Anti-inflammatory, Antibacterial |
| 6. | 31.67 | 1,2 Benzene – dicarboxylic acid, bis (2-ethyl hexyl ester) (CAS) | 390 | $C_{24}H_{38}O_4$ | Benzoic | Antibacterial, used as softens, perfumes and cosmetics. |
| 7. | 37.23 | 1 – Heptatriaco - tanol | 536 | $C_{37}H_{76}O$ | - | - |

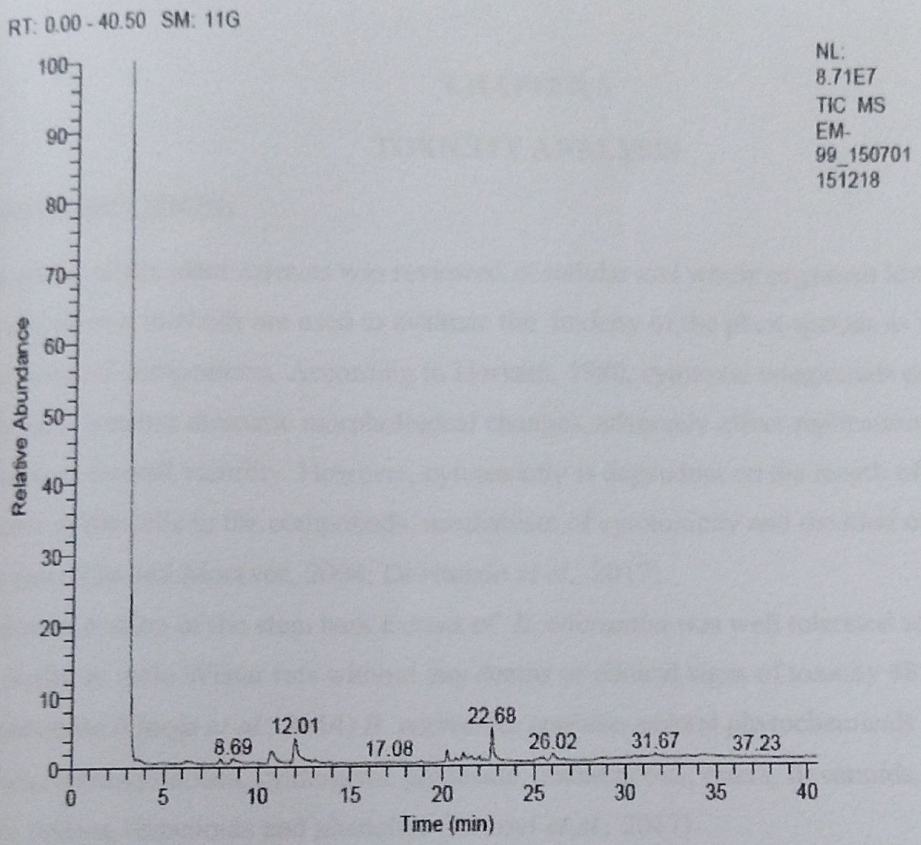



**Figure 4.1 GC-MS Spectrogram for Aqueous extract of *Momordica cymbalaria***

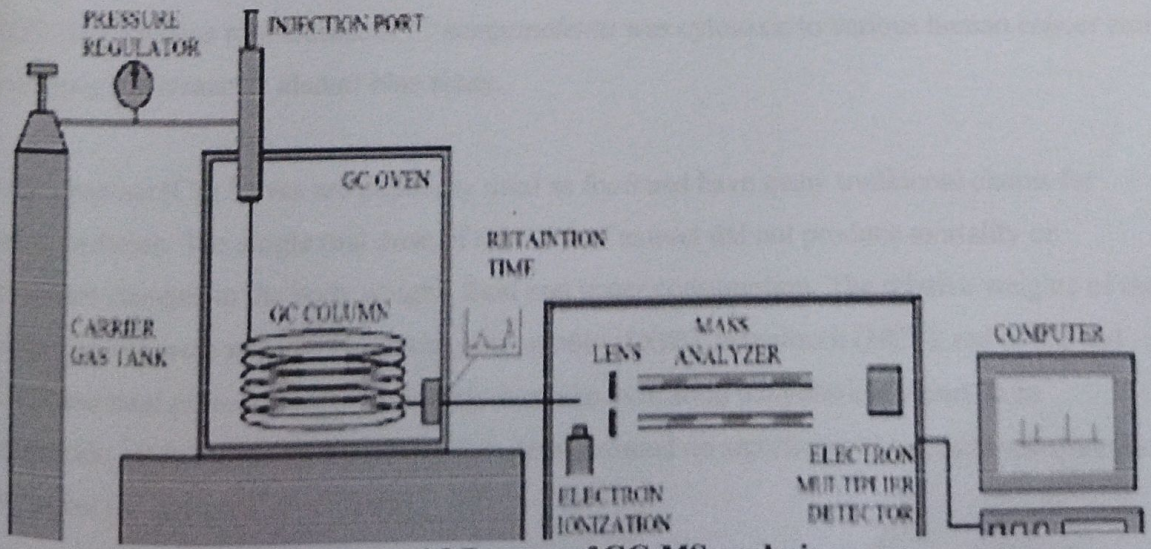



**Figure 4.2 Process of GC-MS analysis**

# CHAPTER 5

# TOXICITY ANALYSIS

## 5.1 INTRODUCTION:

The toxicity of the plant extracts was reviewed at cellular and whole organism level *in vitro* and *in vivo* methods are used to evaluate the toxicity of the plant species as well as their phytochemical composition. According to Horvath, 1980, cytotoxic compounds prevent cellular attachment, because dramatic morphological changes, adversely affect replication rates, or lead to a reduced overall viability. However, cytotoxicity is dependent on the length of time of exposure of the cells to the compounds, mechanism of cytotoxicity and the kind of compound under test (Riss and Moravec, 2004; Di Nunzio *et al.*, 2017).

Oral administration of the stem bark extract of *B. micrantha* was well tolerated at a dose of 2000 mg/kg in male Wistar rats without any deaths or clinical signs of toxicity 48 h after administration (Onoja *et al.*, 2014) *B. micrantha* contains several phytochemicals such as alkaloids, anthraquinones, cyanogenic glycoside, essential oils, esters, flavonoids, saponins, sterols, tannins, terpenoids and phenolics (Maroyi *et al.*, 2017)

*C. sanguinolenta* is popularly used for treating malaria, bacterial respiratory diseases, hypertension, diarrhoea and urinogenital infections (Iwu 1993) skin infections (Anywar et al., 2020). The aqueous root extract of *C. sanguinolenta* was cytotoxic to various human cancer cell lines using the resazurin alamar blue assay.

*Carica papaya* (CP) leaves are popularly used as food and have many traditional claims for herbal medicine. The single oral dose of the CP leaf extract did not produce mortality or significant changes in the body weight, food and water consumption. The relative weights of the internal organs were normal. However, hemoglobin (HGB), hematocrit (HCT), red blood cell (RBC) and total protein were significantly increase indicating dehydration. Apart from triglyceride, other biochemistry parameters demonstrated no significant changes as compared to the control (S. Z. Halim *et al.*,2011).

Winstar albino rats were used under standard conditions for screening of toxicity. Animals were fed with standard lab food and libitum during the study .The protocol has been approved by the government norms (Dhasan *et al.*, 2010).

## 5.2 RESULT AND DISCUSSION:

**Table 5.1 Result of acute toxicity study of sample I and sample II**

| Parameter | 1hr | 2hr | 3hr | 4hr | 24hr | 8th day | 14th day |
|---|---|---|---|---|---|---|---|
| Appearance | N | N | N | N | N | N | N |
| Activity | P | P | P | P | P | P | P |
| Gait | N | N | N | N | N | N | N |
| Reaction to stimulus | | | | | | | |
| a) Sound | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| b) Touch | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| c) Light | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| Lacrimation | A | A | A | A | A | A | A |
| Salivation | A | A | A | A | A | A | A |
| Piloerection | A | A | A | A | A | A | A |
| Stimulant | A | A | A | A | A | A | A |
| Depressant | A | A | A | A | A | A | A |
| Licking of paw | A | A | A | A | A | A | A |
| Convulsions | A | A | A | A | A | A | A |

**N- Normal; A- Absent; P- Present; +=Present Minimum; ++=Present medium**

Toxins may be evaluated qualitatively and quantitatively. Qualitative analysis gives information about the nature of toxins and quantitative analysis gives information about the chemistry and concentration of the drug (S.Parasuram 2011). Toxicological screening is done mostly on animal model in this study albino rats. The rat models were maintained in fasting condition prior to exposure to the aqueous and ethanolic extract of *Momordica cymbalaria*. .The level of dosage

was increased from 5 to 2000 mg/kg of body weight. There were no significant changes in the characteristic or activity of the model. The reaction to stimulus was positive with both extracts up to 14 days. The metabolic changes like lacrimation, salivation, piloerection, stimulant, depression and licking of paw and convulusion was absent in the study. So we can conclude that the extract obtained from *Momordica cymbalaria* is efficient drug without any major side effects and it is safe to be used in human trials with advised dosage according to patient requirement. Further analysis on concentration and dosage of the compounds present in this species can be evaluated

**5.3 CONCLUSION:**

The result revealed that the aqueous and ethanolic extract of *Momordica cymbalaria* showed no mortality at 2000mg/kg.Therefore 2000mg/kg dose was considered as L.D -lethal dose 50 cut off(a safe dose).With regard to the results of the acute toxicity analysis 400mg/kg of body weight was selected for all invitro and *in vivo* experiments as the maximal dose. As per OECD 423 guidelines the dose is said to be "unclassified" under the toxicity scale hence study with higher doses were not executed.

# CHAPTER-6

# ANTIOXIDANT ACTIVITY

## 6.1 INTRODUCTION:

An antioxidant can be defined as: "Any substance that, when present in low concentrations compared to that of an oxidisable substrate, significantly delays or inhibits the oxidation of that substrate"( Halliwell B *et al.*, 1995). Antioxidants are natural or man-made or natural substances that may prevent or delay some types of cell damage. Vegetables and fruits which are good sources of antioxidants, have been found to be healthy , However, research has not shown antioxidant supplements to be beneficial in preventing diseases. Examples of antioxidants include vitamins C and E, selenium, and carotenoids, such as beta-carotene, lycopene, lutein, and zeaxanthin (Christen WG *et al.*, 2010). Antioxidants acts as scavengers in different ways preventing free radical formation by preventing propagation of oxidative chain reaction by being a part of the redox antioxidant network or by regulating gene expression (Braca *et al.*,2001) .Free radicals adversely alter lipids, proteins, and DNA and trigger a number of human diseases. Hence application of external source of antioxidants can assist in coping this oxidative stress. Synthetic antioxidants such as butylated hydroxytoluene and butylated hydroxyanisole have recently been reported to be dangerous for human health. Thus, the search for effective, nontoxic natural compounds with antioxidative activity has been intensified in recent years . They play a important role in various fields such as medicinal field to treat cancer ,cardiovascular disorders and chronic inflammations .In cosmetics for anti ageing process ,In food industries as a food preservative (Nurul Aili Zakaria *et al.*, 2011).

Medicinal plants with antioxidant properties has been extensively used for their ability to treat or prevent several human pathologies. The plant-derived antioxidant compounds with their mechanisms of antioxidant defenses that can help in the prevention of diseases. Finally, both the beneficial and detrimental effects of antioxidant molecules in several human conditions are discussed( Sharifi-Rad Mehdi *et al.*,2020). The physiological role of antioxidants, as this definition suggests, is to prevent damage to cellular components arising as a consequence of chemical reactions involving free radicals. Oxidative stress might be important in the patho physiology of common diseases including atherosclerosis, chronic renal failure, and diabetes mellitus(Young IS *et al.*, 2001). Antioxidant activity in traditionally used plant species is a

method of scientific validation of the medicinal plant use by Indigenous people. Plant species that are used traditionally for multiple symptoms could indicate a high level of antioxidant activity. A plant species that is used for heart disease, diarrhea, urinary issues, circulation, and blood disorders or blood reviver/tonic could be high in antioxidant activity(L.M. McCune 2013). Since focus to harness and harvest the non toxic anti oxidants from plants in indispensible.

## 6.2 MATERIALS AND METHODS

### CHEMICALS AND REAGENTS:

2,2-Diphenyl-1-picrylhydrazyl (DPPH) and ascorbic acid was purchased from Sigma-Aldrich.Phosphate buffer (pH- 7.4) and Methanol.All the chemicals and solvents used were of analytical grade.

### 6.2.1 2,2DIPHENYL-1-PICRYLHYDRAZYL HYDRATE FREE RADICAL SCAVENGING ACTIVITY:

DPPH solution (0.004 %), sample extracts and standard (vitamin C) was prepared in methanol. Sample extract and standard (vitamin C) solution were prepared in different concentrations 20, 40, 60, 80 and 100 µg/ml. 0.5ml of different concentrations of standard solution or sample extracts was taken in different test tubes and then 0.5 ml of DPPH (0.004 %) solution was added and kept in dark for 30 min. and absorbance was recorded at 517 nm. The decrease in absorbance of the DPPH radical caused by antioxidant was due to the scavenging of the radical by hydrogen donation. It was visually noticeable as a colour change from purple to yellow(Majo *et al*., 2008). The percentage inhibition activity was calculated using the formulae below:

$$\% \text{ DPPH free radical scavenging} = \frac{(\text{Absorbance of control} - \text{Absorbance of test}) \times 100}{\text{Absorbance of control}}$$

## 6.3 RESULT AND DISCUSSION:

In this study the scavenging activity with ascending concentration from 20-100µg/ml .The scavenging activity of aqueous extract of *M.cymbalaria* fruits showed good result athwart control with relatively similar to standard .In case of ethanolic extract the scavenging activity

was impressive and almost up to the level of control. The results are summarized in the table 6.1 6.2 and graph fig 6.1,6.2.Vitamin c was used as the standard .The highest scavenging was observed in the 100µg/ml concentration of ethanolic extract 70.26% and the lowest activity was observed in 20µg/ml concentration of aqueous extract 20.26% respectively. The anti oxidant activity may be due to presence of flavonoids, anthroquinones,etc.When compared to aqueous extract ethanolic extract of *M.cymbalaria* showed exemplary results.

*M.cymbalaria* improved the antioxidant defense system in animals and reduced the oxidative stress induced by ischemia reperfusion IR .The scavenging activity was evident from lipid per oxidation and enzymatic antioxidant activities(Deepak jha *et al.*,2018) Phyto constituents like tannin, flavonoids, terpenoids, glycosides, phenol have been implicated as antioxidant in the scavenging radicals (Badami *et al.*,2003)

The ethanolic extract of *M.cymbalaria* exhibited raid scavenging activity but the activity of aqueous extract was to a moderate level. But the rate of activity and this variation may be due to the nature of the solvent or the nature of the *M.cymbalaria* fruit used and concentration of the extract used

**Table 6.1 Antioxidant effect of sample-1 (Aqueous extract)on DPPH .**

| Treatment | Dose (µg/ml) | Absorbance @517 nm | % activity against DPPH radicals |
|---|---|---|---|
| Control | ---- | DPPH control= 0.992 | ---- |
| Vit C | 100 | 0.172 | 82.66 |
| Aqueous extract | 20 | 0.788 | 20.56 |
| Aqueous extract | 40 | 0.692 | 30.24 |
| Aqueous extract | 60 | 0.612 | 38.30 |
| Aqueous extract | 80 | 0.572 | 42.33 |
| Aqueous extract | 100 | 0.496 | 50.00 |

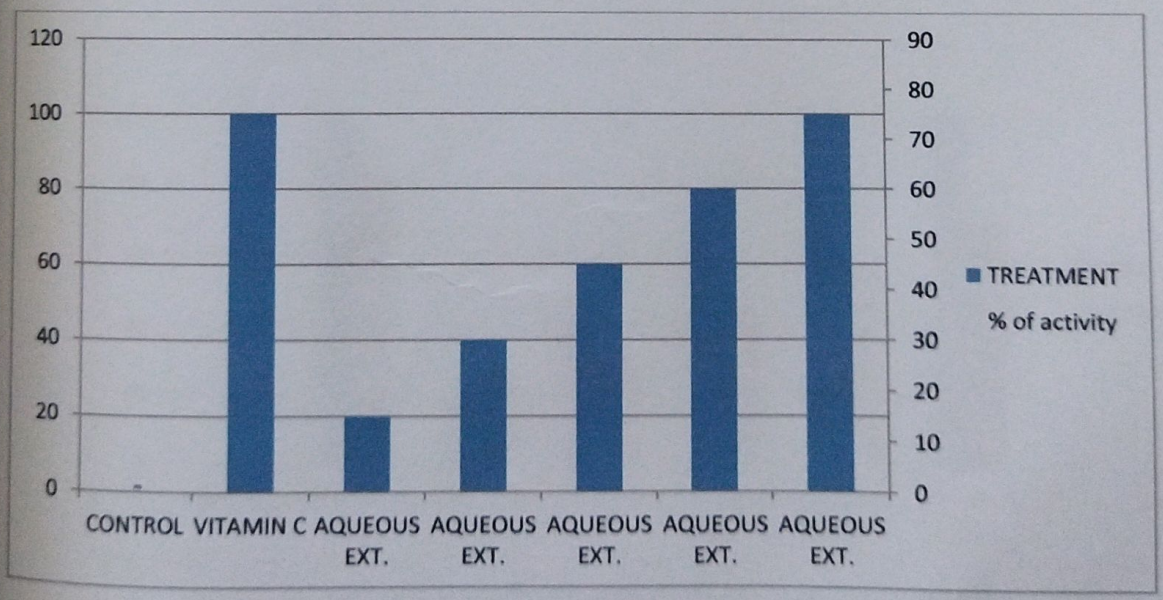



**Figure 6.1 Effect of aqueous extract of *Momordica cymbalaria* fruits**

**Table 6.2 Antioxidant effect of sample-2(Ethanolic extract) on DPPH .**

| Treatment | Dose (µg/ml) | Absorbance @517 nm | % activity against DPPH radicals |
|---|---|---|---|
| Control | ---- | DPPH control= 0.992 | ---- |
| Vit C | 100 | 0.172 | 82.66 |
| Ethanolic extract | 20 | 0.722 | 27.21 |
| Ethanolic extract | 40 | 0.685 | 30.94 |
| Ethanolic extract | 60 | 0.512 | 48.38 |
| Ethanolic extract | 80 | 0.420 | 57.66 |

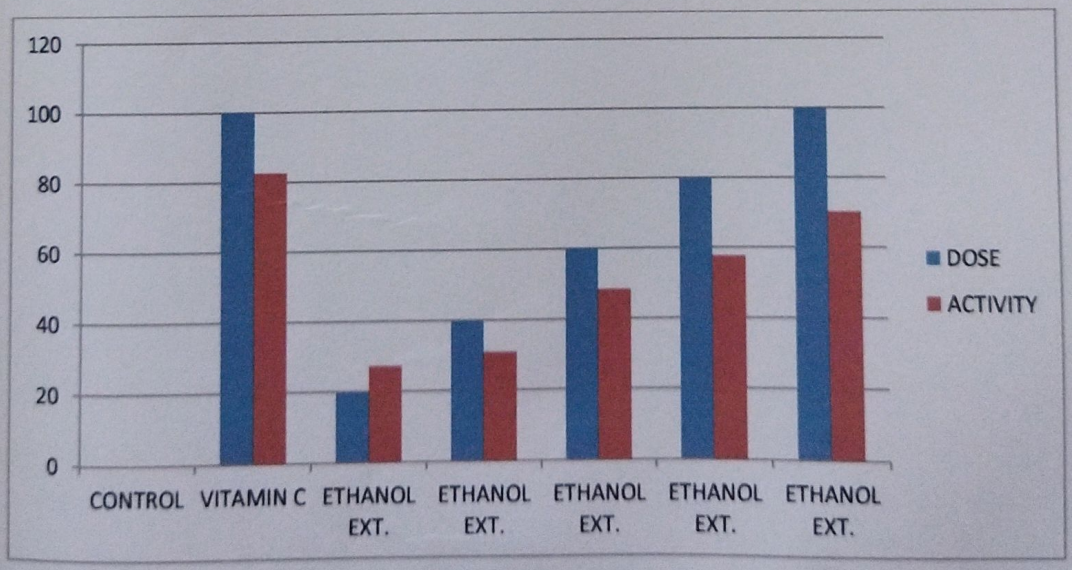



**Figure 6.2 Effect of ethanolic extract of *Momordica cymbalaria***

## 6.4 CONCLUSION:

The result of the test showed that the ethanolic extract of *Momordica cymbalaria* and its antioxidants can be utilized easily as a prominent source of natural antioxidant and it can also be supplemented with food as a medicinal compound .This application finds its root in the pharmaceutical industry. But comparatively the aqueous extract can be used for models with limited dosage and activity than ethanolic extract. Further research is required to isolate the specific antioxidant compound present in the *Momordica cymbalaria* and its constituents.

# CHAPTER -7

# ANTI INFLAMMATION ACTIVITY

## 7.1 INTRODUCTION

Inflammation is a multifaceted biological response shown by living tissues to the injury. It is an inbuilt body defense mechanism evoked by various stimuli such as disease causing organisms, ecological factors, ischemia, immunological reactions, biological factors and free radicals. It eliminates or limits the spread of pathogens (Poli Reddy *et al.*, 2018). Inflammation usually occurs when infectious microorganisms such as bacteria, viruses or fungi invade the body, reside in particular tissues and/or circulate in the blood (Artis D *et al* ., 2015). Inflammation may also happen in response to processes such as tissue injury, cell death, cancer, ischemia and degeneration (Waisman A *et al.*, 2015). Numerous inflammatory mediators are synthesized and secreted during inflammatory responses of different types. Inflammatory substances are usually divided to two main categories: pro- and anti-inflammatory mediators. Some mediators such as interleukin (IL)-12 possess both pro- and anti-inflammatory properties (Vignali D.A *et al.*, 2012). The inflammatory mediators and cellular pathways that have been extensively studied in association with human pathological conditions are cytokines like interferon's, interleukins and tumor necrosis factor α, chemokines which include monocyte chemo attractant protein 1, eicosanoids consisting of prostaglandins and leukotrienes and the potent inflammation-modulating transcription factor nuclear factor κ B.

These mediators are involved in progression of various diseases like cardiovascular and neurodegenerative disorders. Presently for the management of pain and inflammatory conditions the drugs used are either narcotics e.g. opioids or non-narcotics e.g. salicylates and corticosteroids. e.g. hydrocortisone. All of these drugs are highly potent, not suitable for prolonged use and possess many adverse effects. The most widely used drugs throughout the world for inflammation include the non-steroidal anti -inflammatory drugs, but they are associated with undesirable side effects on gastric mucosa, kidney, bronchus and cardiovascular system (Wallace and Vong, 2008), and have limited use (Burke *et al.*, 2006). Inflammatory response occurs in three discrete phases each apparently mediated by different mechanism. Acute inflammation may be an initial response to the harmful stimuli.Cyclooxygenase (COX) is

the key enzyme in the synthesis of prostaglandins ,Prostacyclin and thromboxanses which are involved in inflammation, pain and platelet aggregation.

The response to tissue injury is triggered by the release of several autacoids like, serotonin, bradykinin, prostaglandins, leukotrienes and histamine (Gallin *et al*, 1992). In AD 30, Celsius described the 4 classic signs of inflammation (rubor, calor, dolor, and tumor, or redness, heat, pain, and swelling), and used extracts of willow leaves to relieve them. For many years, salicylate-containing plants were applied therapeutically and lead to the production of a major anti-inflammatory drug - Aspirin. Aspirin, an agent with anti-inflammatory activity, is derived from natural sources, and is used extensively in current clinical practice. Many other aspirin like drugs are now available including the non-steroid anti-inflammatory drugs (NSAIDs) (Vane j *et al*., 1987).

Moreover, synthetic drugs are very expensive to develop and cost of development ranges from 0.5 to 5 million dollars On the contrary herbal me dicines with superior absorption, reduced toxicity and facile availability have been in use since ancient times. According to WHO 80% of the world population rely on pharamacologically active molecules for new drug discovery. It is vital that new efforts are required to develop cheaper and secure analgesic and anti inflammatory drugs.

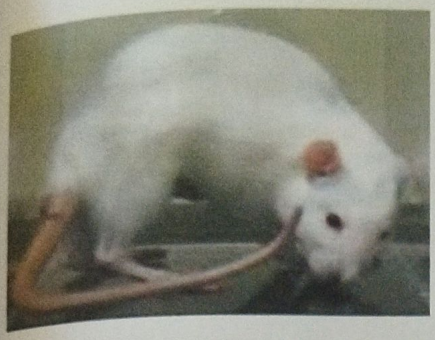
## 7.2 ANTI – INFLAMMATORY TESTING :

### 7.2.1 DRUGS AND REAGENT:

Diclofenac sodium ( Standard for anti-inflammatory) SD fine grade and the solvents methanol of SD fine grade were used in this experiment.

### 7.2.2Animals used

Adult Wistar albino rats of either sex weighing between 150 and 180 gm were used. The selected animals were housed under standard environmental conditions (temperature of 22±10° C) in a 12 hours light- dark cycle. The animals were fed on standard laboratory animal diet (Amruth animal feed company, Sangli, Maharashtra) and the animals were fasted overnight before the experiment.



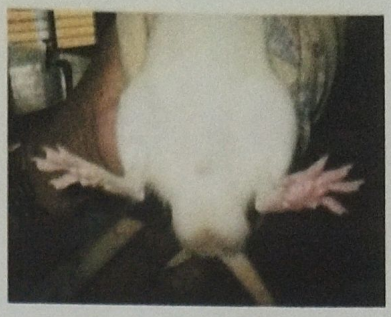


**Figure 7.1 Winstar albino rat used in the present study**

## 7.3 EXPERIMENTAL STUDY

### 7.3.1 ANTI – INFLAMMATORY TESTING :

Anti - inflammatory activity was assessed by the method suggested by Winters et al., (1962) using carrageenan as phlogistic agent. The adult Wister albino rats of either sex weighing between 150 & 180 gm were housed in groups of four animals each. They were starved overnight during the experiment but had free access to water. The volume of paw of each animal was determined before giving any drug. Animals are divided into four groups each consisting of four animals.

Group I served as control which received normal saline [1ml/Kg, (p.o)],

Group II received standard drug Diclofenac sodium [ 10 mg/Kg (p.o)],

Group III received Aqueous extract 400mg/kg p.o. and

Group IV received Ethanolic extract 400mg/kg p.o.

Acute inflammation was produced by sub plantar injection of 0.1 ml of 1% suspension of carrageenan in normal saline, in the right hind paw of the albino rats. One hour after oral administration of the drugs, the paw edema was measured with the aid of a standard plethysmometer (Inco, Chennai) at 1, 2, 3 and 4 hours after the injection of carrageenan. The difference between the readings at time 0 minutes and the different time intervals was taken as the thickness of edema volumes were given in table I. Percentage inhibition of paw edema was calculated by comparing the control for each dose at different hours as given below.

Percentage inhibition= Control OD – (Sample OD / ControLOD)* 100

## 7.4 RESULT AND DISCUSSION:

Anti inflammation activity of aqueous and ethanolic extracts of *Momordica cymbalaria* fruits on carrageenan induced paw edema in albino rats are given in the table and figure .The capacity of the test compound *M.cymabalaria* was evaluated at concentration of 400mg/kg .Edema was reduced by *Momordica cymbalaria* fruit in a time dependent manner .Due to carrageenan administration in all animals swelling reached 1 hr and the swelling was aggregated in the control animal until 4th hr while in treated animal the swelling gradually subsided as the treatment time increased. The swelling was constantly reduced during the 4th hour in the treated rats. *Momordica cymbalaria* fruit aqueous extract showed 13.9% and fruit ethanolic extract showed 81.32% respectively. So the results revealed that the ethanolic compound showed significant anti inflammation against the control group and the aqueous compound showed less significant result when compared to the control. And this effect is because of active principles of phytochemicals.

Anti-inflammatory activity is important for wound-healing procedure. In this phenomena, immune responsive compounds cytokines and interleukins are produced by keratinocytes, B lymphocytes, T lymphocytes and macrophages (Jacob *et al.*, 2012). The anti-inflammatory activity of the leaf extract of *M. dioica* was tested by using carrageenan-induced paw edema. The results illustrated an enhanced antioxidant power of wild species comparable with a commercial variety. It presented a significant anti-inflammatory activity toward carrageenan-induced paw edema in Wistar rats in comparison to indomethacin (10 mg/kg). The contents of flavonoids and total phenolic compounds could be correlated with the antioxidant and enzymes inhibition activities. The major bioactive compounds of phenolic acids and flavonoids such as gallic acid, chlorogenic acid, caffeic acid, ferulic acid, ellagic acid, catechin, rutin, and quercetin could be correlated with the anti inflammatory activity *Momordica* species contains higher anti-inflammatory activities than a commercial variety does, hence tested as drug inflammation-related pathological processes( Gunasekaran Nagarani *et al.*, 2014)

**Table 7.1 Anti inflammation activity of aqueous and ethanolic extract of *Momordica cymbalaria***

| Drug Treatment | Dose mg/kg | Mean changes in paw edema ± SEM % edema inhibition | | | |
|---|---|---|---|---|---|
| | | 1 hour | 2 hours | 3 hours | 4 hours |
| Control Saline | 1 ml/kg | 0.35±0.01 | 0.84±0.00 | 1.26±0.00 | 1.29±0.01 |
| Standard Diclofenac Sodium | 10mg/kg | 0.20±0.00 (42.85%) | 0.28±0.01 (66.6%) | 0.25±0.02 (80.15%) | 0.23±0.00 (82.17%) |
| Aqueous extract | 400mg/kg | 0.32±0.02 (8.57%) | 0.59±0.01 (29.76%) | 1.02±0.01 (19.04%) | 1.11±0.01 (13.9%) |
| Ethanolic extract | 400mg/kg | 0.33±0.02 (5.71%) | 0.28±0.01 (66.66%) | 0.26±0.01 (79.36%) | 0.24±0.01 (81.39%) |

All the data were expressed as mean ± S.E.

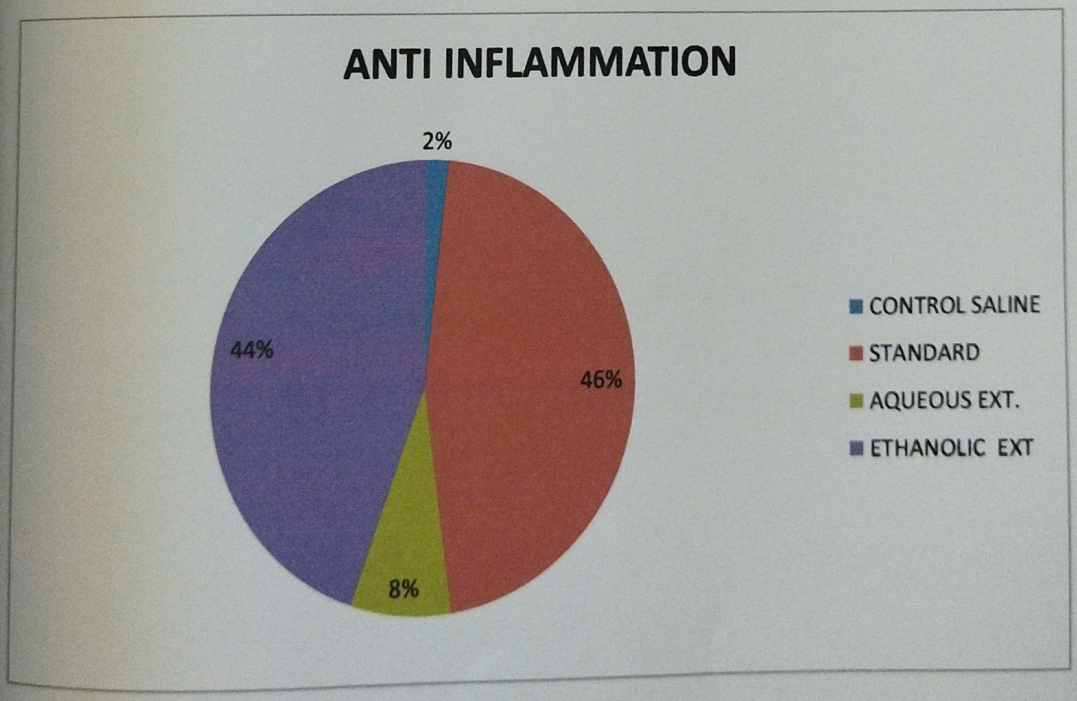



**FIGURE 7.3 Effect of anti inflammation activity of *Momordica cymbalaria***

## 7.5 CONCLUSION:

The disclosure of novel drugs effective of combat inflammation peculiarly through plant origin .Bio active constituent terpenoids and triterpenoids are a promising resource of anti inflammatory substance .Additionally research can be accomplished to ensure a safe therapeutic agent. The ethanolic extract of the *M.cymbalaria* could be considered as a good plant based anti inflammatory drug. In addition, the pure extract could be used as an effective ingredient in herbal based natural compounds.

# CHAPTER -8

# ANTI DIABETIC ACTIVITY

## 8.1 INTRODUCTION:

Diabetes is a metabolic disease that affected 9.3% of adults worldwide in 2019. Its co-occurrence is suspected to increase mortality from COVID-19. The treatment of diabetes is mainly based on the long-term use of pharmacological agents, often expensive and causing unpleasant side effects (Prezor ,*et al.*,2022). The disease is classified into type 1 and type 2 which occurs due to production of insufficient insulin or destruction of βcells .It is one of the common metabolic syndrome since three are 200 million diabetic individuals due to deficiency or abnormalities in insulin action (Dheepak jha,*et al.*, 2019). The blood glucose level in normal rats is in the range of 85-132 mg/dl (Kohn DF ,*et al.*,2002). Fruit powder of *M.cymbalaria* showed anti diabetic effect and hypolipedemic effect .The treatment was given for 15 days, after treatment a significant reduction was observed in fasting blood glucose level in diabetic treated but no hypolipidemic activity in normal rats. *M.cymbalaria* showed significant serum cholesterol and triglycerides .There was significant improvement in hepatic glycogen level as reported by (Deepak jha *et al.*, 2019).

## 8.2 MATERIALS AND METHODS

Animals

Male Albino rats weighing 190-210g were used in the experiments. They were maintained in standard environmental conditions of temperature (25±2°C), relative humidity (55±10%) and 12 hrs dark/light cycle. They were fed with standard diet and water ad libitum.

### 8.2.1 CHEMICALS:

a) Alloxanmonohydrate: Alloxan monohydrate was used as aqueous solution and given in the dose of 150mg/kg of body weight intraperitonially to induce diabetes.

b) Glibenclamide: Glibenclamide is an oral hypoglycemic agent and was obtained as gift sample from Alkem Laboratory, Mumbai. Suspension in distilled water was prepared and administered in the dose of 10 mg/kg of body weight, orally, for standard comparison.

c) Diagnostic instrument Glucometer One Touch, Horizon, LIFESCAN, Johnson and Johnson Ltd. was used to determine the blood glucose level.

**8.2.2 ANTIDIABETIC ACTIVITY:**

Albino rats were divided into five groups each containing five rats and were placed in separate metabolic cages. Except control group, the animals of remaining four groups were fasted for 24 hrs. Diabetes was then induced by alloxan monohydrate (150mg/kg intraperitonially). It takes about 48hrs and after 48hours of diabetes induction the treatment was given. Group I served as control which received normal saline solution through oral route. Group II served as diabetic control. Group III received Glibenclamide (10 mg/kg) and served as reference. Group IV animals received sample I aqueous extract orally at a dose of 400 mg/kg; Group V animals received sample II ethanolic extract orally at a dose of 400 mg/kg. Blood samples were with-drawn at 0hr, 1hr, 2hrs, 3hrs, 4hrs and 24hrs, after treatment, from retro orbital plexus and were analyzed for the blood glucose level, using glucometer. This study was continued for 21 days by taking readings randomly. Blood glucose level was read from digital display of glucometer with its customized test strips. A drop of blood obtained through retro orbital plexus was placed on inserted gluco strips on glucometer. The method is widely used in clinical practice and appears to be sensitive and accurate.

**8.3 RESULT AND DISCUSSION:**

Treatment with alloxan has the blood glucose level to a range of 250-270mg/dl after 5 days .Single dose of administration of extracts at 400mg/kg showed significantly reduced blood glucose level at the 1st 2nd and 3rd hour after single dose administration in diabetes induced rats. Repeated dose administration with *M.cymbalaria* aqueous and ethanolic extract has progressively showed to reduce the level of glucose level contingent on time over a period of 3 weeks when compared to standard the glucose range of the *M.cymbalaria* compared to the standard found some inhibition from 7 to 21 days.

When comparing the standard gibenclamide showed 252 ±1.15% inhibition. The aqueous extract of *Momordica cymbalaria* showed 269±1.10% and ethanolic extract showed224±1.12% respectively. The aqueous extract showed inhibition activity more than the standard and the ethanolic extract showed activity less equal to standard in comparative manner.

**Table 8.1: Effect of single dose treatment of aqueous ext. and Ethanolic ext.**

| GROUP | TREATMENT | DOSE | SERUM GLUCOSE (mg/dL) | | | |
|---|---|---|---|---|---|---|
| | | | Time after glucose administration in minutes | | | |
| | | | 0 hour | 1 hour | 2 hour | 4 hour |
| I | Normal control | 10ml/kg | 93±0.57 | 93±0.57 | 93±0.57 | 93±0.57 |
| II | Control | 10ml/kg | 254±1.14 | 254±1.15 | 254±1.15 | 254±1.15 |
| III | Standard | 10mg/Kg | 252±1.18 | 252±1.15 | 252±1.15 | 252±1.15 |
| IV | Aqueous extract | 400mg/Kg | 282±1.10 | 275±1.18 | 270±1.18 | 269±1.10 |
| V | Ethanolic extract | 400mg/Kg | 270±1.12 | 242±1.14 | 232±1.18 | 224±1.12 |

Value is the mean of 3 observations ±SEM

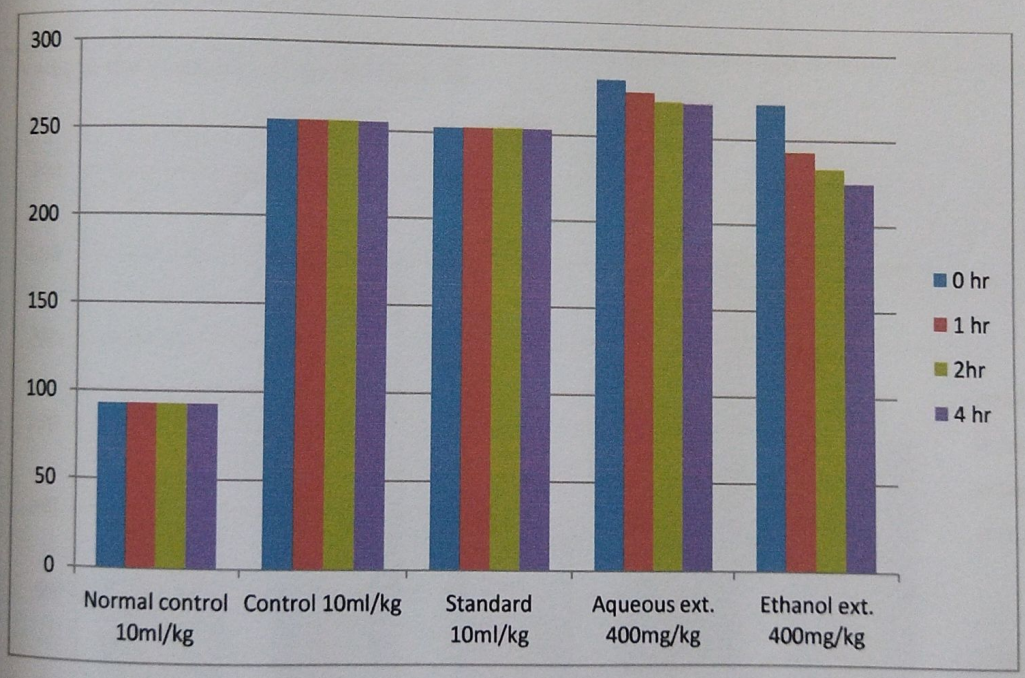



**Figure 8.1 Effect of single dose of *Momordica cymbalaria* aqueous and ethanolic extract**

**Table 8.2: Effect of repeated dose treatment of aqueous extract and Ethanolic on glucose level in alloxan-induced diabetic rats**

| GROUP | TREATMENT | DOSE | SERUM GLUCOSE (mg/dL) TIME AFTER GLUCOSE ADMINISTRATION IN MINUTES | | | |
|---|---|---|---|---|---|---|
| | | | $0^{th}$ day | $7^{th}$ day | $14^{th}$ day | $21^{st}$ day |
| I | Normal control | 10ml/kg | 93±0.57 | 93±0.57 | 93±0.57 | 93±0.57 |
| II | Control | 10ml/kg | 254±1.14 | 254±1.15 | 254±1.15 | 254±1.15 |
| III | Standard | 10mg/Kg | 252±1.18 | 162±1.14 | 122±1.12 | 87±1.18 |
| IV | Sample I Aqueous extract | 400mg/Kg | 282±1.10 | 268±1.18 | 262±1.18 | 260±1.10 |
| V | Sample II Ethanolic extract | 400mg/Kg | 270±1.12 | 216±1.14 | 172±1.18 | 146±1.12 |

Value is the mean of 3 observations ± SEM

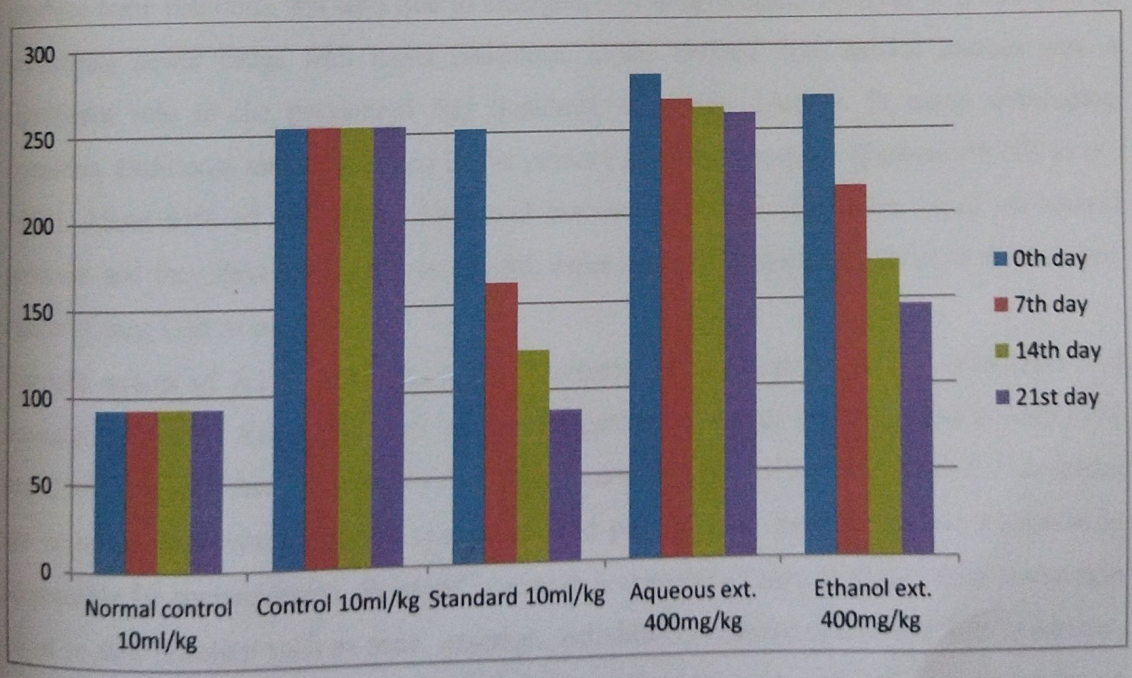



**Figure 8.2 Effect of repeated exposure to Aqueous and ethanolic extract of *Momordica cymbalaria***

# CHAPTER-9

# ANTIBACTERIAL ACTIVITY

## 9.1 INTRODUCTION

Bacterial resistance to antibiotics is a therapeutic problem and the rate at which new antibiotics are being produced is slow (Russell *et al.*, 2002). These drug resistant candidates are more pathogenic with high mortality rate and become a great challenge in the pharmaceutical and healthcare industry. To overcome microbial drug resistant, scientists are looking forward for the development of alternative and novel drugs. Natural sources such as plants, algae and animals provide an array of natural medicinal compounds for the treatment of various infectious diseases (Gaurav Kumar *et al.*, 2005).

Antibiotics are one of our most important weapons in fighting bacterial infections and have greatly benefited the health-related quality of human life since their introduction. However, over the past few decades, these health benefits are under threat as many commonly used antibiotics have become less and less effective against certain illnesses not, only because many of them produce toxic reactions, but also due to emergence of drug-resistant bacteria. It is essential to investigate newer drugs with lesser resistance. Drugs derived from natural sources play a significant role in the prevention and treatment of human diseases. In many developing countries, traditional medicine is one of the primary healthcare systems (Farnsworth NR *et al.*, 1993). About 61% of new drugs developed between 1981 and 2002 were based on natural products and they have been very successful, especially in the areas of infectious disease and cancer (Cragg GM ,*et al* 2005).

Virulent strains of *E.coli* can cause gastroenteritis ,urinary tract infection, septicemia and neonatal meningitis .*Escherichia coli* are gram negative rod shaped bacterium that is commonly found in lower intestine of warm blooded organisms which are endotherms. Most *E.Coli* strains are harmless, but some serotypes can cause food poisoning in humans and are occasionally responsible for contamination. *Staphylococcous aureus* cause a range of illness from minor skin burns to skin infection such as acne, impetigo, cellulitis, carbuncles and scaled skin syndrome, meningitis and threating disease like pneumonia, endocardiac sickness, toxic shock syndrome TSS, bacterimia. *Staphylococcous* is a bacterium that is a member of fimricutes and is frequently found in the human respiratory tract.

## 9.2 MATERIALS AND METHOD:

### BACTERIAL STRAINS:

A total of 3 isolates were taken from diabetic wound including both gram positive and gram negative organisms were selected to assess the susceptibility patterns against aqueous and ethanolic extract of *Momordica cymbalaria*.The bacterial culture as maintained in nutrient slants at 37°C .Each of the culture was freshly cultured prior to testing by tranfering them into separate test tube containing nutrient broth and incubated overnight at 24-48 hrs at 28-37°C.

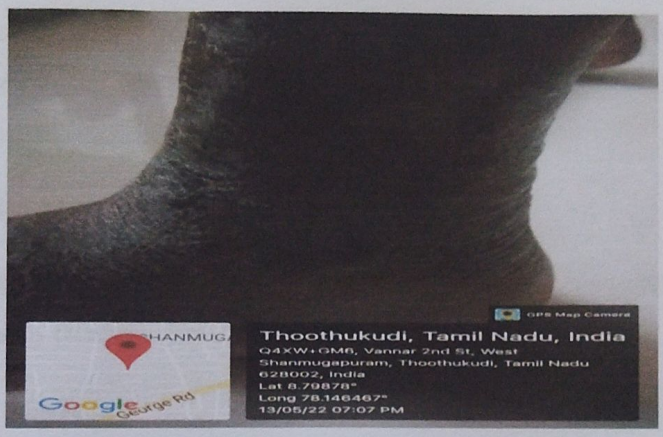



Figure 9.1 Diabetic wound affected area

## 9.3 CHARACTERIZATION / IDENTIFICATION OF ISOLATE:

Isolates were characterized using colony morphology, gram staining and biochemical tests - catalase, voges-proskauer, urease, oxidase described in Collins and Lynne's microbiological methods (Collins *et al.*, 2004)

### 9.3.1GRAM'S STAINING:

The bacterial isolates were gram stained according to the procedure (Cappuccino and Sherman 1996). The isolate was smeared in the slide and then heat fixed. The crystal violet dye was added and was kept for 1 minute and washed in running

water. Gram's iodine was added and kept for 1minute and washed in running water. It was decolorized with ethanol and the counter stain safranin was added. After leaving it for minute it was washed in running water. Then the slide was observed under oil immersion in the binocular microscope (Olumpus - mix- B - Magnus). Gram positive cells appear purple in color and gram negative cells appear reddish to pink in color.

### 9.3.2 HANGING DROP METHOD:

Motility test was performed by hanging drop technique (Gunasekaran, 1996) .A cover slip was coated with Vaseline on its edges. The isolate was transferred into the center of the cover slip. The cavity slide was placed over the cover slip and turned over to prepare hanging drop. It is viewed under microscope. The motility was determined from the swarming movement of the microorganism.

### 9.3.4 BIOCHEMICAL CHARACTERIZATION

The following preliminary biochemical test was carried out according to the method described by (Kannan 1996).

#### a) INDOLE TEST:

Using sterile technique inoculate isolated organism into approximate tube and one tube serve as a control. The tubes are incubated for 24hours at 37°C. Cultures producing a red reagent layer following the addition of Kovac's reagent are indole positive. The absence of color change is indication of negative reaction.

#### b) METHYL RED TEST:

The isolated organism was inoculated into test tube containing MR-VP broth and the tubes were labeled along with control . The tube was incubated for 24 to 48 hours at 37°C the methyl red indicated pH range of four will turn red which is indicative of a positive test . If the indicator turns yellow, it is negative test.

#### c) VOGES-PROSKAUER TEST:

The isolated organism was inoculated into test tube containing MR-VP broth .It was incubated at 37˚C for 24 hours. Development of deep rose color in the culture after 15 minutes following addition of Barrett's reagent is indication of positive result. The absence of rose coloration is a negative result.

**d) CATALASE TEST :**

The isolates were transferred to the test tube 1 to 2 ml of hydrogen peroxide was added catalane positive organism indicate by strong bubble formation. Catalase negative organism was indicated by no bubble formation.

**e) OXIDASE TEST:**

The isolate was transferred to the center of the slide and were and oxidase disc was placed . The positive organisms indicated by deep blue color change. The negative organism was indicated by no change in color.

**f) UREASE TEST:**

The isolate was transferred to the tube for Christensen's urea agar and the urease positive organism was indicated by pink color formation . Urease negative organism was indicated by pale yellow color formation.

**9.4 ANTI -BACTERIAL ACTIVITY ASSAY:**

The evaluation of sensitivity of the test organisms was carried out by using the discs diffusion method modified from Kirby -Bauer test (Cartledge *et al.*, 1992). This disc diffusion susceptibility testing was done on Muller Hington agar plates .The plates were covered and the agar surface was allowed to solidify and dry in horizontal position under the laminar air flow chamber .

The 0.1 ml of culture colonies from the stock were selected and spread using sterile L-rod. Plates were impregnated with well cutter . The plates were properly labeled in conformity with the isolates and the different extracts . After normalizing to room temperature the plates are incubated at 37˚C for 24 hours. Antibiotics like

Amoxycilin were used as the positive control and DMSO was used as negative control can be used against same clinical isolates for comparison. The plates were incubated at 37°C for 24 hrs respectively and after incubation the zone of inhibition were analyzed and results were recorded. The anti bacterial activity was evaluated by measuring the zone of clearance in millimeters by using a graduated scale.

**9.5 RESULTS AND DISCUSSION**:

The isolates were screened morphologically and biochemically using different biochemical test and they were identified as *Staphylococcous aureus* ,*Escherichia coli*, *Klebsiella pneumoniae*. Each of the colony was inoculated and incubated in their respective selective media for confiramation. The antibacterial effect of different extracts of the fruits of *Momordica cymbalaria* against organisms obtained from diabetic wounds sample was determined by measuring the diameter of the zone of inhibition expressed in millimeter (Willkinson,2007) and represented in the table. *Momordica cymbalaria* showed varied differentiation in the antibacterial activity with range of inhibition from 8-15mm against the tested organisms.The degree of antibiotic property depends on several factors like duration of storage, temperature, preparation of media,vitality or organisms, age of plant,etc. Umamaheshwari *et al.,2009*.The ethanolic extract of *Momordica cymbalaria* showed maximum activity against gram negative *E.coli* (15mm) and *Klebsiella pneumonia*(13mm).But it showed minimum activity with gram positive *Staphylococcous aureus* (10mm).The aqueous extract of *Momordica cymbalaria* showed highest activity with Staphylococcous aureus (13mm)and minimum activity with the gram negative *Esherichia coli* and *Klebsiella pneumoniae* (9mm) and (8mm) respectively.The difference in the antimicrobial effect may be due to permeability barrier.In gram negative species the outer membrane is lipid containing and fairly affect the extract and its active components that persist in the plant.(Ravi Kumar *et al.*,2008).The gram negative bacteria comprises of outer membrane that act as a barrier to many environmental substances including antibiotics (Joys Selva Mary Albert,2015). The cell wall of gram negative bacteria prevent the entry of foreign particles and therefore a reason for the performance of the studied extracts in this present study.

**Table 9.1 Morphological and biochemical analysis of bacterial isolates**

| S.NO | TEST | *Esherichia coli* | *Staphylococcous aureus* | *Klebsiella pneumoniae* |
|---|---|---|---|---|
| 1. | SHAPE | Rod | Cocci | Rod |
| 2. | COLOUR | Pink | Purple | Pink |
| 3. | MOTILITY | Motile | Non motile | Non motile |
| 4. | GRAM STAINING | Gram negative(-) | Gram positive (+) | Gram negative (-) |
| 5. | INDOLE | Positive(+) | Negative(-) | Negative(-) |
| 6. | METHYL RED | Positive(+) | Negative(-) | Negative(-0 |
| 7. | VOGES PROSKAUER | Negative(-) | Positive(+) | Positive(+) |
| 8. | CITRATE UTILIZATION | Negative(-) | Negative(-) | Positive(+) |
| 9. | CATALSE | Positive(+) | Positive(+) | Positive(+) |
| 10. | OXIDASE | Negative(-) | Negative(-) | Negative(-) |
| 11. | UREASE | Negative(-) | Positive(+) | Positive(+) |
| 12. | ARRANGEMENT | Single | Clusters | Single, pairs or chains |


Plate 9.1 *Staphylococcous aurues* in MSA agar

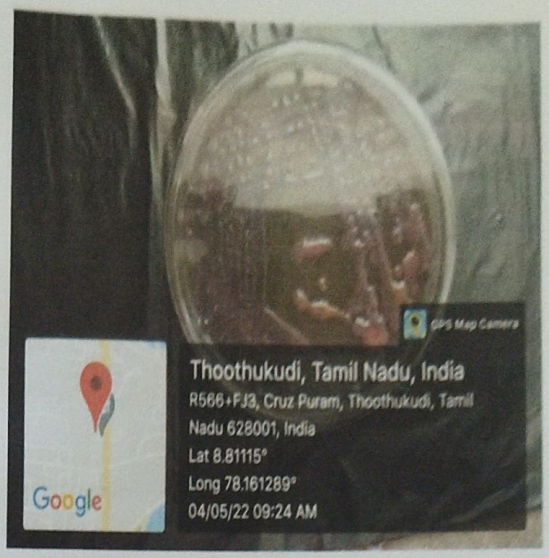



Plate 9.2 *Klebsiella* in EMB Agar

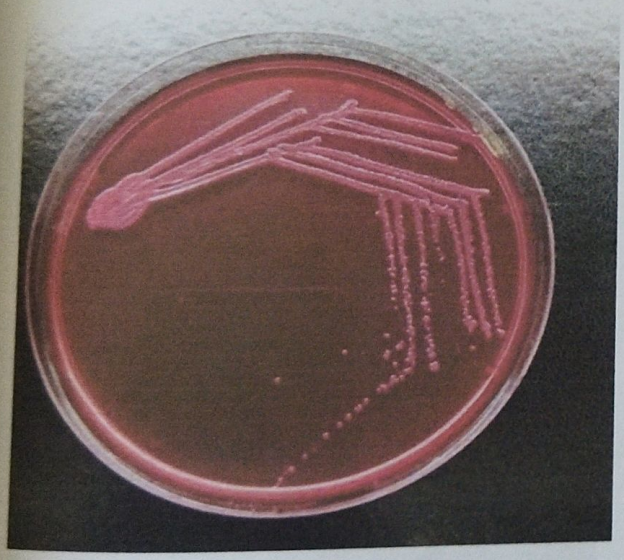


Plate 9.3 *Ecoli* in Mac conkey agar


Plate 9.4 Zone of inhibition against *Klebsiella pneumoniae*

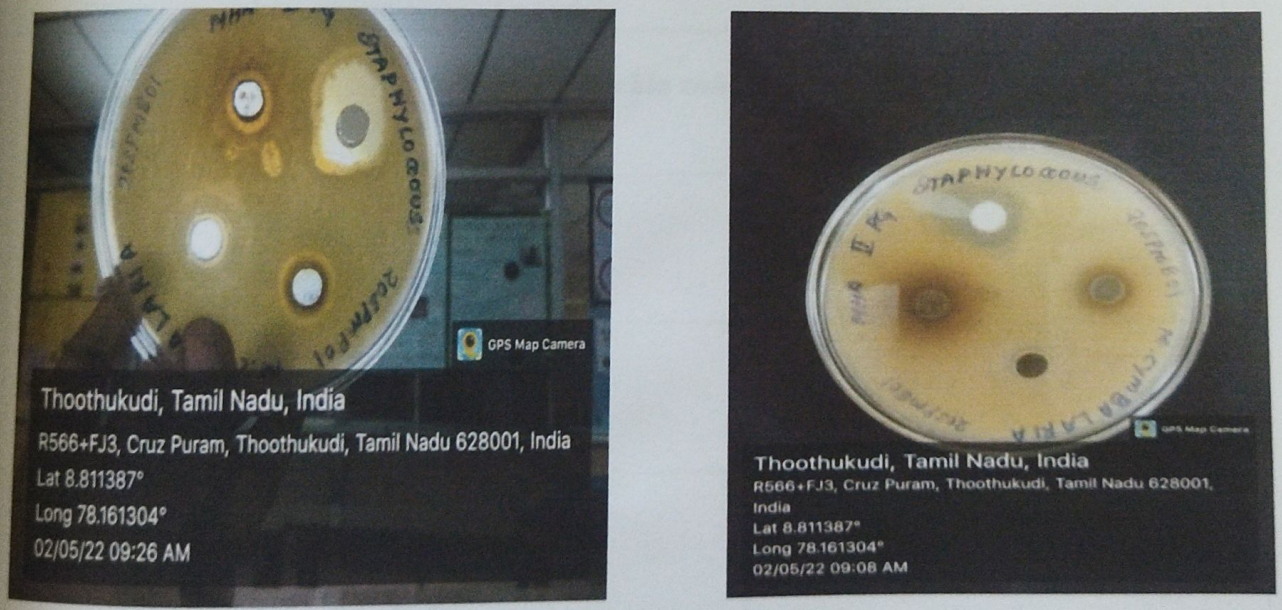
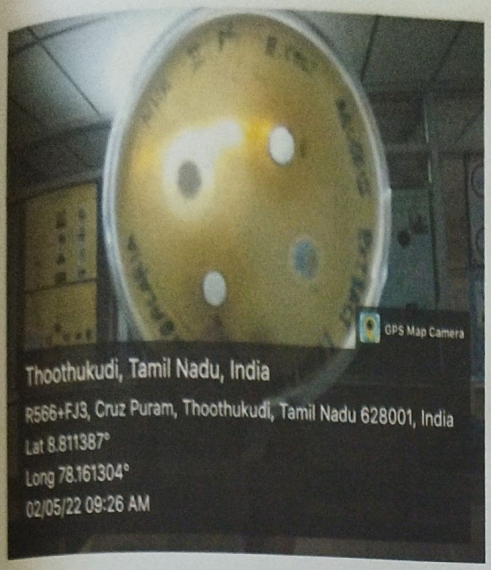



Plate 9.5 Zone of inhibition against *Staphylococcous aureus*



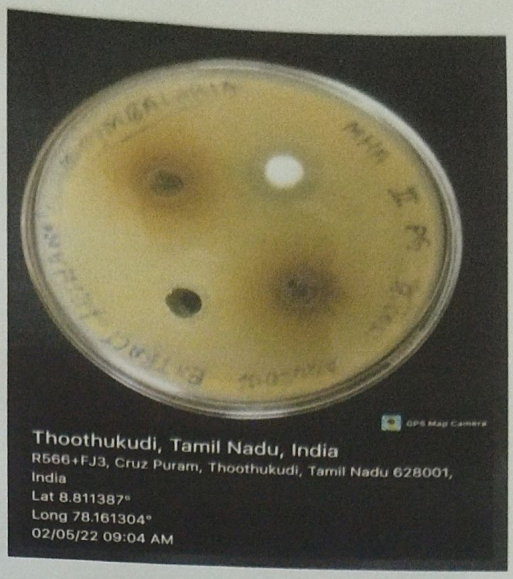




Plate 9.6 Zone of inhibition against

**Table 9.2 Antibacterial activity of *Momordica cymbalaria* extracts.**

| S.NO | SAMPLE | TEST ORGANISMS AND ZONE OF INHIBITION IN (mm) | | |
|---|---|---|---|---|
| | | ***Escherichia coli*** | ***Staphylococcous aureus*** | ***Klebsiella pneumonia*** |
| 1 | Amoxycillin +ve control | 22mm | 25mm | 20mm |
| 2 | DMSO -ve control | - | - | - |
| 3 | *Momordica cymbalaria* aqueous extract | 9 mm | 13mm | 8mm |
| 4 | *Momordica cymbalaria* ethanolic extract | 15mm | 10mm | 13mm |

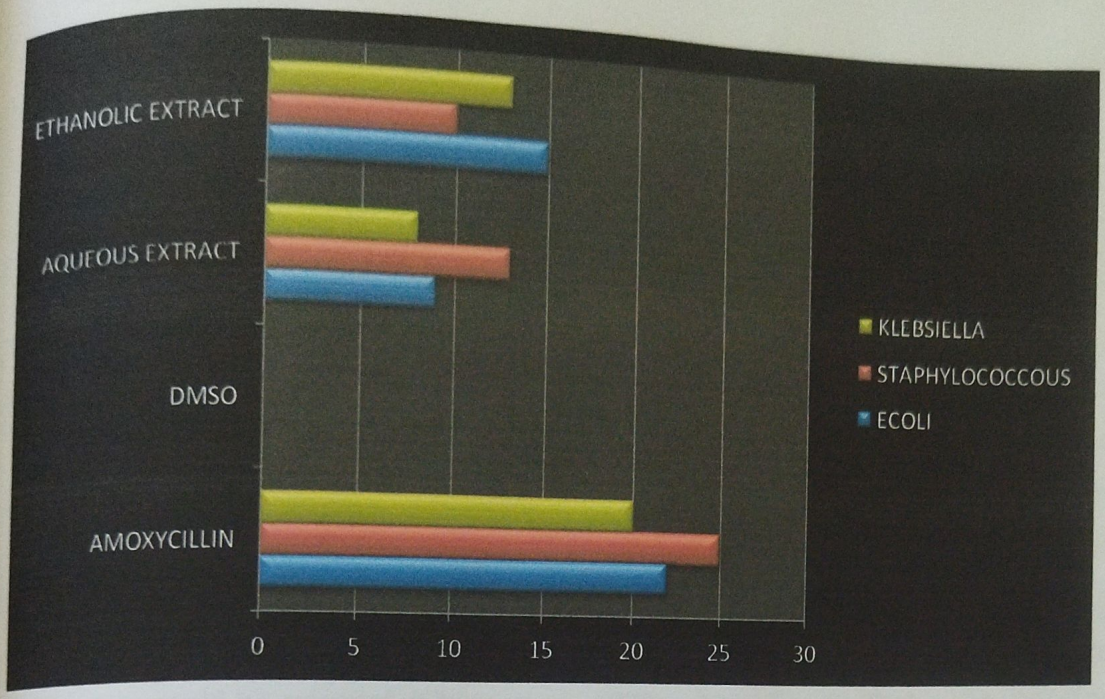



**Figure 9.2 Anti bacterial effect of *Momordica cymbalaria***

## 9.7 CONCLUSION:

The plant species used in the study is usually not used for antibacterial activity and hence the antibacterial nature of the plant against the isolates obtained from diabetic wound is investigated.The isolates in the current investigation are *S.aureus* , *K.pneumoniae*, *E.coli.*The aqueous extract and ethanolic extract have be used in the concentration 100mg/ml . The standard drug amoxycillin was used positive control with the concentration 150mg/ml and Dimethyl Sulfoxide (DMSO) was used as the negative control. The extracts of *Momordica cymbalaria* have high yield of chemical constituents which inhibit the bacterial colonies. The ethanolic extract showed higher range and broad spectrum of antibacterial activity compared to the aqueous extract. Maximum zone of inhibition was observed in *E.coli* with 15mm with ethanol extract of *M.cymbalaria* and minimum inhibition was 8mm with aqueous extract against *Klebseilla pneumoniae* respectively. However further studies are required to evaluate the potential effectiveness of the antibacterial agents from plant species .

# CHAPTER 10

# WOUND HEALING ACTIVITY

## 10.1 INTRODUCTION:

Wound healing is an interaction of complex cascade of cellular and bio chemical actions healing to the restoration of structural and functional integrity with regain of strength of injured tissues. Involves continuous cell – cell interaction and cell matrix interactions that allow the process to proceed in different overlapping phases and process including inflammation , wound contraction , Reepithelialization tissue, re modeling , & formation of granulation tissue with angiogenesis (Martin *et al.*, 1991). Several factors delay reduce the wound healing process including bacterial infection, necrotic tissue, & interference with blood supply, lymphatic blockage & diabetes mellitus, generally if the above factors could be altered by any agent, an increased healing rate could be achieved (Chitra *et al.*, 1998). According to (Dinh *et al.*, 2014) it is a dynamic process that is characterized by the occurance of three independent histopathological stages :inflammation, cell proliferation, and repair and tissue remodeling.

During the process of wound healing ,the cells release growth factors that influence all stages ,so that care for the lesion involves using antibiotics and anti inflammatory agents,which do not always avoid unwanted side effects and toxicity (Barreto RSS ,*et al.*, 2014) this becomes a problem in course of time ,especially for diabetic patients whose wounds heal slowly. Large numbers of cell types—including neutrophils, macrophages, lymphocytes, keratinocytes, fibroblasts, and endothelial cells—are involved in this process. Multiple factors can cause impaired wound healing by affecting one or more phases of the process and are categorized into local and systemic factors. The influences of these factors are not mutually exclusive. Single or multiple factors may play a role in any one or more individual phases, contributing to the overall outcome of the healing process (Guo *et al.*,2010).

Research on wound healing drugs is a developing area in modern biomedical sciences. Scientists who are trying to develop newer drugs from natural resources are looking toward the Ayurveda, the Indian traditional system of medicine. Several drugs of plant, mineral, and animal origin are described in the Ayurveda for their wound healing properties under the term Vranaropaka. Most of these drugs are derived from plant origin. Some of these plants have been screened

scientifically for the evaluation of their wound healing activity in different pharmacological models and patients, but the potential of most remains unexplored (Biswas TK.*et al.*,2003).

Wound is defined as the disruption of the anatomic and cellular continuity of tissue caused by chemical, physical, thermal, microbial, or immunological injury to the tissue. Wound healing processes consist of integrated cellular and biochemical cascades leading to reestablishment of structural and functional integrity of the damaged tissue (J. S. Boateng *et al.*, 2008). Various growth factors such as transforming growth factor beta (TGF-$\beta$), platelet activation factor (PAF), epidermal growth factor (EGF), and platelet-derived growth factors (PDGF) seem to be necessary for the initiation and promotion of wound healing (N. B. Menke 2007). Various treatment options (analgesics, antibiotics, and nonsteroidal anti-inflammatory drugs) are available for the wound management but majority of these therapies produce numerous unwanted side effects (RR Shenoy ,*et al.*, 2011 ,S Ashfaha *et al.*,2000). In recent years, several studies have been carried out on herbal drugs to explicate their potential in wound management and these natural remedies proved their effectiveness as an alternative treatment to available synthetic drugs for the treatment of wound (B. Kumar,*et al.*, 2007). Many natural herbs have been pharmacologically reported possessing potent wound healing activity

## 10.2 MATERIALS AND METHODS

### 10.2.1 Ointment Formulation (British pharmacopoeia, 1993)

A control ointment base was formulated without any drug content. Creams was formulated by using 10% extract ( 10 gm of sample I and Sample II was incorporated in 100gm of cream base). The standard drug for screening wound healing activity is Povidone iodine ointment (5%w/w) which was bought commercially.

### 10.2.2 Animal Model

Albino rats (150-250gm) of either sex were procured from animal house used for the present study. The Albino rats were divided into four groups of six rats. Group I rats were treated with simple ointment base (control). Group II rats were treated with a reference standard Povidone iodine ointment. Group III and IV rats were treated with 10% aqueous and ethanolic ointment respectively.

10.2.3 Wound healing activity:

The excision wound healing activity was studied by the method described by Luisa A DiPietro., (2003) and Farahpour and Habibi.,(2012). The skin area on the dorsal thoracic region of the mice was removed by using a suitable depilatory (Anne French hair removing cream) one day prior to the experiment. Alcohol (70%) was used as antiseptic for the shaved region before making the wound. The surgical procedures were carried out under sterile conditions. The experimental animals were anesthetized with anesthetic ether. After successful anesthesia mice were fixed in a dorsal posture on a surgery table. Circular, full thickness surgical wounds with diameters of 5mm, 1 cm away from the backbone were made using 5mm biopsy punch. Using this excision wound method, the epidermal, dermal, hypo-dermal and panniculus carnosus layers were removed completely. After making surgical wounds, all mice were randomly marked using a non-toxic colour. The animals were divided into the following four groups of six animals each (both male and female) and were treated as given below:-

Group I - Normal control group received petroleum jelly

Group II - Standard group received Povidone iodine ointment

Group III - Drug treated group received 10% w/w of aqueous extract

Group IV - Drug treated group received 10% w/w of ethanolic extract

The drugs were topically applied daily until the formation of complete epithelial layer, starting from the first day of wound excision. All the animals were monitored daily and observed for any wound fluid, evidence of infection and any other abnormalities. The diameters of the wound were measured immediately by using vernier caliper.

The wound area of each animal was measured from the first day of wounding to the days (8th and 16th) until the healing was complete (Table-1). The wound closure was measured at regular intervals of time to see the percentage of wound closure and epithelialization time that indicates the formation of new epithelial tissue to cover the wound.

The percentage of wound contraction was determined using the following formula:

$$\text{Percentage of wound contraction} = \frac{\text{Initial day wound size} - \text{Specific day wound size}}{\text{Initial day wound size.}} \times 100$$

The number of days required for falling of the scar without any residual of the raw wound gave the period of epithelialization.

**Incision wound model** (Potawaleet al., 2007).

Animals were anaesthetized and para vertebral incisions (2.5-3.0 cm long) were made through the entire length of skin. After the incision was made, the parted skin was kept together and stitched with nylon thread at 0.5 cm apart with curved needle . The two test formulations, cream base and povidone iodine ointment were applied on wound once daily for 7 day. The sutures were removed on day 8 and wound tensile strength was measured on day 10 by using constant water flow technique.

10.2.4 **Constant water flow technique**:

On the $10^{th}$ day the animals was secured to the operation table, under light ether anaesthesia. A line was drawn on normal skin on either side of wound, 3 mm away from the wound line. Two Allis forceps were firmly applied on the lines facing each other. On one side the forceps was hooked firmly to metal rod fixed to the operation table. The other forceps was connected to a leak-proof graduated polythene container through a string running over a pulley. The polythene container was connected to water reservoir placed at suitable height through a rubber tube kept occluded with a pinchcock. To measure wound tensile strength, the tube was released to allow a constant and continuous flow of water from the reservoir in to the polythene container. As the weight gradually increases, it acts as a pulling force to disrupt the wound. As soon as the gapping of the wound was observed, the rubber tube was clamped and the polythene container was weighed.

## 10.3 RESULT AND DISCUSSION:

The plant has the potential of wound healing ability and preferred due to its wide spread ability , non toxicity. Absence of side effects and effectivenessas crude preparations (Joys selva mary albert 2016) In excision wound analysis the contraction of wound progress identically with providing iodine ointment and in wound treated with formulation of aqueous and ethanolic extract of *Momordica cymbalaraia* in the four groups helaing was observed on the 16 th day and the test group consisting of the aqueous and ethanolic extract were found to exemplenary and exhibited 85.73 % and 89.39% in comparison to standard with 90.06% healing in excision wound in the table.The tensile strength of group I control was about 121.69±0.33 gm and the

standard showed 128.52±0.30 gm while the Aqueous extract of *Momordica cymbalaria* showed 114.20±3.09 and the ethanolic extract showed 122±4.05 gm with 10% concentration respectively. The surface application of drug is an efficient therapy in destroying the microbial population due to the drug applicable nature at the infected wound site which can enhance wound healing even in diabetic patients. The virulence or pathogenecity of the microbe , amount of inoculums and post immune response ate the major factors during the infection stage (Joys Selva Maryb Albert 2016). The *Momordica* is known to prevent the infraction and stress along with uric acid .Myofilamental alterations and myofibrillar degeneration are reported in ISO treated rats (Vijaya padma,*et al*.,2000). Cardiac sections showed infilteration of inflammatory cells and continuity in muscle fiber when treated with the *M.cymbalaria* extract against myocardial necrotic damage

**Table 10. 1 :- Effect of Topical Application of Aqueous and Ethanolic on Excision Wound Model (wound area mm$^2$)**

| Treatment | % wound contraction in excision wound model | | |
|---|---|---|---|
| | 0 day | 8$^{th}$ day | 16$^{th}$ day |
| Group I<br>Control (simple ointment) | 521.02±3.15 | 316.55±3.30<br>( 39.24) | 119.55±3.23<br>(77.05) |
| Group II<br>Standard | 523.47±2.42 | 218.09±2.00<br>(58.33) | 49.17±0.75<br>(90.06) |
| Group III<br>Sample I 10%<br>(Aqueous extract) | 603.62±2.57 | 425.32±4.13<br>(29.53) | 101.82±1.43<br>(85.73) |
| Group IV<br>Sample II 10%<br>(Ethanol extract) | 613.6±2.07 | 313.57±2.44<br>(48.89) | 65.10±0.84<br>(89.39) |

Values are the mean ±SEM, values showing percentage closure of original excision wound area.

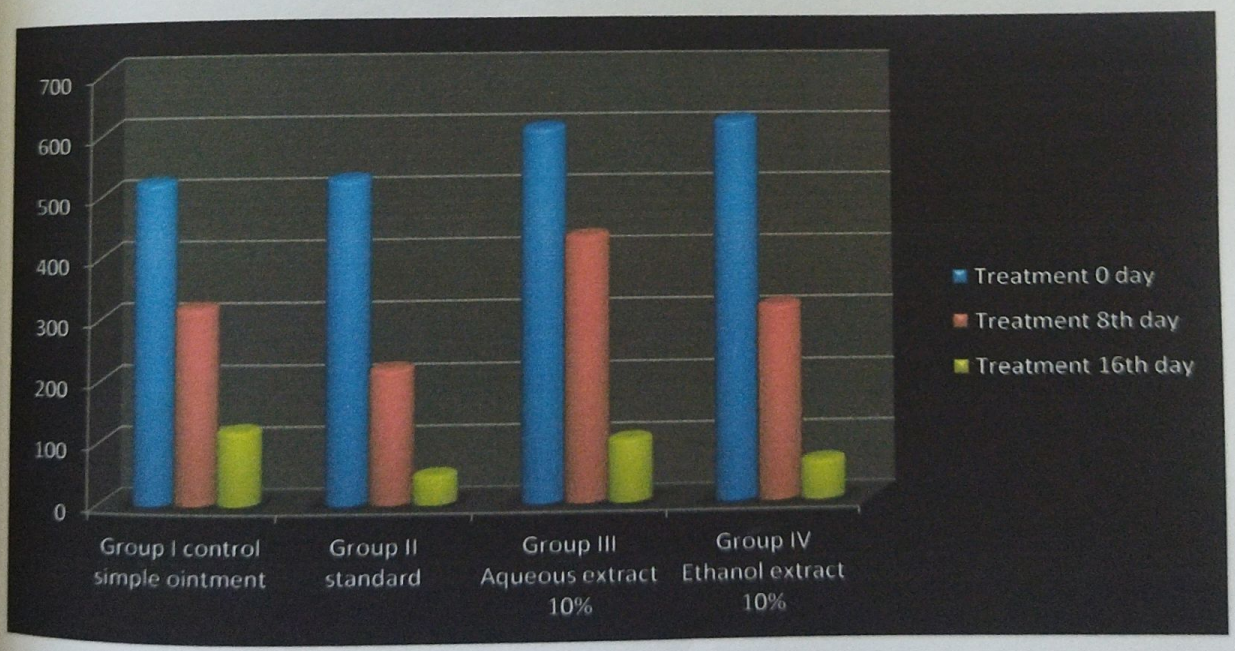



**Figure 10.1 Effect of wound healing activity of aqueous and ethanolic extract of *Momordica cymbalaria* on excision wound**

**Table 10.2 Effect of Topical Application of Aqueous extract and Ethanolic extract on Incision Wound Model**

| Treatment | Tensile strength (gm) |
|---|---|
| Group I<br>Control (simple ointment) | 121.69±0.33 |
| Group II<br>Standard | 128.52±0.30 |
| Group III<br>Aqueous extract 10% | 114.20±3.09 |
| Group IV<br>Ethanolic extract 10% | 122±4.05 |

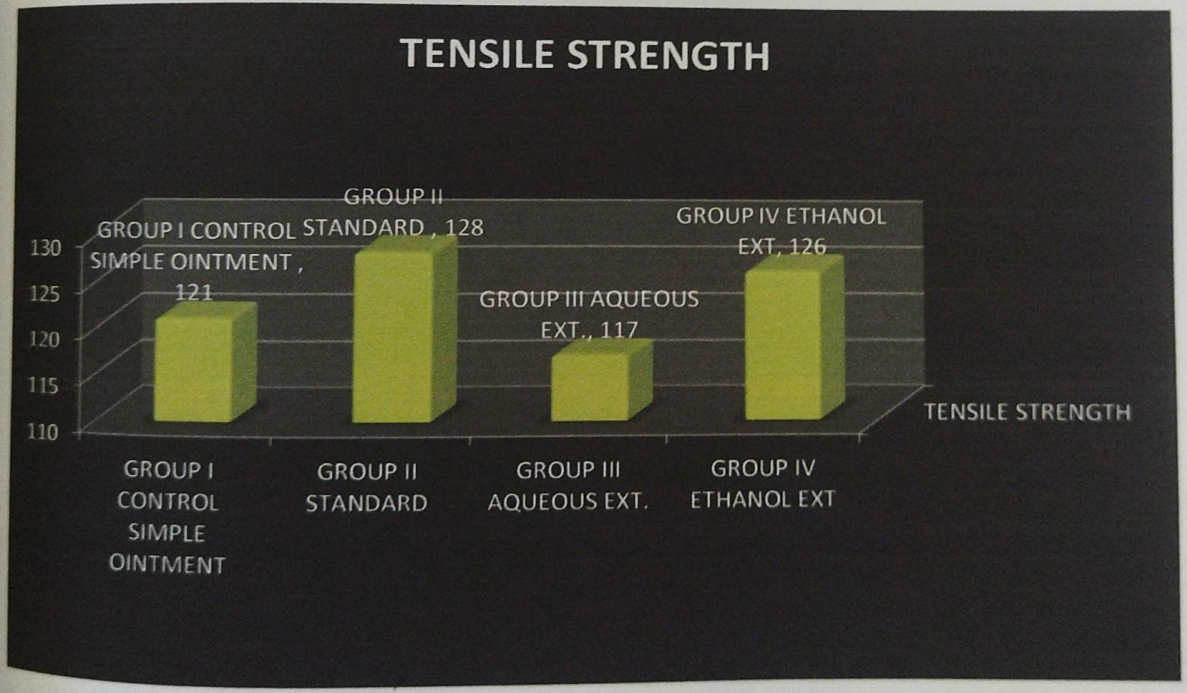



**Figure 10.2 Effect of aqueous and ethanolic extract of *Momordica cymbalaria* on incision wound**

# CHAPTER 11

## SUMMARY

Herbal medicine are used in treatment of varied diseases and these type of drugs are sometimes improperly used. Therefore ,these herbal drugs which serve as an alternate of conventional chemical drugs needs detailed investigation *in vivo* and *in vitro* under modern medicine. Compared to synthetic drug natural drugs have place in therapy recently. Their effect, low cost, easy availability, native species specification and scarce toxicity has earned them an place in the scientific research. Based on the test evidences currently tremendous interest in the potential properties and bio active components isolation on secondary metabolites are into concern. All these new approach will replace synthetic drugs with eco friendly natural substance with less to no side effects (Joys Selva Mary Albert 2018). These extracts of medicinal plants can be used as whole but in case of conventional drugs only the active ingredients is separated and used.

The dissertation entitled "A STUDY OF PHARMACOLOGICAL ACTIVITIES OF *MOMORDICA CYMBALARIA* UNRIPE FRUITS" embodies the native plant variety of Tamil Nadu especially the southern region . The aqueous and ethanolic extracts of the fruits were analyzed phytochemically by various qualitative methods which reported the presence of alkaloids, flavonoids, saponins,coumarins in both and presence of carbohydrates,cardiac glycoside in ethanolic extract. The ethanolic extract was observed as a consortium of many chemical components.

In Gas ChromatographyMass Spectrometry the compounds like Hexadeconic acid, Furancarboxyladehyde, Benzoic acid,etc were reported to be present in the aqueous extract. These components posses various activities like antibacterial, nematicidial, antifungal,uterotonic and used along with disinfectants and softeners.

The toxicity analysis was done to examine the lethal dosage upto which the animal model can stay alive . The results intrepeted that there was no mortality or changes in behavior in the rat model with dose 2000mg/kg of body weight. The reaction to stimulus was normal and so 400mg/kg was selected as the optimal dose for all other *invivo* and *invitro* test in the study.

The ethanolic extract showed comparatively good result in DPPH radical scavenging activity thus contributing to the antioxidant activity or potential of the extract whereas the aqueous extract showed lesser antioxidant activity.

In anti-inflammatory activity the *Momordica cymbalaria* fruit aqueous extract showed 13.9% and fruit ethanolic extract showed 81.32% respectively. So the results revealed that the ethanolic compound showed significant anti inflammation against the control group and the aqueous compound showed less significant result when compared to the control. The presence of terpenoids in variable amount may be the reason for the enhanced anti inflammation activity.

The plant has the potential of wound healing ability and preferred due to its wide spread ability , non toxicity. Absence of side effects and effectivenessas crude preparations .*M.cymbalaria* aqueous and ethanolic extract has progressively showed to reduce the level of glucose level contingent on time over a period of 3 weeks. The aqueous extract in its pure form showed great reduction in the blood serum glucose level with single dose application and also with repeated exposure. But the ethanolic extract showed lesser reduction level. Thus the plant can be used as a anti diabetic drug and utilized under safe doses for type I and type 2 diabetes.

The extracts of *Momordica cymbalaria* showed bactericidal and bacteriostatic activity against the isolates obtained from diabetic wound . The ethanolic extract showed maximum zone of inhibition against *E.coli* within( 15mm). The aqueous extract showed zone of inhibition comparatively less range .

Wound healing effect of aqueous extract and ethanolic extract of *Momordica cymbalaria* was studied . The aqueous and ethanolic extract were found to exemplenary and exhibited 85.73 % and 89.39% in comparison to standard with 90.06% healing in excision wound. Both the extract showed activity less than the control so the extract of this particular plant can be used in negligible amount in wound healing .

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ANNEXURE:

ANNEX 1

DEFINITIONS

Acute oral toxicity refers to those adverse effects occurring following oral administration of a single dose of a substance, or multiple doses given within 24 hours.

Delayed death means that an animal does not die or appear moribund within 48 hours but dies later during the 14-day observation period.

Dose is the amount of test substance administered. Dose is expressed as weight of test substance per unit weight of test animal (e.g. mg/kg).

GHS: Globally Harmonised Classification System for Chemical Substances and Mixtures. A joint activity of OECD (human health and the environment), UN Committee of Experts on Transport of Dangerous Goods (physical–chemical properties) and ILO (hazard communication) and co-ordinated by the Interorganisation Programme for the Sound Management of Chemicals (IOMC).

Impending death: when moribund state or death is expected prior to the next planned time of observation. Signs indicative of this state in rodents could include convulsions, lateral position, recumbence, and tremor (See the Humane Endpoint Guidance Document (9) for more details).

LD50 (median lethal oral dose) is a statistically derived single dose of a substance that can be expected to cause death in 50 per cent of animals when administered by the oral route. The LD50 value is expressed in terms of weight of test substance per unit weight of test animal (mg/kg).

Limit dose refers to a dose at an upper limitation on testing (2000 or 5000 mg/kg).

Moribund status: being in a state of dying or inability to survive, even if treated (See the Humane Endpoint Guidance Document (9) for more details).

Predictable death: presence of clinical signs indicative of death at a known time in the future before the planned end of the experimen ; for example: inability to reach water or food. (See the Humane Endpoint Guidance Document (9) for more details)

## ANNEX 2

## PROCEDURE TO BE FOLLOWED FOR EACH OF THE STARTING DOSES GENERAL REMARKS

For each starting dose, the respective testing schemes as included in this Annex outline the procedure to be followed.

Annex 2 a: Starting dose is 5 mg/kg bw

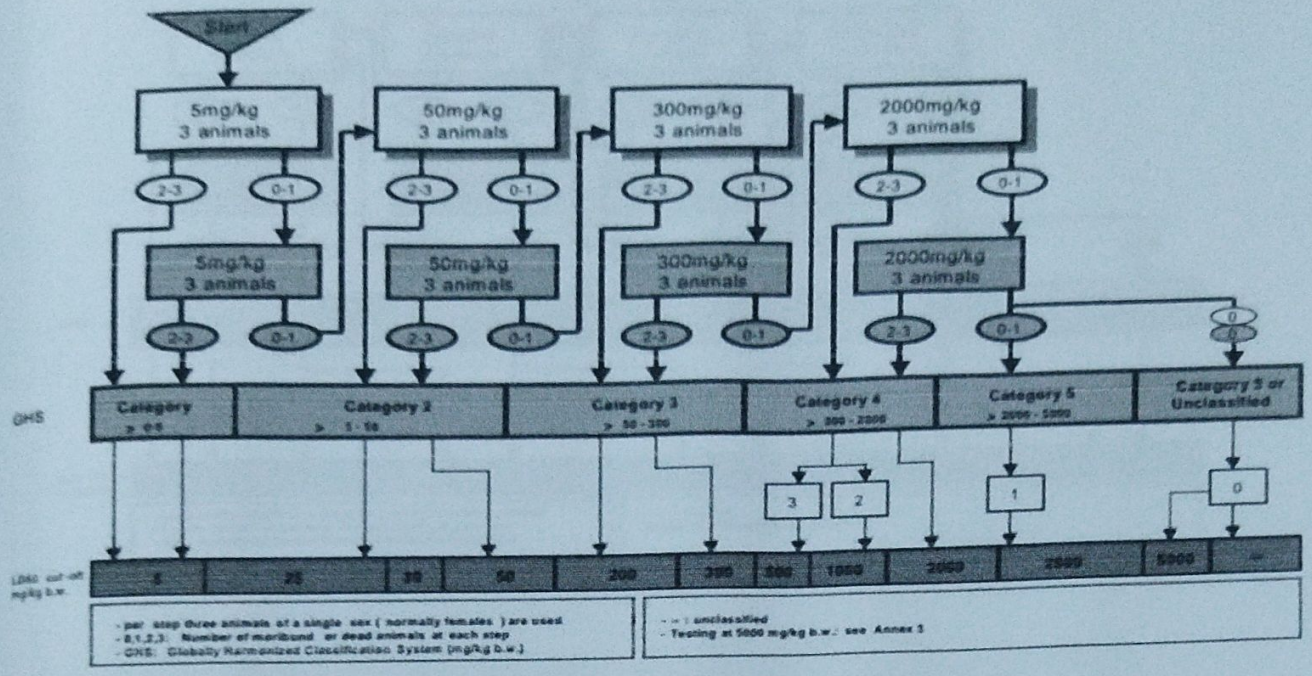
Annex 2 b: Starting dose is 50 mg/kg bw

Annex 2 c: Starting dose is: 300 mg/kg bw

Annex 2 d: Starting dose is: 2000 mg/kg bw

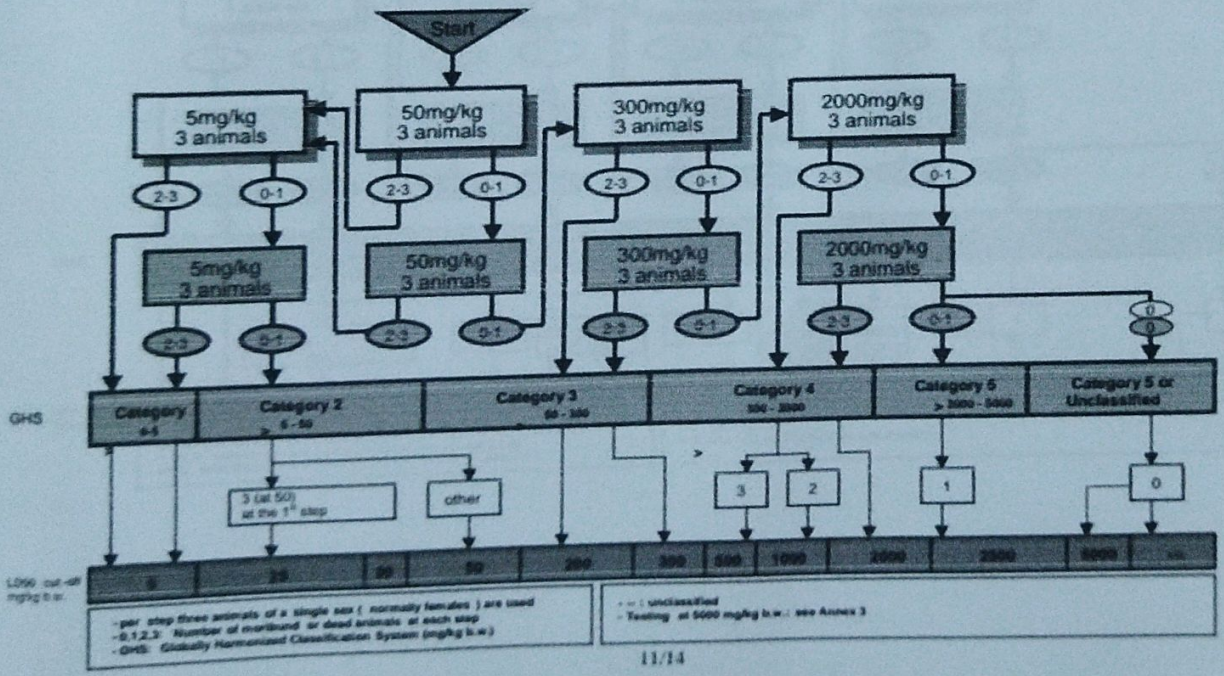
Depending on the number of humanely killed or dead animals, the test procedure follows the indicated arrows.

**ANNEX 2a: TEST PROCEDURE WITH A STARTING DOSE OF 5 MG/KG BODY WEIGHT**

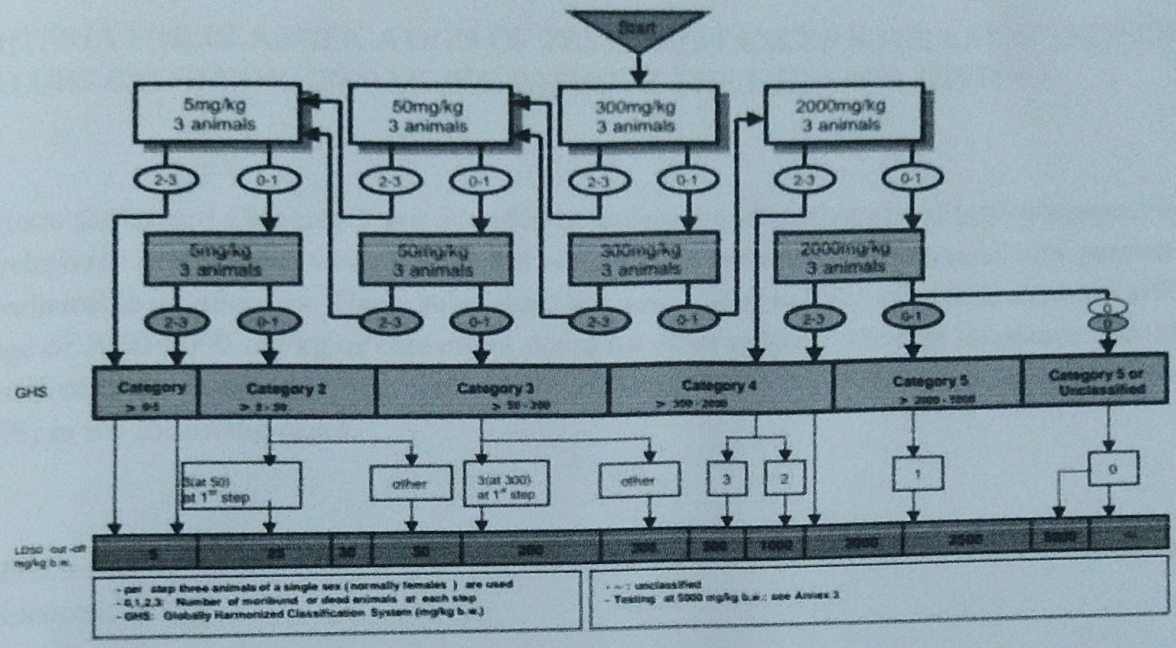




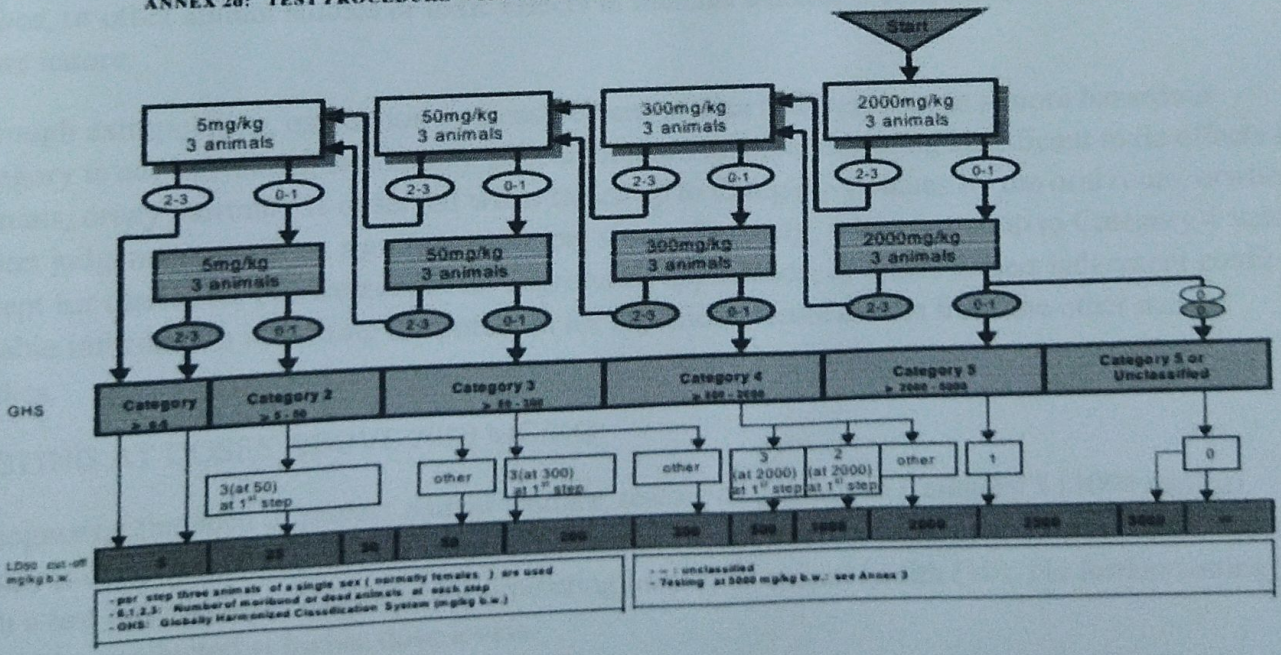

**ANNEX 2b: TEST PROCEDURE WITH A STARTING DOSE OF 50 MG/KG BODY WEIGHT**



**ANNEX 2c: TEST PROCEDURE WITH A STARTING DOSE OF 300 MG/KG BODY WEIGHT**




**ANNEX 2d: TEST PROCEDURE WITH A STARTING DOSE OF 2000 MG/KG BODY WEIGHT**



## ANNEX 3

## CRITERIA FOR CLASSIFICATION OF TEST SUBSTANCES WITH EXPECTED LD50 VALUES EXCEEDING 2000 MG/KG WITHOUT THE NEED FOR TESTING

Criteria for hazard Category 5 are intended to enable the identification of test substances which are of relatively low acute toxicity hazard but which, under certain circumstances may present a danger to vulnerable populations. These substances are anticipated to have an oral or dermal LD50 in the range of 2000-5000 mg/kg or equivalent doses for other routes. The test substance should be classified in the hazard category defined by: 2000mg/kg<LD50<5000mg/kg (Category 5 in the GHS) in the following cases:

If directed to this category by any of the testing schemes of Annex 2a-2d, based on mortality incidences;

if reliable evidence is already available that indicates the LD50 to be in the range of Category 5 values, or other animal studies or toxic effects in humans indicate a concern for human health of an acute nature.

Through extrapolation, estimation or measurement of data if assignment to a more hazardous category is not warranted, andreliable information is available indicating significant toxic effects in humans, orany mortality is observed when tested up to Category 4 values by the oral route, orwhere expert judgement confirms significant clinical signs of toxicity, when tested up to Category 4 values, except for diarrhoea, piloerection or an ungroomed appearance, or where expert judgement confirms reliable information indicating the potential for significant acute effects from the other animal studies.

### TESTING AT DOSES ABOVE 2000 MG/KG

Recognising the need to protect animal welfare, testing of animals in Category 5 (5000 mg/kg) ranges is discouraged and should only be considered when there is a strong likelihood that results of such a test have a direct relevance for protecting human or animal health (10). No further testing should be conducted at higher dose levels.

When testing is required a dose of 5000mg/kg, only one step (i.e. three animals) is required. If the first animal dosed dies , then dosing procedes at 2000mg/kg in accordance with the flow charts in Annex 2. If the first animal survives, two further animals are dosed. If only one of the three animal dies , the LD50 value is expected to exceed 5000mg/kg. If both animals die, then dosing proceeds at 2000mg/kg.

# EFFECT OF OCCIMUM TENUIFLORUM EXTRACT MODULATING APOPTOTIC GENES IN ORAL CANCER

A PROJECT SUBMITTED TO

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**May – 2022**

# CERTIFICATE

This is to certify that the project work entitled **EFFECT OF OCCIMUM TENUIFLORUM EXTRACT MODULATING APOPTOTIC GENES IN ORAL CANCER** submitted to **St Mary's College (Autonomous), Thoothukudi** affiliated to **Manonmaniam Sundaranar University, Tirunelveli** for the partial fulfillment for the award of Master of Science in Microbiology is a bonafide research carried out by **Ms. RABIYA BEGAM M.** under the guidance and supervision of Dr. **Siluvai Kirubagari Aneeshia,** Assistant Professor of Department of Microbiology, St. Mary's College (Autonomous), Thoothukudi, for academic year 2019-2022.

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**DATE :** 25.05.2022

## DECLARATION

I hereby declare that the project work entitled **"Effect of Occimum tenuiflorum extract modulating apoptotic genes in oral cancer"** is a bonafide record of the work completed by me during the academic year 2019-2022 in St. Mary's College (Autonomous), Thoothukudi and submitted as a partial fulfilment of requirements for the award of the Degree of Master of Science in Microbiology prescribed by the Manonmaniam Sundaranar University. I also affirm that this is a original work done by me under the supervision of Dr. **Siluvai Kurubagari Aneeshia,** Assistant Professor of Department of Microbiology, St. Mary's College (Autonomous), Thoothukudi.

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PLACE : THOOTHUKUDI

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In the name of GOD the most beloved and merciful, first and foremost all praise to be GOD for giving me the opportunity, patience, help and guidance for the completion of this.

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To my Parents, and my friends, thank you for bringing me up to be who I am today. My success symbolize and reflects on the undivided support and love from all of you.

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# CONTENTS

## ABBREVIATIONS

| | |
|---|---|
| % | Percentage |
| µg | Microgram |
| µl | Microlitre |
| + | Present |
| - | Absent |
| cm | Centimeter |
| DMSO | Dimethyl sulfoxide |
| DPPH | Diphenyl Tetrazolium Bromide Assay |
| g | gram |
| hr | Hours |
| ha | Hectares |
| in | inches |
| L | litre |
| m | meter |
| mcg | microgram |
| Min | Minutes |
| mm | Millimeter |
| mM | Millimole |
| M.e | Methanolic extract |
| NS | No sensitivity |
| Oc | oral cancer |
| ˚C | Degree Celsius |

| | |
|---|---|
| HPV | Human papilloma virus |
| S.D | Standard deviation |
| Sec | Seconds |
| S.E.M | Standard Error osf the mean |
| sp | Species |
| Vol. | Volume |
| Wt. | Weight. |

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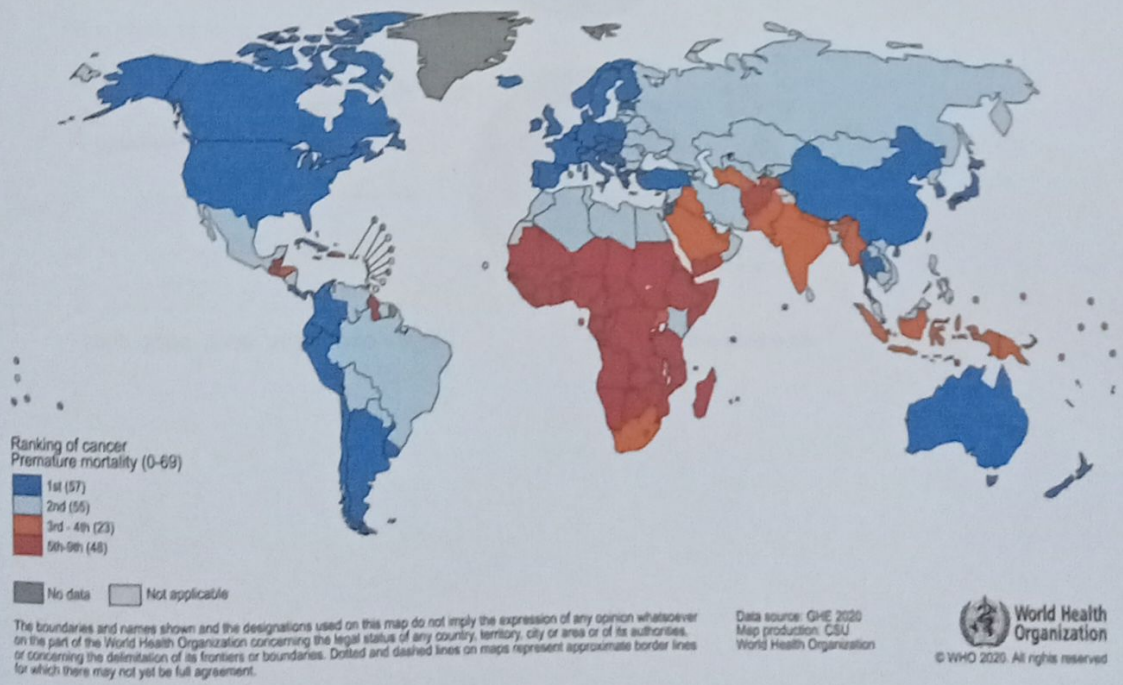
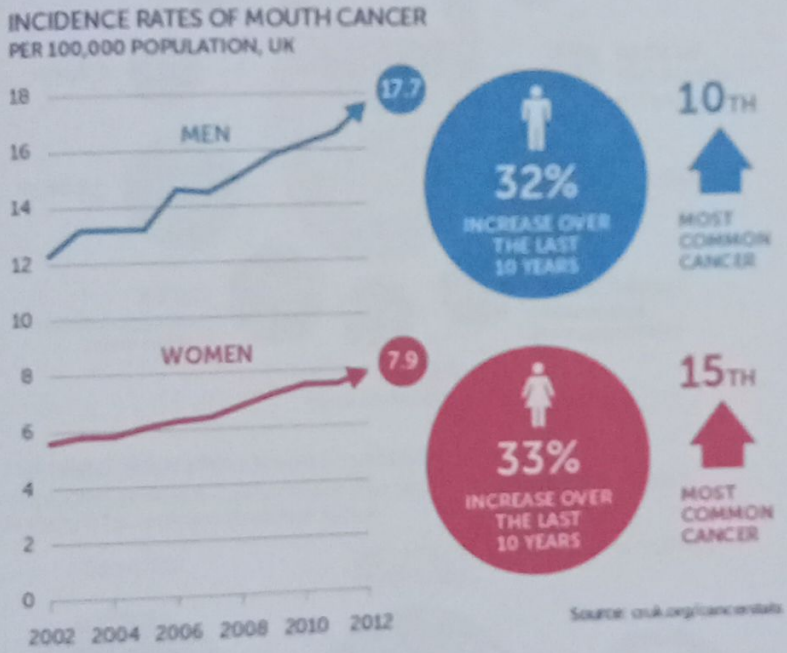
## INTRODUCTION:

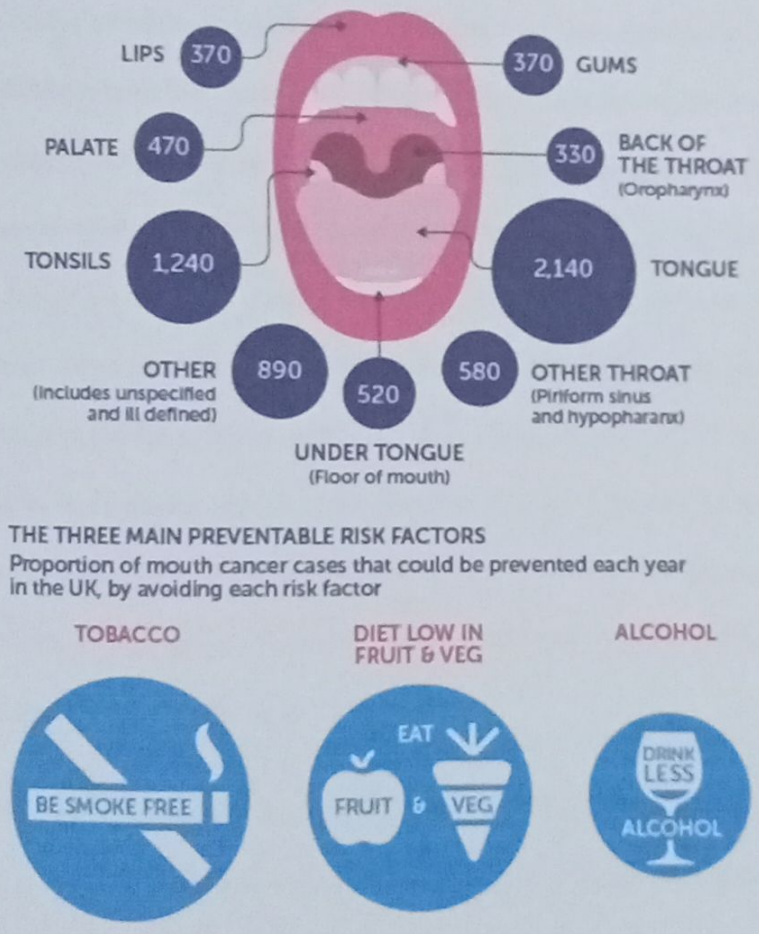
Oral cancer seems to be a leading cause of death worldwide, and its prevalence is very high in developing countries, where people chew tobacco and betel nuts on a regular basis, alcohol use (or both), or infection by the human papilloma virus (HPV). Tobacco and alcohol consumption are two of the most significant risk factors for oral cancer. Oral cancer is likely to occur in people over the age of 40 and affects more than twice as many men when compared to women.




National Ranking of Cancer as a Cause of Death at Ages <70 Years in 2019. The numbers of countries represented in each ranking group are included in the legend. Source: World Health Organization.

# MOUTH CANCER RATES ON THE RISE






According to Globocan 2020, 377,713 new cases are reported every year worldwide and oral cancer has become a common malignancy, with 177,757 deaths each year. Oral cavity cancer is very common in South-Central Asia (e.g., India, Pakistan, and Sri Lanka), with more than one-third of the new cases (135,929) and one-fifth of the deaths (75,290) occurring in India alone

The most commonly used treatment plan includes Radiation-, chemo-, targeted-, immuno-, and hormone-based therapies along with surgery. However, these treatments frequently result in various unwanted short- to long-term side effects. Hence, recent research is looking around for the urgency to evolve suitable chemotherapy consistent with new discoveries in cell biology for the treatment of cancer with less or no toxic effect . Considering the above, there is an urgent need to develop treatment options for oral cancer that have less or no adverse effects. Though there are different groups of drugs that work in different ways to fight cancer cells and shrink tumors, nowadays, herbs are used for cancer remedies. Number of bioactive compounds derived from various plants have recently attracted attention as therapeutic options for cancer treatment. Antioxidants that are found in medicinal plants, such as Vitamins A, C, and E, reduce damage to the mucosa by neutralizing free radicals found in various oral mucosal lesions. Phytochemicals that are found in medicinal plants have the potential to modulate cellular signaling pathways that alter the cellular defense mechanisms to protect normal cells from reactive oxygen species (ROS) and induce apoptosis in cancer cells.

Medicinal plants are commonly used by traditional medical practitioners in their daily practice for the treatment of various diseases and the use of medicinal plants in traditional medicine has been described in literature dating back several 1000 years . There are several chemically synthesized compounds that are present in pants and are used in many biological functions. About 12,000 of such compounds have been isolated so far which constitute only 10% of the total chemical compounds in plants [8]. These chemical compounds contribute their effect on the human body through processes identical to those conventional drugs in terms of how they work. Since time immortal, plants have been used for curing many diseases and maintaining health. There are at least 120 active compounds that have been isolated from plants, which are being used as herbal

medicine today. About 80% similarity has been observed between their traditional use and modern therapeutic use. It is estimated that about two third of the total plant species in the world have medicinal values. In the modern age, at least 7,000 medical compounds have been derived from plants. India is a country that is rich in medicinal herbs and spices consisting of about 2000 species. For about thousands of years, human beings have been using medicinal plants for treating different diseases.

Medical treatment with natural products is preferred because they are more economic and have lesser side-effects. With the widespread of cancer being a threat to humanity, plants have an important role in cancer prevention and in therapy. Many new active chemo-preventive molecules are extracted from medicinal plants. There are several mechanisms responsible for the derivation of plant compounds that have anti-tumor activities. Such mechanisms involve the effects on cytoskeletal proteins which play a key role in cell division, DNA topoisomerase enzymes inhibition, anti-protease or antioxidant activity, stimulation of the immune system etc. Medicinal plants can play a major role to delay or prevent cancer on set and can also support the immune system, thereby improving body resistance to Cancer.

**Pharmacological Importance of Plants in Cancer:**

During the recent years, the focus of cancer treatment by plants and their phytochemicals have increased. Though different plant parts have prospects for curative use and chemoprevention, their mechanisms are very difficult to understand. Therefore, extensive research has been recognised for many targeted molecules which can have potential to be used as an anti-cancer agent. There are instances of modifying abilities of many plants and plant products on many signaling pathways, along with their anti-inflammation and anti-apoptotic target for cancer

therapy. Anti-cancer properties have been observed in several phytochemicals like resveratrol, allicin, lycopene, indole-3-carbinol, vitamin C, gingerol, emodin, natural antioxidant mixture, sulphoraphan, ellagic acid, myrecitin, vanillin and eugenol. They act through one or more signaling pathways [9].

### *Ocimum sanctum* In Cancer Treatment:

One of such medicinal plants having the anti-cancer characteristic is *Ocimum sanctum* which is used traditionally as medicine in south-east Asia. *Ocimum tenuiflorum*, which is also known as *Ocimum sanctum*, Tulsi, or Holy Basil from the family Lamiaceae (tribe ocimeae) has been described as the "Queen of plants" and the "mother medicine of nature" due to its perceived medicinal qualities. It is an aromatic perennial plant that is thought to have originated in North Central India and has been used in the disease's cure and treatment since ancient times. Health effects through modulation of various biological activates has been proved in *Ocimum tenuiflorum*. *Ocimum tenuiflorum* is an accepted name. *Ocimum sanctum* is a heterotypic synonym of O. tenuiflorum. There are no basic differences between them. Therapeutic role has been observed in Ocimum tenuiflorum through its anti-inflammatory, anti-oxidant, analgesic, anti-microbial, antipyretic, anti-diabetic, hepatoprotein, hypolipidemic, immune modulatory, anti-stress activity and wound healing effects. It has been found that *Ocimum sanctum* and some of its phytochemicals such as eugenol, linoleic acid, luteolin, β-sitosterol can prevent skin, liver, oral and lung cancers. Their effects are mediated by increasing the anti-oxidant activity, inducing apoptosis, altering the gene expression, and inhibiting metastasis. Figure 1 represents the morphological appearance of tulsi plant with leaves and flowers.

**Figure 1: Morphological representation of the Tulsi plant with leaves and flowers**

Besides, the components of holy basil have been confirmed to have a noteworthy effect in cancer management through inhibition of cancer development and progression. Furthermore, it has been proved that the components of *Ocimum tenuiflorum* has a synergistic effect when used along with anti-cancer drugs as it reduces the growth of cancer. Long ago, some researchers tried to put some *Ocimum sanctum* leaves over tumor cells which resulted in the inhibition of the growth of tumor cells. Since then, *Ocimum sanctum* has been believed as a medicinal cure to heal Cancer. *Ocimum sanctum* has abundant quantities of Eugenol, the anti-cancerous component and would be a potent weapon discovered so far in fighting against all kinds of Cancer. Now scientists are trying to find new ways in which Eugenol can be produced in larger amounts. If this research succeeds, then *Ocimum sanctum* will be officially declared as anti-Cancer medicine and can be used in curing Cancer. Many research are going on, to genetically modify *Ocimum sanctum* so as to obtain more anti-cancerous compound.

Apoptosis is a systematic and harmonized sequential process causing cell death. This review aims to understand the role of apoptosis and genes associated with it in oral carcinogenesis to aid in early diagnosis, prediction of malignant potential and evaluation of possible treatment targets in oral cancer. The review also provides a comprehensive overview of basil plant extract in modulating the apoptotic genes of oral cancer cell lines- 'KB cell line' (Mouth Epidermal Carcinoma Cells) by cell viability assay, thereby proving the potential to be used as oral cancer therapeutics.

***Ocimum tenuiflorum***, commonly known as **holy basil**, ***tulsi*** or ***tulasi***, is an aromatic perennial plant in the family Lamiaceae. It is native to the Indian subcontinent and widespread as a cultivated plant throughout the Southeast Asian tropics.

The classification of *Ocimum tenuiflorum* is as follows,

**Classification:**

| | | |
|---|---|---|
| Kingdom | : | Plantae |
| Clade | : | Tracheophytes |
| Clade | : | Angiosperms |
| Clade | : | Eudicots |
| Clade | : | Asterids |
| Order | : | Lamiales |
| Family | : | Lamiaceae |
| Genus | : | Ocimum |

Species : O.tenuiflorum

**Morphology:**

Holy basil is an erect, many-branched subshrub, 30–60 cm (12–24 in) tall with hairy stems. Leaves are green or purple; they are simple, petioled, with an ovate blade up to 5 cm (2 in) long, which usually has a slightly toothed margin; they are strongly scented and have a decussate phyllotaxy. The purplish flowers are placed in close whorls on elongated racemes [49]. Figure 6 represents the morphology of *Ocimim tenuiflorum* flowers. Figure 7 represent the magnified tulsi leaf. The three main morphotypes cultivated in India and Nepal are *Ram tulsi* (the most common type, with broad bright green leaves that are slightly sweet), the less common purplish green-leaved (Krishna or *Shyam tulsi*) and the common wild *vana tulsi* (e.g., *Ocimum gratissimum*) [50].

Figure 6: Image showing the *Ocimim tenuiflorum* flowers

Figure 7: Image showing the magnified tulsi leaf

**Origin and Distribution:**

DNA barcodes of various biogeographical isolates of tulsi from the Indian subcontinent are now available. In a large-scale phylogeographical study of this species conducted using chloroplast genome sequences, a group of researchers from Central University of Punjab, Bathinda, have found that this plant originates from North-Central India (Global Biodiversity Information Facility. This basil has now escaped from cultivation and has naturalised into a cosmopolitan distribution [50; 51].

## AIM AND OBJECTIVES:

The Objectives of our study are:

1. To study the anti-proliferative effect of tulsi extract in KB oral cancer cells.

2. Gene Expression analysis of apoptotic genes to understand the apoptotic effect of tulsi extract in oral cancer cells.

## REVIEW OF LITERATURE:

The wide range of therapeutic and pharmacological applications of Tulsi has been the subject of numerous scientific studies of more than one hundred publications during the last decade alone. Numerous in vitro and animal studies attest to tulsi leaf having potent pharmacological actions that include adaptogenic, metabolic, immunomodulatory, anti-cancer, anti-inflammatory, antioxidant, hepatoprotective, radioprotective, antimicrobial, and antidiabetic effects that have been extensively reviewed previously.

Pre-clinical studies have shown that tulsi has increased the swimming survival times in mice and prevents stress-induced ulcers in rats with anti-stress effects comparable to anti-depressant drugs . Similarly, recent studies have reported that the ethanolic and aqueous leaf extracts from tulsi, could protect the rats from stress-induced cardiovascular changes . Few other studies in animal models have further shown that the leaf extract of tulsi possesses anti-convulsant and anxiolytic activities . Over the past fifty years, several animal studies conducted have reported that ingestion of tulsi leaves improves both glucose and lipid profiles in normal and diabetic-induced animal models .. Intra-mammary infusion of aqueous leaf extract of tulsi has also shown promising effect on improving the immune response in bovine models ..

Highest incidences of oral cancer (age-standardized rate of 9.8 per 10 000) has been reported in India and making it the most common cancer among men (men:women ratio 2:1) and accounts for about 30% of all new cases annually .. In India, a recent survey of cancer mortality shows that cancer of the oral cavity is the leading cause of mortality in men and responsible for 22.9% of cancer-related deaths .. There is a trend towards increasing incidence and delayed presentation of oral cancer (about 60% patients present at stage III or IV) . The Indian national cancer registry data show an increasing incidence as per age. However, the incidence of oral cancer

among women is lower than among men. This can be due to the fact of various behavioral pattern and differences in lifestyle between the two genders . In USA, the age group of 55–64 years has the highest incidence of oral. In contrast, in countries such as India, Pakistan and Sri Lanka many patients were <40 years of age in high incidence . The incidence of oral cancer is higher in the lower socio-economic strata of society due to the higher prevalence of risk factors such as use of tobacco. Despite the improvements in diagnostic and management techniques, the age-standardized mortality rates (India, 5.2 per 100 000) have been stable . However, the overall 5-year survival rate for all stages of oral cancer is 60%. These rates are better for localized tumours (82.8%) as compared to tumours with regional (51.8%) or distant metastases (27.8%).

Tobacco is being considered the single most important risk factor for oral cancer. In comparison to people who never smoked, the relative risk of oral cancer is 5.3 for people smoking <15 cigarettes per day, and 14.3 for people who smoked >25 cigarettes per day . In India, smokeless tobacco is used in the form of betel quid (pan) that contains areca nut and lime with dried tobacco leaves. The rampant use of this form of tobacco has been shown to be highly carcinogenic . Traditionally, the pan is placed in the gingival–buccal sulcus and often retained for prolonged durations, which is responsible for the high prevalence of gingivo-buccal cancer. Recently, there has been an increasing popularity especially among the youth, for the use of dried tobacco and areca nut mixtures (pan masala, gutkha, zarda, khainni), owing to their aggressive marketing in India.

Another risk factor which is alcohol consumption, confers to 1.7-fold risk to men drinking 1–2 drinks per day as compared to non-drinkers. The consumption of 25, 50 and 100 g/day of pure alcohol was associated with a pooled relative risk of 1.75, 2.85 and 6.01, respectively, of oral and pharyngeal cancer . A synergistic relationship is shared with tobacco and alcohol together, with

alcohol promoting the carcinogenic effects of tobacco leading to a multifold increase in the risk of oral cancer with combined alcohol and tobacco exposure. Heavy drinkers and smokers have 38 times the risk of oral cancer compared with abstainers.

Cytotoxic effects on the tumor cells have been shown in studies on mouse Lewis lung carcinoma (LLC) cells, A549 (adenocarcinomic human alveolar basal epithelial cells). The study results demonstrated that the apoptosis in A549 cells through a mitochondria caspase-dependent pathway can be induced by ethanolic extract of Tulsi and also inhibits the in vivo growth of LLC in a dose-dependent manner.

A positive cytotoxic ability of Tulsi extract on oral cancer cell lines has revealed the morphological evaluation of O. sanctum treated oral cancer cells line. Morphological changes were typical of apoptosis indicating the anticancer activities of O. sanctum leaves with aqueous extract. Furthermore, the study on a mouse model revealed the O. sanctums, ability to prevent the early events of carcinogen-induced hamster buccal pouch carcinogenesis .

In addition to the extensive literature documenting in vitro and animal research, studies in humans has been systematically reviewed for the use of tulsi as part of a polyherbal formulation However, to date, there are few systematic studies on the clinical efficacy and safety of tulsi as a single herbal intervention in humans with oral cancer. Therefore, the objective of this study was to critically analyze the effect of tulsi extract in treating oral cancer cells.

The majority of breast cancers arise from deregulation of genes involved in the cell cycle or apoptosis. As with all drugs that are manufactured to treat different diseases, it is important for potential natural anti-cancer agents to exhibit a certain level of specificity on their target. The FDA approved chemotherapeutic drugs as mentioned in section 1.5 all exhibit specific activity on their

target at various steps of the cell cycle. Their activity ranges from inhibiting microtubule assembly, to preventing DNA and RNA synthesis whilst some work by preventing DNA replication .

Tumour suppressor genes have now become of great interest in understanding molecular pathogenesis of cancer. The tumours suppressors which have been highly found to be deregulated in oral cancer include BID and Bcl2 genes. In this study, the cytotoxicity assay showed that oral cancer cells exhibited sensitivity to tulsi extract. With the high rate of apoptotic genes getting altered, that have been shown to trigger cancer and the fundamental role these genes play in tumour suppression, we thus aimed to investigate if the tulsi extracts could restore or upregulate the levels of BID and Bcl2 expression. This was done by means of real-time PCR, which can accurately measure changes in gene expression levels.

Real-Time PCR is a highly sensitive and powerful method used for quantitating small changes in gene expression. Its accuracy and the rapid quantification of results make it the ideal method of nucleic acid quantification .

The half-maximal inhibitory concentration- IC50 of the extracts was assessed which showed 50% reduction in surviving cells of KB cell line at 49.7 µg/ml concentration.

In the present study, O. sanctum extracts have also demonstrated apoptosis-induced cytotoxicity. In previous studies the cytotoxic action has been confirmed by inducing apoptosis through Bcl-2 regulated mitochondria caspase-dependent pathways in cancerous cell lines. Along with this, proven free-radical scavenging properties, the O. sanctum extract can be explored for its anticancer potentials.

Cell Viability can be assessed using metabolic viability based high throughput assays like MTT and MTS which are widely used nowadays. However, alteration in both mitochondrial content and metabolism can influence the metabolic viability of cells and radiation is a

mitochondrial biogenesis inducer. Therefore, we tested if MTT assay is a true measure of radiation induced cell death in widely used cell lines.

Radiation induced cellular growth inhibition was performed by enumerating cell numbers and metabolic viability using MTT assay at 24 and 48 hours (hrs) after exposure. Metabolic viability-based assays like MTT and MTS use tetrazolium salts like MTT (3-(4,5-Dimethylthiazol 2-yl) -2,5-diphenyltetrazolium bromide) and MTS (3- (4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) which are the most commonly used method for high throughput screening of anti-proliferative property of compounds on cultured cells . The mitochondrial metabolic rate are measured by the tetrazolium salts used in these assays and indirectly reflect the viable cell numbers. The tetrazolium salt found in MTT is reduced to water insoluble purple formazan crystal in the metabolically active cells by mitochondrial dehydrogenases, predominantly succinate dehydrogenase which can be further measured on spectrophotometers upon solubilization.

The total amount of formazan produced upon MTT reduction is directly proportional to the number of viable cells in the culture. Hence, MTT assay has been widely applied standard method that has been used to evaluate cell viability. As because only living cells having an intact mitochondria and cell membrane can catalyze the reaction, this method is used to measure the remaining viable cells after the treatment induced cell kill. As this assay is easy to perform, affordable and budget-friendly, these assays are used worldwide for analysing metabolic viability and cell proliferation.

To understand the biological alterations involved in disease development, the study of gene expression profile of cancer cells has become an essential tool to determine the new potential Lmarkers, to speculate the clinical outcome, to discover personalized pharmacological therapies

for patients, and to investigate the molecular effects of drug exposure with the aim of improving treatment efficacy [46]. In this regard, Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)-based methods has emerged as the "gold standard" method for a rapid and robust analysis of gene expression [47]. Currently, many PCR arrays are commercially available for the study of gene expression modifications involved in hundreds of molecular pathways.

## MATERIALS AND METHOD:

### Collection of Plant Samples

The tulsi plant were collected from the garden, identified morphologically. Then the collected samples were rinsed with water to remove epiphytes and necrotic parts. The plants were again washed with tap water to remove any associated debris and shade dried at room temperature (28±2°C) for 5-8 days or until they are brittle.

### Plant extract Preparation

The stems and leaves were collected, washed and frozen. The frozen plant material was ground to a fine powder in liquid nitrogen using a warring blender. Once ground, the plant material was weighed and extracted using absolute methanol (1 g/10 ml, w/v) at room temperature for 24 hours. The resulting extract was filtered through a Whatman filter paper, and then the filtrate was dried at 40°C under low pressure using a Büchi rotavapor R-205 (Büchi Labortechnik AG, Switzerland). Once dried, the extract was weighed and dissolved in 100% dimethyl sulfoxide (DMSO, Sigma) to the desired concentration and stored as a stock solution in an airtight container at -20°C until use.

### Cell Lines:

Human oral cancer KB cell lines were obtained from National Center for Cell Science (NCCS), Pune, India. Dulbecco's Modified Eagle Media supplements along with 10% FBS (Sigma-Aldrich, St. Louis, Mo, USA),100 units/ mL penicillin G and 100 µg/mL streptomycin as antibiotic (Himedia, Mumbai, India) were taken in a T25 flask and the cells were nurtured at 37°C. For all the experiments the cells were maintained in 100-mm culture dishes (Nunc) at humidified

5% CO2 /95% atmosphere air (Borg Scientific) at 37°C for 48–72 hours. Every 2-3 days, the cells were sub-cultured to improve the cell number and the cell population was assessed by using haemocytometer, a standard procedure for cell counting. Figure 2 represents the Monitoring the KB Cancer cells in Inverted Phase contrast Trinocular Microscope. Figure 3 represents the $Co_2$ chamber where KB cells were maintained.

Figure 2: Monitoring the KB Cancer cells in Inverted Phase contrast Trinocular Microscope

**Cytotoxicity assay (3-(4,5-dimethythiazol-2-yl) -2,5-diphenyl tetrazolium bromide assay):**

The cytotoxic effect of O. sanctum extracts on KB cell lines was evaluated using MTT cytotoxic assay followed by evaluating the dose-dependent cytotoxic effect of the tulsi extracts and to estimate the half-maximal inhibitory concentration (IC50), the concentration of drug which induces 50% cytotoxicity.

**Principle of the assay:**

This is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The

tetrazolium salts of MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, colored (dark purple) Formazan product. The cells are then solubilized with an organic solvent dimethyl sulfoxide (DMSO) and the released, solubilized Formazan reagent is measured spectrophotometrically. Since the reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells [48].


Figure 3: KB Oral Cancer cells were maintained in $CO_2$ Chamber

**Procedure of 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay:**

In-vitro growth inhibition effect of the test compound was assessed by calorimetric or spectrophotometric determination of conversion of MTT into "Formazan blue" by living cells. The

supernatant from the plate is removed and fresh MEM solution is added and treated with different concentrations of extract or compound appropriately diluted with DMSO. Control group contained only DMSO. In this study, 10, 20, 30, 40 and 50 µg of the stock solution (10 mg/ml prepared in DMSO) were added to respective wells containing 100 µl of the medium. Hence, the final concentrations were 10, 20, 30, 40 and 50 µg/ml. After 48 hours of incubation at 37°C in a humidified atmosphere of 5% CO2, stock solution of MTT was added to each well (20 µl, 5 mg/ml in sterile phosphate-buffered saline) and subjected for further 4 hours of incubation. The supernatant was carefully aspirated, the precipitated crystals of "Formazan blue" were solubilized by adding DMSO (100 µl) and optical density (OD) was measured at the wavelength of 570 nm using a microplate reader. The results represent the mean of five readings. The concentration at which the OD of treated cells was reduced by approximately 50% with respect to the untreated control was determined to be IC50.

Surviving cells were calculated in percentage with the following formula mentioned below and tabulated.

Formula: Surviving cells (%) = Mean OD of test compound × 100.

The % cell viability was calculated using the following formula:

$$\% \text{ inhibition} = \frac{\text{Mean OD of untreated cells - Mean OD of treated cells}}{\text{Mean OD of untreated cells}} \times 100$$

**RNA extraction**

Since RNA is highly susceptible to degradation, safety measures were taken throughout the processing steps to minimize its degradation. Working surfaces, glassware, pipettors and gloves were sprayed with RNaseZAP (Sigma) to eliminate RNase. RNA was isolated from treated and untreated cells using the High Pure RNA Isolation Kit (Roche). This kit is designed to extract total RNA from cultured cells; it includes a DNAse digestion step which aims to remove all contaminating DNA from the RNA. Before RNA extraction, cultured cells were rinsed twice with 2 ml 1x PBS (Sigma) at 37°C then RNA was extracted using RNA Isolation Kit according to manufacturer's specifications, (see Appendix A1). Resulting RNA was quantified using a nanodrop (NanoDrop technologies, USA), reading was taken at 260A, A260/A280 ratio of ~2.0 was regarded as pure.

**Reverse transcription**

For one step Real time PCR (RT-PCR), mRNA had to first be reverse transcribed to cDNA. This process was conducted following RNA extraction and it employs the use of specific primers with sequence complementary to the mRNA site where reverse transcription is meant to initiate. Extracted RNA was reverse transcribed using the PrimeScript RT reagent kit (Takara). Sequence specific primers were selected for use to generate specific cDNAs from the RNA target. The reverse transcription cocktail was prepared in PCR tubes to a final volume of 20 µl. The tubes with the reverse transcription cocktail were briefly vortexed 25 then placed into the T100 thermal cyclers (Biorad) as shown in Figure 4. The RNA was reverse transcribed under the conditions explained as per manufacture protocol. The resulting cDNA was used for Real Time (RT)-PCR.

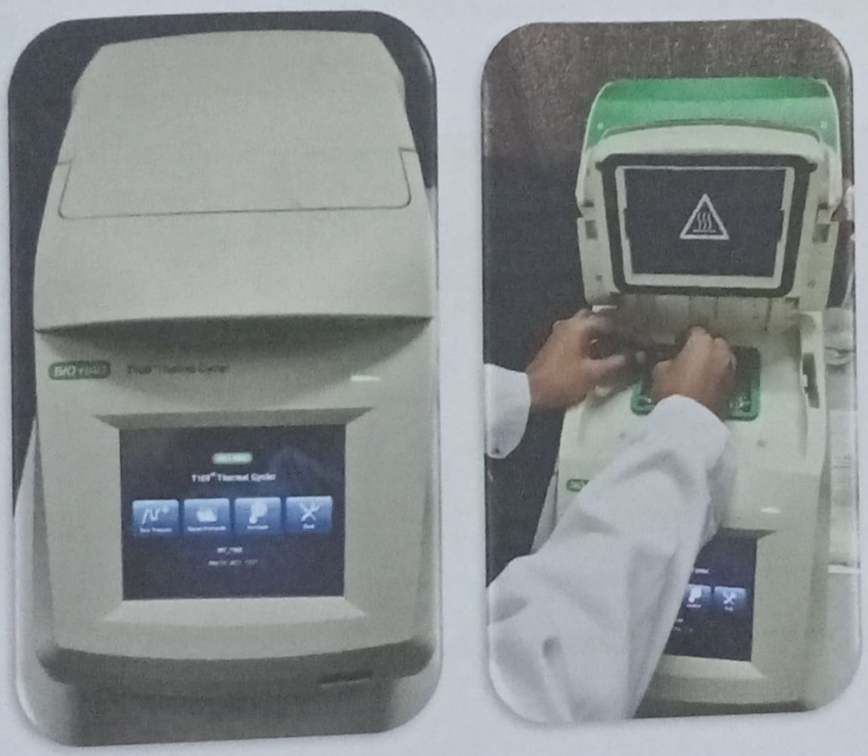


Figure 4: cDNA Conversion Experiment performed in Biorad T100 Thermal Cycler

**Real Time PCR (RT-PCR)**

Real time polymerase chain reaction is the most sensitive technique for simultaneous mRNA detection and quantification. It has been the preferred method of choice for quantitating changes in gene expression patterns. It can be used to quantify mRNA levels from much smaller samples, such as mRNA from a single cell. To monitor cDNA amplification in RT-PCR, SYBR Green (SYBR Premix Ex Taq II, Takara) was used in our study. It contains Taq DNA polymerase and only requires addition of template, primers and water. It is a fluorescent intercalating dye which presents the simplest and cheapest way of detecting PCR product in real time. The dye will only fluoresce when bound to double stranded DNA, thus allowing for fluorescence to increase as the number of double stranded DNA increases, this allows for DNA concentration to be quantified

at each cycle. Quantitative analysis of the mRNA transcripts was carried out using the Qiagen

Figure 5: mRNA Expression Analysis using Qiagen Rotor Gene qRT PCR Instrument

Rotor Gene instrument as shown in Figure 5. The parameters on the machine were set as per manufacturer instructions. The obtained ct values were exported and analyzed in Graphpad Prism software and the values were plotted in a bar diagram.

## RESULTS & DISCUSSION:

### Chemical Composition:

Some of the phytochemical constituents of *tulsi* are oleanolic acid, ursolic acid, rosmarinic acid, eugenol, carvacrol, linalool, and β-caryophyllene (about 8%) (CABI Invasive Species Compendium). *Tulsi* essential oil consists mostly of eugenol (~70%) β-elemene (~11.0%), β-caryophyllene (~8%), and germacrene (~2%), with the balance being made up of various trace compounds, mostly terpenes (IT IS Standard report page).

### Genome Sequence:

The genome of the tulsi plant has been sequenced and reported as a draft, estimated to be 612 mega bases, with results showing genes for biosynthesis of anthocyanins in *Shyama Tulsi*, ursolic acid and eugenol in *Rama Tulsi* [52; 53]. The predicted proteins and other annotations are available [53]

### MTT Assay:

MTT assay is a cell cytotoxicity or viability assay. The tulsi herbal extracts was screened for their possible antiproliferative effect against KB cancer cell lines. The MTT assay method used is described in Methodology Section. Results obtained suggest the crude extracts of the tulsi plant exhibit significant antiproliferative activity against KB oral cancer cell lines, showing very good activity at the highest concentration tested of 49.7 µg/ml, following 24-hour treatment. Cells were treated for 24 hours at increasing concentrations of 10, 20, 30, 40, 50 and 60 µg/ml using the plant aqueous extracts. Figure 8 shows the morphological representation of the KB Oral Cancer cells showing apoptotic changes in the treated group. Figure 9 shows graphical representation of cell

viability assay using MTT Analysis. Table 1 details the data points of Cell viability Assay using MTT Analysis.

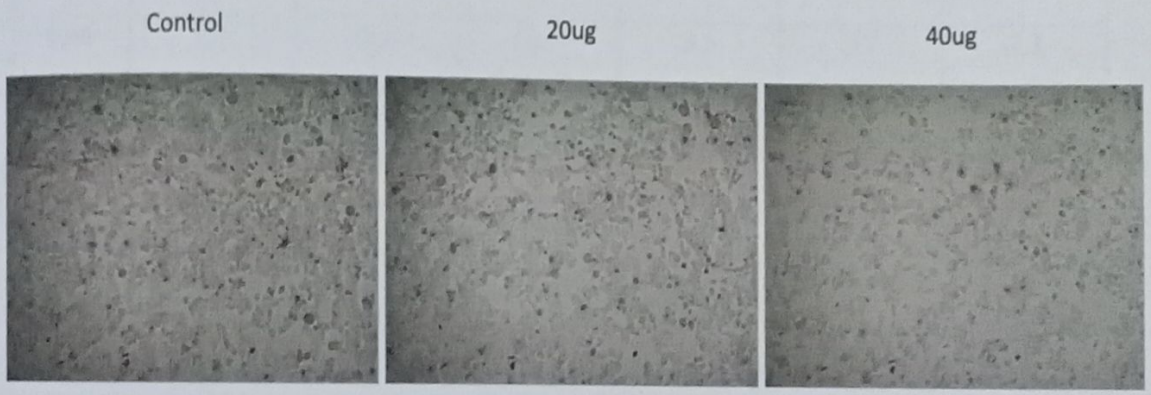



Figure 8: Morphological representation of the KB Oral Cancer cells showing apoptotic changes in the treated group

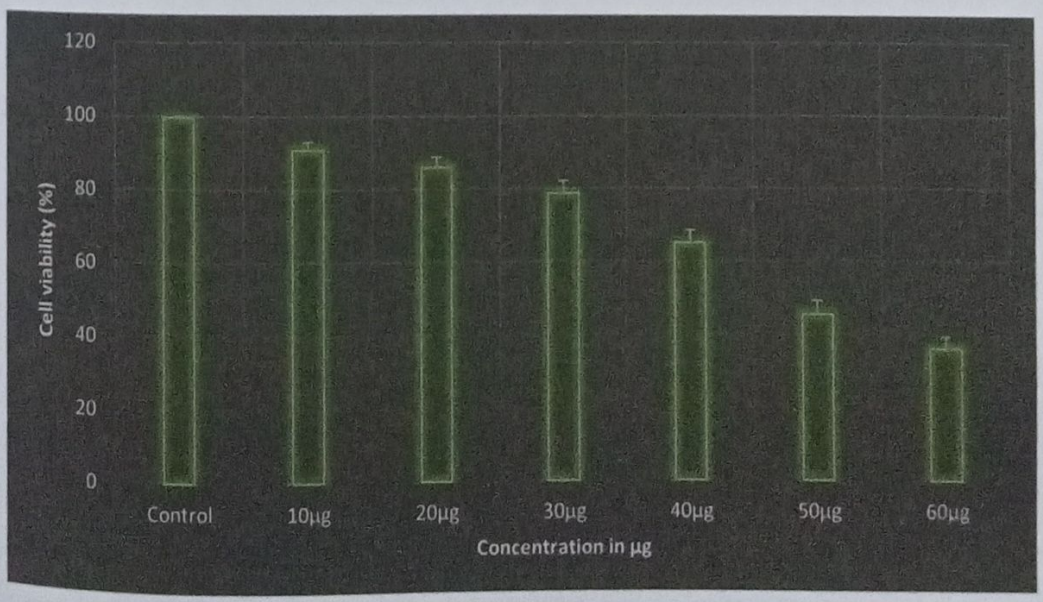



Figure 9: Graphical Representation of Cell viability Assay using MTT Analysis

| | Control | 10µg | 20µg | 30µg | 40µg | 50µg | 60µg |
|---|---|---|---|---|---|---|---|
| | 100 | 88.6 | 83.5 | 78.5 | 67.6 | 46.5 | 35.7 |
| | 99.56 | 93.2 | 89.1 | 82.3 | 60.9 | 41.3 | 33.5 |
| | 99.12 | 90.3 | 85.2 | 75.4 | 66.3 | 48.6 | 39.8 |
| Mean | 99.56 | 90.7 | 85.9 | 78.7 | 64.9 | 45.4 | 36.3 |
| SD | 0.44 | 2.4 | 2.9 | 3.5 | 3.5 | 3.7 | 3.2 |

Table 1: Data points of Cell viability Assay using MTT Analysis

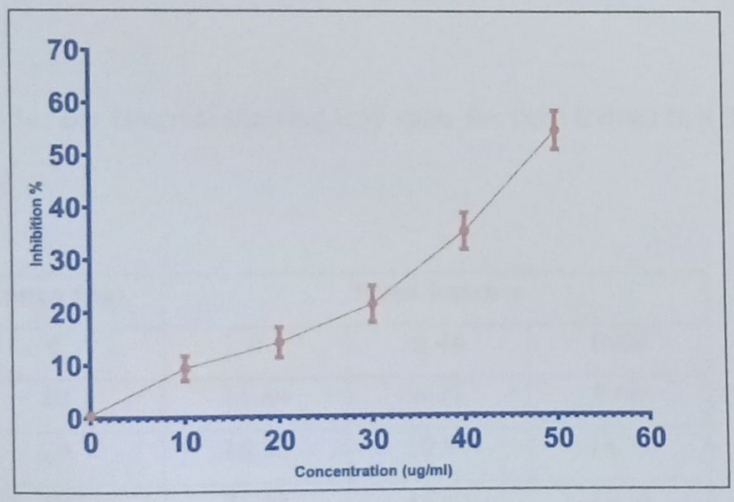



Figure 10: Graphical representation for Inhibition % of KB cells using MTT Assay

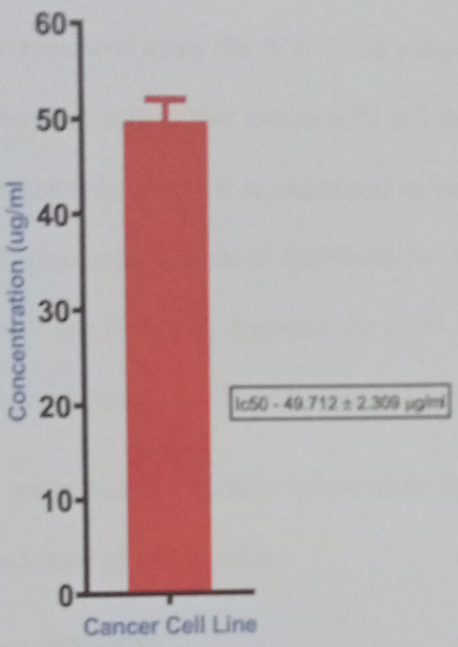



Figure 11: Bar Diagram showing Ic50 value for Tulsi Extract In KB Cells

| Concn (µg) | Plant Extract | | |
|---|---|---|---|
| 0 | 0 | 0.44 | 0.88 |
| 10 | 11.44 | 6.77 | 9.66 |
| 20 | 16.53 | 10.91 | 14.77 |
| 30 | 21.55 | 17.66 | 24.56 |
| 40 | 32.44 | 39.07 | 33.67 |
| 50 | 53.55 | 58.67 | 51.44 |
| 60 | 64.33 | 66.55 | 60.24 |

Table 2: Data points Inhibition % of KB cells using MTT Assay

Cells were treated for 24 hours at increasing concentrations of 10, 20, 30, 40, 50 and 60 µg/ml using the plant aqueous extracts. The antiproliferative activity of the herbal plant extracts on the oral cancer cell lines was measured using the IC50 value principle, which is a principle based on the concentration of the plant extract that causes 50% cell death. The lower the IC50 value of an extract on a cell line, the more potent it is considered to be. The aqueous extracts of the tulsi herbal plants exhibited considerable levels of Inhibition % of KB activity against oral cancer cells presented in Figure 10 and Figure 11 describes the IC50 value for the tulsi extract. Table 2 describes the data points Inhibition % of KB cells using MTT Assay.

The tulsi plant extract were found to exhibit cytotoxicity to oral cancer at IC50 at 49.7µg/ml. These exhibited a high level of cytotoxicity.

**Gene Expression analysis using qRT-PCR:**

Real-Time PCR was employed to investigate the expression levels of the above-mentioned apoptotic genes: BID and Bcl2 in KB oral cancer cells. The designed primer sequences were as follows:

| Gene | Primer | Sequence (5′ to 3′) |
|---|---|---|
| BID | Forward | CAGCTCCGACTCACTCCTG |
| | Reverse | ACAAATACGAATGTGCAGCG |
| BCL2 | Forward | AGGAAGTGAACATTTCGGTGAC |
| | Reverse | GCTCAGTTCCAGGACCAGGC |
| ACTIN | Forward | GATGATGATATCGCCGCGCT |
| | Reverse | CCTCGTCGCCCACATAGGAA |

Table 3: Designed Primer Sequence used for the study

**Relative quantitative real-time polymerase chain reaction:**

Relative quantification, which is based on the relative expression of the target gene versus that of a housekeeping gene, was the preferred mode of quantification for this study. Housekeeping genes are constitutively expressed in all nucleated cells since they are required for basic cell survival. Their mRNA is considered stable even under changing experimental conditions. Housekeeping genes such as glyceraldehyde-3-phosphate dehydrogenase (GADPH), actins, tubulins, cyclophilin, 18S rRNA and 28S rRNA are an absolute prerequisite for use in real-time RT-PCR experiments to ensure accurate normalisation of the experiments. For this study, mRNA expression levels were normalised to the levels of ACTIN B transcripts, which served as our housekeeping or reference genes.

One-step real-time PCR requires a separate step of reverse transcription of mRNA to cDNA before running RT-PCR. This method is ideal in that the starting amount of cDNA can be equalised to ensure the difference in expression level of genes is not due to experimental error. The following mRNA templates were reverse transcribed as in method section.

i. Untreated KB cells

ii. KB cells treated with Tulsi extract

Relative quantitative RT-PCR revealed that the levels of the BID transcript following normalisation with ACTIN B were 1.08 in the untreated KB cells (Figure 11). Treatment with tulsi extract slightly increased expression levels to 0.86. Following normalisation with ACTIN B, the BCL2 levels seemed to have been slightly reduced from 1.02 to 0.97 (Figure 11 and table 4).

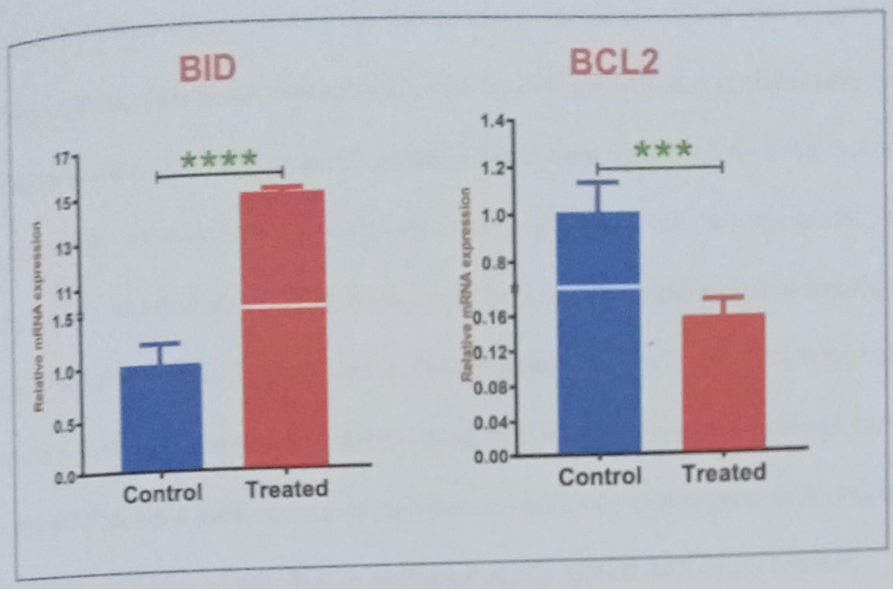



Figure 11. Bar Diagram representing relative mRNA expression of BID and BCL2 by qRT- PCR

| House keeping Gene - ACT | | | | Gene of Interest - BID | | | | | | Status |
|---|---|---|---|---|---|---|---|---|---|---|
| Sample | ct1 | ct2 | Avg ct | ct1 | ct2 | Avg ct | Δ ct | ΔΔ ct | 2^-ΔΔct | |
| Control | 27.37 | 25.73 | 26.52 | 35.29 | 32.99 | 34.14 | 7.62 | 0.00 | 1.00 | Up-Regulated |
| Treated | 25.52 | 25.67 | 25.60 | 28.36 | 30.27 | 29.32 | 3.72 | -3.90 | 14.93 | |
| | | | | | | | Avg Δ ct | 7.62 | | |
| House keeping Gene - ACT | | | | Gene of Interest - BCL2 | | | | | | |
| Sample | ct1 | ct2 | Avg ct | ct1 | ct2 | Avg ct | Δ ct | ΔΔ ct | 2^-ΔΔct | |
| Control | 27.37 | 25.73 | 26.55 | 18.26 | 18.16 | 18.21 | -8.34 | 0.00 | 1.00 | Down-Regulated |
| AM | 25.52 | 25.67 | 25.60 | 20.02 | 19.77 | 19.90 | -5.70 | 2.64 | 0.16 | |
| | | | | | | | Avg Δ ct | -8.34 | | |

Table 4: Data points for the qRT PCR performed for BID and BCL2 genes having ACT as housekeeping gene

Bcl-2:

B-cell lymphoma-2 (Bcl-2) is the primary member of the Bcl-2 group of apoptotic regulatory proteins that assist oncogenesis, not by cell growth and proliferation but by enabling apoptotic resistance. To date, over 15 proteins have been identified to show homology with the Bcl-2 homology domain and are classified into pro-survival, pro-apoptotic, and BH3-only proteins. Bcl-2 was first discovered because of the t (14:18) chromosomal translocations in non-Hodgkin's lymphoma. Bcl-2 was found to be upregulated in non-Hodgkin's lymphomas, small cell lung cancers, follicular lymphoma, chronic lymphocytic leukemia, and macroglobulinemia . A few additional mechanisms such as loss of endogenous miRNAs that repress expression of Bcl-2 gene and gene hypomethylation resulted in malignancies in almost half of all cancers.

Bcl-2 is known to be expressed in normal and breast cancer cells and is reported to be overexpressed when the cells are treated with estrogen. The expression of Bcl-2 in breast cancer is also favourably linked with differentiated and prognostic markers, including ER/PR expression, slow proliferation, small tumor size, and HER2 negativity. A few studies have contemplated the use of Bcl-2 as a prognostic marker for breast cancer. Bcl-2 overexpression was observed exclusively in ER+ disease, but the pathological function is not clear. Teixeira and coworkers showed that the MCF-7 breast cancer cells had enhanced sensitivity to the cytotoxic drug doxorubicin when treated with anti-sense Bcl-2. Furthermore, a group of BH3-mimicking small molecules that functions like the pro-apoptotic BH3-only protein was designed and utilized to counteract the anti-apoptotic proteins' effects Bcl-2, Bcl-XL, and Bcl-W.

In our study, Bcl-2 was expressed in control cells. When treated with the tulsi extract, the Bcl-2 expression was reduced. This massive downregulation of the anti-apoptotic Bcl-2 by the test

compounds, along with improved apoptotic rates in these samples, could explain the therapeutic value of these compounds for cancer therapy.

Bcl-2 like-1 (BCL2-L1) is an isoform of Bcl-xL and part of the Bcl-2 family of anti-apoptotic proteins. Bcl2-L1 regulates apoptosis by binding to and inhibiting voltage-dependent anion channel (VDAC), thereby restricting the mitochondrial release of the caspase activator CYC1. Alternate splicing could lead to Bcl-xL (anti-apoptotic) or Bcl-xS (pro-apoptotic). Bcl2-L1 shares most of its functions and properties with Bcl-xL and are known to be overexpressed in glioblastoma, colorectal cancer, and gastric cancer. Glioblastoma cell lines such as U251 and u87 when treated with miR-342 resulted in the reduction in the expression of anti-apoptotic genes Bcl2-L1 and Mcl-1. Overexpression of miR-342 resulted in enhanced apoptosis, decreased Bcl2-L1 proteins in glioblastoma cells. Expression of Bcl2-L1 was shown to be correlated to 20q gain in colorectal cancer cell lines. The protein expression was enhanced, while the mRNA did not show any change in the expression of Bcl2-L1. Thus, the expression could be regulated at the post-transcriptional level by specific factors on the 20q amplicon . Bcl2-L1 targeted drugs were shown to be highly effective in reducing the size of gastric cancer tumors. Hence, Bcl2-L1 could act as targets for different cancer therapies.

**Bid and Bad:**

BH3-interacting-domain death agonist (BID) and Bcl-2 associated death promoter (Bad) are members of the pro-apoptotic Bcl-2 protein family characterized by the presence of only BH3 domains. Bid's primary function is to interact with Bax and recruit Bax to the outer mitochondrial membrane to form MOMP leading to cytochrome C's release, resulting in apoptosis. Chen *et al.*, revealed that Bid, Bim, and Puma are effective apoptotic inducers in fibroblasts. At the same time, Bad and Noxa are weaker when expressed separately but are more efficient in killing cells. Bid,

Bim, and Puma were known to activate Bax-Bak to enable cytochrome c release. The activator BH3-proteins such as Bid, Bim, Bad, Puma are sequestered by anti-apoptotic Bcl-2 proteins such as Bcl-2, Bcl-xL Mcl-1, thus preventing the formation of MOMP and disabling cytochrome c release. Bad binds to Bcl-2 and Bcl-xL proteins, thus releasing Bid, Bim, and Puma, which might further lead to Bax-Bak activation. Protein kinases usually phosphorylate Bad. During stress conditions, Bad is dephosphorylated and moves to mitochondria, where it activates Bax-Bak, thus triggering apoptosis.

BID and Bad, being pro-apoptotic proteins, was shown to be continuously downregulated in several cancer cell lines. Bid and Bad were involved in enhancing the effectiveness of chemotherapeutic agents on cancer cells in mice. BH3-mimetic agents have been widely used as anticancer drugs. ABT-737 can bind to Bcl-2 anti-apoptotic proteins like Bad but cannot activate Bax and Bak . Bid expression was associated with enhanced tumor cell apoptosis in colon cancer cell lines, while Bad expression had no significant effect. The frequent phosphorylation of Bad could be the reason for its negligible effect on apoptosis. Ranger *et al.*, observed spontaneous tumorigenesis in Bad deficient mice, which died due to radiation-induced thymic lymphomas much quicker than wild-type mice. Bid deficient mice generated a clonal malignancy like human chronic myelomonocytic leukemia. Thus, reduced expression of Bid and Bad is generally correlated with poor prognosis in most cancers.

Higher BID levels in oral cancer cell lines could be correlated with increased apoptotic rates in these cells. In summary, the crude extracts of tulsi induced apoptosis on KB oral cancer cells by altering the key apoptotic genes. As apoptosis is controlled by such a huge network of genes and signalling molecules, it is possible that these tulsi plant extracts control other components of the apoptotic pathway too. Further research that incorporates isolation and

identification of the active compounds would be required to better understand their molecular mechanism and the pathway components they affect.

## SUMMARY

In summary, the crude extracts of tulsi induced apoptosis in oral cancer cells. RT-PCR indicated that their mode of action is in activating the apoptotic pathways. Since apoptosis is controlled by such a huge network of genes and signalling molecules, it is possible that these plant extracts control many other components of the apoptotic pathway too. Further research that incorporates isolation and identification of the active compounds would be required to better understand their molecular mechanism and the molecular pathway that follows this apoptosis induction.

### Future perspectives

Inducers of apoptosis are considered good agents in anti-cancer therapeutics. Small molecules and natural plant extracts have recently attracted attention to modern medical science research with their non-lethal activity. In this study, crude extracts of tulsi plant were screened for its antiproliferative activity against oral cancer cells. This study revealed that not only tulsi plant extracts demonstrate antiproliferative activity to the oral cancer cells, but they induced cell death by apoptosis, rendering them good agents in anticancer drug discoveries.

It could not be determined in this research which components of the apoptotic pathway are being regulated. Hence, further research which includes isolation and characterisation of the active compounds present in these plants is required. Further research into the molecular targets of the isolated active compounds would also provide better insight on the molecular mechanisms of action of these plant extracts.

In conclusion, this study has further suggested the importance of using or targeting traditional plant extracts as potential therapy against several cancers and other illnesses.

## CONCLUSION

Historically, plants, herbs and spices were a folkloric source of medicinal agents, and as modern medicine expanded, many useful drugs were developed from lead compounds discovered from medicinal plants. This approach has provided leads against various pharmacological targets, including cancer, malaria and pain, and remains an important route to new pharmaceuticals. Recent advances in cytotoxic and phytochemical screening have provided scientists with insight into the bioactive properties of medicinal plants, which has led to the development of new medicines. In this study, tulsi plants which are indigenous to South India were screened for their possible antiproliferative and/or pro-apoptotic effect on KB oral cancer cell lines.

MTT assay was used to measure significant variations in the inhibitory activity of the tulsi plant extracts on oral cancer cell lines. The best thing about MTT is that it does not have limitations and one can test as many times as possible. It was used to measure IC50, the concentration of the plant extract that induces 50% cell death on the oral cancer cell lines. Untreated cells were included as a control. From our results as described earlier, we observed interesting results with tulsi extract being able to induce up to 50% cell death in KB oral cancer cells respectively, at a concentration of 49.7 µg/ml.

After the confirmation that the plant extracts indeed managed to induce significant cell death in the oral cancer cell line tested, we had to verify if the type of cell death induced was due to apoptosis, which is genetically controlled or if it was due to necrosis.

Research into the development of novel small-molecule plant-derived extracts and compounds, which may prevent carcinogenesis, curtail its progression, or even cure the disease is still at the forefront of cancer therapeutics. With studies by Creemers et al. (1994) showing compounds such as irinotecan and topotecan derived from Nyssacea Camptotheca accuminata as

the main inducers of apoptosis against colorectal and ovarian cancer. Kedrostis foetidissima, which researchers found to be the most potent of the plant extracts tested, has been found to contain cucurbitacins B, D, E, I, J ,K and L by Konopa et al. (1974) . They further went on to show that K foetidissima exhibits high cytotoxicity to Hela and KB human (Konopa et al. 1974), however the synergistic activity of these cucurbitacins need to be studied further to understand their exact activities on the different cancer cell lines.

As with all potential anti-tumour agents, it is crucial to understand the molecular mechanism underlying their apoptotic activities. In this study, we concentrated on the two genes BID and BCL2.

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# ISOLATION OF ANTIBIOTIC PRODUCING MICROORGANISMS FROM SOIL

A PROJECT SUBMITTED TO

**ST. MARY'S COLLEGE (AUTONOMOUS), THOOTHUKUDI**

**Affiliated by Manonmaniam Sundaranar University**

In partial fulfillment of the requirements for the award of the degree of

MASTER OF SCIENCE IN MICROBIOLOGY

SUBMITTED BY

**N.R. SUTHA**

**(REG. NO. 20SPMB03)**

**Under the Guidance of**

**MS A. MARIA HEARTINA ADLIN VAZ M.SC., SET**

**DEPARTMENT OF MICROBIOLOGY**

**ST. MARY'S COLLEGE (AUTONOMOUS)**

**THOOTHUKUDI- 628001**

**MAY-2022**

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## CERTIFICATE

This is to certify that the project work entitled **"Isolation of antibiotic producing microorganisms from soil"** submitted to in **St. Mary's College (Autonomous), Thoothukudi** affiliated by Manonmaniam Sundaranar University, Tirunelveli for the partial fulfillment for the award of Master of Science in Microbiology is a bonafide research carried out by N.R. SUTHA under the guidance and supervision MS A. Maria Heartina Adlin Vaz M.SC., SET, Assistant Professor of Microbiology St. Mary's College (Autonomous), Thoothukudi, for the academic year 2020-2022

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## DECLARATION

I hereby declare that the project work entitled **"Isolation of Antibiotic Producing Microorganisms from Soil"** is a bonafide record of the work completed by me during the academic year 2020-2022 in St. Mary's College (Autonomous) Thoothukudi and submitted as a partial fulfillment of requirements for the award of the Degree of Master of Science in Microbiology prescribed by the Manonmaniam Sundaranar Universirty. I also affirm that this a original work done by me under the supervision of Ms. A. Maria Heartina Adlin Vaz M.Sc., SET, Assistant Professor of Department of Microbiology St. Mary's College (Autonomous), Thoothukudi.

A. Adlin Vaz

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**PLACE: THOOTHUKUDI**

**DATE:** 25-5-2022

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## ABBREVATION

| | |
|---|---|
| NP | -Natural Product |
| MDRGNB | - multiple drug-resistant Gram-negative staphylococci |
| AMCs | - antimicrobial compounds |
| PRSP | - penicillin-resistant *Staphylococcus aureus* |
| AMPs | - Antimicrobial peptides |
| RPs | - Ribosomal synthesized peptides |
| TPPs | - target product profiles |
| G. S | - Gram staining |
| MR | -Methyl red |
| VP | -Voges Proskauer |
| Ml | -millilitre |
| G | -grams |
| H | -hours |
| Mm | -millimeter |
| MHA | -muller hintor agar |

# 1. INTRODUCTION

Resistance towards antibiotics has become one of the most important issues to be addressed in this century as in recent times, more than 700,000 patients die all over the world because of antimicrobial resistance (AMR). This estimation is going to increase to 10 million by 2050, which will decrease GDP at least by 2.5. (M.M. Konai et al.,2015).

Antibiotic means 'against life' as the name suggest, it is important commercial secondary metabolites produce by wide range of microorganisms' bacteria, fungi, *Streptomyces*, against other microorganisms in an environment to thrive for nutrients. (Sonia Sethi et al.,2013).

Pathogenic bacteria are acquiring resistance to existing antibiotics, most of which are expensive and have been associated with side effects like nephrotoxicity, etc. Bacteria have evolved numerous strategies for resisting the action of antibiotics and antibacterial agents. This is particularly true of those bacteria that are antibiotic producers. Bacteria that produce antibiotics do so to gain a selective advantage over other competing microbes in their natural environment. (M.V.S. Sandhaya et al.,2015).

The emergence of antibiotic-resistant bacteria is a common phenomenon. The emergence of resistance often reflects the evolutionary process that occurs during antibiotic therapy. Antibiotic treatment can select bacterial strains with physiologically or genetically enhanced ability to withstand high doses of antibiotics. Under certain conditions, the drug may inhibit the growth of susceptible strains, while resistant strains may grow preferentially (Levy SB et al., 1994)

In late 1930s, Sir Alexander Fleming hypothesized the presence of penicillin, a molecule produced by a particular mold that kills or stops the growth of certain types of bacteria. When Fleming noticed the spores of the green mold *Penicillium rubens*, he was working on culturing the bacteria that cause the disease. (Pathak A et al., 2020).

Bacterial resistance is the ability of bacterial cells prevents the bacteriostatic or bactericidal effect of antibiotics. Excessive and unintended use of antibiotics contributes to the development of bacterial resistance. For extensive recording and development of microorganisms that are resistant to time and problems as a result, resistant microorganisms emerged. Treatment of specific infections. (Thualfakar Hayder Hasan et al., 2020).

The risk of extensive spread of antimicrobial resistance can reduce positive changes in the modernization of the healthcare organizations. Therefore, not only a novel approach, but also new and effective antibiotics are essential. In a modern technology filled world, different discoveries and health techniques can be used to decrease antimicrobial resistance. New research to speed up the development of new drugs and diagnostic tools is one approach, as well as better monitoring of antibiotic resistance and ways to advance the use of existing antibiotics. There is also a need for a framework to regulate the use of potential new antibiotics to prevent them from being wasted. Lastly, the ongoing pandemic blowout of resistant bacteria demonstrates that the problem can only be talked through international cooperation and thus that any new strategy to manage antibiotic resistance must take into considering the issues of worldwide entry and cost effectiveness. (M.M. Konai et al.,2015).

Soil is the main reservoir of antibiotic-producing microorganisms. Given the high density of microorganisms in the soil, many types of bacteria and fungi have evolved over the years to find ways to prevent the growth of their neighbors and benefit them. No wonder, antibiotics made by one microbe can inhibit many other soil microbes. The genus *Bacillus* and *Streptomyces* of the genus Bacteria, and the genera *Penicillium* and *Cephalosporium* of the genus Fungi are commonly found in soil. The genus *Streptomyces* is the most prolific producer of antibiotics, a bacterium, but a unique subgroup of bacteria called actinomycetes. Soil has historically been used to find new antibiotic producers, but now many of the "old" antibiotics are laboratory-operated and chemically modified to produce new versions of old antibiotics.

The use of antibiotic-producing microorganisms to prevent illness dates back thousands of years, and traditional moldy bread compresses to treat wounds opened in Serbia, China, Greece and Egypt over 2000 years ago. Ebers Papyrus from 1550 BC. BC is the oldest surviving medical document and the list of treatments includes mold bread and healing lands. (R. Emmerich, O. Löw et al, 1899).

Antibiotics between microorganisms were described long before penicillin was discovered. This is due to Louis Pasteur, who suggests that microorganisms may secrete substances to kill other bacteria. By the turn of the 20th century, the production of diffusible and thermostable compounds by bacteria was reported and their usefulness in the fight against infectious diseases has been studied. Perhaps the first clinical use of antibiotics was reported in the 1890s, and Emmerich and Reeve were extracts of *Pseudomonas aeruginosa* to treat hundreds of patients (then known as *Bacillus spicianeus*). (E.E. Hays et al., 1945).

The genus *Bacillus* comprises 377 species (last update in January 2019) of Gram-positive, rod-shaped bacteria (Gordon et al., 1973). Their ability to form endospores, their diversity in physiological properties, as well as their capacity to produce numerous antimicrobial compounds (AMCs) favor their ubiquitous distribution in soil, aquatic environments, food and gut microbiota of arthropods and mammals (Nicholson, 2002)

Bacteria from the *Bacillus subtilis* group consist of small vegetative cells (<1 µm-wide) for which the strain *Bacillus subtilis* subsp. *subtilis* 168 is considered as model organism (Barbe et al., 2009). They are usually mesophilic and neutrophilic, although some can tolerate high pH. The four original species of the group (*Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus pumilus*, and *Bacillus amyloliquefaciens*) were discovered more than 40 years ago (Gordon et al., 1973; Priest et al., 1987).

The potential of *Bacillus subtilis* group strains to produce a wide diversity of secondary metabolites mediating antibiosis was recognized for decades. For any given strain of the *B. subtilis* group, it is now estimated that at least 4–5% of its genome is devoted to antimicrobial compounds (AMCs) production (Stein, 2005). These molecules are mainly antimicrobial peptides (AMPs). Their structures are usually cyclic, hydrophobic and contain peculiar moieties such as D-amino acids (AA) or intramolecular thioether bonds. In addition to AMPs, volatile metabolites also constitute a large family of antimicrobials exhibiting numerous metabolic and functional roles.

Due to the wide diversity of these molecules, their classification is rather complex and can be based on several criteria such as their biosynthetic machinery, sources, biological functions, properties, three-dimensional structure, covalent bonding pattern or molecular targets (Tagg et al., 1976; Wang et al., 2015). Ribosomal synthesized peptides (RPs) are usually derived from short precursors (*ca.* 100 AA) and are processed to mature compounds through post-translational modifications (Oman and van der Donk, 2009).

Of all the antibiotics discovered between 1945 and 1978, 55% were from the genus *Streptomyces*. Several theories have been proposed to explain why soil microorganisms produce so many bioactive NPs (natural products). The most likely explanation is that they have multiple functions, as chemical weapons to kill competitors in the soil, as protection (defense) or predator (attack), as signaling molecules for close relatives. Or mediate insects and plants. This is consistent with evidence that *Streptomyces* and other filamentous actinomycetes evolved about 440 million years ago, almost as soon as the plants first

colonized the land. It is speculated that the filamentous growth of these bacteria provides an advantage in colonizing plant roots and that many of their NPs have evolved or were adopted to mediate these interactions. (M.F. Traxler et al., 2015).

Aside from the scientific difficulties associated with the discovery and development of antibiotics, to protect the potential of existing and future arsenals of clinical compounds, activate antibiotic research and development, and address the challenges posed by AMR. Necessary investment and cultural change. (A. Mullard et al.,2014).

A significant threat to the outcome of the antibiotic era is antibiotic resistance. Resistance is the natural resistance of certain bacterial species (presence before antibiotics are discovered), genetic mutations in microorganisms, the acquisition of resistance by one species from another, and the competitive advantage over mutant strains. Due to selective pressure due to the use of antibiotics given. Non-optimal antibiotic doses, especially due to antibiotic misuse, help in the gradual selection of tolerance. Examples of major resistant pathogens worldwide include penicillin-resistant Staphylococcus aureus (PRSP), methicillin-resistant Staphylococcus aureus (MRSA), and vancomycin-resistant enterococci. (VRE) and multiple drug-resistant Gram-negative staphylococci (MDRGNB) (Yoshikawa TT et al.,2002).

Resistant strains are difficult to treat and require high doses or alternatives that can be more toxic and expensive. According to the Centers for Disease Control and Prevention (CDC), at least 2 million people are infected with antibiotic-resistant bacteria, and more than 23,000 people die each year as a result of these infections. This is expected in more cases. It remains the leading cause of death in developing countries with infectious diseases. This situation is associated with antibiotic resistance worldwide, including emerging and re-emerging infectious diseases, lack of development of new classes of antibiotics, and super bugs (bacteria that have accumulated resistance to almost all available antibiotics). It is getting worse due to the increase.

Bacterial natural antibiotic resistance can be difficult to control as it may precede the discovery of some antibiotics and is one of the natural coping mechanisms for their survival. However, most of the acquired antibiotic resistance is caused by socio-economic and behavioral factors, especially in developing countries. Some of these factors include the abuse of antibiotics by medical professionals, the abuse and improper use of antibiotics by unskilled doctors and the general public. Other factors include substandard medications, the spread of resistant strains due to overcrowding and unsanitary conditions, inadequate infection control practices in

hospitals, inadequate monitoring, poverty, and the implementation of antimicrobial resistance strategies. Lack of resources, lack of political will, etc. (Okeke I N et al., 1999).

Actinomycetes are a rich source of structurally diverse secondary metabolites. Many of them have pharmacologically relevant biological activity. Approximately 23,000 bioactive microbial metabolites have been reported, of which more than 10,000 are derived from actinomycetes, accounting for 45% of all bioactive microbial metabolites. Therefore, the search for new actinomycetes has become an integral part of natural product-based drug research in recently. Antibiotic generating actinomycetes have now no longer been well-investigated. The majority of those microorganisms withinside the soil which are ability drug reassets continue to be unfruitful, and because of this inaccessible for novel antibiotic discovery. This take a look at is anticipated to emerge as a crucial aspect withinside the manufacturing of recent herbal bioactive products. The take a look at become undertaken to isolate and display lawn soil derived actinomycetes for antibiotic activity. (Sudha Sri Kesavan et al.,2015).

Most of the peptide antibiotics produced by *Bacillus* are active against Gram-positive bacteria. However, while compounds such as polymyxin, colistin, and circulin are almost exclusively active against Gram-negative bacteria, basilomycin, mycobacillin, and fungistatin are effective agents against mold. *Bacillus subtilis* species produced antibiotics with soluble protein structures. For commercial production, the current goal is to produce antibiotics such as polymyxin and bacitracin from *Bacillus sps*. Reported members of *Bacillus* generally produce polypeptide type bacteriocines, and these antibiotics generally act on Gram-positive bacteria. (Sonia Sethi et al., 2013).

Fungal-produced antibiotics are widely used especially with current chemotherapy Penicillin, cephalosporins, fusidic acid, has antibacterial and antifungal effects. Range of antibiotics discovered by the inhabitants of the bottom microorganisms including fungi (20% of) isolated antibiotics), actinomycetes (70%) and Authentic bacteria (10%). For the past 30 years new discoveries of microorganisms are possible connect as a potential source of new antibiotics. (M. D. Makut et al., 2011).

Drug-resistant pathogens such as *Aspergillus baumani* and methicillin-resistant *Staphylococcus aureus* (MRSA), or especially infectious pathogens of tuberculosis and adenitis, are attracting attention because they make people sick and difficult or impossible to cure. However, the effects of horizontal gene transfer are not limited to pathogens and humans.

"Most resistance determinants can persist and amplify in symbiotic" reservoirs "rather than clinical isolates. In other words, it is not a bacterium that causes human disease, but a symbiotic bacterium that normally inhabits humans and animals. (Levy and Marshall, 2013).

The four principal forms of antibiotic resistance evolve as; 1) Natural resistance: (Intrinsic, Structural) In this type of resistance, the usage of antibiotics is not associated with the resistance but it caused by the bacteria's structural properties. This occurs as a result of intrinsic resistance, or microorganism which does not follow the target antibiotic structure, or antibiotics which due to its characteristics do not encounter its target. Gram negative bacteria and vancomycin, for example, vancomycin antibiotics does not move through the outer membrane so that these Gram-negative bacteria are naturally insusceptible to vancomycin. Likewise, L-form bacteria that are cell wall-less types of the bacteria, such a Urea plasma and Mycoplasma that are naturally owning beta-lactam antibiotics resistance.

2) Acquired resistance: Regardless of resistance development due to alteration in the genetic features of bacteria, an acquired because it is not affected by the antibiotics it was previously susceptible to it. This form of resistance comes from the main chromosome or extra chromosome structures (plasmids, transposons, etc.). Chromosomal resistance results from mutations that change randomly bacterial chromosome, these mutations can occur by certain physical and chemical factors. This may be due to changes in the composition of bacterial cells, so that may be decreased bacterial drug permeability, or maybe changes to the drug's target in the cell. Streptomycin, aminoglycosides, erythromycin, and lincomycin can develop resistance to these forms. Extrachromosomal resistance relies on extrachromosomal genetic materials that can be transmitted via plasmids, transposons, and integrons. Plasmids are segments of DNA that can replicate independently oof chromosomal DNA. A plasmid is typically responsible for the development of antibiotic inactive enzymes. There are main forms of holding genetic material (resistance genes and plasmids) from bacterial cells, this form are transduction, transformation, conjugation, and mechanism of transposition. The genes with antibiotic resistance on the chromosome or plasmid are intertwined and are situated at the beginning with different integration groups, or integrons. Recombination is very normal in integrons.

3) Cross-resistance It is mean the resistance to a specific antibiotic by specific microorganisms, that work with the identical or related mechanisms and that are also resistant to other antibiotics. This is generally seen when antibiotics have common structures: such as resistance to erythromycin, neomycin, kanamycin, or resistance to cephalosporins and penicillins.

However, cross-resistance can sometimes be seen in a completely distinct group of drugs as well, like a cross-resistance that exists amongst erythromycin-lincomycin, this resistance might be the chromosomal origin or not.

4) Multi-drug and other types of resistance Multidrug-resistant species are typically pathogens that have been resistant to their antibiotics, this ensures that the bacteria will no longer be eliminated or regulated by a single drug. Inappropriate utilization of antibiotics for treatment culminated in the introduction of multidrug resistant pathogenic bacteria. Either of the two mechanisms can induce multidrug resistance in bacteria. Firstly, these bacteria will acquire several genes, each coding for specific drug resistance, this form of resistance usually exists on R-plasmids. Secondly, the form of multidrug resistance may also occur by enhanced gene expression encoding for efflux pumps, enzymatic inactivation for antibiotics, changes in target structure, and others. (Thualfakar Hayder Hasan1, Raad A. Al-Harmoosh,2020).

Fully synthetic antibiotics, beyond introducing novel molecules, enable production at a scale suitable for clinical application. For instance, chloramphenicol became the first fully synthetic antibiotic, whose scaffold originated from a natural product, to reach the clinic in 1949. Unsurprisingly, the rational of semi-synthesis, that of chemically manipulating a scaffold, applies to a fully synthetic antibiotic like chloramphenicol. In fact, replacing the nitro group with methane sulfonyl resulted in thiamphenicol in 1952, which overcame the most concerning toxicity issues and had greater antimicrobial effect, thereby improving its clinical application.

After the successes of the antibiotic golden age, the discovery rate of the underlying ADPs has decreased, along with an increase in class and multidrug-resistance mechanisms, which has weakened the therapeutic efficacy of the antibiotic arsenal and revived the issue of infectious diseases. The need for a new strategy coincided with the genomics era, which redefined the scientific paradigm governing antibiotic discovery and shaped new high-tech platforms. During the genomics era (1995–2004), the total number of sequenced microbial genomes increased from 3 to over 200, Antibiotics in the post-genomics era (2004–2014) reached a staggering 30,000. Given the novelty of the various omics technologies, we are yet to extract their full potential and it seems feasible that these technologies will mature to fulfill this gap. Alternatively, innovative technologies favoring high-throughput may be developed, even by sacrificing molecular sensitivity to some extent. In any case, the increasing need for antibiotics drives the relentless and continuous research on the foreground of antibiotic discovery. This is likely to expand our knowledge on the biological events underlying infectious diseases and,

hopefully, result in better therapeutics that can swing the war on infectious diseases back in our favor (Bernardo Ribeiro da Cunha et al., 2019).

New variants of known classes can be found by screening microbial strains, by varying cultivation procedures or by manipulating the biosynthetic pathway. There is an increasing amount of literature related to pathway manipulation and this trend is likely to continue as methodological advancements result in increased success rates. In some cases, the desired variant might not be a more active compound, but a molecule carrying functional groups suitable for further chemical modifications. As the antibiotics in clinical use belong to a few classes, which have been extensively explored by screening and chemical modification, there is probably little space for finding improved variants within those classes. We provide selected examples of microbial strains producing improved variants of chemical classes not yet in clinical use.

Click chemistry is a new synthetic approach that can accelerate drug discovery by using a few practical and reliable reactions. A 'click' reaction must be of wide scope, giving consistently high yields with various starting materials; it must be easy to perform, insensitive to oxygen or water and use only readily available reagents; finally, reaction work-up and product isolation must be simple, without chromatographic purification. (Stefano Donadio et al., 2010).

Research-led programmes that fail to consider clinical use, manufacturing, regulatory practices, the feasibility of clinical study designs and reimbursement, are not only inefficient but probably doomed to failure. Recently, the WHO published a list of bacteria for which new antibiotics are urgently needed so the next step will be to provide internationally agreed-on target product profiles (TPPs) that will define the properties of suitable antibacterial therapies. Pharmaceutical companies have detailed descriptions of what they consider ideal and acceptable characteristics of new antibacterials such as indication, potency, efficacy, pharmacology, toxicology, safety and dosage. These TPPs could be used by other researchers to ensure that their research is aligned with the most urgent medical needs. TPPs could also be used by funders and investors to select the projects that are most likely to have a clinical impact. If this is not done, research on new antibiotics may well end up failing to address the most urgent needs. (Nicole Jackson et al., 2018).

The last two decades have shown that chasing novelty in terms of targets or compound support has been inefficient and that time is required to establish firm foundations of science upon which to build future activities. We recommend that investment is provided for:

(i) innovative chemistry on, and around, known clinically effective drug

(ii) alternative ways to inhibit the function of clinically validated targets

(iii) understanding resistance mechanisms and how they can be inhibited

(iv) understanding the utility of animal models and the risks around reducing drug-efficacy hurdles

(v) establishing the levels of *in vitro* resistance development that are unacceptable.

Society must not assume short-term solutions can be found and there is no point in prioritizing programs that are unlikely to prove feasible over the next 10 to 30 years. Investment must be prioritized to support feasible projects and, where possible, allocate additional funding to more speculative programs. The natural world remains the largest source of novel drug scaffolds making this a viable option in the search for new antibiotic compounds.

Advances in bacterial culture techniques, molecular biology and metagenomics will make natural product drug discovery easier and more cost-effective, obviating these limiting factors. Screening procedures must include whole-bacterial cell assays, addressing the issue of bacterial permeability and efflux early in the discovery process. Additionally, the generation of training schemes by, and with, pharmaceutical companies that cover all aspects of the pipeline and include natural product drug discovery, are essential and will ensure that expertise is passed on to future researchers. (Shore C K et al., 2016).

The advantages and disadvantages of whole-cell screening have been extensively discussed in recent reviews by Payne and colleagues and these arguments will not be repeated here. It should, of course, be noted that whole-cell screening is now accompanied by a number of new methods, derived from the genomics era, for determining the mode of action of inhibitors. (Gwynn MN et al., 2010).

A particular problem with current whole-cell screening campaigns has been the high incidence of inhibitors that display antibacterial activity through non-specific damage to the bacterial cytoplasmic membrane, which is a strong indicator that such inhibitors would have similar activity again mammalian cells and hence would be cytotoxic. The presence of such inhibitors explains in part why the output from recent whole-cell antibacterial screening programs has been so disappointing. (Payne D J, et al. 2007).

Microbial infections have been the leading cause of death throughout history. This was changed when antibiotics were discovered, causing an increase in life expectancy from 48 years to 72

years. However, this golden era might end very soon. Bacteria have evolved resistance against antibiotics using different pathways. Therefore, restrictive policies about using antibiotics should be implemented by the healthcare system to prevent the further spread of bacterial resistance. However, these policies might not be enough without discovering or synthesizing new antibiotics. Antibiotics synthesis or discovery is a lengthy, tedious multistage process. Moreover, the development of bacterial resistance against any newly developed antibiotics takes around 10 years. Therefore, there is a need to find another strategy to retain the current available antibiotics activity against micro-organisms. Nanotechnology is a cutting-edge science that has been emerged few decades ago, it is concerned with producing fibers or particles in the nanometer scale. In literatures, nanoparticles were shown to improve the drug solubility, bioavailability, modify drug pharmacokinetics, increase drug stability, target drug into certain sites and moreover, were proven to overcome some developed resistance mechanisms against anticancer drug (e.g., Efflux mechanism). Recently, nanotechnology techniques have been applied to combat microbial infections and they were proven to be able to overcome the bacterial developing resistance mechanism. (Yasmin Abo-zei et al., 2021).

Extensive study of the antibiotic resistome throughout the past few decades has enabled us to begin to understand and address existing antibiotic resistance threats. However, it is evident that antibiotic resistance will continue to evolve and spread despite our best efforts to develop new antibacterial agents. Unless current practice is changed, it will be increasingly difficult to establish and maintain even a transient lead over bacteria in this 'arms race'. As research into antibiotic resistance expands, it is important to adopt an explicitly proactive approach to antibiotic resistance identification and surveillance, as well as antibiotic therapy development. This proactive approach involves using a combination of functional metagenomics, next-generation sequencing and cutting-edge computational methods to monitor the evolution and dissemination of resistance before a given resistance determinant emerges in a pathogen or in the clinical setting, as well as proactively developing next-generation therapies that target these resistance determinants. The spread of plasmid-borne carbapenem, quinolone and polymyxin resistance in recent years is a sobering reminder of our need to mitigate existing threats and to anticipate emerging resistance mechanisms before they circulate widely in the clinical setting if we wish to alter the current bleak antibiotic resistance trajectory. Recent advances in the field highlight the promise that the next generation of resistome studies hold for characterizing and countering emerging resistance threats (Terence S. Crofts et al., 2017)

A key aspect that requires investigation in the next generation of antibiotic resistance studies is the proactive development of therapeutic strategies to counteract emerging antibiotic resistance. One side of this approach involves having a greater focus on resistance potential when developing new and improved antibiotics. Current antibiotic discovery and development pipelines only take into account resistance mechanisms that are already prevalent in the clinic. In retrospect, we know that this strategy is doomed to fail, as resistance has been observed for every antibiotic that has been implemented for human use. Going forwards, we should improve on the current reactive strategies by screening promising lead compounds for resistance against diverse functional metagenomic libraries. (Gonzales et al., 2015).

Treatment regimens that induce collateral sensitivity not only enable the elimination of resistant bacteria but also subject resistant bacteria to selective pressure to discard their resistance gene. Inverting the selective advantage of antibiotic resistance is a promising approach for slowing the rate at which resistance evolves in a population. A recent highlight in the search for selection-inverting compounds involved a screen of nearly 20,000 small molecules for compounds that select against the tetracycline efflux pump TetA. This screen identified two candidates that successfully selected for the loss of the tetA gene. Perhaps more important are drugs under development and in early-phase testing that target AcrA–AcrB–TolC efflux complexes in Gram-negative bacteria. Unlike TetA proteins, which are tetracycline specific, AcrA–AcrB–TolC pumps have broad substrate tolerance and can confer multidrug resistance, particularly in Gram-negative bacteria (Stone et al., 2016)

The *Bacillus subtilis* group offers a plethora of antagonistic compounds displaying a broad range of biological functions. In the near future, our understanding of the resistome will be sufficiently mature to enable the development of strategies that actively combat resistance mechanisms themselves in contrast to current strategies that side-step resistance through the expensive development of new antibiotics that immediately select for new mechanisms of resistance. This new approach, in our opinion, provides a higher likelihood of success than searching for a fabled 'silver bullet' antibiotic to which resistance is unlikely to develop. One such strategy is the repurposing of existing antibiotics in synergistic combinations. For example, consider the triple β-lactam combination of meropenem, piperacillin and tazobactam, which synergistically kills methicillin-resistant S. aureus (MRSA) N315 in vitro and in a mouse infection model. The bacteria were collaterally sensitive to the combination of these

three antibiotics, and thus the combination suppressed the evolution of further resistance. (Baym, M et al., 2016)

## 2. AIM AND OBJECTIVE

- To isolate antibiotic producing microorganism from soil.
- To isolate pure cultures of the isolated microorganisms.
- To analyze the morphology of the isolated microorganisms.
- To analyze the biochemical activity of the isolated microorganisms.
- To analyze the antimicrobial activity of the isolated microorganism.
- To determine the minimum inhibitory concentration of the isolated microorganism.

## 3. REVIEW OF LITERATURE

Abraham E. P et al., (1940) associate the beginning of the modern "antibiotic era" with the names of Paul Ehrlich and Alexander Fleming.Even before the extensive use of penicillin, some observations suggested that bacteria could destroy it by enzymatic degradation.

Jones et al., (1944) suggested that this method is similar to Fleming's discovery of penicillin; however, Waksman applied a more systematic, deliberate screening approach, while Fleming's discovery of an antibiotic-producing mold was accidental. This new screening approach, otherwise known as the 'Waksman platform' led to the discovery of an important antibiotic streptomycin, which exhibited in vitro activity against Gram-positive and Gram-negative bacteria.

Waksman SA (1947) and *Landsberg H (1949) also said that* Ehrlich noted certain dyes would colour human, animal, or bacterial cells, whereas others did not. He then proposed the idea that it might be possible to create chemicals that would act as a selective drug that would bind to and kill bacteria without harming the human host. After screening hundreds of dyes against various organisms, in 1907, he discovered a medicinally useful drug, the first synthetic antibacterial organoarsenic compound salvarsan, now called arsphenamine.

Nickell et al., (1959) and Bergmark et al., (1967) both reported that cites the use of a number of plants in wound treatment and it seems possible that these may have been used for many thousands of years. Many plants have properties useful in wound therapy. Many are astringent and some 2500 have antimicrobial effects. The usefulness of many plants may have been suggested by their appearance: knot-grass has leaves that appear bloodstained, while the perforated leaves of St John's wort suggest a use on perforating wounds. Nickell showed that hypericum has antibacterial properties, as do oak-sap and oak-gall or the leaves of lady's mantle, which all contain the astringent tannin. The comfrey contains allantoin which, apart from being antibacterial, is said to be an excellent healing agent, promoting granulation tissue and being able to promote the healing of fractures.

Armelagos, (1969) and Cook et al., (1989) reported the postulated intake of tetracycline in these populations possibly had a protective effect because the rate of infectious diseases documented

in the Sudanese Nubian population was low, and no traces of bone infection were detected in the samples from the Dakhleh Oasis.

Bassett et al., (1980) reported that the traces of tetracycline, for example, have been found in human skeletal remains from ancient Sudanese Nubia dating back to 350–550 CE.

Forrest RD et al., (1982) reported that in many ancient cultures, Greeks and Egyptians used specially selected mold and plant materials to treat infections.

Cook et al., (1989) suggested the distribution of tetracycline in bones is only explicable after exposure to tetracycline-containing materials in the diet of these ancient people. Another example of ancient antibiotic exposure is from a histological study of samples taken from the femoral midshafts of the late Roman period skeletons from the Dakhleh Oasis, Egypt. These samples showed discrete fluorochrome labeling consistent with the presence of tetracycline in the diet at that time.

Keller and Stiehm, (2000) suggested that the antibiotic treatment choices for already existing or emerging hard-to-treat multidrug-resistant bacterial infections are limited, resulting in high morbidity and mortality rates. Although there are some potential alternatives to antibiotic treatment such as passive immunization.

MacNeil et al., (2001) reported that accessing the uncultivated portion of microbiota through the metagenomic approach.

Chopra et al., (2002) reported that this strategy of modification of the existing antimicrobials was initiated (and successfully implemented) during the period, when the rate of discovery of novel drug classes suddenly dropped in the 1970s, and the growing resistance problem enforced researchers to look into the possible modification of the existing arsenal that could confer improved activity, less sensitivity toward resistance mechanisms, and less toxicity.

Rappe and Giovannoni (2003) and Schloss and Handelsman (2004) reported that after yielding diminished returns, the platform was abandoned. Still, many experts now advocate for a revival of this platform, as synthetic approaches have been unable to replace the success of natural product drug discovery. Furthermore, soil and marine environments may still be promising untapped sources for antibiotic compounds. Metagenomic analyses have shown that 99% of bacteria from soil and marine samples are "uncultured," meaning they do not grow under normal laboratory conditions.

Levin and Bull (2004) and Monk et al., (2010) reported that phage therapy can be performed.

Pillai et al., (2005) suggested that use of combination regimens for the prevention or reversal of antibiotic resistance is already a common strategy, but is typically performed with the combination of two antibiotics, rather than an antibiotic plus antibiotic adjuvant. The goal with co-administration of two antibiotics is to achieve synergistic activity, or when the sum of in vitro activity of two drugs is greater than with either agent alone.

Schatz et al., (2005) suggested that building on the work of Fleming, microbiologist Selman Waksman sought to find more sources of antibiotic-producing microbes from soil. His approach involved the screening of soil-derived bacteria (mostly Actinomycetes spp.) against susceptible test organisms and evaluating zones of inhibited growth on an overlay plate.
Hancock and Sahl (2006) reported that borrowing antimicrobial peptides and compounds from animals and plants.

Makovitzki et al., (2006) suggested that mimicking the natural lipopeptides of bacteria and fungi.

Van Epps HL (2006) reported that in 1939, coinciding with the start of World War II, Dubos had reported the discovery of the first naturally derived antibiotic, tyrothricin, a compound of 20% gramicidin and 80% tyrocidine, from Bacillus brevis. It was one of the first commercially manufactured antibiotics and was very effective in treating wounds and ulcers during World War II.

Clatworthy (2007) suggested that the comparison of metabolic pathways in commensal and pathogenic bacteria and drugs targeting the pathogenic traits may help to identify the novel drug/target combinations in pathogens and thus the novel paradigm of antimicrobial therapy as targeting virulence.

Payne et al., (2007), Thomas et al., (2017) suggested that while this news was initially encouraging, further investigation revealed a more sobering outlook. First, only approximately 20-30% will translate to a marketable product, given the success rates of an antibiotic moving through development.
Calderon CB and Sabundayo BP (2007) synthetic antibiotic chemotherapy as a science and development of antibacterials began in Germany with Paul Ehrlich in the late 1880s.

Bosch F et al., (2008) and Wright PM (2014) reported that the first sulfonamide and the first systemically active antibacterial drug, Prontosil, was developed by a research team led by Gerhard Domagk in 1932 or 1933 at the Bayer Laboratories of the IG Farben conglomerate in Germany, for which Domagk received the 1939 Nobel Prize in Physiology or

Medicine. Sulfanilamide, the active drug of Prontosil, was not patentable as it had already been in use in the dye industry for some years. Prontosil had a relatively broad effect against Gram-positive cocci, but not against enterobacteria. Research was stimulated apace by its success. The discovery and development of this sulfonamide drug opened the era of antibacterials.

Robertson et al., (2008) reported that the use of the complete synthetic route pioneered during the early years of the antibiotic era. The latter approach becomes dominant in the search for drugs aimed at the newly identified targets in a bacterial cell. Other strategies may include drugs engineered to possess dual target activities, such as a rifamycin–quinolone hybrid antibiotic.

Martínez (2008) and Wright (2010) reported that the history of massive production and use of antibiotics by humans is very short on the evolutionary scale, but even this short-term (albeit large-scale) practice has produced very interesting results demonstrating the interminable adaptive capabilities of bacteria, which allow them to withstand massive antibiotic insults and generate some formidable examples of hard-to-treat infections that we call "superbugs." Although no broad baseline data collection on antibiotic resistance genes has been performed at the onset of antibiotic use, recent studies suggest that the most likely source of these genes is the environmental antibiotic resistome.

Boucher et al., (2009), Thomas et al., (2017) reported that most of these antibiotics do not have a novel mechanism of action but are instead modifications of existing antibiotic classes and only 38% of the antibiotics in development are expected to be active against ESKAPE pathogens, which have been regarded as high priority for more than a decade.

Falkinham et al., (2009) reported that tetracyclines are unique among antibiotics in that they are strong chelators and are incorporated into the hydroxyapatite mineral portion of bones as well as tooth enamel and thus provide permanent markers of metabolically active areas during tetracycline exposure. Traces of exposure to other antibiotics in ancient populations are much more difficult to detect, and only surviving customs and anecdotal evidence may point to these occurrences.

Cui and Su, (2009) suggested that another possibility of exposure to antimicrobials in the pre-antibiotic era could be through the remedies used for millennia in traditional/alternative medicine, in particular in traditional Chinese medicine (TCM). The best-known example is the discovery of a potent anti-malarial drug, qinghaosu (artemisinin), which was extracted in the

1970s from *Artemisia* plants, used by Chinese herbalists for thousands of years as a remedy for many illnesses.

Williams KJ (2009) suggested that this heralded the era of antibacterial treatment that was begun with the discovery of a series of arsenic-derived synthetic antibiotics by both Alfred Bertheim and Ehrlich in 1907.

Lu and Collins (2009) reported that combination therapy coupling antibiotics with an antibiotic-enhancing phage, for example, has demonstrated the potential to be a promising antimicrobial intervention.

Nichols et al., (2010) suggested to using a method similar to the Waksman platform, but with a modified technique for isolating and growing uncultured bacteria.
French (2010), Lushniak (2014), Rossolini et al., (2014), Brown and Wright (2016), Martens and Demain (2017) reported that the emergence and spread of resistant bacteria, coupled with the paucity of new antibiotics, has evolved into a global health crisis.
Hughes and Fenical (2010) and Rahman et al., (2010) also suggested that some possible approaches to tap the novel antimicrobial diversity is the exploration of ecological niches other than soil, such as the marine environment.

Kohanski et al., (2010) reported that the intervention strategies aimed not only at the targets but rather at biological networks may help to create new antibacterial therapies.

Gartin et al., (2010) reported that the situation is different in countries where the sales of antibiotics are inadequately regulated, and antibiotics are available without prescription. In the absence of regulation, the personal decisions on antibiotic purchase and use are governed by cultural and economic reasons.

Simoens et al., (2012), Kwok and Koenigbauer (2015) Established with the intention to streamline antibiotic development, LPAD allows faster access to antibiotics for patients with serious or life-threatening bacterial infections in which no appropriate treatment options exist. The drug's safety and effectiveness can be studied in significantly smaller, more rapid, and less expensive clinical trials using this mechanism, which is similar to the orphan drug approval process.
Lewis (2013) and Lyddiard et al., (2016) suggested that though penicillin was highly effective and in frequent use at the time, its antibacterial activity was primarily limited to Gram-positive bacteria. Streptomycin, the first of the aminoglycoside antibiotic class, was also the first drug

with activity against Mycobacterium tuberculosis. After the successful launch of streptomycin, the Waksman platform quickly became the quintessential tool for antibiotic discovery at the time, and ultimately the most successful and widely adopted antibiotic discovery platform to date. Discovery of other antibiotics occurred shortly thereafter, and continued over the next 20 years, famously referred to as the 'golden age' of antibiotics.

Walsh and Wencewicz (2014) reported that during the golden age of antibiotics, from 1940 through 1960s, the antibiotic development pipeline flourished.

Wright (2014) and Lewis (2017) reported that the rapidity of new antibiotics discovered at the time appeared to be outpacing the spread of antibiotic resistance. However, the majority of antibiotics developed during this period were through natural product discovery, a few synthetic antibiotic classes or "scaffolds" were also developed with success and remain in used today like fluoroquinolones, sulfonamides etc.

Ling et al., (2015) and Fiers et al., (2017) reported that the investigators unveiled the discovery of a new antibiotic, teixobactin.

Tan et al., (2015) and Pathak A et al., (2020) reported that in 1928, Sir Alexander Fleming postulated the existence of penicillin, a molecule produced by certain moulds that kills or stops the growth of certain kinds of bacteria. Fleming was working on a culture of disease-causing bacteria when he noticed the spores of a green mold, Penicillium rubens, in one of his culture plates. He observed that the presence of the mould killed or prevented the growth of the bacteria. Fleming postulated that the mould must secrete an antibacterial substance, which he named penicillin in 1928.

Stone (2015), Sinha and Kesselheim (2016) suggested that in addition to expedited approval programs, another pathway, the Limited Population Antimicrobial Drug (LPAD) pathway, was signed into law in 2016 as a provision to the 21st Century Cures Act.

Katz and Baltz (2016). This drug discovery platform was a success for approximately 20 years. Unfortunately, mining through soil microbes eventually led to frequent re-isolation or rediscovery of known compounds.

Sojib Bin Zaaman et al., (2017) has reported that in late 19$^{th}$ century, treatment for infectious disease were primarily based on traditional beliefs of medicine. To treat infectious disease, many mixtures with antimicrobial properties were used described over 2000 years ago.

Luepke and Mohr (2017), Luepke et al., (2017) reported that industry sponsors, regulatory agencies, and organizations at the national and international level are taking action to overcome hurdles that led to a dry antibiotic pipeline. Just as the Oxford group discovered during their

wartime efforts of mass-producing penicillin, public-private collaborations are critical to successfully revive the antibiotic pipeline and bring new antibiotics to patients in need.

Evdokia Syranidou et al., (2022) Microplastics develop distinct interactions with antibiotics. Hydrophobic, Van der Waals forces, electrostatic and π-π interactions rule these interactions. Microplastics selectively enrich antibiotic resistant genes within the biofilm.

# 4. METHODOLOGY

## COLLECTION OF SAMPLES

The soil sample were collected from different areas of the garden of St. Mary's College (Autonomous), Tuticorin, Tamil Nadu, India.

Soil sample was collected in such a way to get the soil of crust and depth of at least 10 cm with the help of sterile spatula and placed in sterile petri plate for transportation to laboratory.

The soil sample was sieved to extract fine soil particles which measured about 10 g.

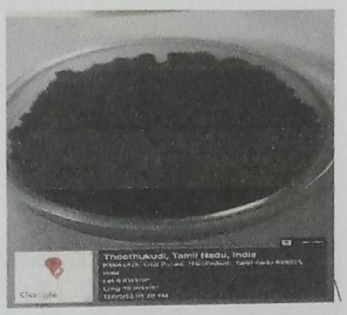


**Plate:4.1 Soil sample**

## PREPARATION OF SOIL SAMPLE

One gram of soil was weighed and mixed in 10 ml of sterile distilled water in a conical flask. The conical flask was mixed thoroughly by vigorous shaking for few minutes and allow the sediment to settle. This mixture gives 1:10 dilution and therefore this solution is stock solution. One ml of this solution was taken and transferred to the test tube containing 9 ml of sterile distilled water to give 1:100 that is $10^{-2}$. Then this process was repeated till 1:10000 dilution.

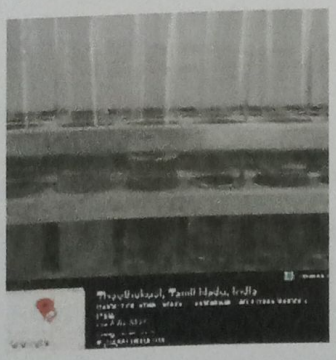


**Plate:4.2 Serial dilution**

## ISOLATION OF MICROORGANISMS

The media was prepared for isolation of bacteria and appropriate media is nutrient agar. An amount of 100 ml of distilled water was added to the conical flask and 2.8 g of nutrient agar powder was dissolved in it. The conical flask was sterilized using autoclave method at 121°C for 15 min and allowed to cool down for little while. Then aseptically the media was poured into the sterile petri plates and allowed to solidify.

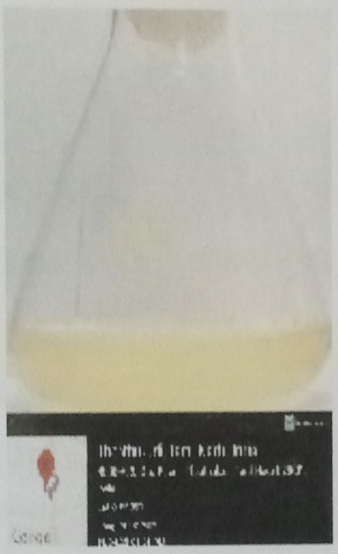


**Plate:4.3 Conical flask with media**

## INOCULATION OF SAMPLE

0.1 ml of soil inoculum from each dilution above prepared was inoculated separately onto labelled petri plates in duplicate for bacterial species isolation and the plates with inoculum were spread evenly with the help of sterile glass rod. This procedure is also called crowded plate technique. The petri plates were inverted and placed at 37°C for 24 h in the incubator.

## SUB-CULTURING OF MICROORGANISMS

Bacterial colonies with clear margin or zone of clearance were picked and sub-cultured on fresh NA or NB using sterile inoculation loop, streak the petri plates or the test tube slants can be used in laminar air flow to purify the isolates and incubate at 37°C for 24 h.

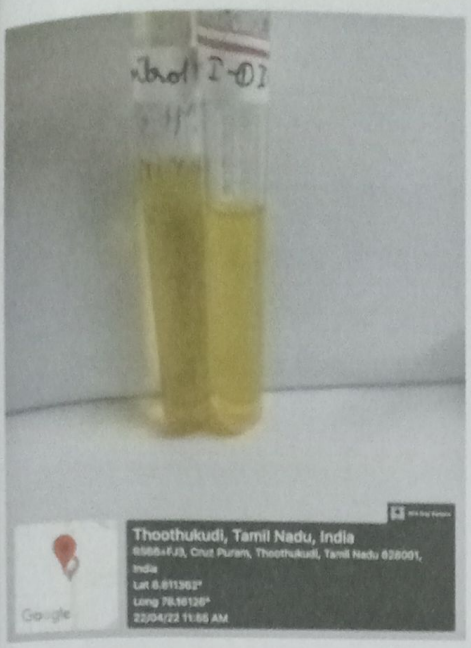



Plate:4.4 Isolates 1 and 2 subculture in NB

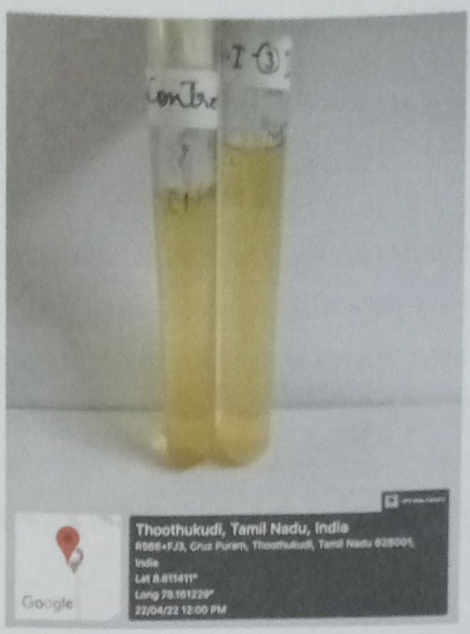



Plate:4.5 Isolates 3 and 4 subcluture in NB

## PRESERVATION OF ISOLATES

Total of four isolates were isolated which were preserved in the slants of nutrient agar by keeping them into the refrigerator at 4°C and maintained for longer period of time by serial sub-culturing.

Plate:4.6 Subculturing of NA slants for preservation

## IDENTIFICATION OF THE ISOLAES OR CHARACTERIZATION OF ISOLATED MICROORGANISMS

### (1) COLONY CHARACTERIZATION

Colony morphology is the visual culture characteristics of a bacterial colony on an agar plate. Observing colony morphology is an important skill used in the microbiology laboratory to identify microorganisms. Colonies need to be well isolated from other colonies to observe the characteristic shape, size, color, surface appearance, and texture. Another important characteristic of a bacterial colony is hemolysis. Hemolysis is the result of the lysis of the blood cells that are a component of Blood Agar.

### (2) STAINING CHARACTERIZATION

Cell staining is important in the diagnosis of microorganisms because bacteria can be identified by the color differentiation of stains (dyes). Microscopic examination of stained cell samples allows examination of the size, shape, and arrangement of organelles, as well as external appendages such as the whip-like flagella, which are the cell's organs of motion. When sample cells are stained to show their chemical composition, it is called differential staining.

Gram staining: A smear of selected strain was prepared on a clean glass slide and the smear was allowed to air-dry and then heat-fixed. The heat-fixed smear was flooded with crystal violet and after one minute, it was washed with water and flooded with mordant Gram's iodine. The smear was decolorized with 95 % ethyl alcohol, washed with water and then counter-stained with safranin for 45 sec. After washing with water, the smear was dried with tissue paper and examined under oil immersion.

### (3) BIOCHEMICAL CHARACTERIZATION

Biochemical tests are the tests used for the identification of bacteria species based on the differences in the biochemical activities of different bacteria. These differences in carbohydrate metabolism, protein metabolism, fat metabolism, production of certain enzymes, ability to utilize a particular compound etc. help them to be identified by the biochemical tests.

## (a) IMVIC TEST

The IMViC tests are a group of individual tests used in microbiology lab testing to identify an organism in the coliform group.

### (i) INDOLE TEST

Indole test detects the production of indole from the amino acid tryptophan. One percent tryptophan broth in test tube glucose was inoculated with bacteria colony. After incubation period of 37°C for 48 hours, one milliliter (1ml) of Kovac's reagent was added to the broth. The test tube was shaken gently and then allowed to stand for 20 minutes. The formation of red coloration at the top layer indicated positive and yellow coloration indicates negative test.

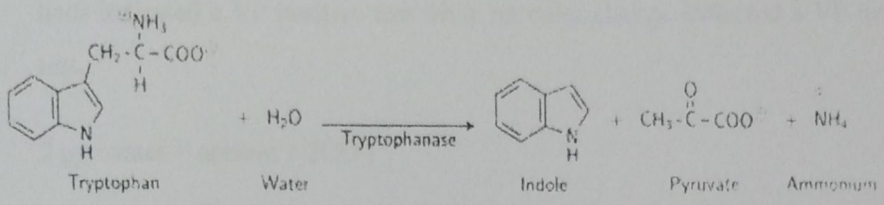



**Figure:4.1 Indole Reaction**

### (ii) METHYL RED TEST

Methyl red (MR) is a pH indicator to determine whether the bacterium carries out mixed acid fermentation. Five milliliters (5ml) of MR VP broth were inoculated with the test organism and incubated for 48-72 hours at 37°C after which, one milliliter (1ml) of broth was transferred into a small tube. Small amount (2-3 drops) of methyl red was added. A red color formation on addition of the indicator signified a positive methyl red test while yellow color signified a negative test. To the rest of the broth in the original tube 4-5 drops of 4% KOH were added followed by 5% naphthol in ethanol. The test tube (sealed with cotton plug) was shaken and placed in a sloping position.

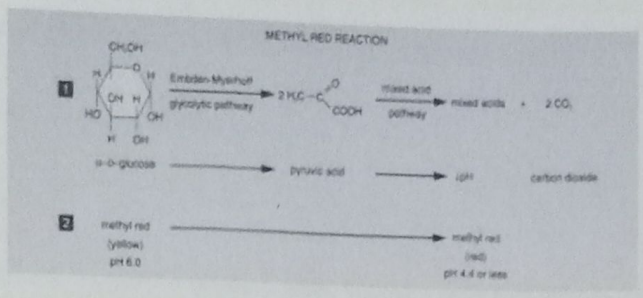



**Figure:4.2 Methyl Red Reaction**

### (iii) VOGAS- PROSKAUER TEST

VP (Voges-Proskauer) used to detect the production of acetoin. The development of red color started to appear from the liquid-air interface within 1 hour indicated a VP positive test while no color change indicated a VP negative test.

2 pyruvates = acetoin + $2CO_2$

acetoin + NADH + $H^+$ = 2,3-butanediol + $NAD^+$

### (iv) CITRATE TEST

This test determines whether or not the bacterium can use sodium citrate as a sole source of carbon. This is carried out by inoculating the test organism in test tube containing Simon's citrate medium and this was incubated for 24 hours to 72 hours. The development of deep blue color after incubation indicated a positive result.

Citrate → Oxaloacetic acid → Pyruvic acid + $CO_2$

Excess of sodium from sodium citrate + $CO_2$ + $H_2O$ → $Na_2CO_3$

## (b) CATALASE TEST

Catalase test used to detect the presence of catalase which converts hydrogen peroxide to water and oxygen. This test was carried out by putting a drop of hydrogen peroxide on a clean slide. With the edge of another slide, a colony of the microorganism

was picked and allowed to contact to hydrogen peroxide. Presence of bubbles indicates positive reaction while absence of bubble indicates negative reaction.

$$H_2O_2 \xrightarrow[\text{catalase}]{} H_2O + O_2$$

## (c) UREASE TEST

Urea medium, whether a broth or agar, contains urea and the phenol red as a pH indicator. Many organisms, especially those that cause urinary tract infections, produce the urease enzyme, which catalyzes the splitting of urea in the presence of water to release two molecules of ammonia and carbon dioxide. The ammonia combines with the carbon dioxide and water to form ammonium carbonate, which turns the medium alkaline, turning the indicator from its original orange-yellow color to bright pink. This test is performed as part of the identification of several genera and species of the *Enterobacteriaceae* family.

$$\underset{\text{Urea}}{(NH_2)_2CO} + 2\,H_2O \xrightarrow{\text{Urease}} \underset{\text{Carbon dioxide}}{CO_2} + \underset{\text{Water}}{H_2O} + \underset{\text{Ammonia}}{2\,NH_3}$$

**Figure:4.3 Urease reaction**

## ISOLATION OF TEST STRAINS

Three bacterial strains, namely Staphylococcus species, Escherichia coli, and Klebsiella species used in this study were isolated on selective media from skin, dirty water and wound sample collected from student, sewage and wounded patient respectively. Biochemical tests were performed to confirm the identity of these strains. Two fungi were isolated, namely Aspergillus and Mucor both from bread which was allowed to spoil on purpose and identified through microscopic analysis of spores and fungal hyphae. The purpose for selecting the above bacteria and fungi was because all of them are potential opportunistic pathogens commonly associated with bacterial and fungal infections.

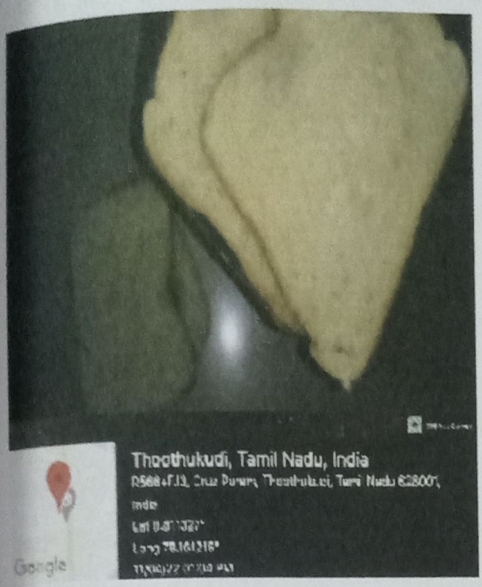



Figure:4.4 Contaminated bread

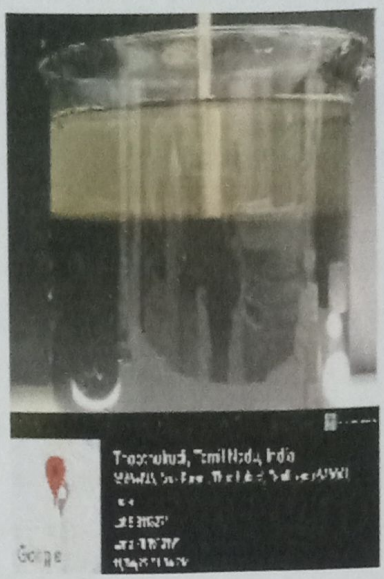


Figure:4.5 Beaker of sewage water

## ANTIMICROBIAL ACTIVITY BY AGAR DIFFUSION METHOD-SECONDARY SCREENING

Agar well diffusion method is widely used to evaluate the antimicrobial activity of plants or microbial extract that to check the culture for the production of antimicrobial metabolites. Similarly, to the agar plate surface is inoculated by spreading a volume of the microbial inoculum over the entire agar surface. For antibiotic production, Muller Hinter Agar (MHA) was prepared by adding 3.8 g of MHA media in 100 ml of distilled water and autoclaved at 121°C for 20 min. After sterilization, the media was cooled and poured into sterile petri plates and kept in incubation 37°C for 24 h to check its sterility. 24 h fresh cultures in NB of the test organisms, that are bacterial species, Staphylococcus, E. coli, Klebsiella, fungal species Aspergillus and mucor were centrifuged and the supernatant was used. After incubation, sterilized cotton buds were dipped in the Eppendorf tube with the supernatant and swabbed on the MHA plates. Wells were made on MHA plates using sterile borer. The Isolates 1, 2, 3 and 4 were also centrifuged and the supernatant were poured into specific labelled MHA plates with positive control which is Amoxicillin for bacterial positive control and Fluconazole for fungal positive control. The petri plates were incubated at 37°C for 24 h for observing zone of inhibition.

## MINIMUM INHIBITORY CONCENTRATION

The minimum inhibitory concentration (MIC) is the lowest concentration of a chemical, usually a drug, which prevents visible growth of a bacterium or bacteria. MIC depends on the microorganism, the affected human being (in vivo only), and the antibiotic itself. It is often expressed in micrograms per milliliter (μg/mL) or milligrams per liter (mg/L). There are three mains reagents necessary to run this assay: the media, an antimicrobial agent, and the microbe being tested. MIC test was performed when the cultures entered stationary phase as evidenced by growth curve. The culture broth that is MHA broth was centrifuged at high speed for 15 min. The supernatant was filtered and used for the MIC test. In a series of test tube having test strains (E. coli and S sps), the centrifuged broth was added with different concentrations and incubated at 37o C for 24 h. The test tubes were then observed for turbidity. The lowest concentration at which turbidity is not seen is taken as MIC.

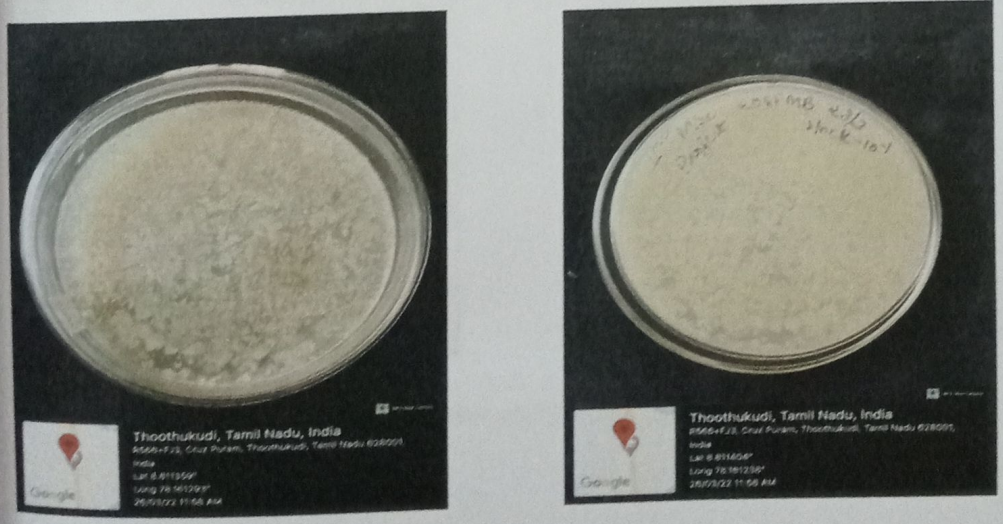
## 5. RESULT AND DISCUSSION

In this study, the isolate producing antibiotic is isolated using the methodology.

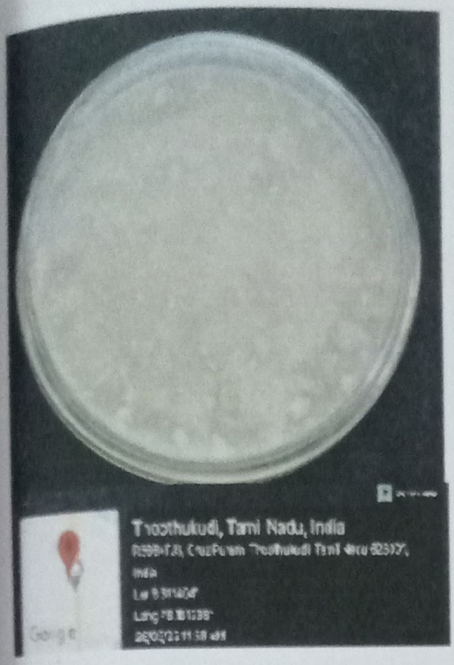
### ISOLATION OF MICROORGANISMS FROM SOIL BY CROWDED PLATE TECHNIQUE:

Plates were observed for the presence of any colony with a clear zone around it that is Zone of Inhibition. Plates approximately 300-400 colonies were selected that showed crowd but well demarcated colonies. Four different types of colonies from dilution 1:10 and 1:10000 were found to show clear Zone if Inhibition around them.


**Plates:5.1 Isolation of microorganisms from soil by crowded plate technique**

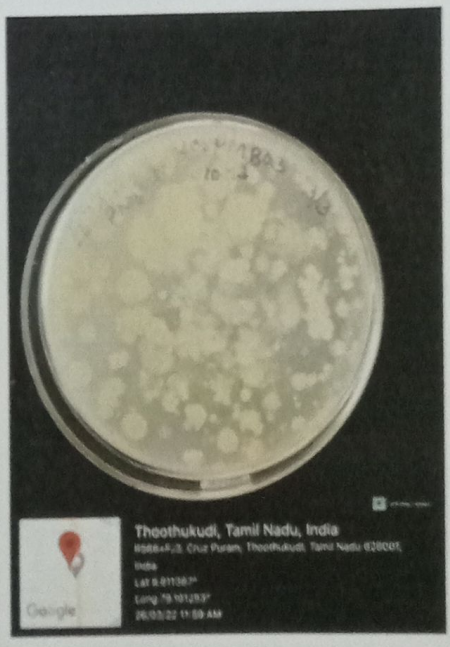



**Plates:5.2 Petri plates from serially diluted soil sample i.e. $10^{-1}$**

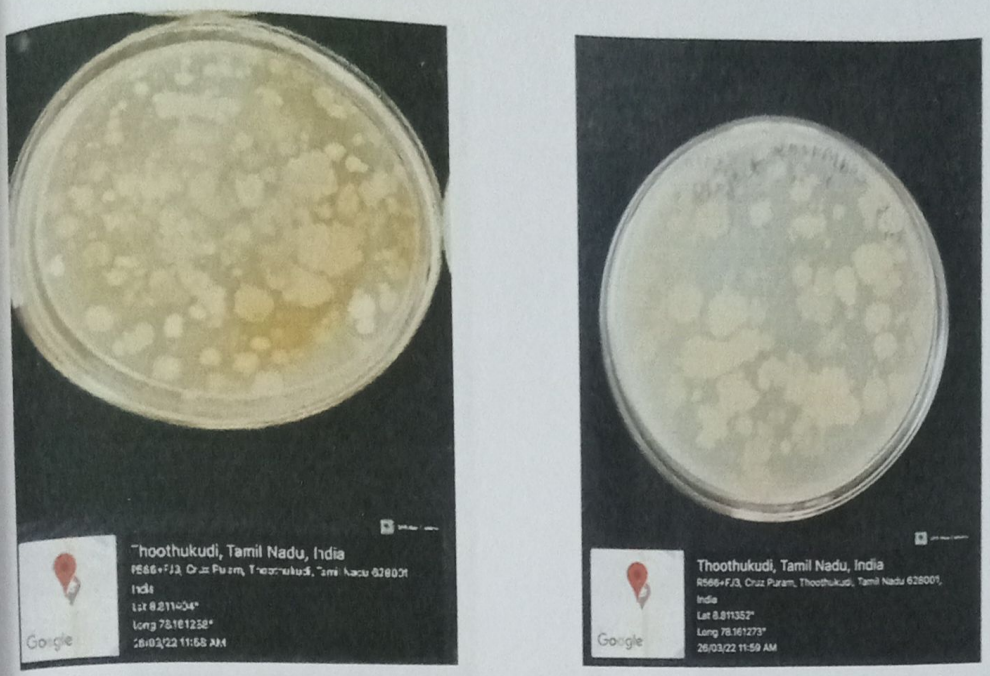




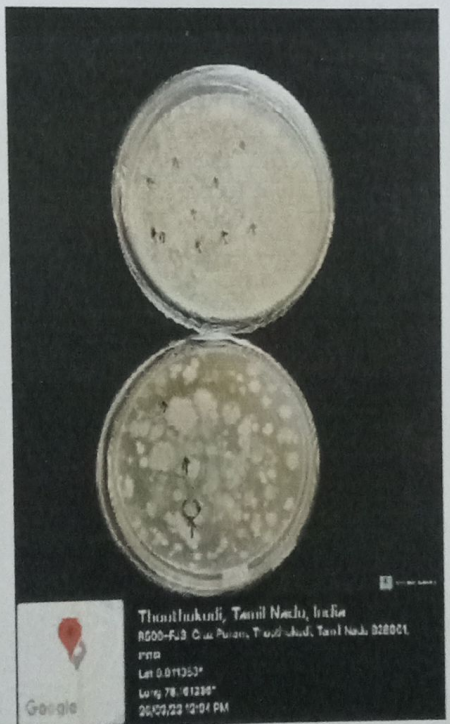
**Plates:5.3 Petri plates from serially diluted soil sample i.e. $10^{-2}$**

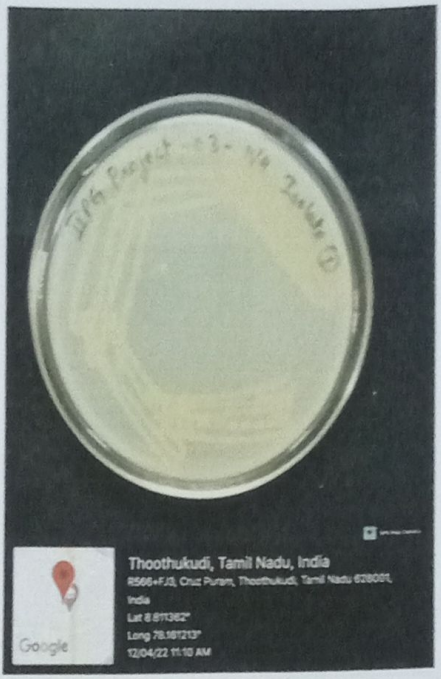

**Plates:5.4 Petri plates from serially diluted soil sample i.e. $10^{-3}$**




**Plates:5.5 Petri plates from serially diluted soil sample i.e. $10^{-4}$**



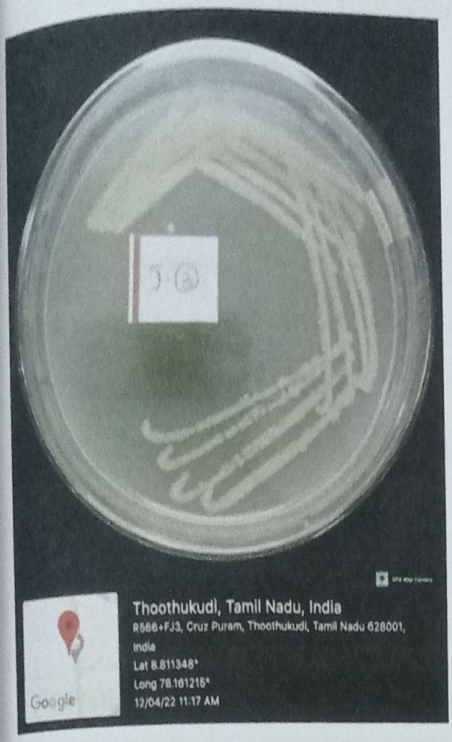

**Plates:5.6 Isolates were selected from these plates**


**Plates:5.7 Isolate 1**

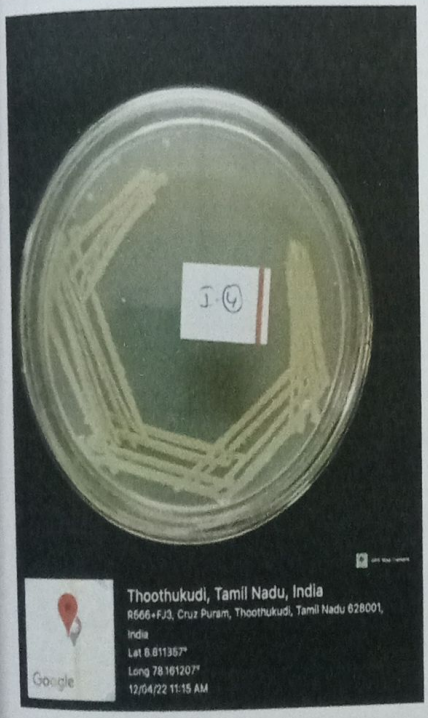


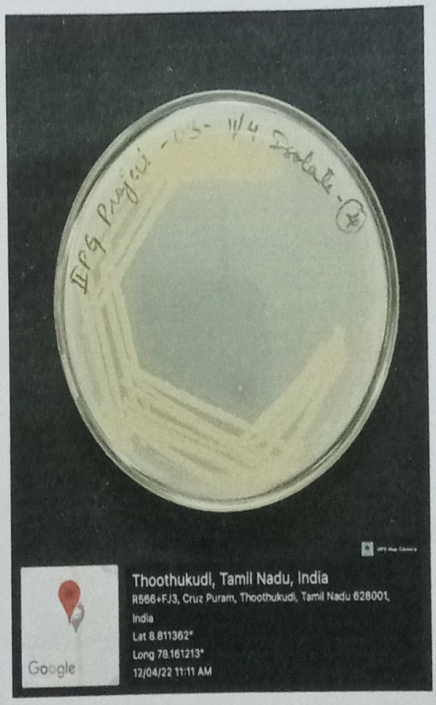
**Plates:5.8 Isolate 2**





**Plates:5.9 Isolate 3**




**Plates:5.10 Isolate 4**

## IDENTIFICATION OF THE ISOLAES OR CHARACTERIZATION OF ISOLATED MICROORGANISMS

### COLONY CHARACTERIZATION

The isolate 1 shows small size colonies which are circular in shape with white to brown coloration. The edges are entire and the colonies are elevated convex. The isolate 2 shows medium colonies which are round in shape with whitish coloration. The edges are entire and elevated slightly convex, this colony features represent *Bacillus* sp strain.

The isolate 3 shows small in size which are white to creamy in color with circular and irregular. The edges are undulate and the colonies are flat. The isolate 4 shows medium colonies and round irregular shape with creamy color. Entire to undulate edges and no elevation is observed.

| Isolates codes | Size | Type or Shape | Color | Edge | Elevation |
|---|---|---|---|---|---|
| Isolate 1 | Small | Circular | White to brown | Entire | Convex |
| Isolate 2 | Medium | Round | Whitish | Entire | Slightly convex |
| Isolate 3 | Small | Circular and irregular | White to creamy | Undulate | Flat |
| Isolate 4 | Medium | Round and irregular | Creamy | Entire to undulate | Flat |

**Table:5.1 Colony characterization**

## STAINING CHARACTERIZATION

The Gram's staining showed that the isolate 1,3 and 4 are G-ve. The Gram's staining of isolate 2 showed that it is G+ve again speculating that it can be *Bacillus sp* strain.

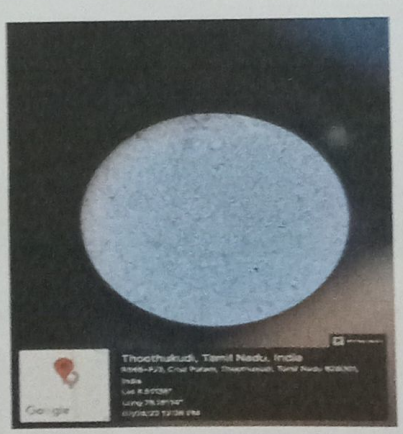
| ISOLATES | GRAM'S STAINING |
|---|---|
| ISOLATE 1 | -VE |
| ISOLATE 2 | +VE |
| ISOLATE 3 | -VE |
| ISOLATE4 | -VE |

Table:5.2 Gram Staining


Plates:5.11 Gram Staining of Isolate 1


Plates:5.12 Gram Staining of Isolate 2


Plates:5.13 Gram Staining of Isolate 3




Plates:5.14 Gram Staining of Isolate 4

## ISOLATION OF TEST STRAINS

### BACTERIAL STRAINS

1) *Escherichia coli*

Sample was collected from sewage and serially diluted after which in sterile condition inoculated in EMB agar and observed for green metallic sheen. Biochemical test was performed to conform the organisms and Gram's staining was done.

| TESTS | RESULTS |
|---|---|
| INDOLE TEST | +VE |
| MR TEST | +VE |
| VP TEST | -VE |
| CITRATE TEST | +VE |
| GRAM'S STAINING | -VE |

**Table:5.3 Biochemical Test for *Escherichia coli***

**Plate:5.15 G.S of *E. coli***

**Plate:5.16 Indole test for *E. coli***

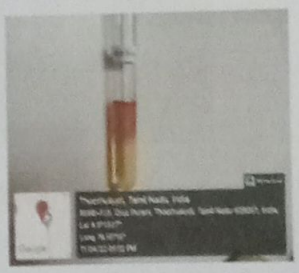


**Plate:5.17 MR test for *E. coli***

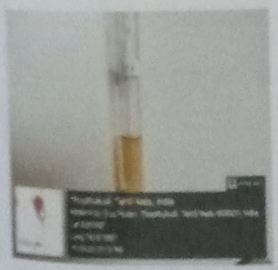


**Plate:5.18 VP test of *E. coli***

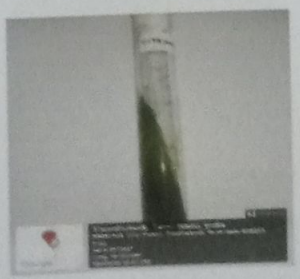


**Plate:5.19 Citrate test for *E. coli***

1) *Staphylococcus* species

Sample was collected from a wounded individual and inoculated in MSA agar and observed for discoloration of the media. To confirm biochemical test were conducted and Gram's staining was done.

| TESTS | RESULTS |
|---|---|
| INDOLE TEST | -VE |
| MR TEST | +VE |
| VP TEST | +VE |
| CITRATE TEST | +VE |
| GRAM'S STAINING | +VE |

**Table:5.4 Biochemical Test for *Staphylococcus* species**

**Plate:5.20 G.S of *S. sp***

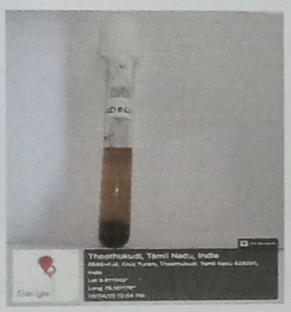


**Plate:5.21 Indole test for *S. sp***

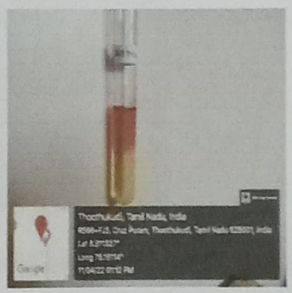


**Plate:5.22 MR test for *S.sp***

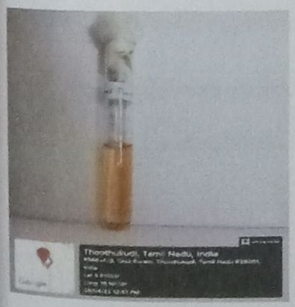


**Plate:5.23 VP test of *S. sp***

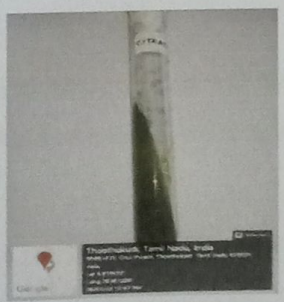


**Plate:5.24 Citrate test for *S. sp***

2) *Klebsiella* species

Sample was collected from urine and inoculated in Mac-Conkey agar. To confirm biochemical test were conducted and Gram's staining was done.

| TESTS | RESULTS |
|---|---|
| INDOLE TEST | -VE |
| MR TEST | -VE |
| VP TEST | +VE |
| CITRATE TEST | -VE |
| GRAM'S STAINING | -VE |

**Table:5.5 Biochemical Test for *Klebsiella species***


**Plate:5.25 G. S for *Klebsiella sp.***


**Plate:5.26 Biochemical test of *Klebsiella sp.***

FUNGAL STRAINS

1) *Aspergillus* species

Sample was collected from molded bread. It was confirmed by microscopic studies with Lactophenol cotton blue dye specific for fungi.


**Plate:5.27 LPCB staining for *Aspergillus sp***

2) *Mucor* species

Sample was collected from molded bread. It was confirmed by microscopic studies with Lactophenol cotton blue dye specific for fungi.

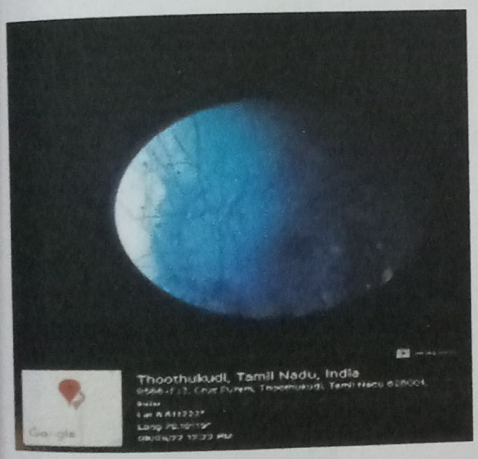



**Plate:5.28 LPCB staining for *Mucor sp***

## BIOCHEMICAL CHARACTERIZATION OF THE 4 ISOLATES

## IMVIC TEST: INDOLE TEST

All the isolates were negative to indole test means did not use tryptophan as carbon source to convert into indole.

| ISOLATES | INDOLE TEST |
|---|---|
| ISOLATE 1 | -VE |
| ISOLATE 2 | -VE |
| ISOLATE 3 | -VE |
| ISOLATE4 | -VE |

**Table:5.6 Indole test results**

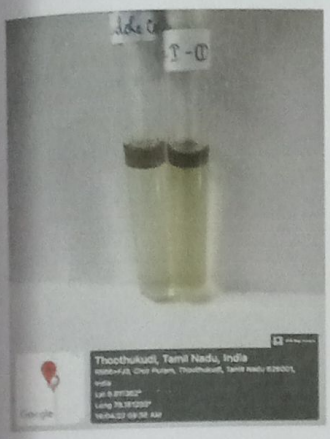



**Plate:5.29 Indole test for Isolate 1**


**Plate:5.30 Indole test for Isolate 2**


**Plate:5.31 Indole test for Isolate 3**


**Plate:5.32 Indole test for Isolate 4**

## METHYL RED TEST

All the isolates showed +ve result after addition of methyl red.

| ISOLATES | METHYL RED TEST |
|---|---|
| ISOLATE 1 | +VE |
| ISOLATE 2 | +VE |
| ISOLATE 3 | +VE |
| ISOLATE 4 | +VE |

**Table:5.7 MR test results**

**Plate:5.33 MR test for Isolate 1**

**Plate:5.34 MR test for Isolate 2**

**Plate:5.35 MR test for Isolate 3**

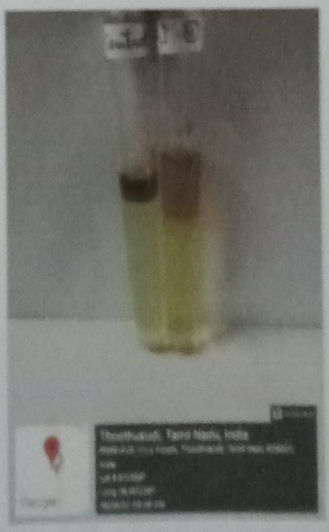


**Plate:5.36 MR test for Isolate 4**

## VOGES- PROSKAUER TEST

Voges- Proskauer test was answered +ve by only isolate 2 and isolates 1, 3 and 4 answered negative.

| ISOLATES | VOGAS- PROSKAUER TEST |
|---|---|
| ISOLATE 1 | -VE |
| ISOLATE 2 | +VE |
| ISOLATE 3 | -VE |
| ISOLATE 4 | -VE |

**Table:5.8 VP test results**


**Plate:5.37 VP test for Isolate 1**


**Plate:5.38 VP test for Isolate 2**


**Plate:5.39 VP test for Isolate 3**


**Plate:5.40 VP test for Isolate 4**

## CITRATE TEST

Isolate 1, 2 and 3 are positive for citrate test and isolate 4 shows negative result.

| ISOLATES | CITRATE TEST |
|---|---|
| ISOLATE 1 | -VE |
| ISOLATE 2 | -VE |
| ISOLATE 3 | -VE |
| ISOLATE 4 | +VE |

**Table:5.9 Citrate test results**

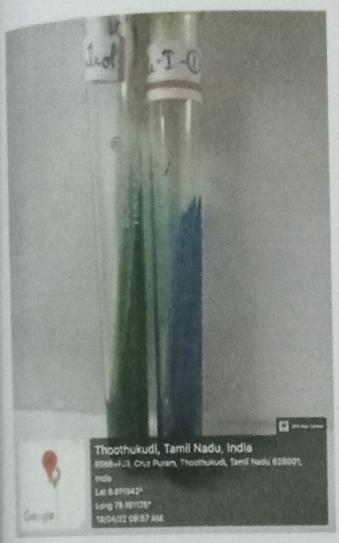



**Plate:5.41 Citrate test for Isolate 1**


**Plate:5.42 Citrate test for Isolate 2**


**Plate:5.43 Citrate test for Isolate 3**


**Plate:5.44 Citrate test for Isolate 4**

## CATALASE TEST

Catalase test was answered positive by isolate 2, 3 and 4. The negative result was observed in isolate 1.

| ISOLATES | CATALASE TEST |
|---|---|
| ISOLATE 1 | -VE |
| ISOLATE 2 | +VE |
| ISOLATE 3 | +VE |
| ISOLATE 4 | +VE |

**Table:5.10 Catalase test results**

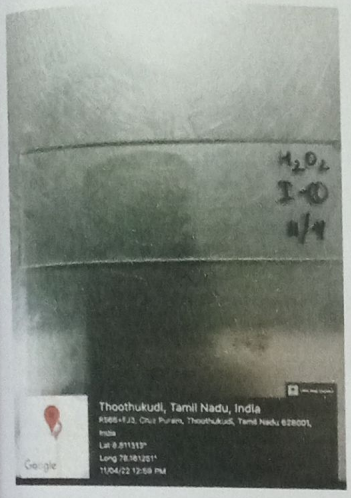



**Plate:5.45 Catalase test for Isolate 1**

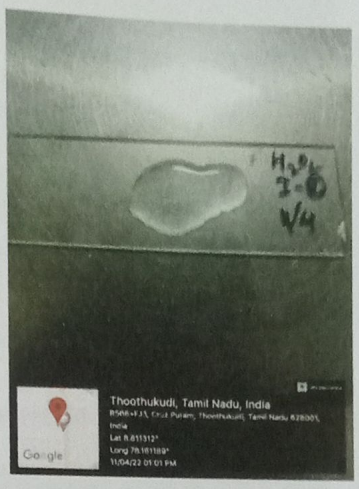



**Plate:5.46 Catalase test for Isolate 2**

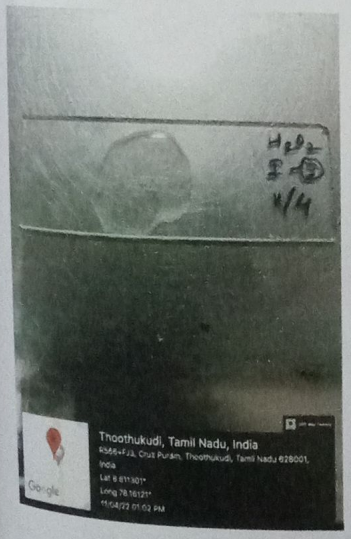



**Plate:5.47 Citrate test for Isolate 3**

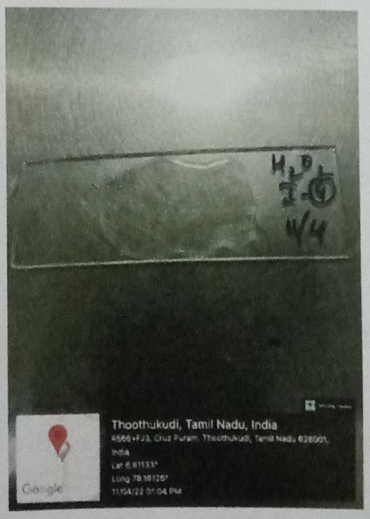



**Plate:5.48 Citrate test for Isolate 4**

## UREASE TEST

Urease test was positive for isolates 1, 2 and 3, and for isolate 4 the test was negative.

| ISOLATES | UREASE TEST |
|---|---|
| ISOLATE 1 | +VE |
| ISOLATE 2 | +VE |
| ISOLATE 3 | +VE |
| ISOLATE 4 | -VE |

**Table:5.11 Urease test results**


**Plate:5.49 Urease test for Isolate 1**


**Plate:5.50 Urease test for Isolate 2**


**Plate:5.51 Urease test for Isolate 3**


**Plate:5.52 Urease test for Isolate 4**

*Table:5.12 Biochemical results of all isolates.*

| TESTS | ISOLATE 1 | ISOLATE 2 | ISOLATE 3 | ISOLATE 4 |
|---|---|---|---|---|
| INDOLE TEST | -VE | -VE | -VE | -VE |
| METHYL RED TEST | +VE | +VE | +VE | +VE |
| VOGAS-PROSKAUER TEST | -VE | +VE | -VE | -VE |
| CITRATE TEST | +VE | +VE | +VE | -VE |
| CATALASE TEST | -VE | +VE | +VE | +VE |
| UREASE TEST | +VE | +VE | +VE | -VE |

Soil sample was collected, four bacterial colonies having zone of inhibition were picked and streaked on nutrient agar plates to get pure cultures. Out of the four isolates only one bacterial isolate according to the table which concludes from the biochemical tests performed that, Isolate 2 could be of genus *Bacillus*. The major aim of current research was to identify and characterize bacterial strains isolated from soil that has the ability of producing natural antibiotic.

## ANTIMICROBIAL ACTIVITY BY AGAR DIFFUSION METHOD-SECONDARY SCREENING

Sample that is Isolate 2 was subjected to agar well diffusion assay, using *Escherichia coli, Staphylococcus species, Klebsiella species, Aspergillus species* and *Mucor species* as test organisms. Antimicrobial activity was measured in terms of zone of inhibition. The incubated samples were evaluated and optimum antimicrobial activity of inoculum of Isolate 2 that is *Bacillus species* was ensured at 48 h with control Amoxicillin. Amoxicillin was added for only bacterial plates and for fungi plates Fluconazole was added to provide positive result that is zone of inhibition.

Amoxicillin is a penicillin antibiotic. It is used to treat bacterial infections, such as chest infections (including pneumonia) and dental abscesses. Fluconazole is an antifungal medication used for a number of fungal infections. This includes candidiasis, blastomycosis, coccidiodomycosis, cryptococcosis, histoplasmosis, dermatophytosis, and pityriasis versicolor.

Plate:5.53 Isolate 2 *against*

*Escherichia coli*

Plate:5.54 Isolate 2 *against*

*Staphylococcus species*

Plate:5.55 Isolate 2 *against*

*Klebsiella species*

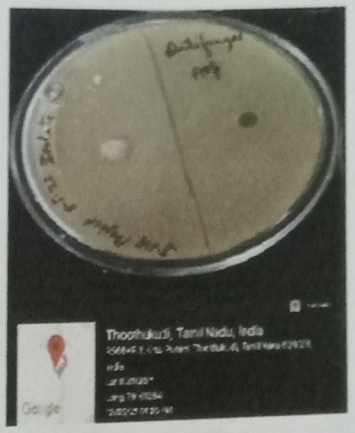


Plate:5.56 Isolate 2 *against*

*Aspergillus species*

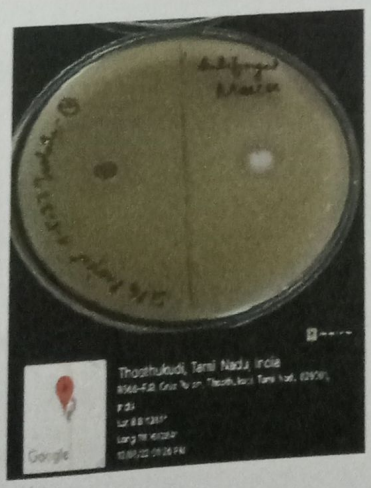


Plate:5.57 Isolate 2 *against*

*Mucor species*

**Table:5.13 Antibacterial activity of the Isolate 2**

| Test Organism | Zone of inhibition for Positive Control (in mm) | Zone of inhibition for Isolate 2 (in mm) |
|---|---|---|
| *Escherichia coli* | 4mm | 4.5mm |
| *Staphylococcus species* | 4mm | 5.5mm |
| *Klebsiella species* | 4mm | 3.5mm |
| *Aspergillus species* | 3mm | No Zone of Inhibition |
| *Mucor species* | 3mm | No Zone of Inhibition |

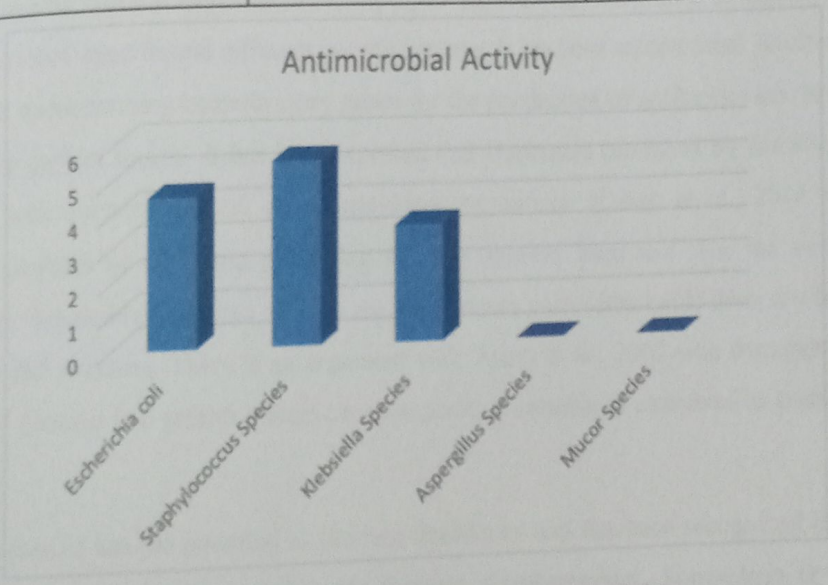



**Figure:5.1 Graph for Antimicrobial Activity**

Observation showed that against only bacterial strains the zone of inhibition was present. The growth against *Escherichia coli* little more than positive control amoxicillin and for *Staphylococcus species* showed most zone of inhibition. Against *Klebsiella species* the zone of inhibition is less than control. The fungus showed growth against the Isolate 2 that is no zone of inhibition was observed.

*Bacillus* metabolites showed activity against *Staphylococcus aureus* measured as zone of inhibition of 13.8mm in Sonia Sethi et al., 2013. Anhar Al-Turk et al., 2020 suggested that

Bacillus metabolites showed activity against *Staphylococcus aureus* measured as zone of inhibition of 10.33 and for *Staphylococcus pneumonia* measured as zone of inhibition of 13.33mm. And for *Escherichia coli* no zone of inhibition was observed.

The *Bacillus species* are known for the synthesis of secondary metabolite with remarkable diversity both in structure and function. (SiloSuh et al., 1994). For instance, cerecin7, Tochicin, Thuricin 7, thuricin439 and entomocidus 9 (Cherif et al.,2003; Cherif et al.,2001) and few may be ribosomal in origin including subtilin (Zhang et al.,2004), sublancin (Paik et al.,1998), subtilosin A (Babasaki et al., 1985), and TasA (Stover et al.,1999) polymyxin, difficidin, subtilin, mycobacillin, bacitracin, barnase, etc. Polymyxins are anti-Gram-negative; difficidin is broad spectrum. The antibiotic produced by the presently isolated strains needs to be further characterized by HPLC, NMR, etc., to pinpoint its nature and properties.

On agar media cultural characteristics displayed by bacteria, were used to identify bacteria because of their specific and different growth patterns. It has been reported that *Bacillus species* and other spore forming bacteria carry genes for the production of antibiotics and breakdown of diverse carbon source. It has been reported that Bacitracin produced by *Bacillus species* inhibits both *Escherichia coli* and *Staphylococcus aureus*. Hassan et al., 2014 identified fourteen isolates of antibiotic producing *Bacillus species* from soil. For the synthesis of secondary metabolites *Bacillus species* are well known with remarkable diversity both in its function and structure. There is an argument with Aslim et al., 2002 who documented those strains of *Bacillus* had greater effects on gram positive bacteria as compared to gram negative bacteria.

*Bacillus subtilis* has the potential to produce antibiotics and has been recognized for past 50 years. *Bacillus subtilis* is an endospore forming rhizobacterium. Sonenshein et al., 2002 collected several wild type *Bacillus subtilis*, having the potential to produce more than two dozen of antibiotics. *Bacillus subtilis* C126 strain from sugar cane fermentation have the potential to produce polypeptide antibiotic, Bacitracin. Production of Bacitracin by *Bacillus subtilis* is a pH dependent which gave maximum production at pH of 7.8 - 8. Strains of *Bacillus cereus* from a soil sample have the ability to produce Bacteriocin and was active against most gram positive but not against gram negative bacteria. M15 strain of *Bacillus cereus* possesses inhibitory effect against both gram positive and gram-negative bacteria. *Bacilli* are predominant soil bacteria widely used in industrial applications, particularly antibiotics production having medically, agriculturally and veterinary importance. *Bacillus species*

preferred hosts for the production of many improved and new products used in genomic and proteomics. To enhance the yield of Bacitracin it is possible to clone and amplify the gene coding for some key enzymes in the biosynthetic pathways of Bacitracin.

According to Prescott et al. spore forming bacteria and other members of the *Bacillus* genus possess genes for the catabolism of diverse carbon source and antibiotic synthesis. Muaz and Shahida, in research discovered the production of bacitracin and subtilin by *Bacillus sp.* Prescott et al., reported that Bacitracin produced by *Bacillus sp.* inhibits *Escherichia coli* and *Staphylococcus aureus* which collaborated the result in the present study.

*Bacillus lentus* and *Bacillus alvei* also shows antibacterial activity against *Staphylococcus aureus*. *Bacillus pumillus* only show slight zone of inhibition on *Proteus spp* while it is inactive against others was reported by Sonia Sethi et al., 2013.

## MINIMUM INHIBITION CONCENTRATION

The minimum inhibitory concentration was performed against test organisms *Escherichia coli* and *Staphylococcus species* only.

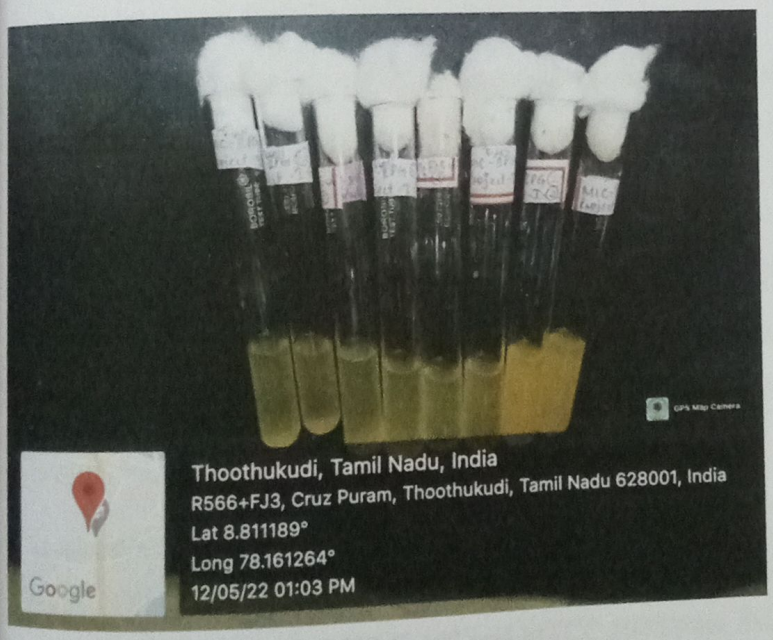



**Plate:5.58 MIC for Isolate 2 against *Escherichia coli***

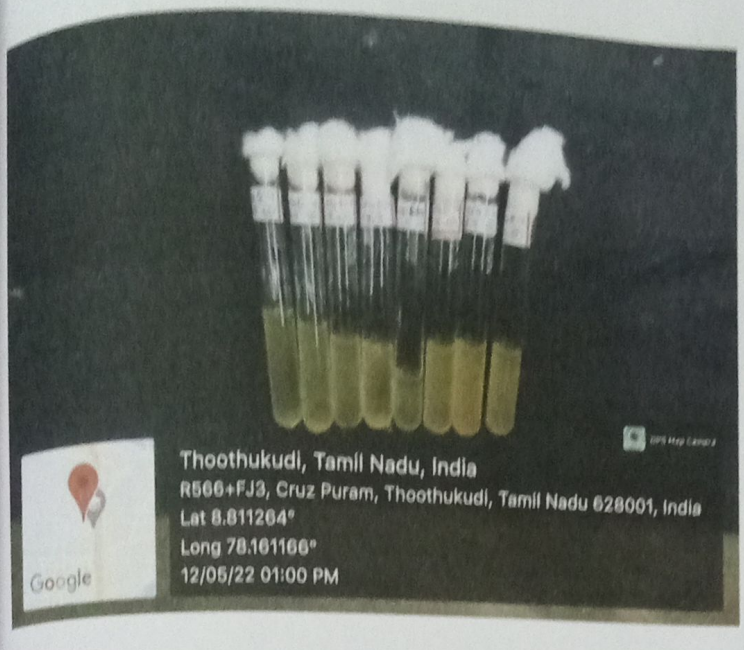



**Plate:5.59 MIC for Isolate 2 against *Staphylococcus* species**

The minimum inhibitory concentration of dialysed concentrate for gram-negative bacteria *Escherichia coli* was found to be 7 ml and for gram-positive bacteria *Staphylococcus* species was found to be 6 ml. MIC against *Klebsiella* species was not considered because the zone of inhibition was lesser than the positive control.

One study (Tang-Liu *et al.*, 1994) showed that 4 hours after instillation, tobramycin has an average concentration of 8µg/mL in human tears. Therefore, this concentration would still be effective against most strains tested in this study, since most strains had an MIC of 1.00 µg/ml. However, four strains obtained higher MICs (8 µg/mL, 48 µg/mL and two isolated with 256 µg/mL). These MICs suggest poor response to treatment *in vivo* with tobramycin considering the possible MICs that can be achieved in the most common eye drop treatment protocols. Therefore, it is recommended to increase the frequency of administering tobramycin from every four to approximately every two hours.

It is essential to determine the MIC values of the substances to evaluate the antimicrobial efficacy. MIC and minimum bactericidal concentration (MBC) values of partially purified compound for S. pyogenes MTCC 442 and MTCC 1928 were found to be similar. But in the case of B. cereus and Enterococcus faecalis, MBC values were higher as compared to MIC values. However, MIC and MBC values for Staphylococcus epidermidis ATCC 12228 were found to be very high (>200 µg/mL). Bacillomycin F which effectively inhibited C. albicans (MIC, 40 µg/mL) and C. tropicalis (MIC, 40 µgml−1) exhibited modest inhibition against Micrococcus luteus (MIC, 200 µg/mL) having no inhibitory effect on other bacteria tested (e.g.,

MIC > 400 µg/mL: E. coli K12, Streptomyces albus G., and S. aureus). Likewise in our investigation, narrow-spectrum activity against gram-positive bacteria like S. pyogenes, S. epidermidis, B. cereus, and E. faecalis were found in partially purified antimicrobial compound reported by studies conducted by Kaye *et al.* (2009) showed that topical administration of an antimicrobial to the cornea may achieve a different concentration and bioavailability in the tissue than the serum levels. In the treatment of bacterial keratitis, the MIC values are an important measure for evaluating the potential effectiveness of topically applied antimicrobials (Sueke *et al.*, 2010).

# 6. SUMMARY

In this study, the soil is the chosen for isolation of antibiotic producing microorganisms as it contains large microbial community with potential of antibiotic producer. The antibiotic producing microorganisms were isolated from the soil which was collected from the college campus and then inoculated in NA media as crowded plate technique through serial dilution from $10^{-1}$, $10^{-3}$ and $10^{-4}$. The colony with zone of inhibition was noted and isolated. Four isolates were isolated.

The pure cultures of those isolates were obtained in NA plates. These isolates were named Isolate 1, 2, 3, and 4. The colony morphology of each isolate was studied. The biochemical test was performed on each isolate to identify the isolates' genus like IMVIC test, Catalase test, Urease test. With colony morphology and biochemical test, the isolates were evaluated. The evaluation predicted that the Isolate 2 could be of genus *Bacillus* species.

Antimicrobial activity by agar diffusion method, that is, secondary screening was done against test organisms. Test organisms were both bacterial, *Escherichia Coli, Staphylococcus species, Klebsiella species,* and fungal, *Aspergillus species* and *Mucor species.* Test organisms were tested against Isolate 2. The zone of inhibition was observed least against *Klebsiella species,* more against *Staphylococcus* species and medium towards *Escherichia Coli* compared to positive control Amoxicillin. The zone of inhibition was absent against fungal test organisms that is *Aspergillus* and *Mucor* species compared to positive control Fluconazole.

MIC is an important test to determine the minimum concentration at which the growth of the test organism is inhibited by the Isolate 2. Considering only *Staphylococcus* species and *Escherichia Coli* as test organisms for MIC as it produced less diameter of zone of inhibition compared to positive control. The turbidity was less to observe at 6 ml for *Staphylococcus* species and for *Escherichia Coli* it appeared around 7 ml.

Summarizing that Isolate 2 can be *Bacillus* species as this study proves with morphological, biochemical test and antibacterial proof. Further elucidation of structure of the compound, the species identification, purification of the compound, etc. Growing resistance in microorganisms has driven more identification of new antibiotics which can be novel in their action.

# 7. CONCLUSION

In search of new antibiotics, relatively simple and rapid methods have been developed for screening microorganisms for antibiotic producing ability. Soil samples are commonly employed in the isolation of antibiotic producing organisms. The miss use of antibiotics has led to the evolution of pathogens with resistance to major available antibiotics.

The mainstream approach relies on the discovery and development of newer, more efficient antibiotics. Declining private investment and lack of innovation in the development of new antibiotics are undermining efforts to combat drug-resistant infections, says the World Health Organization (WHO). Antibiotic resistance is still a public health threat during the COVID-19 pandemic. CDC experts are closely monitoring the possible effects of COVID-19 on the national state of antibiotic resistance and antibiotic use.

The detection of these antagonistic substances revealed interesting properties that justify its importance and its study on potential application. The biochemical nature and the best conditions to produce the substances studied in this work are being investigated to further purification experiments. Many microorganisms have been evaluated for the production of antimicrobial substance. However, the high cost and low yields have been the main problem for its industrial production.

In this study, the microbial isolates with antimicrobial activity from soil were isolated. Among all screened isolates, Isolate 2 which is *Bacillus* sp. strain metabolite showed zone of inhibition against both G +ve and G-ve bacteria, against fungi did not show any zone of inhibition. The MIC determination helped in knowing at which concentration the antibacterial activity starts. Production of antibiotic by microorganisms from soil is affected by many factors including nitrogen and carbon source. Therefore, there is a great need to optimize with different substrates that provides maximum production of antibacterial substances.

Hence it can be concluded from this study that the antibiotics produced by Isolate 2 *Bacillus sp* observed to be broad spectrum, this could be further exploited to know their efficacy compared with currently available antibiotics. This study may contribute in providing information on the antibiotic producing microorganisms in soil. Further characterization, purification, and structural elucidation are recommended to know the novelty, quality and commercial value of these antibiotics.

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## ANNEXURE I

### NUTRIENT AGAR

| | |
|---|---|
| Peptone- | 5 g |
| Beef extract- | 3 g |
| Sodium chloride- | 10 g |
| Agar- | 15 g |
| Distilled water- | 1000 ml |

### NUTRIENT BROTH

| | |
|---|---|
| Peptone- | 5 g |
| Beef extract- | 3 g |
| Sodium chloride- | 10 g |
| Potassium nitrate- | 5 g |
| Distilled water- | 1000 ml |

### PEPTONE BROTH

| | |
|---|---|
| Peptone- | 20 g |
| Sodium chloride- | 5 g |
| Distilled water- | 1000 ml |

### MR-VP BROTH

| | |
|---|---|
| Peptone- | 7 g |
| Dextrose- | 5 g |
| Potassium phosphate- | 5 g |
| Distilled water- | 1000 ml |

### SIMMON CITRATE AGAR

Ammonium di hydrogen phosphate- 1 g

Di potassium phosphate- 1 g

Sodium chloride- 5 g

Sodium citrate- 2 g

Magnesium sulphate- 0.2 g

Agar- 15 g

Distilled water- 1000 ml

**UREA AGAR**

Urea- 20 g

Sodium chloride- 5 g

Monopotassium phosphate- 2g

Peptone- 1 g

Dextrose- 1 g

Phenol red- 0.012 g

Agar- 15 g

Distilled water- 1000 ml

**MULLER-HINTON AGAR**

Peptone- 15 g

Meat extract- 2 g

Starch- 1.5 g

Agar- 17 g

Distilled water- 1000 ml

**EMB AGAR**

| | |
|---|---|
| Peptone- | 10 g |
| Lactose- | 5 g |
| Sucrose- | 5 g |
| Dipotassium phosphate- | 2 g |
| Eosin Y- | 0.4 g |
| Agar- | 15 g |
| Methylene blue- | 0.065 g |
| Distilled water- | 1000 ml |

**PDA AGAR**

| | |
|---|---|
| Potato (infusion form)- | 20 g |
| Dextrose- | 2 g |
| Agar- | 15 g |
| Distilled water- | 1000 ml |

**MAC- CONKEY AGAR**

| | |
|---|---|
| Peptone- | 17 g |
| Proteose peptone- | 3 g |
| Lactose monohydrate- | 10 g |
| Bile salts- | 1.5 g |
| Sodium chloride- | 5 g |
| Neutral red- | 0.03 g |
| Crystal violet- | 0.001 g |
| Agar- | 15 g |
| Distilled water- | 1000 ml |

## MANNITOL SALT AGAR

| | |
|---|---|
| Enzymatic digest of casein- | 5 g |
| Enzymatic digest of animal tissue- | 5 g |
| Beef extract- | 1 g |
| D- mannitol- | 10 g |
| Sodium chloride- | 10 g |
| Phenol red- | 0.025 g |
| Agar - | 15 g |
| Distilled water- | 1000 ml |

ANNEXURE II

## BARITT'S REAGENT

Solution A

Alpha- naphthol- 5 g

Ethanol (95%)- 95 ml

Solution B

Potassium hydroxide- 40 ml

Creatine- 0.3 g

Distilled water- 1000 ml

## CRYSTAL VIOLET

Solution A

Crystal violet (90% dye content)- 2 g

Ethyl alcohol- 20 ml

Solution B

Ammonium oxalate- 0.8 g

Distilled water- 80 ml

## GRAM'S IODINE

Potassium iodide- 2 g

Iodine- 10 ml

Distilled water- 1000 ml

## HYDROGEN PEROXIDE

Hydrogen peroxide- 10 ml

Distilled water- 90 ml

**KOVAC'S REAGENT**

P- dimethylaminobenzaldehyde- 5 g

Amyl alcohol- 75 ml

Hydrochloric acid- 25 ml

**METHYL RED SOLUTION**

Methyl red- 0.1 g

Ethyl alcohol- 300 ml

Distilled water- 200 ml

**SAFRANIN**

Safranin- 0.5 g

Distilled water- 1000 ml

**LACTOPHENOL COTTON BLUE**

Phenol- 50 g

Lactic acid- 50ml

Glycerol- 100 ml

Cotton blue (Aniline Blue)- 0.125 g

70% Ethanol- 70 ml

Distilled water- 50 ml