Characterization of plant extracts and evaluation of in-vitro applications

A Dissertation for

MIC -651 Discipline Specific Dissertation

16 credits

Submitted in partial fulfilment of Master's Degree

MSc. in Microbiology

By

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DECLARATION

I hereby declare that the data presented in this Dissertation report entitled "CHARACTERIZATION OF PLANT EXTRACTS AND EVALUATION OF IN-VITRO APPLICATIONS" is based on the results of the investigations carried out by me in microbiology at the School of Biological Sciences and Biotechnology, Goa University under the supervision of Dr. Trupti Asolkar and the same has not been submitted elsewhere for the award of a degree by me. Further I understand that Goa University or its authorities will not be responsible for the correctness of observational/ experimental or other findings given in the dissertation.

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This is to certify that the dissertation report **"Characterization of plant extracts and evaluation of in-vitro applications"** is a bonafide work carried out by Ms. Rachel Vanessa D'Souza under my supervision in partial fulfilment of the requirements for the award of the degree of **M.Sc. in Microbiology** in the microbiology programme at the School of Biological Sciences and Biotechnology, Goa University.

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CHARACTERIZATION OF PLANT EXTRACTS AND EVALUATION OF IN-VITRO APPLICATIONS

By:
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SCHOOL OF BIOLOGICAL SCIENCES AND BIOTECHNOLOGY
MICROBIOLOGY PROGRAMME
2023-2024

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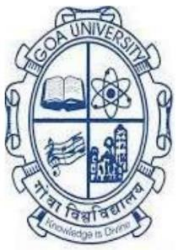

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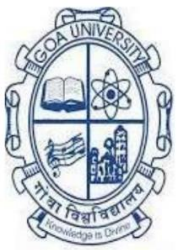

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PREFACE

Antibiotics have revolutionized healthcare, but the rise of multi-drug resistant bacteria, poses a grave threat to their efficacy. This challenge has spurred interest in ethnopharmacology and exploring plant-based remedies. Plants have long been integral to healthcare, offering diverse medicinal compounds and traditional medicinal practices, spanning cultures, provide valuable insights into plant properties. With nearly 80% of the world relying on traditional medicine, the importance of plant-based remedies is evident as they offer unparalleled chemical diversity, driving interest in natural therapeutic drugs. Plants have already yielded treatments for various conditions, highlighting their potential in healthcare. As modern pharmaceuticals increasingly originate from natural sources, plants remain central to drug development. Advances in pharmacology research and modern technology now shines the spotlight on plants as a promising source for novel drugs, offering hope in the fight against antibiotic resistance.

ACKNOWLEDGEMENT

Albert Schweitzer once said ‘At times our own light goes out and is rekindled by a spark from another person. Each of us has cause to think with deep gratitude of those who have lighted the flame within us”

And so I would like to express my sincerest gratitude to my research guide, Assistant Professor Dr. Trupti Asolkar for her valuable guidance and constant motivation during the course of my dissertation that enabled me to complete my research work.

I also extend my gratitude to Dr. Lakshangy Charya, Programme Director of M.Sc. Microbiology, Goa University and all the teaching staff of Microbiology, SBSB for their help as well as their encouragement and advice that spurred me towards greater accomplishments.

I also thank all the non-teaching staff, Mr. Surendra Velip, Mrs. Robertina Fernandes, Mr. Bhagwant Karpe, and Mr. Domingos Dias, for all their help in providing necessary laboratory equipment as well as for procuring the necessary reagents for my work.

To my family, my siblings, who have been my pillars of support and inspire me every day to be better and fuel all my hopes and dreams, I’m forever grateful for your existence!! You guys are the light and spark that kindled my passion for science.

Lastly I owe a special mention of gratitude to Arzoo, Saloni, Divika, Apurva , Eden , Sainil, Nikita , Bhavana, Sarvasvi, Manswi, and others for looking out for me and making all those hours spent working in the laboratory all the more bearable and enjoyable by being in it.

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ABBREVIATIONS USED

entity	Abbreviations
Centimetres	cm
Colony forming units	Cfu/ml
Culture control	CC
Dimethyl sulfoxide	DMSO
DMSO control	DC
Grams per litre	g/L
MBC	Minimum bactericidal concentration
MIC	Minimum inhibitory concentration
Micro litres	µL
Microliters per ml	µL/mL
Millilitres	mL
Millimetres	mm
Optical density	OD

ABSTRACT

With the rise in antibiotic resistant organisms as well as the evolutionary emergence of new disease causing organisms, a global health concern has arisen leading to the exploration of new pharmaceuticals from plants. Plants have been used for centuries as traditional medicine for treatment of various ailments. The aim of this study was to evaluate the antimicrobial activity of plant extracts and *Simarouba glauca (Lakshmi taru), Garcinia indica (*Kokum) and *Piper nigrum* (Pepper) were selected for this study. A standard method was used for preparation of methanolic extract and the best antimicrobial activity was shown by *S. glauca* against bacterial plant pathogens such as *Ralstonia solanacearum* in plate as well as liquid based assays and fungal pathogens such as *Fusarium oxysporum f.sp.solani.* MIC of *Ralstonia* was determined to be 5 µL/ 5ml of media. Phytochemical screening indicated the presence of compounds like alkaloids, tannins and flavonoids. Scanning electron microscopy showed disrupted cell membranes and 'bubble' like texture of the organism. Highest antioxidant activity was exhibited by Lakshmi Taru>Kokum>Pepper. Soil population studies conducted showed significant reduction in pathogen population.

Keywords: Lakshmi Taru, Kokum, Pepper, *Ralstonia*, antimicrobial activity, plant pathogens

CHAPTER 1:

INTRODUCTION

1.1 BACKGROUND

Antibiotics are one of man's greatest weapons in the fight against a variety of bacterial infections and since their discovery, have vastly improved the health-related quality of human life. (Rakholiya & Chanda, 2012)

However, an emerging and looming public health care concern is the alarming rise of multi-drug resistant pathogenic bacteria and the lack of effective antimicrobial treatment in cases of infection caused by these pathogenic bacteria. (Manandhar et al., 2019)

Hence due to this alarming decrease in the potency of pharmacological derivatives against these resistant microorganisms, the potential of ethnopharmacology is being explored. (Sasidharan et al., 2010)

Holistic or herbal medicine has been one of the oldest forms of healthcare known to humankind. Nature has been man's greatest inspiration, and most forms of modern medicine are proof of the vast knowledge that has been obtained from understanding the complexities of Mother Nature.

From ancient literature to modern scientific records of traditional medicinal knowledge, there lies a truth- that plants provide the sole medicinal source in case of healthcare in various developing Asian countries. Plants have long been a source of medicinal compounds, providing humanity with remedies for various ailments since ancient times. From the traditional Chinese 'Sheng-Nongs' herbal book to Ayurveda in India, plants have been revered for their healing properties. These ancient practices have stood the test of time and have been considered an important cultural heritage, all while providing valuable insights into the medicinal properties of plants that continue to captivate modern researchers (Sheng-Ji, 2001).

The World Health Organization describes that almost 80% of the world's population relies on traditional medicine for their basic healthcare needs. The need for chemical diversity which is an unmatched availability in plants, has led to increased interest in naturally derived therapeutic drugs (Sasidharan et al., 2011). They also define a medicinal plant as "any plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes, or which are precursors for chemo-pharmaceutical semi synthesis". (Doughari, 2012)

The growing interest in plant metabolites as possible antimicrobials can also be attributed to the alarming rate of plant species extinction which could result in irretrievable loss of potentially useful phytochemical structures that could be invaluable for chemical synthesis. (Lewis et al., 1995)

In current times, plants have been a source of analgesics, anti-inflammatories, asthma medication, antiarrhythmic agents as well as antihypertensives (Webster, 2008). A rough estimation of about 14-18% of higher plant species are medicinally used and around 74% of pharmacologically active plant derived components were discovered after following up on ethno-medicinal use of the plants (Ncube et al., 2008). Most modern clinical pharmaceuticals are of natural product origin and such natural products play key roles in the development of novel drugs in the pharmaceutical industry. The limits of synthetic drugs to control major diseases are being brought to light and therefore, havw led the ongoing pursuit of discovery of new molecular structures using plants as inspiration. (Preethi et al., 2010)

Therefore, with advancements in scientific research and technology, the spotlight has turned to the vast reservoir of bioactive compounds present in plants, promising novel avenues for drug discovery and development. (Manandhar et al., 2019)

1.2 AIMS AND OBJECTIVES

- Screening of plant extracts for antimicrobial and antioxidant activity.
- Partial characterization of plant extracts using phytochemical screening.

1.3 HYPOTHESIS

Untapped potential of native ethno-medicinal plants can be a research opportunity for exploration of antimicrobial effects, as a viable option for therapeutics such as a replacement for antibiotics, as well as components for biodegradable polymers and as a source of antioxidant activity. (Homayouni et al., 2017)

1.4 SCOPE

Future scope could include identification of bioactive compounds present in Lakshmi Taru with further purification using Thin Layer Chromatography, Column chromatography and Gas Chromatography Mass spectrometry. Plant-based greenhouse inoculation studies could be carried out to determine the effectivity, when host plant for pathogen is present. Successful identification and purification of bioactive compounds can help in chemical synthesis and potential novel antimicrobials. Biodiesel production is another scope for future studies.

CHAPTER 2:

LITERATURE

REVIEW

“Relentless and Dizzying Rise of Antimicrobial Resistance” (Nordberg et al, 2004) is one of the leading causes of lasting infections and as a result is a major cause of morbidity and mortality in human healthcare.

Antibiotic resistance can be attributed to genetic changes that enable the bacteria to overcome the effects of the antibiotic with various mechanisms. Some of these mechanisms can be summarized as antibiotic inactivating enzymes e.g. β-lactamases, impaired uptake of antibiotics, drug efflux, modification of the target or development of an alternate metabolic pathway. (Raghunath, 2008)

The increased use of chemical pesticides has introduced problems of health as well as environmental hazards within the agriculture industry and therefore, natural plant derivatives are proving to be the best biorational options of today. (Tiwari et al., 2007)

Because of the vast number of plant species that can be studied, it is important to have effective systems of reliable methods to evaluate the effectiveness of medicinal plants for antimicrobial agents. Evaluation for antimicrobial agents of plant origin starts with an in-depth biological analysis of plant extracts for efficacy and safety, followed by the identification of active principle, dosage formulation, efficacy, and pharmacokinetic profile of a novel drug. (Tanaka et al., 2006).

2.1 ANTIMICROBIAL STUDIES OF PLANT EXTRACTS

Methanolic extracts of *Oxalis corniculata*, *Artemisia vulgaris*, *Cinnamomum tamala*, and *Ageratina adenophora* were tested and screened against twelve pathogenic microorganisms with the best potential being observed against *Escherichia coli, Salmonella typhi, MDR Salmonella typhi, Klebsiella pneumoniae, and Citrobacter koseri.* Antifungal properties of *Ageratina adenophora* have also been reported against certain *Rhizopus spp*. (Manandhar et al., 2019)

Plant antimicrobials have been found to be synergistic when used in conjunction with standard drugs i.e. enhance the effect of the drug even if on its own, it does not possess antimicrobial effects. In a study that carried out testing the synergistic interaction of two plant extracts namely *C.papaya* and *T.catappa*, it was proved that since plant extracts consist of a blend of compounds, these compounds can potentially enhance the effects of antibiotics. (Rakholiya & Chanda, 2012)

Antimicrobial potential of ethanolic and water extracts of roselle *(Hibiscus sabdariffa*), rosemary (*Rosmarinus officinalis*), clove (*Syzygium aromaticum*), and thyme (*Thymus vulgaris*) on known food pathogens and spoilage organisms such as *Escherichia coli, Salmonella enteritidis, Vibrio parahaemolyticus* with ethanolic extract of *Hibiscus sabdariffa,* showed significant antibacterial activity against most of the bacterial strains. The decline in internal pH (pH_{int}), and membrane potential of plant extract treated cells, indicated effective activity and increased prospective use as a food preservative. (Gonelimali et al., 2018)

Antifungal potential of aqueous extracts of *Fragaria virginiana*, *Epilobium angustifolium* and *Potentilla simplex* has been reported against yeasts such as *Candida glabrata* and *Saccharomyces cerevisiae* with *Fragaria virginiana* showing the best results overall against various dermatophyte and mold isolates at concentrations of 800 mg/l and less at 72 hours. Others such as *Potentilla simplex* showed inhibition at concentration as low as 25 mg/ml at 48 hours against *C. glabrata*. (Webster, 2008). Other antifungal studies carried out using extracts included methanolic extracts of *Solanum xanthocarpum* and *Datura metel* against *Aspergillus fumigatus, A. flavus, A. niger* and exhibited in vitro MICs of 1.25–2.50 mg/ml by both micro broth dilution and percent spore germination assays. Disc diffusion assays of *Datura* showed inhibitory activity at 0.062 mg/disc against *Aspergilli*.(Dabur et al., 2004)

2.2 PHYTOCHEMICAL COMPOUNDS

It has been reported that the major contributing factor towards the antimicrobial activity displayed by the plant is due to its innate limitless ability to produce a variety of aromatic secondary metabolites. The major classes of these compounds include flavonoids, flavones, tannins, coumarins, quinones, and others, with most of these compounds acting as part of a plant's natural defense mechanism against a variety of pathogenic organisms. (Das et al., 2010)

2. 2.1 Phenolics

Many types of plant materials contain phenolic compounds and extracts that have bioactive properties. Many plant extracts enriched with phenolic compounds and individual phenolics also have promising anti-quorum sensing potential and can inhibit the formation of biofilms and the production of toxins associated with food-borne pathogens. Antioxidant activity, as well as lipid peroxidation, are just some of the physiological activities exhibited by such compounds and their mechanisms of action differ from those of traditional antibiotics and hence could make these plant phenolics effective against drug-resistant pathogens. (Takó et al., 2020)

Cinnamic and caffeic acids are the most common representatives of a wide group of phenylpropane-derived compounds. Essential oils are a class of phenolic compounds that show the presence of a C3 side chain at a lower level of oxidation and lack oxygen and are often described as antimicrobial as well. (Cowan, 1999)

2.2.2 Flavonoids

Flavones, flavonoids, and flavanols are phenolic compounds with one carbonyl group and are a plant's response to microbial infection (Dixon et al., 1983) and are hence often found effective in vitro as antimicrobial substances against a diverse region of microorganisms (Das et al., 2010)

Their activity can be attributed to their ability to complex with extracellular and soluble proteins and form complexes with bacterial cell walls while more lipophilic flavonoids may have the ability to disrupt microbial membranes.

Catechins are an important mention of these types of compounds and are primarily found to be present in oolong tea. A mixture of these catechin compounds was found to inhibit pathogenic organisms such as *Vibrio cholerae, Streptococcus mutans, Shigella*, etc. (Borris, 1996)

2.2.3 Coumarins

Coumarins are phenolic substances made of fused benzene and α-pyrone rings and are known to have a characteristic odour, with several of them displaying antimicrobial properties too. Warfarin is an example of a coumarin that is widely used as an oral anticoagulant and even as a rodenticide. (O'Kennedy and Thornes, 1997).

2.2.4 Terpenoids

Terpenes are secondary metabolites that are isoprene structure-based compounds and are terpenoids when the compound contains an additional element such as oxygen. These compounds are associated with the plant's natural scent and fragrance. It was also reported that 60% of essential oil derivatives were inhibitory to fungi while 30% inhibited bacteria. Triterpenoid betulinic acid is an example of a terpenoid having antiviral activity. (Chaurasia and Vyas, 1977)

2.2.5 Alkaloids

They are a large group of ammonia compounds comprising basically of nitrogen bases and oxygen. Their function includes plant defense against herbivores and pathogens.

They have also been exploited for stimulants, pharmaceutical drugs, narcotics, and poisons attributed to their potent biological activities. (Doughari, 2012)

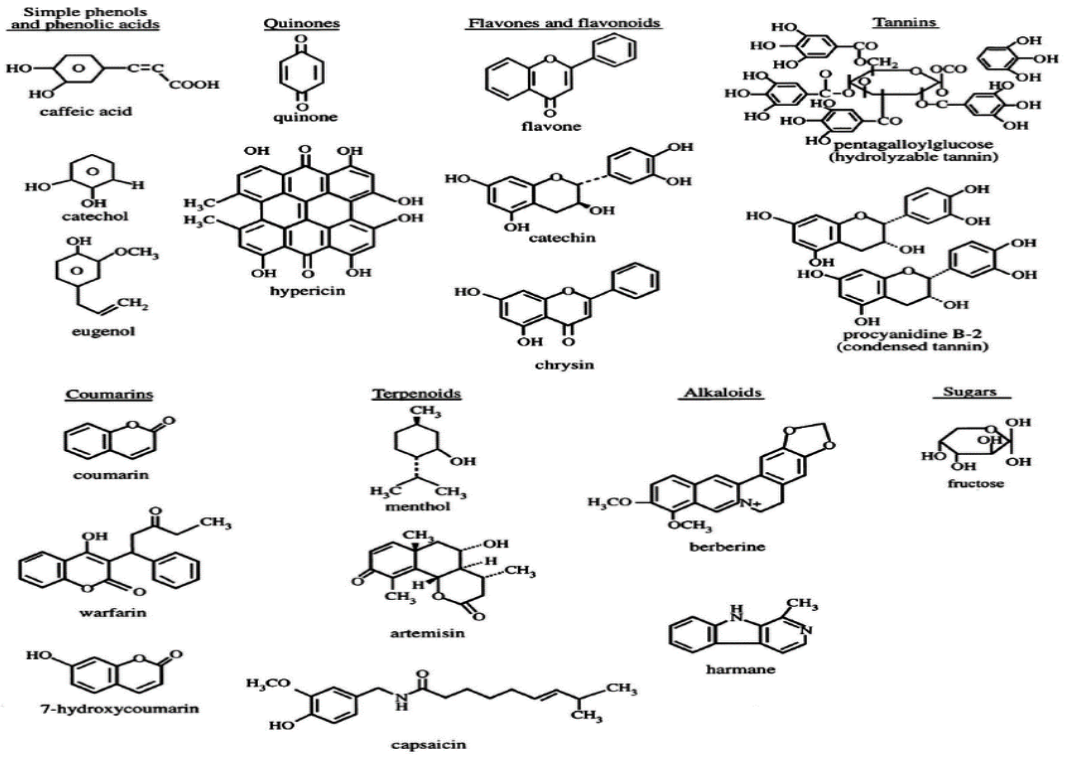

Figure 2.1: Chemical structures of some phytochemical compounds.

(Cowan et al., 1999)

2.3 MODE OF ACTION OF PHYTOCHEMICAL COMPOUNDS

Phytochemicals demonstrate different modes of action against bacteria, some of which include, interference with the phospholipid bilayer of the cell membrane which can result in loss of permeability by increase or decrease in cellular constituents, damage to specific enzymes involved in structural synthesis. The mechanism of action is generally considered to be the disturbance of cytoplasmic membrane by inducing cellular membrane perturbations, disrupting the proton motive force, electron flow, active transport, and coagulation of cell contents. (Kotzekidou et al., 2008)

2.3STUDY OF PLANT PATHOGENS

2.4.1 Ralstonia solanacearum

Ralstonia solanacearum is a soil-borne pathogen and a Gram-negative, aerobic ,non-fermentative rod-shaped organism. It is the primary cause of bacterial wilt in plants. Its major hosts belong to the *Solanaceae* and *Musaceae* families. The most common external symptoms of infected plants are wilting, stunting and yellowing of the foliage. Due to its wide host range, economic losses can be high.(Alvarez et al., 2010)

Treatment of *Ralstonia solanacearum* pathogen is known to be difficult due to antibiotic resistance to β-lactams and most of the aminoglycosides. No single treatment is available, however use of essential oils such as thymol are being explored as a possible treatment for reduction of pathogen populations. (Pradhanang et al., 2003)

2.4.2 Xanthomonas

Xanthomonas sp is the causative agent of black rot disease in cruciferous plants belonging to *Brassicaceae* family such as cauliflower, broccoli, cabbage etc., producing V-shaped necrotic lesions on the foliar margins and blackened veins (Alvarez, 2000). Such an infection is especially harmful due to the formation of biofilm, which demonstrates various degradative extracellular enzymes as well as other virulence factors. (Dow et al., 2003)

Xanthomonas species are Gram-negative, rod shaped, motile and appear as large yellow mucoid colonies due to the production of the pigment xanthomonadin which is characteristic of the genus (Bradbury, 1984). Current crop protection strategies include bacteriophage therapy. (Papaianni et al., 2020)

2.4.3 Fusarium

Fusarium is a soil-borne vascular pathogen that causes diseases such as crown rot, head blight, and scab on cereal grains, vascular wilts etc, in plants and are filamentous fungi belonging to

the Class *Ascomycetes* and Family *Hypocreaceae*. Symptoms of *Fusarium* wilt range from stunted growth, yellowing, discolouration of xylem to root and stem decay. (Okungbowa and Shittu, 2012)

Most common methods for control of these pathogenic fungi include breeding of resistant cultivars as well as the use of rhizospheric fungi such *Streptomyces griseoviridis* for control.

2.5 ETHNOMEDICINAL STUDIES OF INDIAN PLANTS

India is in a position of extraordinary bio-variety with its rich wellspring of therapeutic plants dispersed among the different geological and natural climates inside the country. The nation has an improved history in regards to the utilization of conventional medication from homegrown and non-natural sources which is well recorded and comprehensively rehearsed. (Sawant et al., 2015). Native information on involving therapeutic plants for recuperating human diseases is, be that as it may, at risk for gradual disappearance since this information is passed on orally from one age to another without any textual guide, and also because numerous conventional healers do not keep written documentation (Kaido et al., 1997). Goa, represented as the smallest state of India, has a wide knowledge of medicinal plants and their uses. The rural areas of Goa are inhabited by socio-economically backward communities like Kunbi, Velip, Gawde, Chambhar, Mhar, Kansar, etc most of whom use a large number of plants for the treatment of various diseases (Estbeiro, 2001).

2.5.1 Kokum

Garcinia indica commonly known as Kokum, is used widely in culinary practices. Its extract is also known to have both antifungal as well as antibacterial properties and therefore is being viewed as a bio-preservative in food applications (Varalakshmi et al., 2010). The juice is a soothing drink in summer months and is known to provide relief from gastric disorders as well as being traditionally used to treat sores, skin ailments such as rashes caused by allergies,

dermatitis and to relieve sunstroke. It is also used as a remedy for diarrhoea, piles, dysentery, and tumours. It aids in digestion, purifies the blood and fights cholesterol (Mishra et al., 2006).

Some important compounds are hydroxy citric acid [HCA], garcinol and the pigment anthocyanin (cyanidin -3-glucose). Garcinol is reported to have strong antioxidant activity due to the presence of phenolic hydroxyl groups and a β-diketone moiety (Padhye et al., 2009) while also simultaneously exerting anti-inflammatory effects (Liao et al., 2005).

Kokum is reported to show antibacterial activity against Gram-positive and Gram-negative organisms such as *Pseudomonas aeruginosa* and this antibacterial activity is attributed to the presence of furfural in kokum extract. (Sutar et al., 2010)

2.5.2 Lakshmi Taru

Simarouba glauca, commonly called Lakshmi Taru or "paradise tree", belongs to the family *Simaroubaceae* with quassinoids being one of its taxonomic markers with the phytochemicals present in leaf, fruit, pulp and seeds. It is well known to possess medicinal properties such as antimicrobial, anti-analgesic, anticancer, antiviral, astringent and is also used as a stomach tonic. Quassinoids from S. *glauca* seed including glaucarubin, glaucarubinone, glaucarubol and glaucarubolone have shown in vitro cytotoxic activity against certain cancers (Soumya and Nambisan, 2018). They also reported that methanolic extract exhibited moderate potential for antibacterial activity against S. *aureus, K. pneumoniae, P. aeroginosa, S. typhimurium* and *P. vulgaris* however due to lack of relevant literature no comparison could be made. Other uses of *S. glauca* include the production of Biodiesel, wherein trans-esterification method is used to prepare SG biodiesel from seeds. It has been reported that performance and emissions results yielded better results with blends of *Simarouba glauca* as compared to other diesel fuels. (Krutheesh and Dhanabal, 2021)

2.5.3 Pepper

Piper nigrum, known as black pepper, belongs to the *Piperaceae* family and is commonly found on the east coast of India. It is reported that this species is used in herbal Ayurvedic formulations (Trikatu) due to the presence of several classes of secondary metabolites such as lignans, neolignans, flavones, flavanones with alkaloids and amides being the most biologically relevant. Piperine (1-piperoyliperidine), an alkaloid has been reported to have anticancer, anti-inflammatory, analgesic, gastro protective, antioxidant, and antiseptic properties. Piperine also exhibits activity in prostate cancer as well as inhibition of the activity of liver enzymes. Microbial inhibition of *Staphylococcus aureus* and *Salmonella* sp. with MIC < 100 μg/mL reported with macerated ethanolic extract. (Alves et al., 2022)

Ethanolic extract of the plant displayed a broad antimicrobial spectrum and exerted significant antibacterial effect against both Gram-positive and Gram-negative bacteria tested including *S. aureus* and *B. subtilis* as well as *E. coli* and *K. pneumoniae* respectively. *S. epidermidis* was reported as the most sensitive with a MIC of 78.12 μg/ml. (Zarai et al., 2013)

CHAPTER 3:

METHODOLOGY

3.1 COLLECTION OF SAMPLES

Plant selection was based on use in herbal medicine, traditional knowledge passed down from elders as well as availability. The plants selected were Lakshmi Taru, kokum and pepper.

Tender leaves of *Garcinia indica* (Kokum), *Simarouba glauca* (Lakshmi Taru), *Piper nigrum* (Pepper) were collected in a clean ziplock bag. Samples were washed and kept for initial drying. The samples were then kept in the incubator for further drying for about 2 weeks. After the samples were completely dried, they were made into powder by maceration with mortar & pestle and some by blender.

3.2 PREPARATION OF EXTRACTS

Methanolic extract of *Simarouba glauca* (Lakshmi Taru): 12.5g of Lakshmi Taru powder was weighed and added to 100ml methanol in a conical flask, plugged and kept on a rotary shaker at 150-190 rpm overnight. The extract obtained was filtered using Whatman filter paper No 1 and the filtrate was stored in centrifuge tubes at 4°C. Concentration of extract was calculated to be around 0.125g/ml.

Methanolic extract of *Garcinia indica* (Kokum): 5g of kokum powder was added to 30 ml methanol in a conical flask, plugged and kept on a rotary shaker at 150-190 rpm overnight. Filtrate was obtained using Whatman filter paper No 1 and stored in centrifuge tubes at 4°C. Concentration of extract was calculated to be around 0.166g/ml.

Methanolic extract of *Piper nigrum* (Pepper): 5g of pepper powder was added to 50 ml methanol and similar procedure repeated. Concentration of extract was calculated to be around 0.10g/ml.

(Preethi et al., 2010) (Soumya and Nambisan, 2018),(Gonelimali et al., 2018), (Manandhar et al ., 2019)

Note: 8 ml of filtrate was kept in autoclaved glass vials and kept for complete evaporation and then resuspended in 4ml DMSO (1X). This was used for experimental use.

3.3 CULTURE PREPARATION

Cultures used: Unidentified Gram-positive culture, *Ralstonia solanacearum*, *Xanthomonas* sp. Loopful of culture from 48-hour old streaked plate was inoculated in respective broth medium i.e. BG broth for *Ralstonia solanacearum* and Nutrient Broth for GGram-positiveand *Xanthomonas* sp, and kept overnight on a rotary shaker.

3.4 ANTIBACTERIAL ASSAY

Plate based	Liquid based
A) Disc diffusion assay Filter paper discs of 8mm diameter were cut and autoclaved. 0.1 ml overnight grown culture was spread plated on MH agar plates. The discs were placed on the agar and loaded with 10uL of extract with methanol or DMSO maintained as control. The experiment was also conducted using 0.1ml spread plated culture.	A) With incubation 75 µL of overnight grown culture was added into sterile eppendorf tubes. 25 µL extract was added to each tube in duplicates and kept for 2 hours incubation on a shaker. Culture control and solvent control was kept. After 2 hours contents were transferred into sterile tubes containing 5ml sterile BG broth or nutrient broth depending on culture requirement and kept overnight on the shaker. The OD was measured at 600 nm using UV- Vis spectrophotometer.

B) Well diffusion MH media was seeded by adding 0.2 ml overnight grown culture per 100 ml of molten media and pouring into sterile empty petri plates. After solidification, wells were bored using a cork borer. 30ul of extract was added to respective wells as well as control. The experimental setup was also replicated using 0.1 ml spread plated culture.	B) Direct inoculation 75 µL of overnight grown culture was added into sterile tubes containing 5ml respective media. 25 µL of extract added to each tube in duplicates. Solvent control and culture control were kept and left overnight on the shaker. The OD was measured at 600 nm using UV-Vis Spectrophotometer

3.5 MINIMUM INHIBITORY CONCENTRATION AND MINIMUM BACTERICIDAL CONCENTRATION

Based on the results of the antibacterial testing, the most efficient crude extract was chosen to identify its MIC. Various amounts of the crude extract were added in duplicates to broth tubes to determine the lowest amount that inhibited growth. (Soumya and Nambisan, 2018)(Zarai et al., 2013) 0.1 ml of highest volume of extract was diluted and spread plated to check for growth.

3.6 ANTIFUNGAL ASSAY (POISONED FOOD METHOD)

Fungal cultures used- *Fusarium oxysporum f.sp.solani, Fusarium oxysporum f.sp.niveum* and *Sclerotium gerbera.*

50 and 100 µL of extract was spread plated onto PDA plates. Using a cork borer, a plug of evenly grown fungal culture was cut and placed in the centre of the plate. The plate was left at RT for 3-4 days and compared with a non-treated plate for visible reduction in colony diameter. Culture control as well as solvent controls were kept. (Quiroga et al., 2001)

3.7 SEM ANALYSIS

SEM slides were autoclaved and a smear was made of control and test under sterile conditions and left to dry completely. Slides were flooded with 2.5 % Glutaraldehyde and left overnight. After fixing, the slides were washed with distilled water and then dried with ethanol gradients from 20-100%. Each gradient was kept for 10 mins and then taken for imaging. (Golinejad and Mirjalili, 2020)

3.8 DPPH (1, 1-DIPHENYL -2-PICRYL HYDRAZYL) SCAVENGING ASSAY (ANTIOXIDANT ASSAY)

200 µL of 10mg/ml concentration of all 3 extracts was added to the microtiter plate and diluted with methanol. 100 µL of DPPH was added to each well. Positive control was ascorbic acid and methanol the blank. The plate was read using an ELISA microtiter plate reader at 540 nm.

% DPPH scavenging = [OD of control – OD of sample / OD of control] x 100

(Soumya and Nambisan, 2018)

3.9 QUALITATIVE ANALYSIS OF PHYTOCHEMICALS

3.9.1 Test for Alkaloids; (Wagner test)

Approximately, 1 ml of crude extract was mixed with 2 ml of Wagner's reagent (Appendix II). Reddish brown colour precipitate indicates the presence of alkaloids

3.9.2 Test for Phenols

To 1 ml of the extract, 3 ml of distilled water followed by a few drops of 10% aqueous Ferric chloride solution was added. Formation of blue or green color indicates the presence of phenols.

3.9.3 Test for Tannins

1ml extract was mixed with a few drops of 10% lead acetate. Formation of white precipitate indicates the presence of tannins.

3.9.4 Test for Flavonoids: (alkaline reagent test)

1ml of crude extract was mixed with a few drops of 20% NaOH. Yellow color indicates a positive test.

3.9.5 Test for Terpenoids

5ml of crude extract was mixed with 2ml of chloroform. 3 ml sulphuric acid was added drop wise along the sides. Formation of reddish brown precipitate at the interface of the layers is indicative of a positive test.

(Loganathan et al., 2021) (Bhandary et al, 2012)

3.10 SOIL POPULATION STUDIES

5g of autoclaved garden soil was taken in 3 sterile beakers. 1ml of 0.1 OD culture (overnight grown *Ralstonia solanacearum*) was added to all and mixed well. To the test 500 µL of Lakshmi taru extract was added and mixed well. To the solvent control 500 µL of DMSO was added and mixed well. After 4 days, 1g of soil was weighed and dilutions were prepared and plated. Colony count was recorded and observed for reduction in soil population.

RESULTS

PRELIMINARY STUDIES

Table 3.1: Diameter of zones of inhibition (mm) of plant extracts against unidentified Gram positive organism

Extract	(Methanolic extract) Zone of inhibition(mm)				(DMSO extract) Zone of inhibition(mm)	
	Spread plate		Pour plate		Spread plate	
	Plate 1	plate 2	Plate 1	Plate 2	Plate 1	Plate 2
Lakshmi Taru	15	16	23	22	15	16
	16	15	23	22	18	16
	16	16	22	21	16	17
Kokum	-precipitate	-	-	-	-	-
Pepper	-	-	-	-	-	-

Note: 8 mm Disc were used.

Plate-based studies showed effective inhibition of Gram-positive culture with both methanolic as well as DMSO Lakshmi Taru extract while Kokum showed a ring of precipitation around the well and disc. Pepper showed no visible zone of inhibition.

Table 3.2: Diameter of zones of inhibition (mm) of plant extracts against *Xanthomonas* sp by Disc diffusion method

Extract	(Methanolic extract) Zone of inhibition(mm)				(DMSO extract) Zone of inhibition(mm)	
	Spread plate		Pour plate		Spread plate	
	Plate 1	plate 2	Plate 1	Plate 2	Plate 1	Plate 2
Lakshmi Taru	15	12	-	-	16	15
	16	16	-	-	17	15
	14	15	-	-	17	15
Kokum	-	-	-	-	-	-
Pepper	-	-	-	-	-	-

Similar results were obtained for *Xanthomonas* with Lakshmi Taru showing zone of inhibition. Further studies were carried out using liquid based assays for confirmation.

Table 3.3: Diameter of zones of inhibition (mm) of plant extracts against *Ralstonia solanacearum* by Disc diffusion method

Extract	Zone of inhibition(mm) (DMSO extract)	
	Spread plate	
	Plate 1	Plate 2
Lakshmi Taru	26	25
	27	26
	26	24
Kokum	-	-
Pepper	-	-

Experimental results showed extremely potent inhibition of *Ralstonia solanacearum* with DMSO Lakshmi Taru extract. In comparison with other extracts, Lakshmi Taru proved to be the most effective antibacterial extract and broth-based assays were used for further studies.

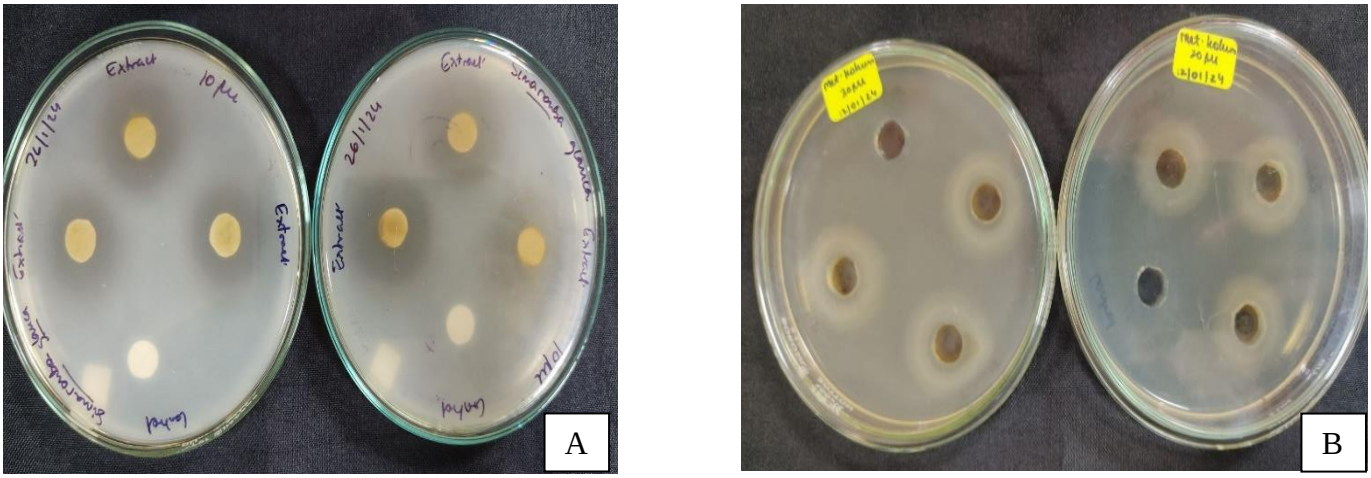

Figure 3.1: A) Disc diffusion of methanolic extract of Lakshmi Taru against seeded unidentified Gram-positive culture. B) Well diffusion of methanolic extract of Kokum against unidentified Gram-positive culture seeded agar showing precipitation around the well.

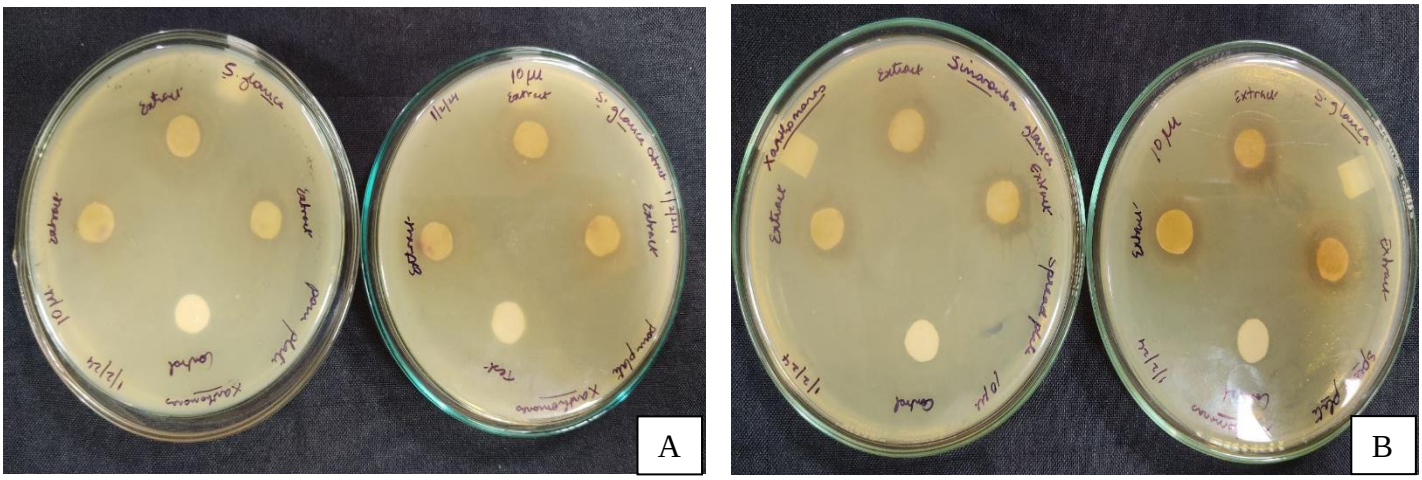

Figure 3.2: A) Disc diffusion of methanolic Lakshmi Taru against *Xanthomonas* sp seeded Agar. B) Disc diffusion of methanolic Lakshmi Taru against spread plated *Xanthomonas* sp showing zone of inhibition.

Table 3.4: Antifungal activity of plant extract against *Fusarium* spp

	Fusarium oxysporum f.sp.solani				*Fusarium oxysporum f.sp niveum*			
Extract used	Diameter of colony(in cms)				Diameter of colony(in cms)			
	Volume spread plated(µL)				Volume spread plated(µL)			
	50	100	100 DMSO	Culture control	50	100	100 DMSO	Culture control
Lakshmi Taru	4.5	4	4.8	5	2.8	2.6	3	3.5
Kokum	4	3.7	4.6	4.8	2.6	2.5	2.8	3.3
Pepper	4	4	4.9	5	2.6	2.5	3	3.5

Note: 8mm cork borer was used for fungal plug

Lakshmi Taru showed good inhibition of both the *Fusarium* species as seen as the reduction in colony diameter as compared to the DMSO and Culture controls with 100 µL of extract. Kokum and pepper both showed colony diameter reduction of *Fusarium oxysporum f.sp.solani* but did not show inhibition of *Fusarium oxysporum f.sp niveum* and were ineffective.

Table 3.5: Antifungal activity plant extract against *Sclerotium gerbera*

Extract used	Size of colony (in cm)			
	Volume spread plated (μL)			
	50	100	100 DMSO	Culture control
Lakshmi Taru	2.7	2	3	4
Kokum	2.8	2.5	3	4.5
Pepper	2.1	1.8	2.2	3.5

All 3 extracts showed increased inhibition with increase in volume of extract against *Sclerotium*, with kokum and Lakshmi Taru showing similar amount of reduction in colony diameter. The best antifungal activity could be stated as Lakshmi Taru >Kokum>Pepper

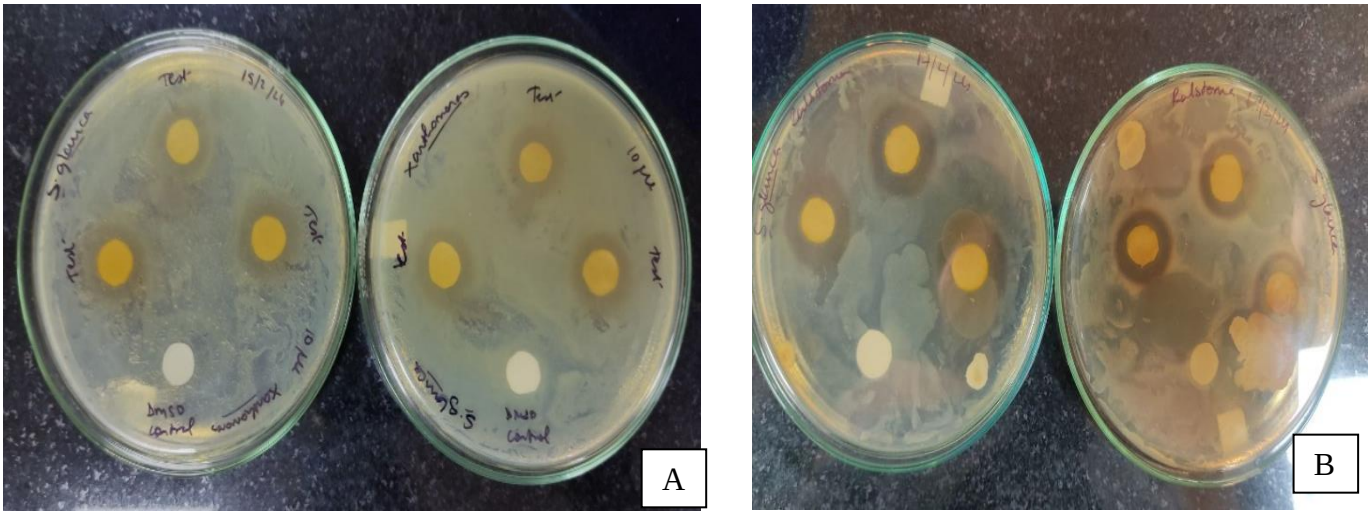

Figure 3.3: A) Disc diffusion of DMSO Lakshmi Taru with zone of inhibition against spread plated *Xanthomonas* sp. B) Disc diffusion of DMSO Lakshmi Taru with zone of inhibition against spread plated *Ralstonia solanaecearum*.

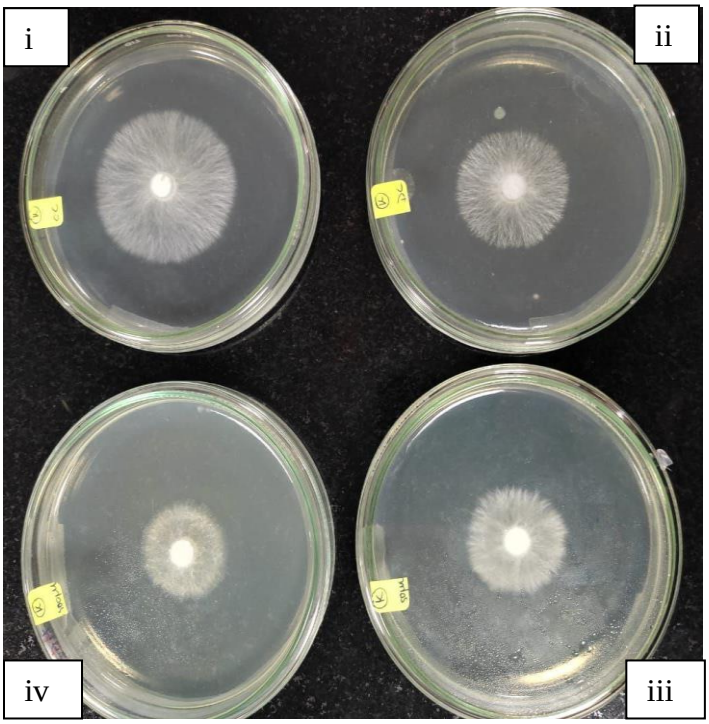

Figure 3.4: Antifungal assay of Kokum against *Sclerotium* where i) Culture control ii) DMSO control iii) 50 µL spread plated extract iv) 100 µL spread plated extract

Table 3.6: Antimicrobial activity in liquid medium for *Ralstonia solanacearum* and *Xanthomonas* sp with direct inoculation

Extract	OD at 600 nm		
	Ralstonia solanacearum	*Xanthomonas* sp (25 μL extract)	*Xanthomonas* sp (30 μL of extract)
Lakshmi Taru	0.336	0.503	0.646
	0.36	0.51	0.64
Kokum	0.609	0.541	0.634
	0.69	0.522	0.6
Pepper	0.747	0.493	0.585
	0.64	0.592	0.6
DMSO control	0.704	0.508	0.706
	0.88	0.5	0.766
Culture control	0.899	0.709	0.715
	0.835	0.775	0.723

From the data in table 3.6, Lakshmi Taru showed significant inhibition against *Ralstonia solanacearum* while pepper showed inhibition towards *Xanthomonas* sp in both volumes of extract tested as compared to their respective controls. For further analysis, a 2-hour incubation was set up in order to determine if further inhibition occurs due to closer interaction between extract and cells.

Table 3.7: Antimicrobial activity in liquid medium unidentified Gram-positive culture, *Ralstonia solanacearum* and *Xanthomonas* sp with 2-hour incubation

Extract	OD at 600 nm		
	Unidentified Gram positive	*Ralstonia solanacearum*	*Xanthomonas* sp
Lakshmi Taru	0.173	0.176	0.546
	0.182	0.15	0.55
Kokum	0.31	0.254	0.566
	0.31	0.248	0.568
Pepper	0.412	0.803	0.59
	0.41	0.947	0.57
DMSO control	0.38	1.24	0.689
	0.347	1.11	0.603
Culture control	0.428	1.084	0.622
	0.363	1	0.642

On maintaining a 2-hour incubation, Lakshmi Taru showed a further reduction in OD for *Ralstonia solanacearum* as well as a prominent inhibition of the unidentified Gram-positive culture while Kokum extract also showed a reduction in OD on comparison with the culture and solvent controls. Pepper however showed no significant improvement in reduction of OD of *Xanthomonas* sp. Moreover, since kokum and Lakshmi Taru proved to be ineffective as well, further study of *Xanthomonas* sp was not carried out. Hence with these preliminary findings, Lakshmi Taru was determined to be the most effective against *Ralstonia* and the unidentified Gram-positive culture in comparison with the other 2 plant extracts and therefore, was used for determining the MIC.

Table 3.8: MIC of unidentified Gram-positive culture.

Volume of extract (µL /5 ml)	OD at 600 nm
5	0.858
	0.704
10	0.853
	0.704
15	0.678
	0.576
20	0.495
	0.48
25	0.46
	0.47
30	0.439
	0.644
DMSO control	0.809
	0.967

Culture control	0.877
	0.902

With the volume removed from a stock of 0.125 g/ml and a 1X resuspension with DMSO and a 2-hour incubation, the MIC was determined to be 15 μL. Analysis of Gram-positive culture ended here with the determination of MIC.

Table 3.9: MIC of *Ralstonia solanacearum* with Lakshmi Taru, direct inoculation and 2-hour incubation

Volume of extract (µL /5 mL)	OD at 600 nm	
	With 2 hour incubation	Direct inoculation
5	1.43	1.457
	1.25	1.527
10	0.886	0.74
	0.92	0.71
15	0.288	0.66
	0.304	0.6
20	0.224	0.54
	0.213	0.58
25	0.157	0.564
	0.159	0.55
30	0.125	0.511
	0.142	0.49

35	Not Done	0.483
		0.471
40	Not Done	0.465
		0.43
45	Not Done	0.411
		0.409
50	Not Done	0.4
		0.394
DMSO control	1.608	1.628
	1.84	1.61
Culture control	1.659	1.641
	1.82	

On comparison of both the data sets, the Minimum inhibitory concentration of *Ralstonia solanacearum* was found to be 5 μL. Based on these observations, the determination of MBC of the same was carried out.

Table 3.10: MBC of *Ralstonia solanacearum* with direct inoculation of Lakshmi Taru

Volume of extract (µL /5 ml)	OD at 600 nm
60	0.772
	0.694
80	0.31
	0.298
100	0.308
	0.301
120	0.325
	0.33
130	0.342
	0.348
140	0.318
	0.342
150	0.29
	0.32

160	0.4
	0.405
DMSO control	1.1
Culture control	1.12

Table 3.11: Viable count of MBC plated dilutions

Volume of extract (µL/ml)	dilution	Colony count	Viable count Cfu/ml
Experiment 1			
70	10-4	140	$140x10^5$
110	10-5	79	$79x10^6$
Experiment 2			
150	10-4	>300	-
	10-5	matt	-
160	10-4	154	$154x10^5$
	10-5	30	$30x10^6$

After experimental analysis for determination of MBC, it was observed that the extract was able to show a significant reduction of growth indicating that inhibition was up to a threshold level point but was unable to kill the pathogen completely despite the large volume of extract added as seen in Figure 3.5. The experiment was performed twice, with the spread plating of the dilutions of the highest volume still showing colony growth.

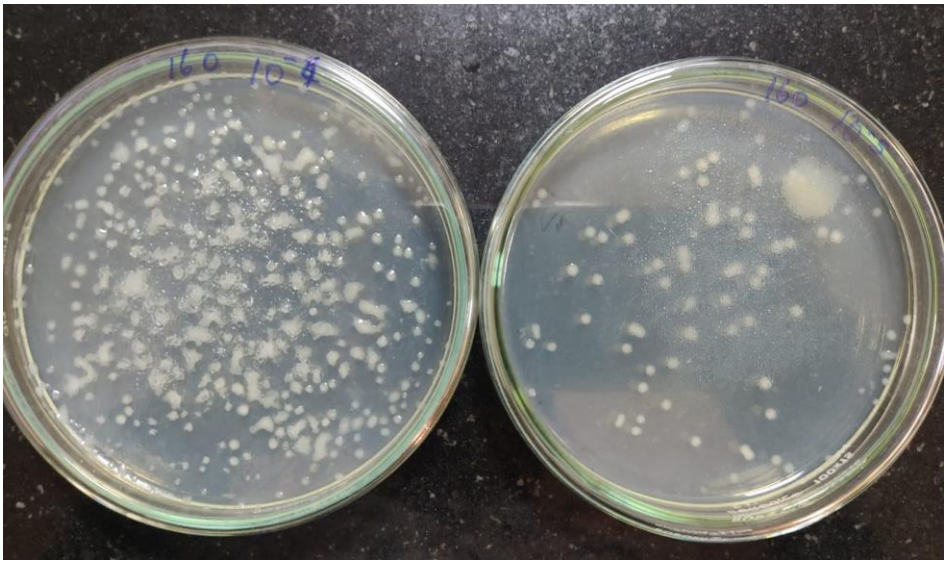

Figure 3.5: MBC plating of 160µl extract

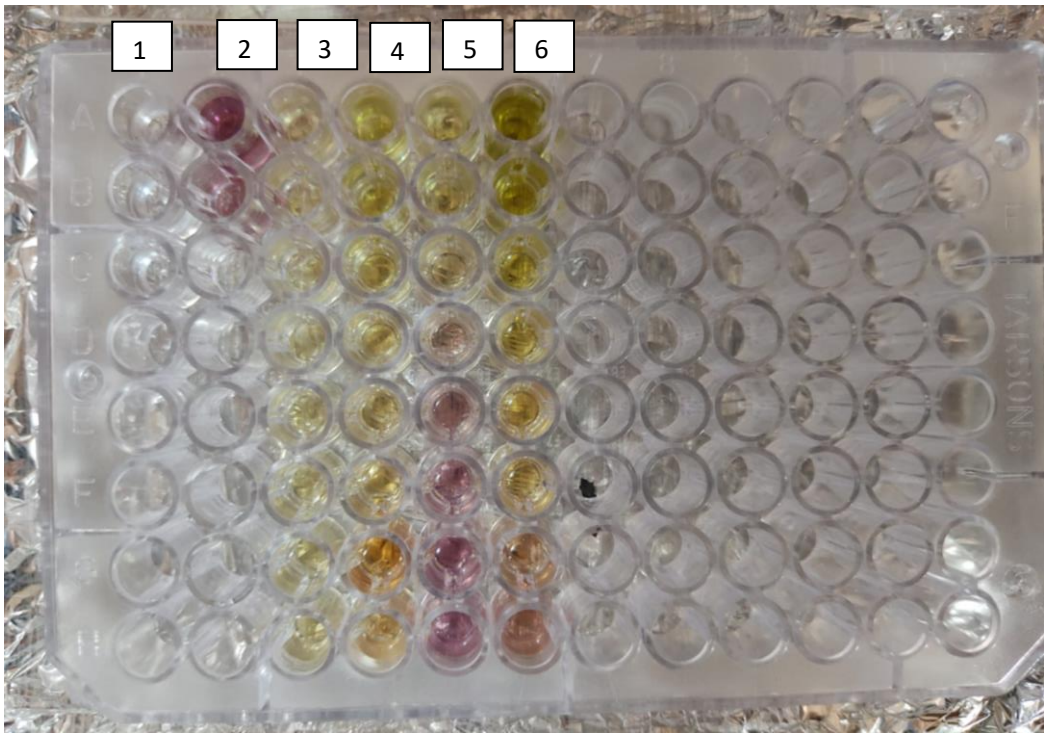

Figure 3.6: DPPH scavenging microtitre plate where 1) blank methanol 2) DPPH 3) Ascorbic acid (positive control) 4) 10 mg/ml Lakshmi Taru 5) 10 mg/ml Kokum 6) 10 Pepper mg/ml

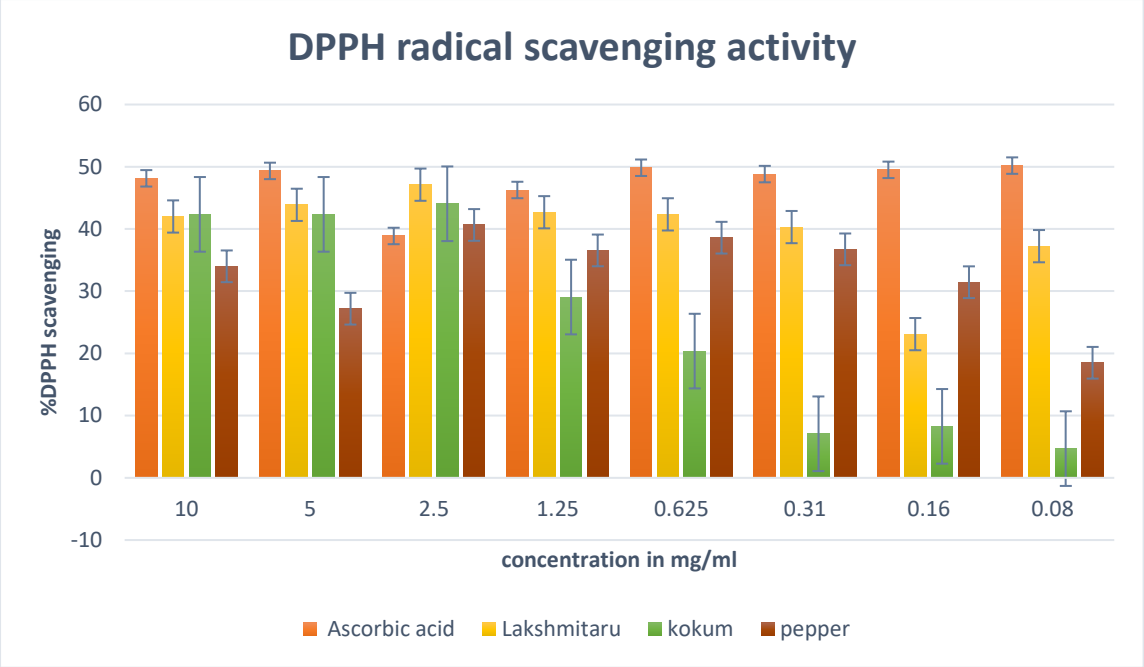

Figure 3.7: Comparison of %DPPH scavenging activity of standard with all 3 test extracts.

From Figure 3.7, on comparison of all 3 extracts with an ascorbic acid standard, it can be said that Lakshmi Taru showed high percentage radical scavenging activity for most concentrations followed by Kokum with slight decrease at 1.25 mg/ml. Pepper showed its highest activity at 2.5 mg/ml.

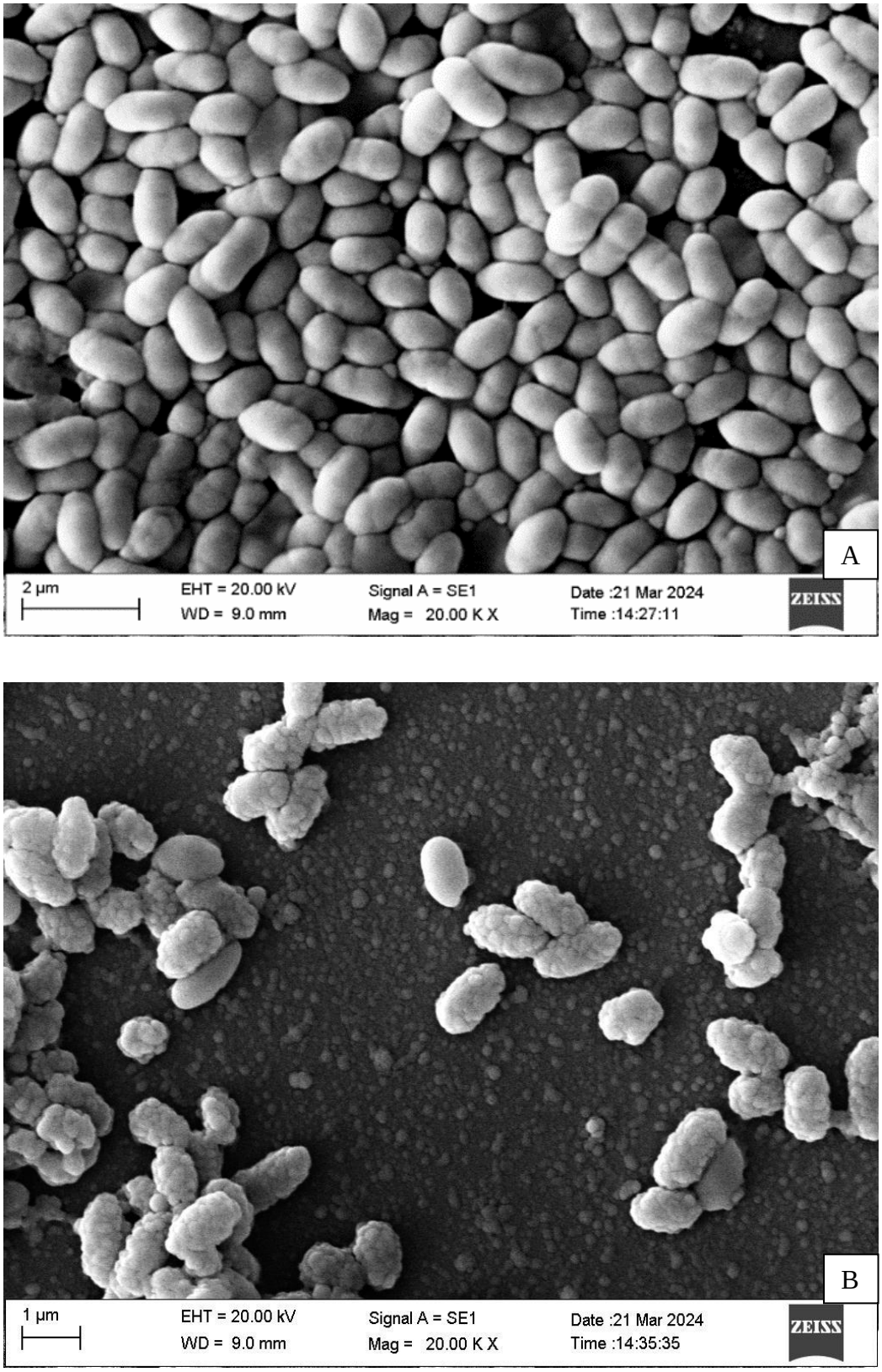

Figure 3.8: Scanning electron microscopy of A) untreated *Ralstonia solanacearum* cells

B) Treated *Ralstonia solanacearum* cells

Table 3.12: Soil pathogen population count

		Colony count		Viable count in Cfu/ml
	Dilution	Plate 1	Plate 2	
Test	10^{-2}	0	0	-
	10^{-3}	0	0	-
	10^{-4}	0	0	-
Culture control	10^{-4}	61	44	$53x10^5$
	10^{-5}	10	4	$7x10^6$
DMSO control	10^{-2}	0	0	-
	10^{-3}	0	0	-
	10^{-4}	0	0	-

From the experiment conducted twice, it was concluded that the population of 0.1 OD inoculated *Ralstonia solanacearum* in soil was reduced significantly on treatment with Lakshmi Taru extract. No colonies were seen on DMSO plates upto 10^{-2} dilution. Culture control showed viable colonies of $53x10^5$

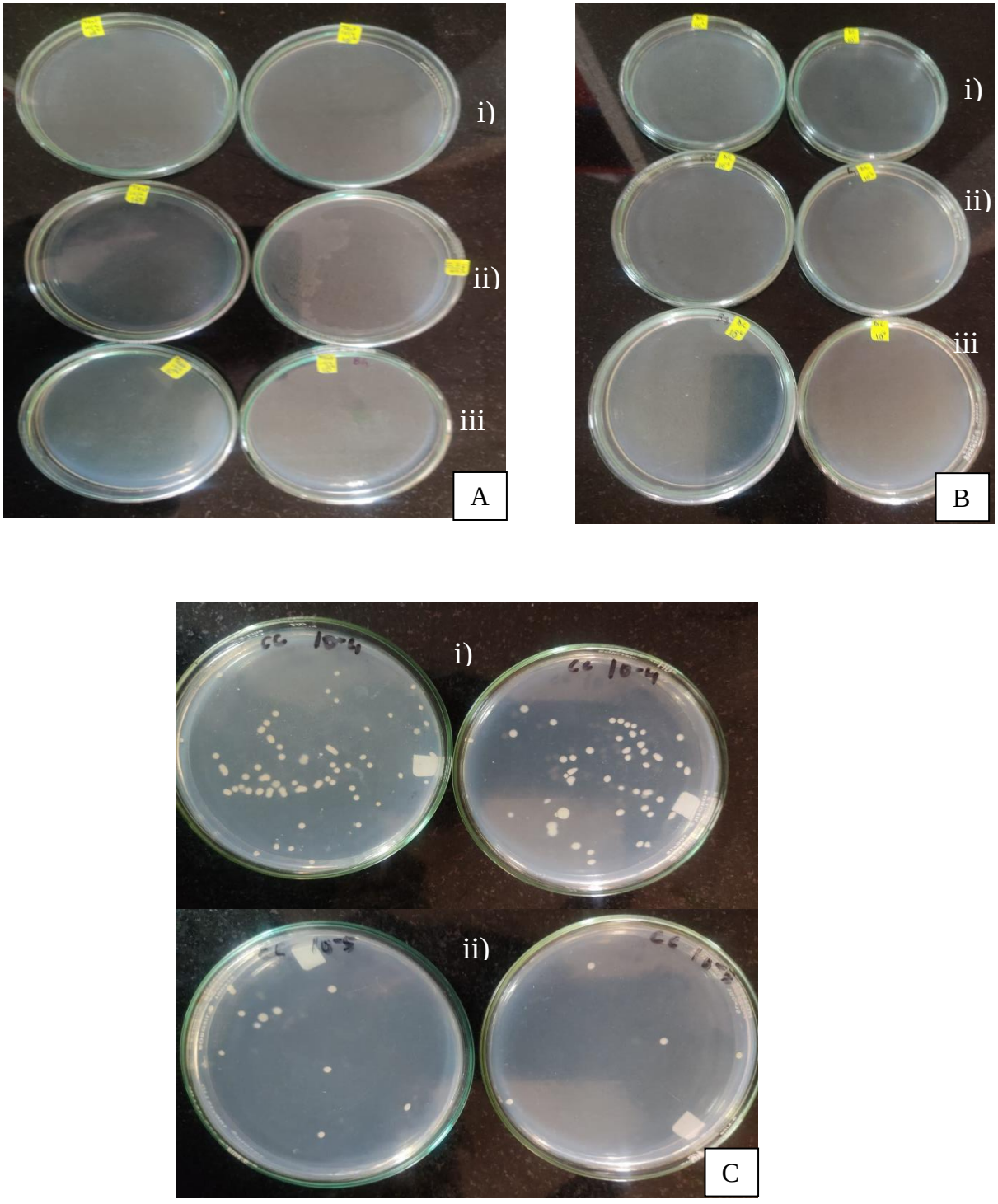

Figure 3.9: Soil population studies showing no growth on A) test sample treated with Lakshmi Taru where i) 10^{-2} ii) 10^{-3} iii) 10^{-4} , B) DMSO control where i) 10^{-2} ii) 10^{-3} iii) 10^{-4} and showing growth on C) culture control where i) 10^{-4} and ii) 10^{-5}

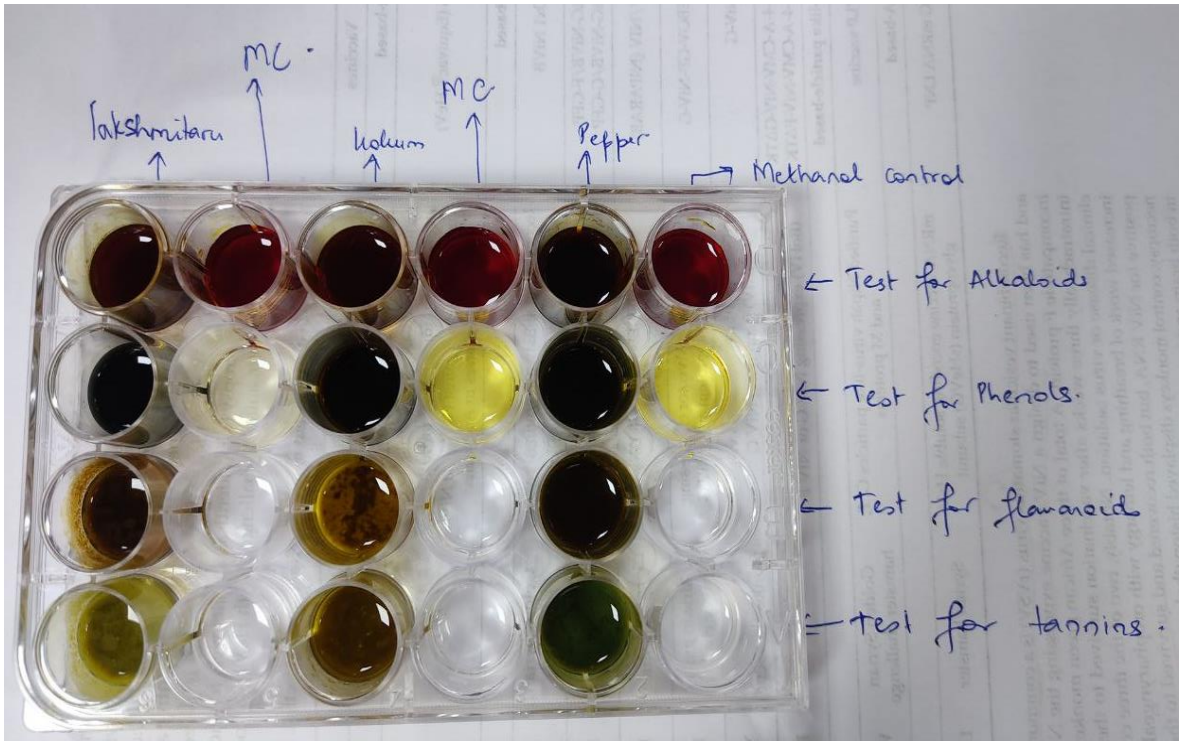

Figure 3.10: Phytochemical screening of all 3 methanolic extracts.

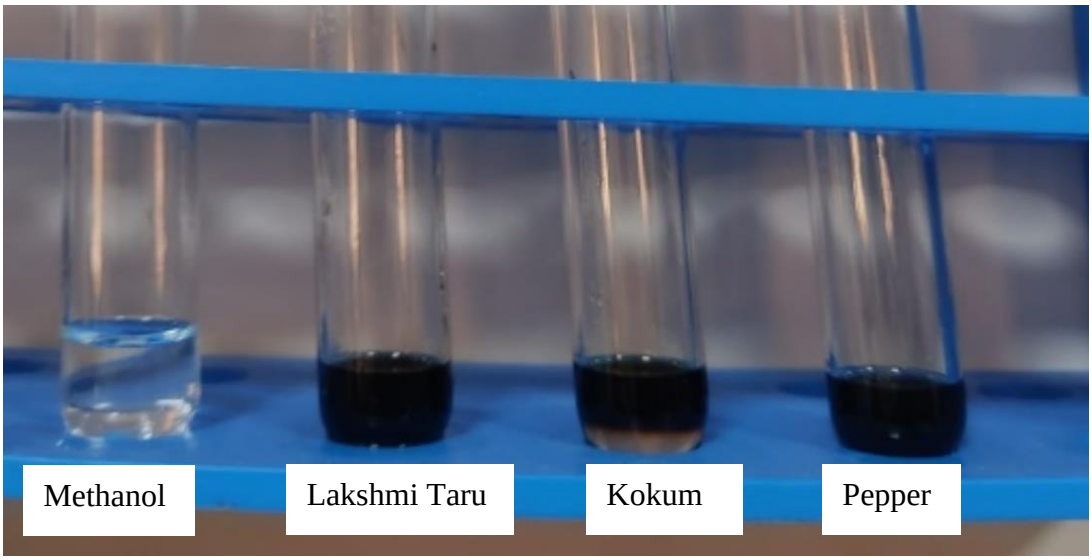

Figure 3.11: Screening test for Terpenoids

CHAPTER 4:

ANALYSIS AND

CONCLUSIONS

The antimicrobial activity of 3 plant extracts (*Simarouba glauca, Garcinia indica, Piper nigrum*) were tested against bacterial plant pathogens such as *Ralstonia solanacearum* and *Xanthomonas* sp as well as fungal plant pathogens, namely *Fusarium oxysporum f.sp.solani, Fusarium oxysporum f.sp.niveum* and *Sclerotium gerbera.* The methanolic extracts of all 3 plants showed antimicrobial activity with highest inhibition activity against *Ralstonia solanacearum* by *Simarouba glauca* with zone of inhibition of 26 mm with 10ul of extract. A 6 mm zone of inhibition of ethanolic extract of *S. glauca* against *Pseudomonas aeruginosa* was reported by Hussain et al and a 11 mm zone with methanolic leaf extract of the same against *E. coli* reported by Jangale et al. MIC was determined to be around 5µL (1µL/ml of media). This can be correlated to studies conducted by Soumya and Nambisan where lowest MIC of 0.16ug/ml was obtained with *S.aureus* using *S.glauca.* MBC value of *Ralstonia solanacearum* was proved to be greater than 160 µL. Extract was found to be ineffective against *Xanthomonas* sp in liquid based assay.

Phytochemical screening resulted in positive tests for alkaloids, phenols and tannins which was also reported by Mathew et al. Kumar et al reported higher yields of phytochemicals present in methanolic extracts as compared to ethanolic. T.G Umesh et al phytochemical investigations revealed *Simarouba glauca* leaf has only 0.14 to 0.18% of flavonoids, 250-400 µg/mg phenolics and 67-200 µg/mg tannin content. Antifungal activity of the same was seen against *Fusarium oxysporum* species which is supported by the results obtained by Mikawlrawng et al with fresh methanolic leaf extracts. Scanning electron microscopy of the treated sample and control showed membrane perturbation which could be the initial stages of lysis and similar observations were observed by Soumya and Namibasan with *E. coli, K. pneumoniae, P. aeruginosa, P. vulgaris, S. aureus.* No study with *Ralstonia solanacearum* has been conducted. Best Antioxidant activity was shown by Lakshmi Taru>Kokum>Pepper.

Soil-based population studies carried out indicated an evident decrease in population on treatment with extract as seen on plates which showed no growth on test and growth on culture control plates. However, if the reduction of the population was due to the extract or DMSO was not conclusive due to time constraints and inability to repeat the experiment. No comparison could be made due to lack of literature available.

Methanolic *Garcinia indica* extract displayed a precipitate around the well which, based on other studies, could be a consequence of the high amount of tannins present that could complex with proteins present in the media displaying a visible ring of precipitation as reported by Kraus et al. The extract also showed some antifungal activity against *Sclerotium gerbera and Fusarium* spp with the test colony diameter showing reduction in growth as compared to culture control with no treatment.

Methanolic *Piper nigrum* was ineffective against most test organisms in broth as well as plate based assays. Marginal inhibition was shown in broth-based studies and some antifungal activity was observed against *Fusarium.*

Garcinia indica displayed the presence of flavonoids, tannins and terpenoids with its antimicrobial activity being primarily reported due to presence of furfural by Sutar et al.

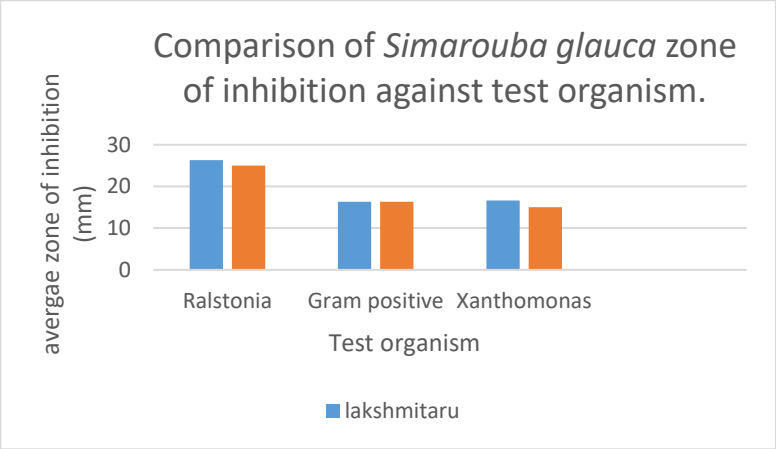

Figure 3.12: Comparison of effectivity of *Simarouba glauca* extract against test organisms

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APPENDIX I

MEDIA

1) Nutrient agar

Ingredients	g/Litre
Peptone	5
Sodium chloride	5
Beef extract	1.5
Yeast extract	1.5
agar	15
pH	7.4

1. Mueller Hinton agar

Ingredients	g/Litre
Beef infusion	300
Casein acid hydrolysate	17.5
Starch	1.5
agar	17
pH	7.3

2. BG agar

Ingredients	g/Litre
Peptone	10
Yeast extract	1.0
Tryptone	1.0
Glucose	5.0
agar	15g

3. Potato dextrose agar

Ingredients	g/Litre
Infusion from potatoes	200
Dextrose	20
Agar	15
pH	5.6

APPENDIX II

REAGENTS

1) 10% Lead acetate solution

1g of lead acetate powder weighed and added to 10ml distilled water. Freshly prepared and Shaken properly before use.

2) 20% NaOH

2g of sodium hydroxide pellets added to 10ml distilled water. Shake vigourously before use

3) 10% Ferric chloride

1g of ferric chloride powder added to 10ml distilled water and used freshly.

4) Wagner reagent

2gm of iodine and 6 gm of potassium iodide added to 100ml of distilled water.

5) DPPH (254 mm stock solution)

0.1g of DPPH powder added to 1 ml 95% methanol. 0.2 ml of this stock solution was added to 1.8 ml 95% methanol. From this solution 1.6 ml was added to 20 ml methanol

6) 1% ascorbic acid

0.1 g of L-Ascorbic acid added to 10 ml methanol giving final concentration of 10mg/ml