

EXPLORATION OF BIOLOGICALLY ACTIVE MEDICINAL PLANTS FOR FOOD INDUSTRIAL APPLICATIONS

Submitted in partial fulfilment of the requirements for the award of
Bachelor of Technology degree in Biotechnology

By

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SATHYABAMA

**INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)**

Accredited with Grade "A" by NAAC

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BONAFAIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **Sabitha S (38230028)**, who carried out the project entitled "**EXPLORATION OF BIOLOGICALLY ACTIVE MEDICINAL PLANTS FOR FOOD INDUSTRIAL APPLICATIONS**" under my / our supervision from **January 2022** to **April 2022**.

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ABSTRACT

BACKGROUND. Post-harvest losses of fresh produce continue to be a problem in the agricultural sector, as they are highly perishable and this loss is due to microbial contamination, improper handling, temperature, and transportation. Today, synthetic materials are used to protect fresh produce, which has side effects for both humans and the environment. Thus, there is an increasing demand for organic protectants containing plant bioactive components to preserve and protect fresh produce.

MATERIALS AND METHODS. The present study was undertaken to prepare the Bio wash and Biodegradable wrappers to preserve the fresh produce. Biologically active medicinal plants were collected, processed, formulated and extracted using ethanol, methanol and aqueous. The disc diffusion method was employed to determine the antimicrobial activity of the different formulations. The effective formulation was tested for its phytochemical property and antioxidant property by DPPH assay. The antimicrobial activity of the formulated Bio wash and its ingredient was carried out by the agar well method. Its efficacy and physicochemical property were evaluated and compared with commercial Bio wash. A Biodegradable wrapper was prepared and antimicrobial activity was evaluated. Physical, chemical, and biodegradability tests were also conducted.

RESULTS. From the evaluation of antimicrobial activity, the combination of Neem, lemon peel and Tulsi was employed for the preparation of protectants. Phytochemical analysis showed the presence of secondary metabolites. Bio wash had a zone of inhibition of 17 mm and 12 mm against *S. aureus* and *P. aeruginosa*, respectively while commercial Bio wash had a 13 mm zone of inhibition against *S. aureus*. After Bio washing, bacterial growth was reduced compared to washing with regular water and the reduction in bacterial growth was observed from the third minute. It has good stability and organoleptic properties with no particulate. The IC₅₀ value was determined to be 129.45 µg/ml, which was almost close to the IC₅₀ value of the standard 110.89 µg/ml. The prepared wrapper has 14mm and 9 mm zone of inhibition against *S. aureus* and *P. aeruginosa* respectively. It showed varying characteristics with thickness 0.198 mm, density 2.731 g/cm³, moisture content

10.47%, and water absorption percentage 18.94 %. It exhibited a pleasant color, odor, and resistance to chemical solvents. The wrapper was almost degraded within 15 days of being buried in the soil while the synthetic wrapper exhibited no change.

CONCLUSION. In the current study, we have formulated the Bio wash and Bio wrappers for fresh produce that possess antimicrobial, antioxidant, phytochemical and physicochemical properties. They are considered safe because they do not contain soap, detergents, perfume, and alcohol. It could pave the way as an alternative to synthetic protection. It can be used in the food industry to protect and prolong the shelf life the fresh produce. As a result, food waste and loss are reduced, and food safety is improved. Further, toxicity studies have to be investigated to extend their applicability.

LIST OF CONTENTS

CHAPTER NO	TITLE	PAGE NO
	ABSTRACT	i
	LIST OF ABBREVIATIONS	vii
	LIST OF FIGURES	viii
	LIST OF TABLES	x
1	INTRODUCTION	2
1.1	Consequences of Food Spoilage	2
1.1.1	Impact on economy	3
1.1.2	Impact on human health	3
1.2	Biologically active medicinal plants for protection of fresh produce	4
1.2.1	Washing of Fresh produce	6
1.2.2	Packaging of Fresh produce	6
2	LITERATURE SURVEY	9
3	AIM AND SCOPE OF THE PRESENT STUDY	14
3.1	Aim	14
3.2	Objective	14
3.3	Scope	14
4	MATERIALS AND METHODS	16
4.1	Sample Collection	16
4.2	Formulation of herbal plants	16
4.3	Preparation of herbal Extracts	17
4.3.1	Solvent extraction using Ethanol	17
4.3.2	Solvent extraction using methanol	17
4.3.3	Aqueous extraction	18
4.4	In Vitro Screening for antimicrobial activity against bacterial and fungal pathogens	18
4.4.1	Test organisms	18
4.4.2	Preparation of inoculum	18

4.4.3	Preparation of Nutrient agar plate	18
4.4.4	Preparation of Extract disc	18
4.4.5	Antimicrobial Activity of different formulations using disc diffusion method	19
4.4.6	Antimicrobial Activity of aqueous extract of F1 using agar well diffusion method	19
4.4.7	Synergistic Antimicrobial Activity of ethanol and methanol extracts	19
4.4.8	Antimicrobial Activity by 96 well plate method	20
4.5	In Vitro Antioxidant assay of extract	20
4.5.1	Reagent preparation	21
4.5.1.1	0.1mM DPPH	21
4.5.1.2	Standard Stock solution	21
4.5.1.3	Sample Stock solution	21
4.5.1.4	Standard and Sample working solution	21
4.5.2	Working Procedure	21
4.6	Qualitative phytochemical analysis	22
4.6.1	Test for alkaloids	22
4.6.2	Test for carbohydrates	22
4.6.3	Test for flavonoids	22
4.6.4	Test for Phenols and Tannins	23
4.6.5	Test for Terpenoids	23
4.6.6	Test for Saponins	23
4.6.7	Test for Glycosides	23
4.6.8	Test for Steroids	23
4.6.9	Test for Quinones	23
4.6.10	Test for Anthraquinones	23
4.7	Application of selected Biologically active medicinal plants for food industrial applications	24
4.7.1	Preparation of Bio wash	24
4.7.1.1	Evaluation of formulated Bio wash	24
4.7.1.1.1	Antimicrobial activity of formulated Bio wash and its ingredients	24
4.7.1.1.2	Determination of Efficacy of Bio wash	25

4.7.1.1.3	Microbial load in effluent	26
4.7.1.1.4	Efficacy of Bio wash on time intervals	27
4.7.1.2	Physiochemical characterization of formulated Bio wash	27
4.7.1.2.1	Organoleptic Test	27
4.7.1.2.2	pH Evaluation	27
4.7.1.2.3	Shelf life of Bio wash	27
4.7.1.3	In Vitro Antioxidant assay of Bio wash	28
4.7.2	Preparation of Biodegradable wrapper	28
4.7.2.1	Evaluation of prepared Biodegradable wrapper	29
4.7.2.1.1	In Vitro Screening for antimicrobial activity of prepared Bio wrapper	29
4.7.2.1.1.1	Test organisms	29
4.7.2.1.1.2	Preparation of inoculum	29
4.7.2.1.1.3	Preparation of test Bio wrapper	30
4.7.2.1.1.4	Antimicrobial Activity of Bio wrapper	30
4.7.2.1.2	Clarity and transparency	30
4.7.2.1.3	Flammability test	30
4.7.2.1.4	Air test	30
4.7.2.1.5	Thickness	30
4.7.2.1.6	Density	31
4.7.2.1.7	Moisture content	31
4.7.2.1.8	Water absorption percentage	31
4.7.2.1.9	Chemical resistance	31
4.7.2.1.10	Biodegradable test by soil burial method	32
5	RESULTS AND DISCUSSION	33
5.1	Sample Collection	33
5.2	Preparation of herbal Extracts	34
5.3	In Vitro Screening for antimicrobial activity against bacterial and fungal pathogens	36
5.3.1	Test organisms	36
5.3.2	Preparation of inoculum	37
5.3.3	Antimicrobial Activity of different formulations using disc diffusion method	37

5.3.4	Antimicrobial Activity of aqueous extract of F1 using agar well diffusion method	39
5.3.5	Synergistic Antimicrobial Activity of ethanol and methanol extracts	40
5.3.6	Antimicrobial Activity by 96 well plate method	43
5.4	In Vitro Antioxidant assay of extract	45
5.5	Qualitative phytochemical analysis	47
5.6	Application of selected biologically active medicinal plants for food industrial applications	48
5.6.1	Preparation of Bio wash	48
5.6.1.1	Evaluation of formulated Bio wash	49
5.6.1.1.1	Antimicrobial activity of formulated Bio wash and its ingredients	49
5.6.1.1.2	Determination of Efficacy of Bio wash	51
5.6.1.1.3	Microbial load in effluent	52
5.6.1.1.4	Efficacy of Bio wash on time intervals	54
5.6.1.2	Physiochemical characterization of Formulated Bio wash	54
5.6.1.3	In Vitro Antioxidant assay of Bio wash	55
5.6.2	Preparation of Biodegradable wrapper	56
5.6.2.1	Evaluation of the prepared Biodegradable wrapper	57
5.6.2.1.1	In Vitro Screening for antimicrobial activity of prepared Bio wrapper	57
5.6.2.1.2	Clarity and transparency	58
5.6.2.1.3	Flammability and air test	59
5.6.2.1.4	Thickness and Density	60
5.6.2.1.5	Moisture content and Water absorption percentage	60
5.6.2.1.6	Chemical resistance	61
5.6.2.1.7	Biodegradable test by soil burial method	62
6	SUMMARY AND CONCLUSION	64
7	REFERENCES	65

LIST OF ABBREVIATIONS

ACRONYM	EXPANSION
D/W	Distilled water
G	Gram
µg	Microgram
mg	Milligram
µl	Microlitre
ml	Milliliter
mm	Millimeter
cm	Centimetre
mM	Millimolar
%	Percentage
Hrs	Hours
Mins	Minutes
DPPH	1,1-Diphenyl-2-Picryl Hydrazyl
CFU	Colony-forming unit

LIST OF FIGURES

FIGURE NO	TITLE	PAGE NO
4.1	Methodology to prepare Bio wash and Bio wrapper	16
4.2	Plate preparation for In Vitro Antioxidant assay	22
4.3	Demonstration to evaluate the efficacy of Bio wash	26
4.4	A pictorial representation of Bio wrapper preparation	29
5.1	Sample preparation	35
5.2	Preparation of herbal extracts	36
5.3	Test organism Subcultured	37
5.4	Inoculum Preparation	37
5.5	Antimicrobial Activity of different formulations	38
5.6	Graphical representation of Antimicrobial Activity of different formulations	40
5.7	Synergistic Antimicrobial activity of ethanol and methanol extracts	41
5.8	Graphical representation of Synergistic Antimicrobial activity of ethanol and methanol extracts	43
5.9	Graphical representation of antimicrobial Activity by 96 well plate method	44
5.10	Graphical representation of In Vitro Antioxidant activity by DPPH assay	46
5.11	IC50 values of tested samples	46
5.12	Qualitative phytochemical analysis	47
5.13	Formulated Bio wash	49
5.14	Antimicrobial activity of Bio wash and its ingredients	50
5.15	Appearance of apple before and after Bio washing	52
5.16	Determination of Efficacy of Bio wash	52
5.17	Microbial load in effluent	53
5.18	Graph plotted between Cfu/ml and dilution factor for testing the microbial load in effluent	53
5.19	Efficacy of Bio wash on time intervals	54
5.20	In Vitro Antioxidant assay of Bio wash	56

5.21	IC ₅₀ Value of Bio wash	56
5.22	Biodegradable wrappers	57
5.23	Antimicrobial activity of Bio wrappers	58
5.24	Flammability Test	59
5.25	Air test	60
5.26	Chemical resistance test	62
5.27	Biodegradable test by soil burial method	63

LIST OF TABLES

TABLE NO	TITLE	PAGE NO
1.1	Information about the samples selected for the Current Study	5
4.1	Formulation of herbal plants	17
4.2	Composition of the film-forming solution	28
5.1	Antimicrobial Activity of different formulations using disc diffusion method	39
5.2	Antimicrobial Activity of aqueous extract of F1 using agar well diffusion method	39
5.3	Synergistic Antimicrobial activity of ethanol and methanol extracts	41
5.4	Antimicrobial Activity by 96 well plate method	44
5.5	In Vitro Antioxidant activity by DPPH assay	45
5.6	Qualitative phytochemical analysis	47
5.7	Formulation of Bio wash	49
5.8	Antimicrobial activity of Bio wash and its ingredients	51
5.9	CFU/ml of effluent	53
5.10	Physiochemical characterization of Formulated Bio wash	55
5.11	In Vitro Antioxidant assay of Bio wash	55
5.12	Antimicrobial activity of Bio wrappers	58
5.13	Clarity and transparency	59
5.14	Flammability test	59
5.15	Thickness and Density	60
5.16	Moisture content and Water absorption percentage (%)	61
5.17	Chemical resistance	62

CHAPTER 1

INTRODUCTION

1 INTRODUCTION

Fruits and vegetables are key parts of a healthy diet because they include phytonutrients and micronutrients that aid the body's biological functioning (Bhilwadikar *et al.*, 2019). India presently produces 71 million tonnes of fruit and 133 million tonnes of vegetables across a total area of 6 million hectares and 8 million hectares, respectively. The global production of fruits and vegetables is 550 million tonnes and 910 million tonnes, respectively. While every year India produces 64 million tonnes of fruit and 126 million tonnes of vegetables, accounting for about 12 and 14 % of global production, respectively (Jain, 2019). According to the United Nations Food and Agriculture Organization (FAO), approximately one-third of the produced food is wasted across the globe and this loss is due to contamination, improper handling, harvesting, temperature, humidity, and transportation (Sagar *et al.*, 2018, Wunderlich *et al.*, 2018). Microorganisms, on the other hand, are responsible for the majority of food spoilage, as they colonize quickly, multiply rapidly, and produce harmful compounds that make them intended for human consumption, as they compete with people for scarce and vital food resources (Hammond *et al.*, 2015). About 45% of fruits and vegetables are spoiled due to their fragile nature and short shelf life (Matar *et al.*, 2018). Food deterioration lowers the energy, nutrient value, and color of the food (Han *et al.*, 2018). We discuss the consequences of food spoilage, its impact, and possible ways to reduce it through an organic and eco-friendly manner.

1.1 CONSEQUENCES OF FOOD SPOILAGE

As the world's population rises, food demand also increases. Healthy living necessitates high-quality, safe food. Food deterioration, on the other hand, has led to food insecurity in several parts of the world (Odeyemi *et al.*, 2020). Food spoilage happens when the food becomes unpalatable, hazardous, and incompatible for human consumption and also affects the food's color, flavor, texture and odor or quality (Pawar *et al.*, 2021, Sahu *et al.*, 2016). Food waste and losses occur at every stage of the food supply chain, from planting to harvesting, processing, storing, and marketing, all the way to consumers and the food service industry (Goossens *et al.*, 2019). It can be caused by a variety of physical, microbiological, or chemical processes. Major factors contributing to food spoilage include pH, temperature, nutrients, air, microorganisms, and chemical reactions such as oxidation,

proteolysis, putrefaction, and hydrolytic rancidity (Amit *et al.*, 2017). Bacterial species like *Escherichia coli*, *Salmonella* spp., *Staphylococcus aureus*, *Listeria* spp., *Klebsiella* spp., *Vibrio*, and fungal species like *Fusarium* spp., *Blastomyces*, *Saccharomyces* species, *Mucor* spp., *Aspergillus* spp., *Cladosporium* spp., are some disease-causing microbes found in food (Gizaw, 2019, Lianou *et al.*, 2016). This food deterioration can impact the economy and health.

1.1.1 Impact on economy

It is also an economic loss when the time and resources spent on production processes are taken into account, along with the use of land, fertilizers, and other costs associated with agricultural production (Vilariño *et al.*, 2017). Both farmers and consumers suffer significant financial losses as a result of food spoilage. About 7000 crores of fruits and vegetables are produced in India per year of which 2100 crores are lost to wastage (Rawat, 2015). According to the FAO, 1.3 billion tons of food waste per year accounts for a massive financial burden (Petruzzi *et al.*, 2017). With such an amount, about one-eighth of the global population would be provided with enough food to overcome malnourishment and meet the growing global food demand which is expected to reach 150–170% of present levels by 2050 (Ishangulyyev *et al.*, 2019). In both product and revenue terms, it's estimated that microbial deterioration contributes to more than 20% of all fruit and vegetable losses (Bautista, 2014). Every 1% decrease in loss saves 5 million tonnes of fruit and vegetables each year (Sahu *et al.*, 2016). Lowering post-harvest losses decreases production, distribution, and trade costs, as well as lowers consumer prices, and promotes farmer revenue (Rawat, 2015).

1.1.2 Impact on human health

In addition to economic losses, food waste causes problems in other areas like environmental loss and social damage (Mustamin *et al.*, 2020). The World Health Organization estimates that every year 600 million individuals nearly one out of every ten people on the earth become unwell upon consuming tainted food, with 4,20,000 deaths, leading to the loss of 33 million healthy lives. Each year 550 million people get sick from diarrhea caused by consuming contaminated food, and 2,30,000 die from it (WHO, 2020). Reduced food spoiling will help to alleviate world

hunger and increase food security. Additionally, food nutrition and security would be improved (Vilariño *et al.*, 2017).

1.2 BIOLOGICALLY ACTIVE MEDICINAL PLANTS FOR PROTECTION OF FRESH PRODUCE

As previously discussed, food spoilage is a big issue that harms both humans and the economy by causing the food to lose its appearance and nutritional value. To maintain the quality of food and prevent it from deteriorating, a preservation technique is the best option (Md. Sharif *et al.*, 2017). Currently, food preservation and safety are ensured by proper handling, the deployment of the cold chain, and the inclusion of chemical preservatives. While, synthetic preservatives cause a lot of side effects, which makes natural preservatives more appealing. Herbal medicines are more readily available, less expensive, time-tested, and thought to be safe with no adverse effects than synthetic ones (Dubey *et al.*, 2018). There are several phytochemicals in medicinal plants, such as flavonoids, alkaloids, tannins, and terpenoids, which have antimicrobial and antioxidant properties (Gonelimali *et al.*, 2018). It exhibits therapeutic properties such as antioxidant, antibacterial, anti-inflammatory, anticancer, and antiviral (Parham *et al.*, 2020). Exploring the natural antimicrobials offers a chance to reduce foodborne illnesses. The supplementation of essential oils with medicinal plants extracts offers a significant opportunity because the extracts possess bioactive phytochemicals that may make the food safer by inhibiting the growth of food spoilage-causing pathogens and therefore limit the spread of foodborne illnesses (Ullah *et al.*, 2020). In the current study, nine medicinal plants drew our attention as potential sources of extracts for food protection. Table 1.1 shows the information about the selected plants for this study. Our main emphasis is to prepare Bio wash and Bio wrapper to decontaminate the microbes and extend the shelf life of fresh produce using the biologically active medicinal plants.

Table:1.1 Information about the samples selected for the Current Study

S. No	Samples	Scientific Name	Family Name	Other names	Origin	Bioactive Compounds
1.	Karpuravalli Leaves	<i>Plectranthus amboinicus</i>	<i>Lamiaceae</i>	Indian Borage, Indian Mint	India, Arabian Peninsula	Carvacrol, Thymol, Caryophyllene, α -Humulene, γ -Terpinene, eugenol
2.	Tulsi Leaves	<i>Ocimum tenuiflorum</i>	<i>Lamiaceae</i>	Vrinda (holy basil)	North central India	Oleanolic, Ursolic, Rosmarinic acid, Eugenol, Carvacrol, Linalool
3.	Neem leaves	<i>Azadirachta indica</i>	<i>Meliaceae</i>	Nim, Margosa	India, South Asia	Azadirachtin, nimbin, ascorbic acid, Salanin, nimbandiol
4.	Guava leaves	<i>Psidium guajava</i>	<i>Myrtaceae</i>	guyava, Amaruud	Mexico, India, Central America	Coumarins, psoralins, cineol, triterpenes, tannins, flavonoids, malic acid
5.	Lemon peel	<i>Citrus limon</i>	<i>Rutaceae</i>	Citron, Nimbu	Northeast India, Myanmar	D-limonene, pinene, terpinene, flavonoids, phenolic acids, coumarins
6.	Dried ginger-Chukku	<i>Zingiber officinale</i>	<i>Zingiberaceae</i>	Sonth	Maritime Southeast Asia	Gingerols, shogaols, paradols, terpene, vitamin C
7.	Omam seed powder	<i>Trachyspermum ammi</i>	<i>Apiaceae</i>	Ajwain, carom, thymol	India and Iran	Carbohydrates, saponins, phenolic compounds, thymol, γ -terpinene
8.	Vasambu powder	<i>Acorus calamus</i>	<i>Acoraceae</i>	Sweet flag, Muskrat root	Asia	β -asarone, β -cadinene, benzoquinone, calamendiol, spathulenol, β -Sitosterol
9.	Nilavembu powder	<i>Andrographis paniculate</i>	<i>Acanthaceae</i>	Creast, green chiretta	India and Sri Lanka	Alkaloids, flavonoids, phenols, tannins, coumarin
10.	Clove oil	<i>Syzygium aromaticum</i>	<i>Myrtaceae</i>	Nail, Clavus	Indonesia	Eugenol, eugenyl acetate, α -humulene, β -caryophyllene, 2-heptanone

1.2.1 Washing of Fresh produce

Vegetables are usually cleaned in water that contains free chlorine in the range of 0 to 30 ppm. Chlorine and chlorinated derivatives have been used in the food industry for decades as a sanitizer in the food industry. Many researchers believe that excessive chlorine use can be harmful because residual chlorine reacts with organic matter to produce carcinogenic disinfection by-products such as trihalomethanes, chloropicrins, chloramines, haloacetic acids and halo ketones. The use of chlorine in the food sector is prohibited in European countries such as the Netherlands, Sweden, Germany, and Belgium due to the risks it poses (Bilek and Turantas, 2013). Apart from Chlorine, chlorine dioxide, ethanol, ozone, organic acids, hydrogen peroxide, and electrolyzed water are used as disinfectants on fresh produce but are not advisable to use in large quantities (Feliziani *et al.*, 2016). Premier R, (2012) examined the vegetable washing chemicals Citox, Aussan, Citrofresh, Nylate, stabilized chlorine dioxide, and Peroxyacetic Acid, each of which has its own set of benefits and drawbacks. Medicinal plants can be exploited as a substitute for synthetic preservatives in the food industry. Antimicrobial, antioxidant, and anti-inflammatory properties of medicinal plants have paved the way for the prevention of a wide range of infectious diseases, as well as societal benefits (Dubey, 2018). Alkaloids, Flavonoids, and polyphenolic compounds found in indigenous medicinal plants have antiseptic, disinfecting, and antibacterial properties (Alghamdi, 2021). Until now, no studies have been conducted on our herbal combinations for food industry applications. As far as we know, there hasn't been any previous research published on herbal Bio-wash for fruits and vegetables.

1.2.2 Packaging of Fresh produce

Food packing is typically thought to be a waste of money. In the wrong eyes, it is seen as an added economic and environmental burden, rather it is an effective tool to prevent food losses by extending the shelf life of food (Guillard *et al.*, 2018). Packaging material for food is designed to protect it from microorganisms, heat, light, pressure, and moisture, also extend the shelf life and preserve its quality (Biji *et al.*, 2015). In addition to protecting food items, packaging materials need to be able to maintain our ecosystem (Nidhin *et al.*, 2019). Various materials used for packaging food include biobased/biodegradable, synthetic/non-biodegradable,

plastics, glass, paper, and metal (Teck Kim *et al.*, 2014). Decomposition of polymer plastic materials can take up to 100 years. It could take more than 20 years for the landfilled polymer to degrade (Chisenga *et al.*, 2020). According to *PlasticsEurope's Market Research and Statistics Group* (PEMRG) and *Conversion Market & Strategy* (GmbH), almost 370 million tons of plastics were produced worldwide in 2019 out of which 39.6% were utilized for packing. In 2018, plastic waste from post-consumer packaging amounted to 17.8 million tons. Of the waste collected, only 42% was recycled, while the rest was deposited in landfills, causing pollution (Plastics-The facts 2020). As a result, efforts are underway to replace plastic with biodegradable alternatives (Chisenga *et al.*, 2020). Biodegradable materials can be effectively employed in food packaging where biodegradability is emphasized as a major feature. Renewable resources such as biomass and agricultural products can be used to produce biodegradable polymers (Nidhin *et al.*, 2019). Sustainable, biocompatible, biodegradable, readily available, and non-toxic attributes make bio-based polymer wrappers highly advantageous (Helanto, 2019). Bio food packaging material has mechanical, optical, and thermal properties (Keskin *et al.*, 2017). Also, it has good antimicrobial properties as well as barrier properties to chemicals, vapor, and odor (Hong *et al.*, 2021). There are many packaging strategies available such as scavengers of oxygen, carbon dioxide, ethylene, moisture, etc., but in this study, we will focus on antimicrobial packaging (Jung & Zhao, 2016). Antimicrobial packaging is a type of active packaging that involves incorporating antimicrobial agents into the film material to eradicate food-borne pathogens, extending shelf life to extremes (Malhotra *et al.*, 2015). Natural antimicrobial agents have broader use than synthetic antimicrobial agents because they are natural and do not harm humans or the environment (Ahmed *et al.*, 2020). Huang *et al.*, (2019) discussed natural antibacterial agents that are employed in food packaging. Herb extracts and essential oils can serve as excellent materials for food packaging, as these are rich in active components and meet the requirements for sustainability (Flores *et al.*, 2021). In this study, synergistic extracts and essential oils are used for the preparation of antimicrobial food packaging.

CHAPTER 2

LITERATURE SURVEY

2 LITERATURE SURVEY

- Preethi R *et al.*, (2009) investigated the antibacterial and antioxidant efficiency of plants such as *Ficus religiosa*, *Holarrhena antidysenterica*, *Psidium guajava* and *Leucas aspera*, in the control of food-borne infections. Methanol, Ethanol and aqueous extracts were used. *P. guajava* methanol extract had excellent antibacterial activity against food-borne pathogens such as *B. Subtilis*, *S. typhi*, and *Pseudomonas species*. The antifungal property of *H. antidysenterica* was higher, with a maximum inhibition zone of 23mm against *Aspergillus spp.* *Psidium guajava* has a strong antioxidant activity of 36 µg/100 mg.
- Mostafa A.A *et al.*, (2018) investigated the antimicrobial property of ethanolic extract of *S. aromaticum*, *T. vulgaris*, *P. granatum*, *Z. officinales* and *C. cyminum* against foodborne pathogens including *S. aureus*, *P. aeruginosa*, *S. typhi*, *B. cereus*, *E. coli* by using the disc diffusion method. Cuminum cyminum was active against *S. aureus*. Ethanolic extracts of *S. aromaticum* and *P. granatum* showed efficient bactericidal and bacteriostatic properties against *S. aureus* and *P. aeruginosa* with MBCs of 5.0 mg/ml and 10 mg/ml and MICs ranges 2.5 mg/ml - 5.0 mg/ml, except for *P. aeruginosa*, which was less susceptible and had an MBC of 12.5 mg/ml *S. aromatic*.
- Institute of Pesticide Formulation Technology- IPFT (2020), has successfully developed a Disinfectant spray for Vegetables & fruits. IPFT has prepared formulations with water to make fruits and vegetables completely safe for human consumption. Vegetables and fruits were decontaminated with the formulation simply by soaking them in diluted formulation for 15-20 minutes and then rinsing them under fresh water. This easy technique removes all pesticides from fruits and vegetables.
- Feliziani E *et al.*, (2016) reviewed some of the disinfectants that can be used to prevent fruit and vegetable disease after harvest. It delves into the history, chemicals, and regulations of some of the most commonly used disinfectants today. Disinfectants are discussed in terms of practical uses, experiments, residues, phytotoxicity, modes of action advantages, and disadvantages.

Chlorine, chlorine dioxide, ethanol, ozone, organic acids, hydrogen peroxide, and electrolyzed water are all examples of disinfectants. The many aspects in this review lead to the conclusion that disinfection is an important strategy for managing postharvest degradation of fresh food.

- Zhang Z *et al.*, (2015) showed that Cinnamic acid (CA) was effective in controlling *Botrytis cinerea*, which causes gray mold on table grapes. CA inhibits the mycelial growth of *B. cinerea* on potato dextrose agar. By staining spores with fluorescent dyes 7-dichlorodihydrofluorescein diacetate and propidium iodide, the strategies by which CA inhibited fungal growth were investigated. The study revealed that CA can disrupt the plasma membrane's integrity and increase *B. cinerea*'s intracellular reactive oxygen species levels, resulting in a decrease in growth rate. Meanwhile, CA treatment increased the activity of peroxidase and polyphenol oxidase, two enzymes that are linked to plant resistance. Overall, this research found that CA was efficient at controlling gray molds in table grapes during the postharvest period by inhibiting pathogen growth and increasing host resistance.
- Ukuku D *et al.*, (2015) developed a natural-compound-based sterilizing wash that could minimize the foodborne infections caused by *E. coli*, *Salmonella*, and *Listeria* on fresh-cut produce. He made a nisin-EDTA base solution that kills surface bacteria on honeydew melons, cantaloupes, and other products which performs better than water, chlorinated water, or hydrogen peroxide. During the fresh-cut preparation process, no bacterial pathogens were transmitted from entire cantaloupes treated with the nisin-based sanitizer to fresh-cut pieces. Furthermore, no colony was found in fresh-cut pieces maintained at 5°C for 7 days,
- Dubey N *et al.*, (2018) reviewed the Washing Chemicals for Fruits and Vegetables. Aussan is an antibacterial disinfectant made from bioflavonoids and organic acids extracted from fruits and vegetables. After a 10-minute exposure, test findings show that it can kill 99.86 % of *P. aeruginosa* and 99.99 % of *S. aureus*. CitroFresh is composed of malic acid, citric acid, bitter orange extract, and vegetable glycerine obtained from plant extracts and is used as a food wash

and natural preservative. It can retain produce fresh for up to 2-3 times longer than regular methods. Effective against fungi, viruses, and bacteria, including *S. aureus*, *E. coli*, *L. monocytogenes*, *S. choleraesuis* and *C. botulinum*. Citrox is a soluble bioflavonoid compound derived from citrus fruits. Effective against pathogenic bacteria, fungus, and yeast.

- Nair M.S *et al.*, (2017) studied the Guava quality chitosan and alginate coatings in combination with pomegranate peel extract (CHE) to maintain food quality. Respiration rate, Ripening Index, Colour, Total phenolics, total flavonoids, Ascorbic acid, and Antioxidant activity were determined. Moreover, it preserved the nutritional characteristics of guava by retarding the respiration rate, thus delaying senescence. CHE samples had reduced losses of ascorbic acid, total phenolics, total flavonoids, and antioxidant activity of 29 %, 8 %, 12 %, 12 % (DPPH), and 9 % (FRAP). Between different phytochemicals and AOA, a higher degree of correlation ($r>0.918$) was observed. PPE-enhanced coatings were found to be effective in preserving the quality of guavas during a 20-day storage period at low temperatures.
- Kim K.M *et al.*, (2006) investigated the antibacterial action of soy protein isolate (SPI) film containing green tea extract (GTE) against *S. aureus*, *S. typhimurium*, *P. aeruginosa* and *E. coli*. The SPI film incorporating GTE's physical and mechanical properties was also investigated. *S. mutans* and *S. aureus* were resistant to the SPI film containing GTE. With increasing GTE concentrations up to 4%, the antibacterial activity of the SPI film containing GTE against *S. mutans* increased. The tensile strength and elongation of SPI films containing GTE were lower, and the total soluble matter was higher than those of control SPI films. As a result, GTE could be utilized in food packaging films as an antibacterial agent.
- Nisar T *et al.*, (2017) developed antimicrobial films by incorporating varying quantities of clove bud essential oil into the citrus pectin. The properties of physical, mechanical, optical, antibacterial, antioxidant, FTIR, DSC, and XRD of the film were studied. According to the findings, adding CEO to pectin has a significant impact on the physicochemical properties of the films, such as

increased thickness. A reduction in moisture content and WVP values, implying higher water barrier properties, substantiated the hydrophobic character of films. DSC analysis improved the mechanical characteristics and thermal stability of the material. *S. aureus*, *E. coli*, and *L. monocytogenes* inhibition zones were 8.50 mm to 30.27 mm, 12.53 mm to 21.20 mm, and 14.67 mm to 26.43 mm, respectively.

- Hu X *et al.*, (2019) used a casting technique to develop *Ginkgo biloba* extract (GBE) integrated gelatin films. The films' mechanical properties, moisture content, solubility, water vapor permeability, film microstructure, infrared spectroscopic features, light barrier property, antioxidant property, and antibacterial property were studied. The study revealed that the incorporation of the GBE into gelatin films reduced moisture content, film solubility and WVP while improving thermal stability, antibacterial against *S. aureus* and *C. albicans*, and antioxidants activities ranged 24.7% to 85.6%. Increased tensile strength with decreased elongation at break was observed.
- Patel J *et al.*, (2019) developed the agar-based bioplastic with glycerol which served as the plasticizer. It exhibited high strength and elasticity. With different glycerol concentrations, it has a 0.0020 cm thickness. It has 335.0 kg/cm³ of tensile strength and 17.5% of elongation of break. FTIR result reveals it has a high bending strength.

CHAPTER 3

AIM AND SCOPE OF THE PRESENT STUDY

3 AIM AND SCOPE OF THE PRESENT STUDY

3.1 AIM

The aim of the study was to explore the biologically active medicinal plants for food industrial applications

3.2 OBJECTIVE

- To collect the medicinal plant
- To formulate and prepare the plant extract using ethanol, methanol and distilled water
- To evaluate the antimicrobial activity and synergistic antimicrobial activity of the extract
- To determine the Antioxidant property by DPPH Assay and phytochemical analysis of the extract
- To formulate, evaluate, characterize and assess the physicochemical properties of the Bio wash
- To prepare and evaluate the Biodegradable wrapper
- To compare the prepared Bio wash and Bio wrapper with the commercial product

3.3 SCOPE

- Bio wash and Bio wrapper developed are safe since they are free of preservatives, detergents, perfumes, and alcohol. The product is 100% natural, eco-friendly, and cost-effective
- It helps to prevent spoilage, extend the shelf life of fresh produce and reduce Food-borne infection. Therefore, food safety is enhanced and wastage is reduced
- The formulated Bio wash can also be used as a hand sanitizer, surfactant, or disinfectant.
- The application of prepared Bio wrappers can also be extended to protect meat and poultry products

CHAPTER 4

MATERIALS AND METHODS

4 MATERIALS AND METHODS

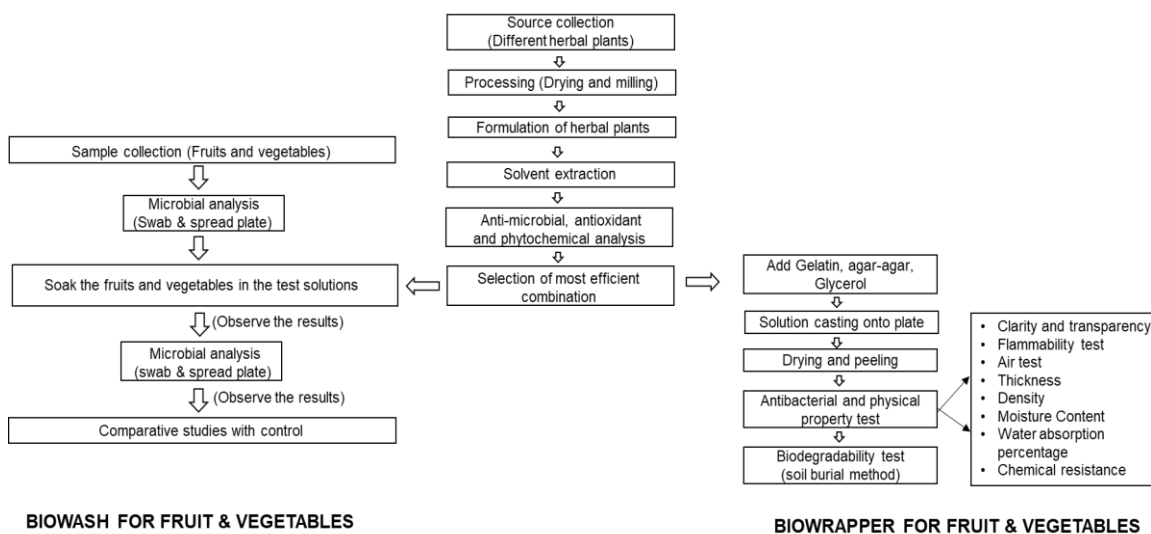


Fig:4.1 Methodology to prepare Bio wash and Bio wrapper

4.1 SAMPLE COLLECTION

The fully matured leaves of *Azadirachta indica* (neem), *Ocimum tenuiflorum* (Tulsi), *Plectranthus amboinicus* (Karpuravalli), *Psidium guajava* (Guava), were collected. Similarly, *Citrus limon* (lemon) peel and dried *Zingiber officinale* (ginger) ie., Sukku were also collected. Then the collected leaves samples were washed under running tap water to remove any dirt before the drying process. The leaves were shade dried for 20 days, then ground into a powder and stored in airtight bags (Hassan and Ullah, 2019). *Trachyspermum ammi* (Omam) seed powder, *Acorus calamus* (Vasambu) powder, *Andrographis paniculata* (Nilavembu) powder were purchased from the herbal store in Chennai, Tamil Nadu, India.

4.2 FORMULATION OF HERBAL PLANTS

Based on random assumption, three different samples were combined in such a way to form five different formulations. 5g of each sample were weighed and placed in the beaker. The following Table 4.1 were the various combinations that were tried. For bulk production, 50g of each sample was used. The most efficient formulation was determined based on the results of the antimicrobial and antioxidant activity which was used for further studies.

Table:4.1 Formulation of herbal plants

Formulation Number	Ingredients
S1	Neem
S2	Lemon peel
S3	Tulsi
F1	Neem, Lemon peel, Tulsi
F2	Tulsi, Lemon peel, Karpuravalli
F3	Tulsi, Karpuravalli, Neem
F4	Omam, Vasambu, Guava leaf
F5	Sukku, Lemon peel, Neela Vembu
F6	Neem, Lemon peel, Tulsi, Turmeric

4.3 PREPARATION OF HERBAL EXTRACTS

The solvent extraction process was carried out to prepare the herbal extracts. Three different solvents i.e., Ethanol, Methanol and Aqueous were used for solvent extraction (Manandhar *et al.*, 2019). The solvent was optimized based on antimicrobial results.

4.3.1 Solvent extraction using Ethanol

For the extraction process, the powdered sample was soaked with an equal volume of ethanol for 24 hrs at room temperature. After 24 hrs, the content was filtered through grade 1 Whatman filter paper and the filtrate was centrifuged to get the particle-free extract, then the supernatant was left for evaporation at room temperature to concentrate the extracts. The resulting extracts were stored in an Eppendorf tube for further analysis.

4.3.2 Solvent extraction using methanol

For the extraction process, the powdered sample was soaked with an equal volume of methanol for 24 hrs at room temperature. After 24 hrs, the content was filtered through grade 1 Whatman filter paper and the filtrate was centrifuged to get the particle-free extract, then the supernatant was left for evaporation at room

temperature to concentrate the extracts. The resulting extracts were stored in an Eppendorf tube for further analysis.

4.3.3 Aqueous extraction

For the extraction process, the powdered sample was soaked with an equal volume of distilled water (D/W) for 24 hours at room temperature. After 24 hrs, the content was filtered through sterilized muslin cloth and the filtrate was centrifuged to get the particle-free extract, then the supernatant was left for evaporation at room temperature to concentrate the extracts. Alternatively, the formulation was boiled to get the extract. The resulting extract was stored in an Eppendorf tube for further analysis (Stéphane F. F. Y et al., 2021).

4.4 IN VITRO SCREENING FOR ANTIMICROBIAL ACTIVITY AGAINST BACTERIAL AND FUNGAL PATHOGENS

4.4.1 Test organisms

The antimicrobial properties of sample extracts were tested against Gram-positive bacteria including *Staphylococcus aureus* 29213 (*S.au*), *Staphylococcus aureus* 25923 (*S.au* 23) and Gram-negative bacteria including *Escherichia coli* 25922 (*E.co*), *Pseudomonas aeruginosa* 27853 (*P. a*), *Klebsiella pneumoniae* 13439 (*K. pm*), *Enterococcus faecalis* 29212 (*E. fa*) as well as one fungal pathogen *Candida albicans* (*C.a*)

4.4.2 Preparation of inoculum

Before an antimicrobial activity, the loopful of overnight microbial culture was added to 1 ml nutrient broth and incubated for 30 mins at room temperature.

4.4.3 Preparation of Nutrient agar plate

A nutrient agar medium was prepared and sterilized in an autoclave. Under aseptic conditions, media was poured into a sterile Petri plate and allowed to solidify, which served as the base agar for bacterial growth.

4.4.4 Preparation of Extract disc

The disc was prepared by punching 6 mm diameter holes in Whatman filter paper, which was then sterilized in an autoclave. With a pipette, 20 µl of extract were dispensed on both sides of the disc and allowed to dry.

4.4.5 Antimicrobial Activity of different formulations using the disc diffusion method

The antimicrobial activity of each plant extract was determined using the disc diffusion method described by (Balouiri *et al.*, 2016). With a sterile cotton swab, each inoculum of the test organism was swabbed onto a nutrient agar plate. The extract impregnated disc was placed on the plate with sterile forceps. Disc dispensed with ethanol, methanol, and aqueous were served as the controls for their respective solvent extracts. All the procedures were performed in a laminar airflow chamber. The plate was then incubated in an incubator for 24 hrs at 37°C. An antimicrobial agent disperses through the nutrient agar thereby it inhibits the growth of the pathogens. The diameter (in mm) of the inhibitory zone around the disc was measured after 24 hrs to assess the antimicrobial activity.

4.4.6 Antimicrobial Activity of aqueous extract of F1 using agar well diffusion method

Agar well diffusion method was performed for aqueous extract of F1 against *S. aureus* and *P. aeruginosa*. Nutrient agar plate and inoculum were prepared as the method described in 4.4.2 - 4.4.3. With a sterile cotton swab, the inoculum of the test organism was swabbed onto a nutrient agar plate. Well with 5mm diameter was punched in each of these plates using a sterile cork borer. About 50 µl of the extract was added to the respective wells with a micropipette without disturbing the nearer well. All the procedure was performed in a laminar airflow chamber. The Plate was then incubated in an incubator for 24 hours at 37°C. An antimicrobial agent disperses through the nutrient agar thereby it inhibits the growth of the pathogens. The diameter (in mm) of the inhibitory zone around the well was measured after 24 hours to assess antimicrobial activity (Gonelimali *et al.*, 2018).

4.4.7 Synergistic Antimicrobial Activity of ethanol and methanol extracts

The disc diffusion method was used to determine the *In-Invitro* synergistic antimicrobial activity. Here, two different formulations of the same solvent were combined to improve the effectiveness of antimicrobial activity (Stefanović, 2017). In a 2ml Eppendorf tube, 500 µl of one ethanolic formulation was mixed with 500 µl of another ethanolic formulation and left to react for 5 hrs. Similarly, This was also done with the methanol extract. Then with a pipette, 20 µl of extract were dispensed on both sides of the disc and allowed to dry. Nutrient agar plate and inoculum were prepared as the method described in 4.4.2 - 4.4.3. With a sterile cotton swab, the inoculum of the test organism was swabbed onto a nutrient agar plate. The extract impregnated disc was then placed on the plate with sterile forceps. Disc dispensed with ethanol, methanol was served as the control for their respective solvent extracts. All the procedure was performed in a laminar airflow chamber. The plate was incubated in an incubator for 24 hrs at 37°C. An antimicrobial agent disperses through the nutrient agar thereby it inhibits the growth of the pathogens. The diameter (in mm) of the inhibitory zone around the discs was measured after 24 hrs to assess the synergistic antimicrobial activity.

4.4.8 Antimicrobial Activity by 96 well plate method

Antimicrobial activity was measured in 96-well plates using the method described by Bala *et al.*, 2011, with slight modification. In 96 well plates, 100 µl of inoculum of *S. aureus* was added to each well. Also, the different sample extracts with different concentrations (20, 40, 60, 80, 100 µg/ml) were added to the well. Nutrient broth and pathogen without extract were used as control. All the procedures were carried out in a laminar airflow chamber. Plates were covered with an adhesive seal and incubated in an incubator for 24 hrs at 37°C. Then, after 24 hrs the absorbance value of the sample was read at 517 nm using a UV spectrophotometer to check for bacterial inhibition.

4.5 IN VITRO ANTIOXIDANT ASSAY OF EXTRACT

The Antioxidant or the free radical scavenging activity of the methanolic extract of S1, S2, S3 and F1 was determined by using 1,1-Diphenyl-2-Picryl Hydrazyl (DPPH) assay according to the method described previously by (Kibiti and Afolayan, 2015, De Torre *et al.*, 2019) with some modification.

4.5.1 Reagent preparation

4.5.1.1 0.1mM DPPH

0.1mM DPPH solution was prepared by dissolving 0.197mg of DPPH in 5ml of methanol and the conical flask was covered with Aluminium foil to prevent its exposure to light.

4.5.1.2 Standard Stock solution

Dissolved 2 mg of ascorbic acid in 2 ml of methanol.

4.5.1.3 Sample Stock solution

Dissolved 1 mg of sample extract (S1, S2, S3, and F1) to 10 ml of methanol.

4.5.1.4 Standard and Sample working solution

From the stock solution, 100, 200, 300, 400, and 500 µg/ml concentrations were prepared respectively.

4.5.2 Working Procedure

In 96 well plate, add 100 µl of 0.1mM DPPH solution to 100µl of samples and 100 µl of standards which are prepared as working solution with different concentrations (100, 200, 300, 400, 500 µg/ml). 100 µl of DPPH solution and 100 µl of methanol was added as control whereas methanol served as the blank for antioxidant assay. The reaction mixture was mixed, wrapped with aluminum foil and allowed to react at room temperature for about 30 mins. Then, the absorbance value of the samples was read at 517 nm using a UV spectrophotometer. The inhibition of radical scavenging activity (%) was determined by using the formula:

$$\text{Radical Scavenging activity (\%)} = \frac{A_0 - A_1}{A_0} \times 100$$

Where, A_0 - Absorbance of the control

A_1 - Absorbance of the sample extract or standard

In Vitro Antioxidant assay was demonstrated as shown in Figure 4.2. The IC_{50} values of extract and standard were calculated from the graph by plotting % of inhibition against concentration. Where IC_{50} is the concentration of sample extract or standard that scavenge 50% of DPPH radicals (Xiao *et al.*, 2020).

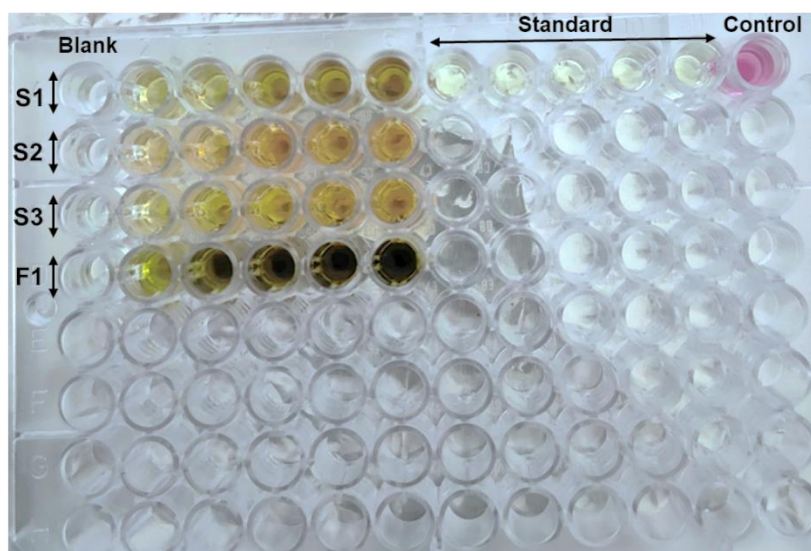


Fig:4.2 Plate preparation for In Vitro Antioxidant assay

4.6 QUALITATIVE PHYTOCHEMICAL ANALYSIS

Phytochemical analysis was performed on the methanol extract of S1, S2, S3, and F1 (Neem, Lemon peel, Tulsi) to determine the presence and absence of phytochemical compounds by visually examining the appearance of colour or frothing by using the following methods described by (Panchal and Parvez, 2019, Deshmukh and Theng, 2018, Yadav and Agarwala, 2011) and phytochemical testing was done for alkaloids, carbohydrates, flavonoids, terpenoids, saponins, glycosides, steroids, quinones, anthraquinones, Phenols and Tannins.

4.6.1 Test for alkaloids

Dragendorff test - To 1ml of extract, one drop of dragendroff reagent was added. The appearance of orange-red color indicates the presence of alkaloids.

4.6.2 Test for carbohydrates

Benedict's test - To 1ml of extract, a few drops of benedict's reagent was added and heated in a water bath. The presence of reducing sugars was indicated by the presence of red, yellow, or green precipitate.

4.6.3 Test for flavonoids

Lead acetate test- To 1ml of extract, a few drops of 10% Lead acetate was added. The presence of flavonoids was indicated by yellow precipitate.

4.6.4 Test for Phenols and Tannins

Lead acetate test - To 1ml of extract, a few drops of 1% Lead acetate was added. The presence of Phenols and Tannins was indicated by the formation of the precipitate.

4.6.5 Test for Terpenoids

To 1ml of extract, 2ml of chloroform was added and evaporated until dry after which 2ml of concentrated Sulfuric acid was added and heated for 2 mins. The presence of Terpenoids was indicated by the formation of a greyish color.

4.6.6 Test for Saponins

Foam test - To 1 ml of extract, 20 ml distilled water was added and shaken vigorously for 15 mins in a graduated cylinder. The production of foam up to 1cm in length indicated that saponins were present.

4.6.7 Test for Glycosides

Salkowski's test - To 1 ml of extract, 2ml of chloroform was added. After that, 2ml of concentrated Sulfuric acid was cautiously added and gently shaken. The steroidal ring, or glycone portion of the glycoside, was indicated by a reddish-brown color.

4.6.8 Test for Steroids

To 1 ml of extract, 2 ml of chloroform was added and to this concentrated Sulfuric acid was cautiously Sideways. The presence of steroids was indicated by the formation of red color at a lower layer of chloroform.

4.6.9 Test for Quinones

To 1 ml of extract, 1 ml of concentrated sulphuric acid was added and the formation of red color indicates the presence of quinones.

4.6.10 Test for Anthraquinones

Borntragers test - To 1 ml of extract, 5 ml of chloroform was added, and the tubes were shaken for 5 mins. Then an equal volume of 10% ammonia solution

was added and blended. The presence of anthraquinone was indicated by the formation of pink-violet or red color at a lower layer.

4.7 APPLICATION OF SELECTED BIOLOGICALLY ACTIVE MEDICINAL PLANTS FOR FOOD INDUSTRIAL APPLICATION

Aqueous extract of formulation 1 (F1_{aq}) i.e., a combination of Neem, lemon peel and Tulsi was used for the preparation of Bio wash and Biodegradable wrapper as this combination exhibited good antimicrobial and antioxidant effect.

4.7.1 Preparation of Bio wash

The Bio wash was made by boiling the F1 formulation with distilled water for 30 mins at 100°C. The mixture was filtered through a muslin cloth and centrifuged to get a particle-free extract, and the supernatant was used for further examination. As a preservative and to enhance the antibacterial activity, a few drops of clove oils were added. F1_{aq} and F1_{aq} + Clove oil are two separate bio-washes that were prepared, shortly B1 and B2 and used for comparative study with commercially available Bio wash (Cortés-Rojas *et al.*, 2014).

4.7.1.1 Evaluation of Formulated Bio wash

Formulated Bio wash was subjected to the antimicrobial test, efficacy and physicochemical studies.

4.7.1.1.1 Antimicrobial activity of formulated Bio-wash and its ingredients

The antimicrobial activity of prepared Bio wash and its ingredients was evaluated against *S. aureus* 25923 (S.au 23), *P. aeruginosa* 27853 (P. a) and *C. albicans* (C.a). Nutrient agar plate and inoculum were prepared as the method described in 4.4.2 - 4.4.3. With a sterile cotton swab, the inoculum of the test organism was swabbed onto a nutrient agar plate. Wells with 5mm diameter were punched in each of these plates using a sterile cork borer. About 50 µl of the samples were loaded to the respective wells with a micropipette. All the procedures were carried out in a laminar airflow chamber. The Plate was then incubated in an incubator for 24 hrs at 37°C. An antimicrobial agent disperses through the nutrient agar thereby it inhibits the growth of the pathogens. The diameter (in mm) of the

inhibitory zone around the well was measured after 24 hours to assess antimicrobial activity (Gonelimali *et al.*, 2018). Commercially available fruit and vegetable wash were also assessed as experimental controls.

4.7.1.1.2 Determination of Efficacy of Bio wash

The efficacy of formulated Bio wash and control was assessed using a test sample, an apple purchased from a local market in Chennai. The procedure was carried out as previously described by Mahapatra *et al.*, (2015) with some modifications.

Step 1: The apple's surface (S) was swabbed with a sterile cotton swab and streaked onto the nutrient agar plate.

Step 2: The apple was soaked in the normal water (NW) for 5 mins and its surface was swabbed and streaked onto the plate using the sterilized cotton swab.

Step 3: The apple was soaked in the test Bio wash (BW) for 5 mins and using the sterilized cotton swab its surface was swabbed and streaked onto the nutrient agar plate.

Step 4: Then the same Bio washed apple was soaked in normal water for 5 mins (ABW) and its surface was swabbed with a sterilized cotton swab and streaked onto the plate.

The process described above was performed in a single Petri plate with four divisions. Then the plate was incubated in an incubator for 24 hrs at 37°C. After 24 hrs the difference in bacterial growth was noted. The same procedure was carried out with other formulated bio-wash and control. A demonstration to evaluate the efficacy of Bio wash was shown in Figure 4.3.



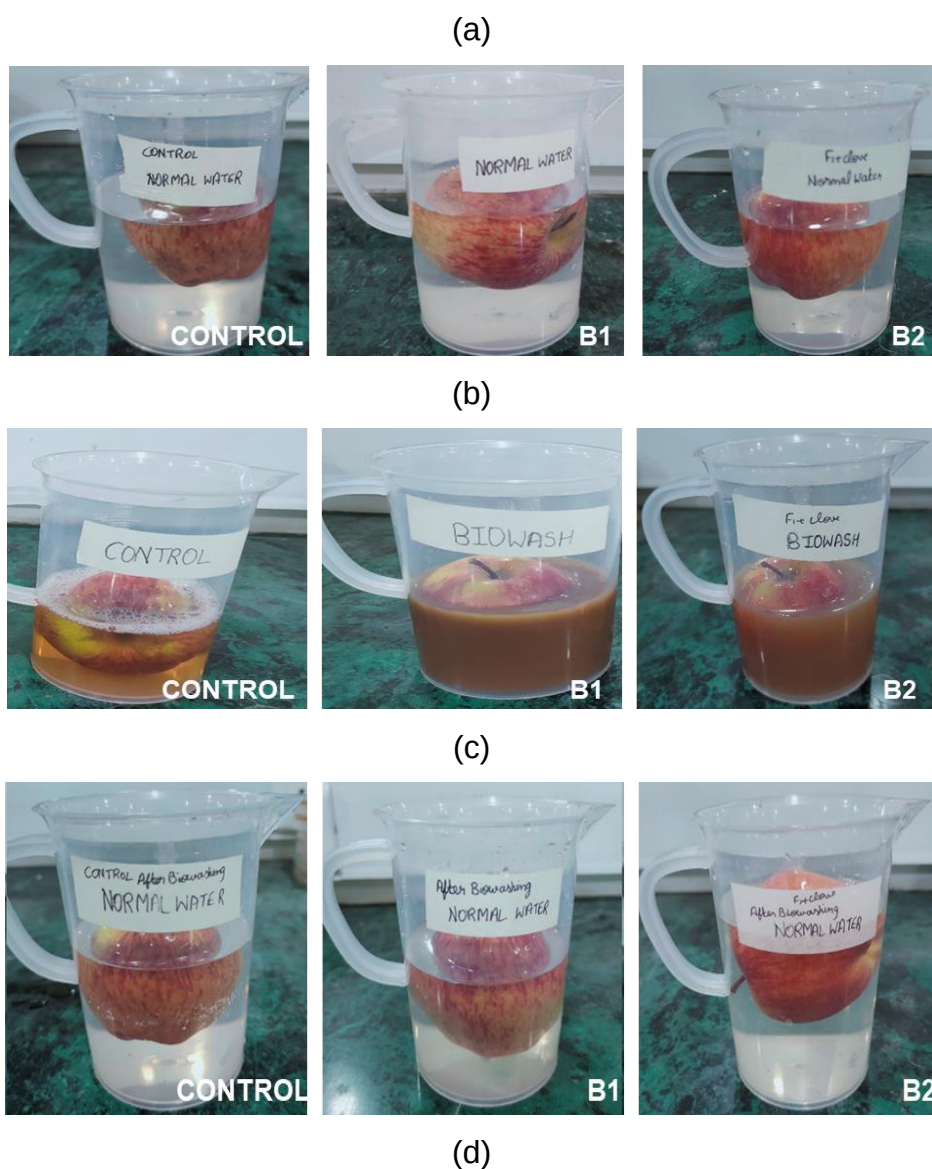


Fig:4.3 Demonstration to evaluate the efficacy of Bio wash

a) Swabbing apple surface; b) Apple soaked in normal water; c) Apple soaked in formulated Bio wash; d) Apple soaked in normal water after Bio washing

4.7.1.1.3 Microbial load in effluent

The microbial load in the effluent i.e, the water and test Bio wash after being washed on the apple was determined using a serial dilution followed by the spread plate method. Serial dilution was done up to 10^{-5} dilution factors with normal water wash, before and after the Bio wash solution. After serial dilution, the solution was spread onto the respective nutrient agar plate using L-rod. The plate was incubated in an incubator at 37°C for 24 h. The microbial load was calculated using

CFU/ml and the formula was given below (Blaize *et al.*, 2022).

$$\text{CFU/ml} = (\text{no. of colonies} \times \text{dilution factor}) / \text{volume of the culture plate}$$

4.7.1.1.4 Efficacy of Bio wash on time intervals

The following procedure was carried out to test the efficacy of the Bio wash and the control.

Step 1: Using a sterilized cotton swab, the apple surface was swabbed and streaked onto the nutrient agar plate.

Step 2: The apple was soaked in the test Bio wash for 1 min and its surface was swabbed with a sterilized cotton swab and streaked onto the nutrient agar plate. Then again, the apple was soaked on the same test Bio wash.

Step 3: Step 2 was repeated for up to 5 minutes, after which the plate was incubated at 37°C for 24 hrs. After 24 hrs bacterial growth was observed. The same procedure was carried out with the formulated Bio wash and control.

4.7.1.2 Physiochemical characterization of formulated Bio wash

4.7.1.2.1 Organoleptic Test

The formulated Bio wash and control were visually evaluated for color, texture, odor, appearance and homogeneity (Booq *et al.*, 2021).

4.7.1.2.2 pH Evaluation

The pH was determined by using pH paper (Shaikh *et al.*, 2020).

4.7.1.2.3 Shelf life of Bio wash

The shelf life of the formulated Bio wash was examined every week for homogeneity at three different temperatures of 4°C, 27°C and 40°C (Chandravanshi *et al.*, 2021).

4.7.1.3 In Vitro Antioxidant assay of Bio wash

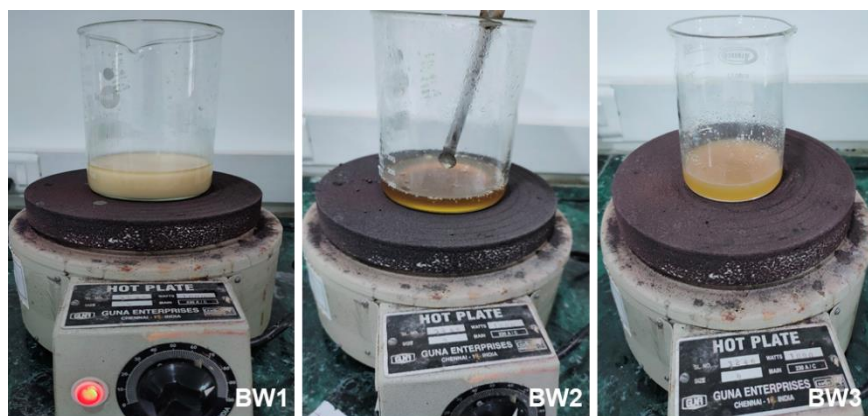
The DPPH free radical scavenging assay was used to assess the antioxidant activity of the Bio wash. This was performed using the same procedure as in section 4.5.

4.7.2 Preparation of biodegradable wrapper

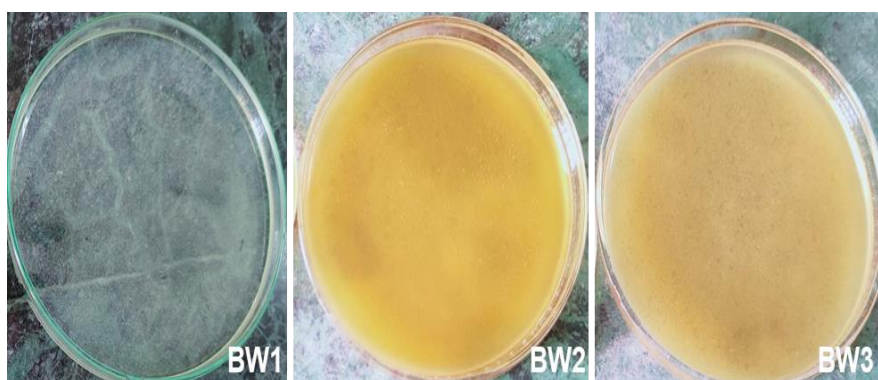
The Biodegradable wrapper was prepared by the casting technique. The composition of the film-forming solution was listed in Table 4.2. The film was prepared by dissolving the respective amount of agar-agar, gelatin, and glycerol in distilled water and boiling it at 80 ° C on a hot plate. The mixture was stirred for 20 min at room temperature to ensure homogeneity of the reaction. After continuous stirring, the solution was poured onto a glass Petri plate and spread uniformly with a glass rod. It was allowed to dry at room temperature for 24 hrs or until the films were completely dry. Then the film prepared was peeled off from the Petri plate and preserved for further experimental investigations. Likewise, three Biowrapper were prepared one with water, one with F1_{aq} extract, and one with F1_{aq} extract and clove oil, where the clove oil served as a preservation and antibacterial activity enhancer and was named BW1, BW2, and BW3 respectively (Marichelvam *et al.*, 2019, Patel *et al.*, 2019, Seeta Uthaya Kumar *et al.*, 2019). A pictorial representation of Bio wrapper preparation was shown in Figure 4.4.

Table:4.2 Composition of the film-forming solution

S. No	Different Biodegradable wrapper	Compositions					
		D/W (ml)	Agar Agar (g)	Gelatin (g)	Glycerol (ml)	Extract (ml)	Clove oil (ml)
1.	With water (BW1)	30	0.75	0.2	1	-	-
2.	With F1 _{aq} (BW2)	15	0.75	0.2	1	15	-
3.	With F1 _{aq} + Clove Oil (BW3)	15	0.75	0.2	1	14.5	0.5



(a)



(b)

Fig:4.4 A pictorial representation of Bio wrapper preparation

a) Boiling of film-forming solution; b) Drying of film

4.7.2.1 Evaluation of prepared Biodegradable wrapper

Antimicrobial analysis, clarity, transparency, flammability, and an air test were all performed on the prepared Bio wrapper. It was also assessed in terms of thickness, density, moisture content, water absorption, chemical resistance, and biodegradability.

4.7.2.1.1 In Vitro Screening for antimicrobial activity of prepared Bio wrapper

4.7.2.1.1.1 Test organisms

The antimicrobial properties of the film were tested against Gram-positive bacteria *Staphylococcus aureus* 25923 (S.au 23) and Gram-negative bacteria *Pseudomonas aeruginosa* 27853 (P.a)

4.7.2.1.1.2 Preparation of inoculum

Before an antimicrobial activity, the loopful of overnight microbial culture was added to 1 ml nutrient broth and incubated for 30 minutes at room temperature.

4.7.2.1.1.3 Preparation of test Bio wrapper

Each Bio wrapper was cut into 3 pieces of 7x7 mm using the blades and placed in a laminar airflow chamber for exposure to UV light for 15mins.

4.7.2.1.1.4 Antimicrobial Activity of Bio wrapper

The antibacterial potential of the prepared film was examined against *S. aureus* and *P. aeruginosa* by the agar disc diffusion technique. The test organism's inoculum was swabbed onto a nutrient agar plate with the help of a sterile cotton swab. Then using the sterilized forceps, the film piece was placed onto the plate. Polyethene film served as the control. The above procedure was performed in a laminar flow chamber. Plates were then incubated at 37°C for 24 h. The diameter (in mm) of the inhibitory zone around the wrapper was measured after 24 hrs to assess the antimicrobial activity.

4.7.2.1.2 Clarity and transparency

The Bio wrapper was evaluated visually for clarity and transparency (Jack *et al.*, 2017).

4.7.2.1.3 Flammability test

In the flammability test, the Bio wrapper was cut into 2x2 cm and was burnt with the candle. Then the Burning time was noted (Jack *et al.*, 2017).

4.7.2.1.4 Air test

The Bio wrapper was cut into 2x2 cm squares and left open for 7 days. A change in size was observed (Jack *et al.*, 2017).

4.7.2.1.5 Thickness

The thickness of the wrapper was measured at 5 different locations using a micrometer (screw gauge). And the result was estimated by taking the average value of thickness at different locations (Sharma *et al.*, 2017).

4.7.2.1.6 Density

The Bio wrapper was cut into 1x1 cm pieces and measured its side length (a), weight (M), and thickness(d) in 3 different locations. The density was then calculated using the following formula (Jarolimkova, 2015).

$$\text{Density } (\rho) = \frac{M}{da^2}$$

4.7.2.1.7 Moisture content

The Bio wrapper was cut into 1x1 cm square pieces were weighed to measure the initial weight (W_i). Then they were dried in an oven at 85°C for 24 h. The samples were weighed one more to measure the final weight (W_f). The moisture content was then determined using the following formula (Shafqat *et al.*, 2021).

$$\text{Moisture Content (MC)} = \frac{(W_i - W_f)}{W_i} \times 100$$

where W_i is the initial weight and W_f is the final weight.

4.7.2.1.8 Water absorption percentage

The Bio wrapper was cut into 1 cm x 1 cm square pieces and dried in an oven at 60°C. The initial mass of the samples was measured and the wrapper was immersed in water for 24 hrs. The mass of the samples after immersion was measured. The Water absorption percentage was then determined using the following formula (Krishnamurthy and Amritkumar, 2019).

$$\text{Water absorption percentage} = \frac{(W_1 - W_0)}{W_0} \times 100$$

Where W_0 and W_1 are the weight of before and after immersion of the wrapper

4.7.2.1.9 Chemical resistance

The Bio wrappers were immersed in different solvents Acid, base, salt and alcohol for 48 hrs, and their resistance was ascertained by measuring the change

in appearance. A polyethylene cover was used as the control (Krishnamurthy, and Amritkumar, 2019)

4.7.2.1.10 Biodegradable test by soil burial method

Bio wrappers of size 4x4 cm were weighed to measure. The samples were buried under the soil. The soil was kept moist, and after a week it was visualized for changes. A polyethylene cover was used as the control (Shafqat *et al.*, 2021, Shellikeri *et al.*, 2018).

CHAPTER 5

RESULTS AND DISCUSSION

5 RESULT AND DISCUSSION

5.1 SAMPLE COLLECTION

The plants employed in this study were chosen based on their antimicrobial activity reported in a research paper. The collected fresh leaves were dried and coarse into a fine powder. The sample collection and processing are depicted in Figure 5.1.

Karpuravalli leaves (*Plectranthus amboinicus*)



(a)



(b)



(c)



(d)

Tulsi leaves (*Ocimum tenuiflorum*)



(a)



(b)



(c)



(d)

Neem leaves (*Azadirachta indica*)



(a)



(b)



(c)



(d)

Guava leaves (*Psidium guajava*)



(a)



(b)



(c)



(d)

Lemon peel (*Citrus limon*)



(a)

(b)

(c)

(d)

Dried ginger-Sukku (*Zingiber officinale*)



(a)

(d)

**Omam seed powder
(*Trachyspermum ammi*)**



**Vasambu powder
(*Acorus calamus*)**



**Nilavembu powder
(*Andrographis paniculata*)**

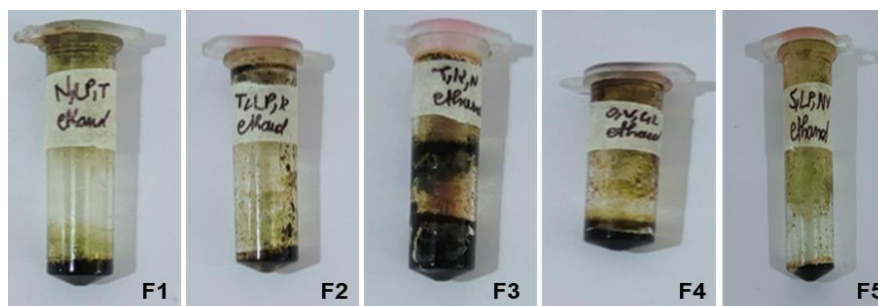


Fig:5.1 Sample preparation

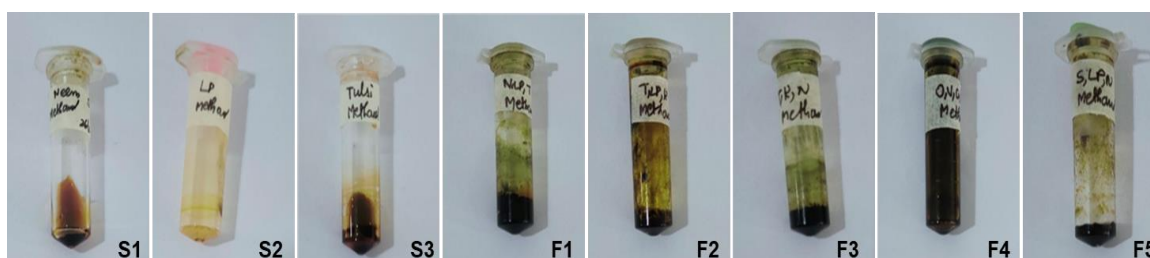
a) - Fresh leaves; b) - Trimmed leaves; c) - Dried leaves; d) - Powdered leaves

5.2 PREPARATION OF HERBAL EXTRACTS

Solvent extraction was performed using ethanol, methanol and distilled water to get the plant extract. The final extract was kept in an Eppendorf tube after soaking, filtering, and evaporation, as shown in Figure 5.2. Component extraction was better with ethanol and methanol solvents.



(a)



(b)



(c)

Fig:5.2 Preparation of herbal extracts

- a) Solvent extraction using ethanol; b) Solvent extraction using methanol;
c) Aqueous extract

5.3 IN VITRO SCREENING FOR ANTIMICROBIAL ACTIVITY AGAINST BACTERIAL AND FUNGAL PATHOGENS

5.3.1 Test organisms

The test organism subcultured and used for this study was given in Figure 5.3.



Fig:5.3 Test organism subcultured

5.3.2 Preparation of inoculum

Prepared inoculums for seven test organisms for antimicrobial activity are shown in Figure 5.4.

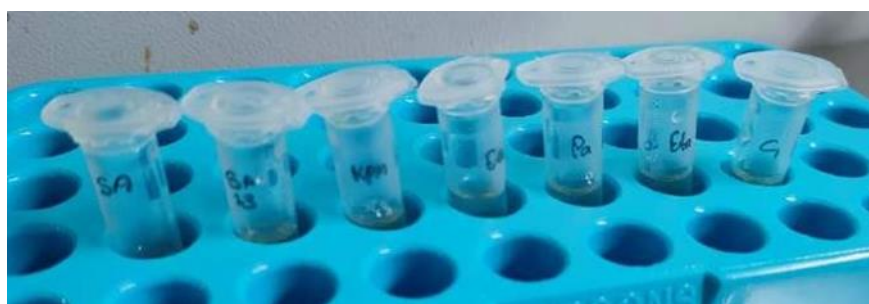
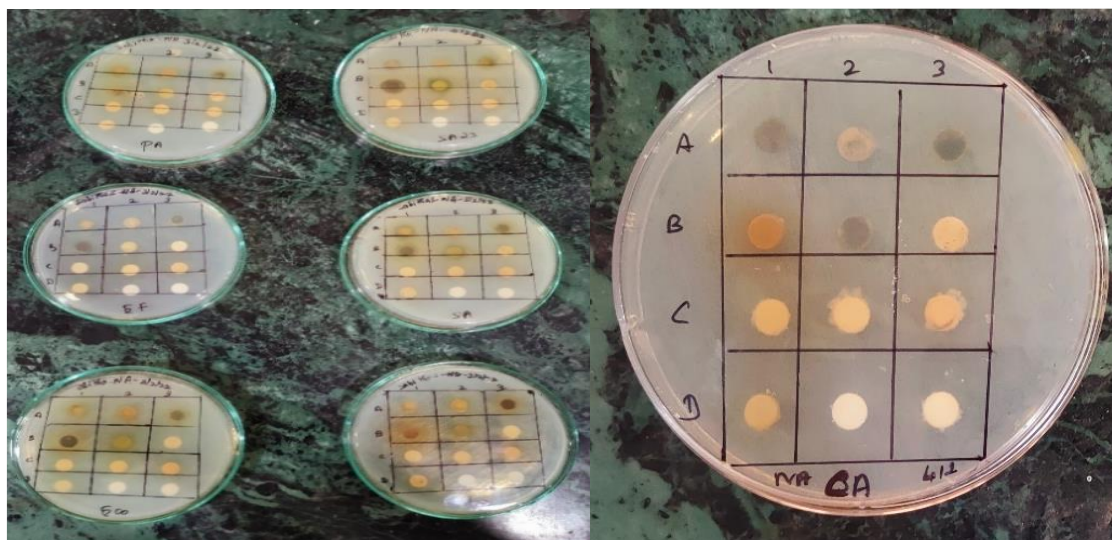


Fig:5.4 Inoculum Preparation

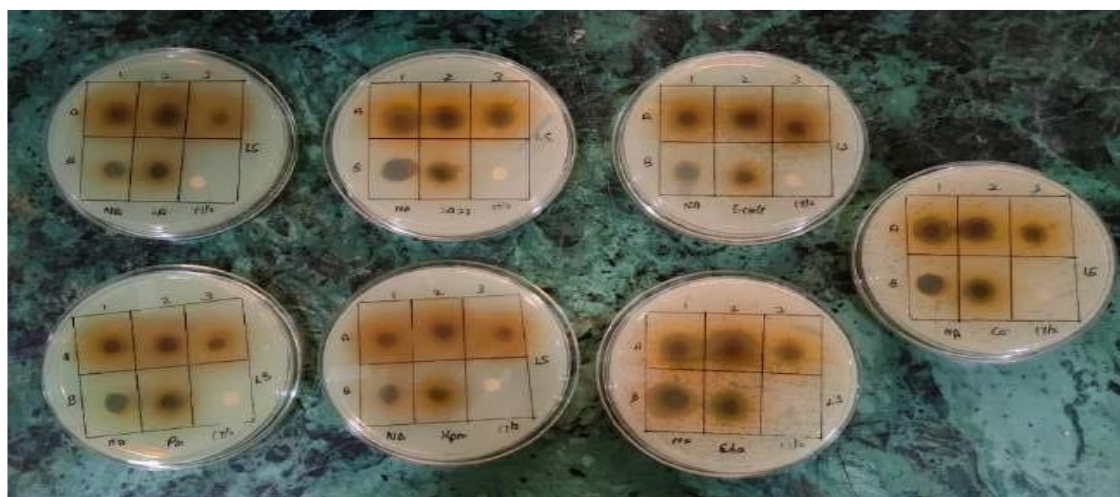
5.3.3 Antimicrobial Activity of different formulations using disc diffusion method

In this study, we have tested the ethanolic, methanolic and aqueous extracts of different formulations for their antimicrobial activity against seven test organisms namely *Staphylococcus aureus* 29213 (*S.au*), *Staphylococcus aureus* 25923 (*S.au* 23), *Escherichia coli* 25922 (*E.co*), *Pseudomonas aeruginosa* 27853 (*P.a*), *Klebsiella pneumoniae* 13439 (*K. pm*), *Enterococcus faecalis* 29212 (*E. fa*) and *Candida albicans* (*C.a*). The evaluation of antimicrobial activity for various formulations were displayed in Figure 5.5 and Table 5.1. Ethanolic extract of F4 and F5 showed antimicrobial activity against *S. aureus* with zones of inhibition of 11 mm and 9 mm respectively. All the methanolic formulations except F4 exhibited varying zones of inhibition (>8 mm) against test organisms except *K. pneumoniae*. In comparison to all other formulations, the methanolic F1 showed a greater zone of

inhibition against all tested organisms except *K. pneumoniae* with the highest zone of 14 mm against *E. faecalis* and *C. albicans*. However, the aqueous extract of different formulations did not exhibit any antimicrobial activity against the 7 tested pathogens but slight diffusion was visualized in F1 against *S. au* 23 so this formulation was again tested by the agar well diffusion method. Furthermore, no zones were found in the control disc, implying that the zone observed in the formulation was due to the plant's bioactive compounds rather than the solvents.



(a)



(b)

Fig:5.5 Antimicrobial Activity of different formulations using disc diffusion method

- a) Antimicrobial activity of ethanol and aqueous extract
- b) Antimicrobial activity of methanol extract

Table:5.1 Antimicrobial Activity of different formulations using disc diffusion method

Formulations	Solvents	Zone of Inhibition (mm)						
		Test organisms						
		<i>S. au</i>	<i>S. au</i> 23	<i>K.pm</i>	<i>E.co</i>	<i>P. a</i>	<i>E. fa</i>	<i>Candida</i>
F1	Ethanol	-	-	-	-	-	-	-
	Methanol	12	10	-	13	10	14	14
	D/W	-	-	-	-	-	-	-
F2	Ethanol	-	-	-	-	-	-	-
	Methanol	11	9	-	13	10	18	11
	D/W	-	-	-	-	-	-	-
F3	Ethanol	-	-	-	-	-	-	-
	Methanol	11	10	-	11	-	9	9
	D/W	-	-	-	-	-	-	-
F4	Ethanol	-	11	-	-	-	-	-
	Methanol	-	-	-	-	-	15	-
	D/W	-	-	-	-	-	-	-
F5	Ethanol	-	9	-	-	-	-	-
	Methanol	8	9	-	7	8	13	12
	D/W	-	-	-	-	-	-	-
Control	Ethanol	-	-	-	-	-	-	-
	Methanol	-	-	-	-	-	-	-
	D/W	-	-	-	-	-	-	-

5.3.4 Antimicrobial Activity of aqueous extract of F1 using agar well diffusion method

Due to the appearance of slight diffusion in the disc with F1 aqueous extract, it was examined again with a boiled sample using the agar well diffusion method. As can be seen from Figure 5.15 and Table 5.2, it has a zone of inhibition of 11 mm and 9 mm against *S. aureus* 23 and *P. aeruginosa* respectively. The negative result for antibacterial activity using the disc diffusion method on F1 aqueous extract could be due to incomplete extraction or insufficient extraction time (Golam Rasul, 2018). Figure 5.6 shows the graphical representation of the antimicrobial activity of different formulations.

Table:5.2 Antimicrobial Activity of aqueous extract of F1 using agar well diffusion method

Extract	Zone of Inhibition (mm)	
	Test organisms	
	<i>S. aureus</i> 23	<i>P. aeruginosa</i>
F1 _{aq}	11	9

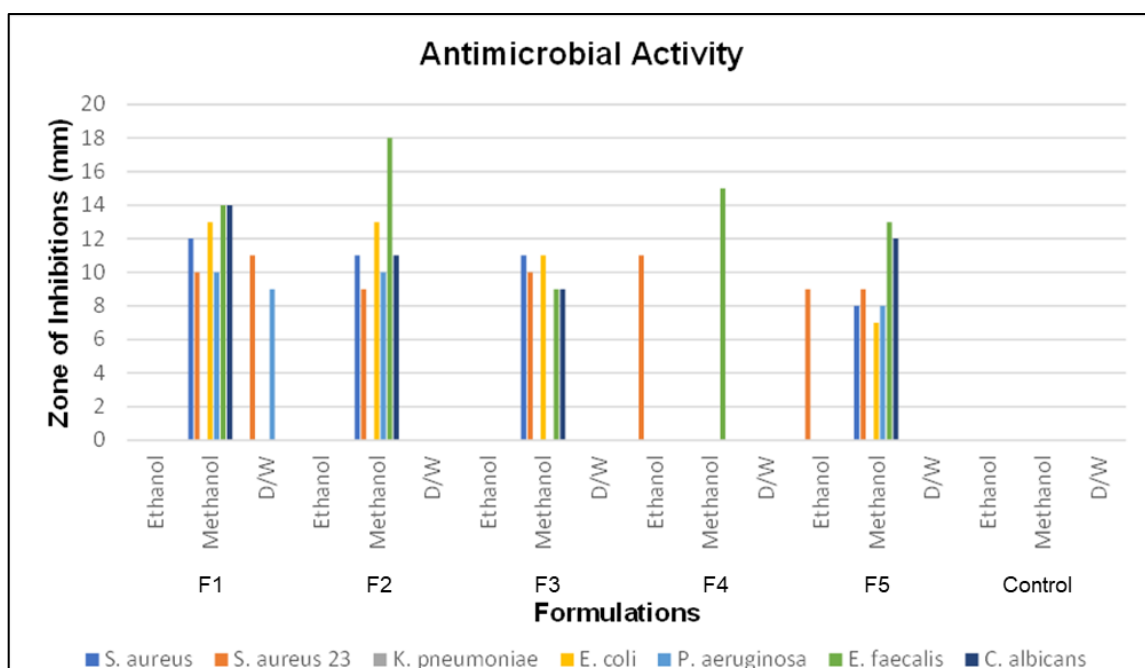
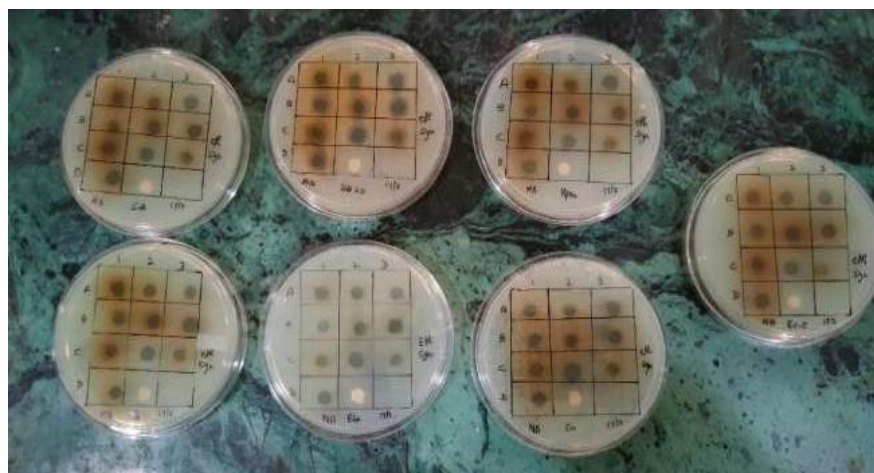


Fig:5.6 Graphical representation of Antimicrobial Activity of different formulations

5.3.5 Synergistic Antimicrobial Activity of ethanol and methanol extracts

In Synergistic Antimicrobial Activity, both ethanol and methanol extract enhanced their antimicrobial activity, as illustrated in Figure 5.7 and Table 5.3. A graphical representation of the Synergistic Antimicrobial activity of ethanolic and methanolic extracts results was given in Figure 5.3. The methanol extract was more effective than the ethanol extract since most methanol formulations had a variable zone of inhibition ranging between 6 mm and 18 mm, whereas the ethanol extract had a zone ranging from 6 mm to 11 mm. Most of the formulations were active against *S. aureus* 23, *K. pneumoniae*, and *C. albicans*. Nevertheless, the

formulation with F1 has the maximum zone and inhibits all pathogens, except *E. Coli*. After analyzing the antibacterial and synergistic antimicrobial activities of different formulations, it was determined that F1, a combination of neem, lemon peel, and tulsi, had the best antimicrobial activity. As a result, this formulation was employed in further studies.



(a)



(b)

Fig:5.7 Synergistic Antimicrobial activity of ethanol and methanol extracts

- a) Ethanol extracts
- b) Methanol extracts

Table:5.3 Synergistic Antimicrobial activity of ethanol and methanol extracts

Synergistic Formulations	Solvents	Zone of Inhibition (mm)						
		Test organisms						
		<i>S. au</i>	<i>S. au</i> 23	<i>K.pm</i>	<i>E.co</i>	<i>P. a</i>	<i>E. fa</i>	<i>Candida</i>
F1+F2	Ethanol	11	10	6	-	10	-	-
	Methanol	-	8	-	-	8	13	10
F1+F3	Ethanol	-	11	-	-	-	-	-
	Methanol	6	8	-	-	8	12	10
F1+F4	Ethanol	10	8	-	-	-	-	-
	Methanol	9	9	-	-	8	14	9
F1+F5	Ethanol	-	9	-	-	-	-	-
	Methanol	-	8	-	-	6	13	11
F2+F3	Ethanol	11	8	-	-	9	9	-
	Methanol	-	9	-	-	6	18	9
F2+F4	Ethanol	11	9	-	-	-	-	-
	Methanol	9	10	-	-	-	12	8
F2+F5	Ethanol	9	8	9	-	10	-	-
	Methanol	-	-	-	-	7	12	8
F3+F4	Ethanol	-	10	-	-	-	-	-
	Methanol	7	10	-	-	-	13	6
F3+F5	Ethanol	-	7	-	-	-	-	-
	Methanol	-	-	-	-	-	9	8
F4+F5	Ethanol	-	10	-	-	-	-	-
	Methanol	8	8	-	-	9	13	8
Control	Ethanol	-	-	-	-	-	-	-
	Methanol	-	-	-	-	-	-	-

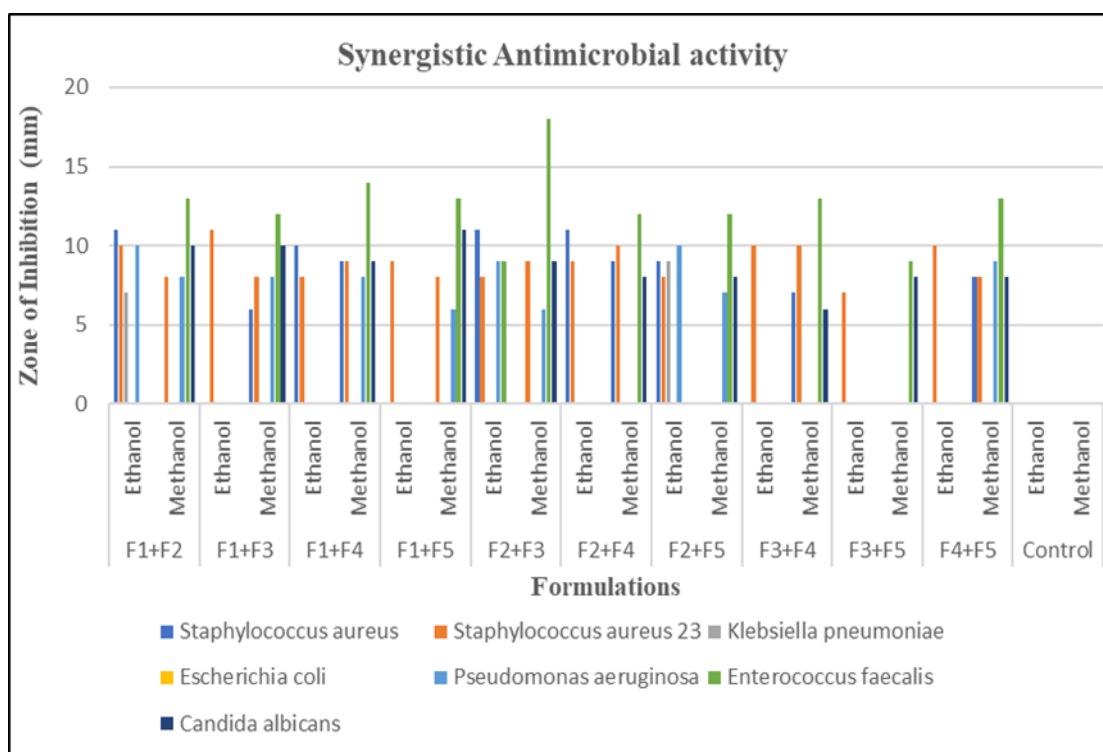


Fig:5.8 Graphical representation of Synergistic Antimicrobial activity of ethanol and methanol extracts

5.3.6 Antimicrobial Activity by 96 well plate method

The antimicrobial activity of methanolic extracts of S1, S2, S3, and F1 at concentrations of 20-100 ($\mu\text{g/ml}$) was determined against *S. aureus* 23 using the 96-well plate method, and the absorbance readings were recorded in Table 5.4 and mapped to the graph in Figure 5.9. Absorbance was directly proportional to the cell number (Stevenson *et al.*, 2016). The absorbance value decreased as the concentration of the extract increased, thus inhibiting bacterial growth. As the concentration of extract increase, bacteria growth decrease. They showed less inhibition individually, but when all three extracts were combined, they showed higher inhibition. On comparing all the samples, F1 showed a greater decline in the absorbance value from 3.541 to 1.846 thus the growth of the pathogen was inhibited. The order of inhibition was as follows **F1>S2>S1>S3**. This confirms that formulation 1 had good antimicrobial properties and this was used for further studies.

Table:5.4 Antimicrobial Activity by 96 well plate method

S. No	Samples	Absorbance (517 nm)				
		Concentration ($\mu\text{g/ml}$)				
		20	40	60	80	100
1.	S1	3.261	3.190	3.119	2.814	2.565
2.	S2	3.593	3.187	2.798	2.362	2.157
3.	S3	3.597	3.536	3.347	3.083	2.839
4.	F1	3.541	3.150	2.459	1.968	1.846

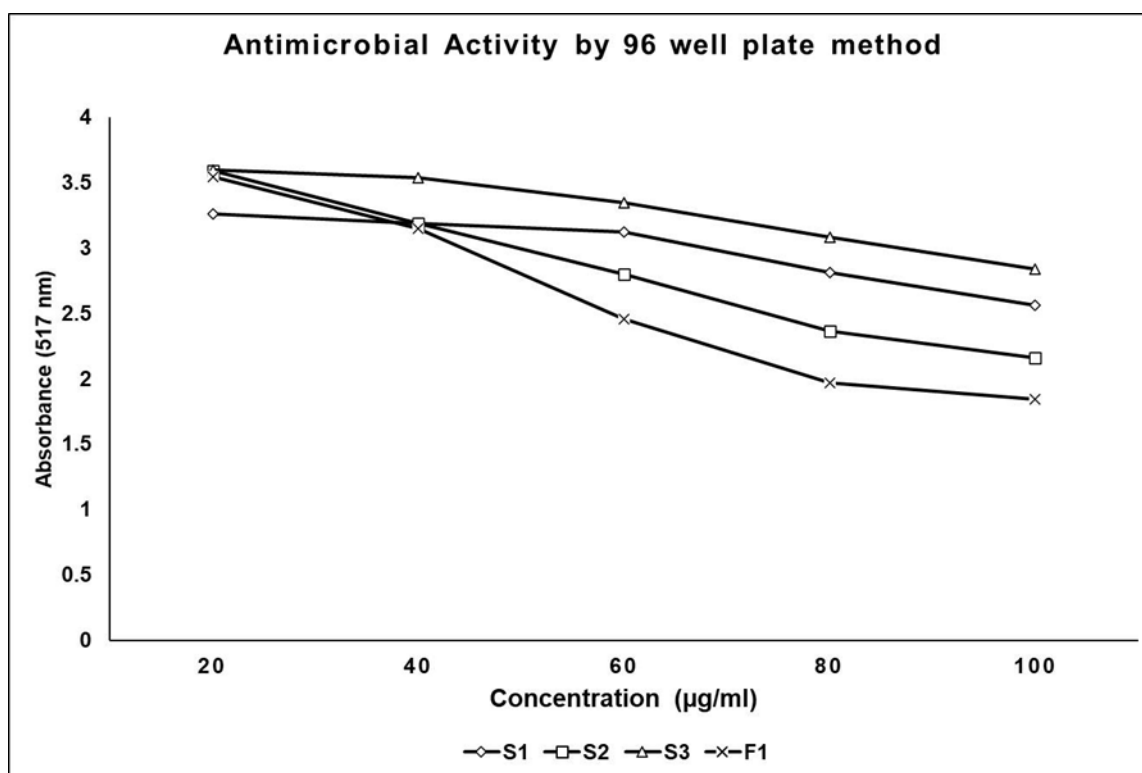


Fig:5.9 Graphical representation of antimicrobial Activity by 96 well plate method

5.4 IN VITRO ANTIOXIDANT ASSAY OF EXTRACT

The Free radical scavenging assay for S1, S2, S3 and F1 was performed by the DPPH method and the result was summarized in Table 5.5 and Figure 5.10. As shown, all the extracts exhibited various levels of antioxidant activity and the IC₅₀ values of the extracts have been determined in Figure 5.1. A lower IC₅₀ value indicates higher antioxidant activity which signifies the inhibition of 50% of free radicals. The IC₅₀ of methanolic extracts of S1, S2, S3 and F1 was 245.31 µg/mL, 172.34 µg/mL, 298.14 µg/mL and 148.23 µg/mL, respectively. Individually, the extracts have a high IC₅₀ value, but when combined, they have a lower IC₅₀ value, indicating that the combined extracts have more antioxidant effects. The IC₅₀ of standard 108.17 µg/ml and was almost the same as that reported in John and Madhumitha, (2017) which was 99.225 µg/ml. Among all the tested samples, a standard had the lowest IC₅₀ value (108.17 µg/ml) followed by F1 (148.23 µg/ml). The order of DPPH scavenging was found to be in the order of **standard > F1 > S2 > S1 > S3**. Hence, F1 act as a good free radical scavenger at low concentration.

Table:5.5 In Vitro Antioxidant activity by DPPH assay

S. No	Samples	Absorbance (517 nm)					% of inhibition					IC ₅₀ (µg/ml)
		Concentration (µg/ml)					Concentration (µg/ml)					
		100	200	300	400	500	100	200	300	400	500	
1	Standard	0.357	0.281	0.187	0.12	0.062	48.33	59.33	72.94	82.63	91.03	108.17
2	S1	0.394	0.383	0.329	0.283	0.212	42.98	44.57	52.39	59.04	69.32	245.31
3	S2	0.381	0.335	0.279	0.233	0.189	44.86	51.52	59.62	66.28	72.65	172.34
4	S3	0.436	0.389	0.357	0.284	0.257	36.9	43.7	48.34	58.9	62.81	298.14
6	F1	0.368	0.317	0.249	0.176	0.093	46.74	54.12	63.97	74.53	86.54	148.23

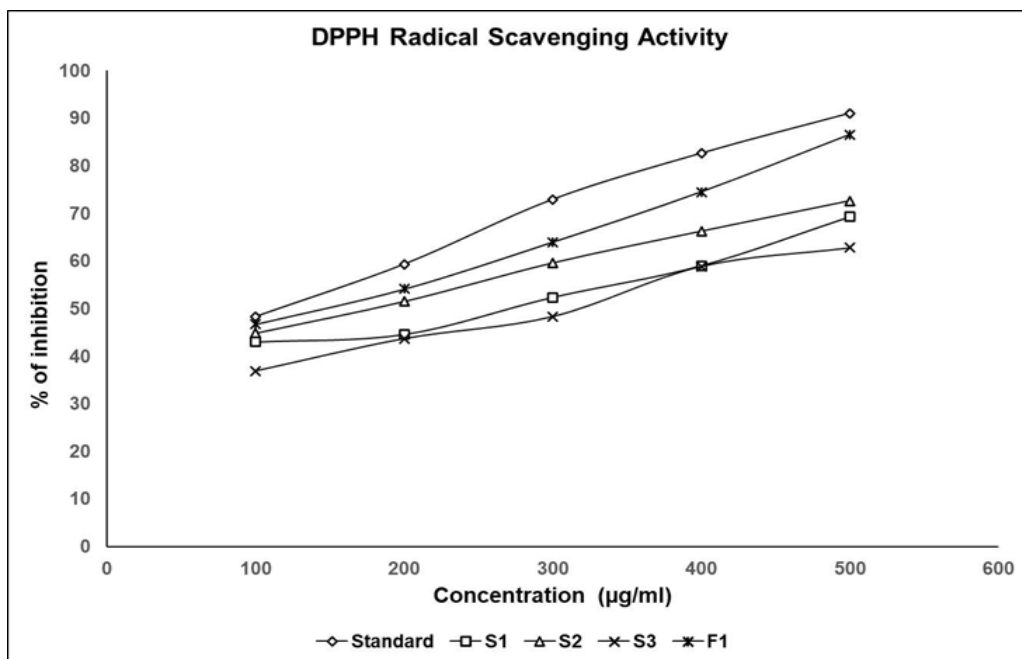


Fig:5.10 Graphical representation of In Vitro Antioxidant activity by DPPH assay

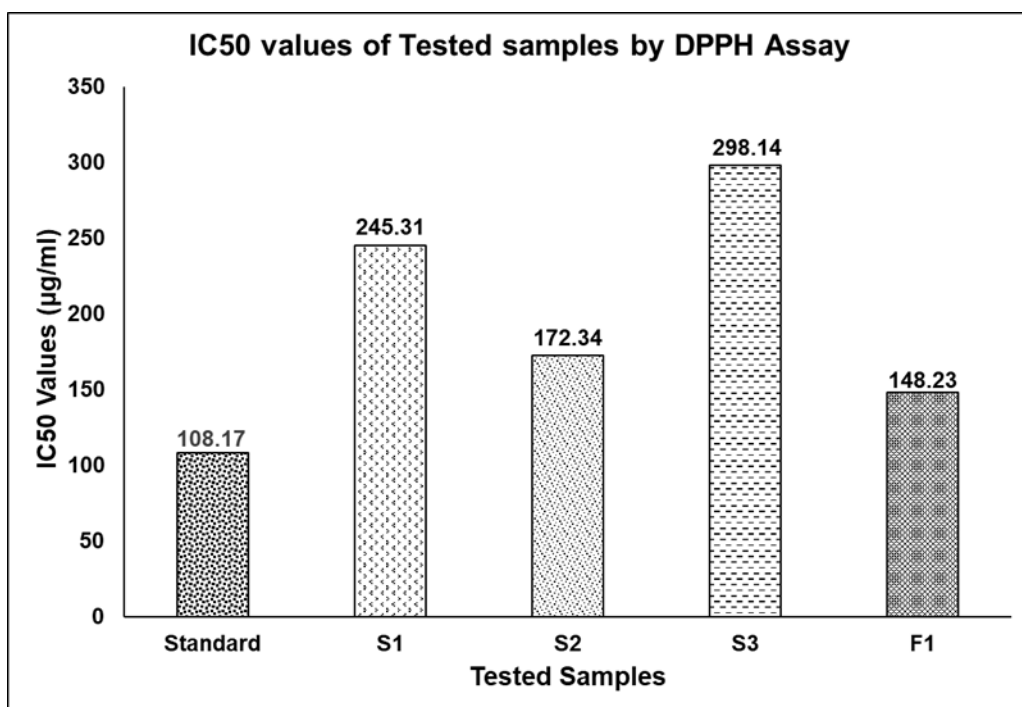


Fig:5.11 IC₅₀ values of tested samples

5.5 QUALITATIVE PHYTOCHEMICAL ANALYSIS

Figure 5.12 and Table 5.6 summarize the qualitative results for phytochemical analysis, where (+) denotes the presence and (-) denotes the absence of phytochemicals. The phytochemical test of the methanol extract of formulation 1 (Neem, Lemon peel, Tulsi) revealed the presence of Alkaloids, Carbohydrates, Flavonoids, Phenols and Tannins, Terpenoids, Glycosides, Steroids, Anthraquinones. It indicates that these compounds were present in the methanolic extract of F1. Saponins and Quinones were given negative results it indicating that those compounds were not present.

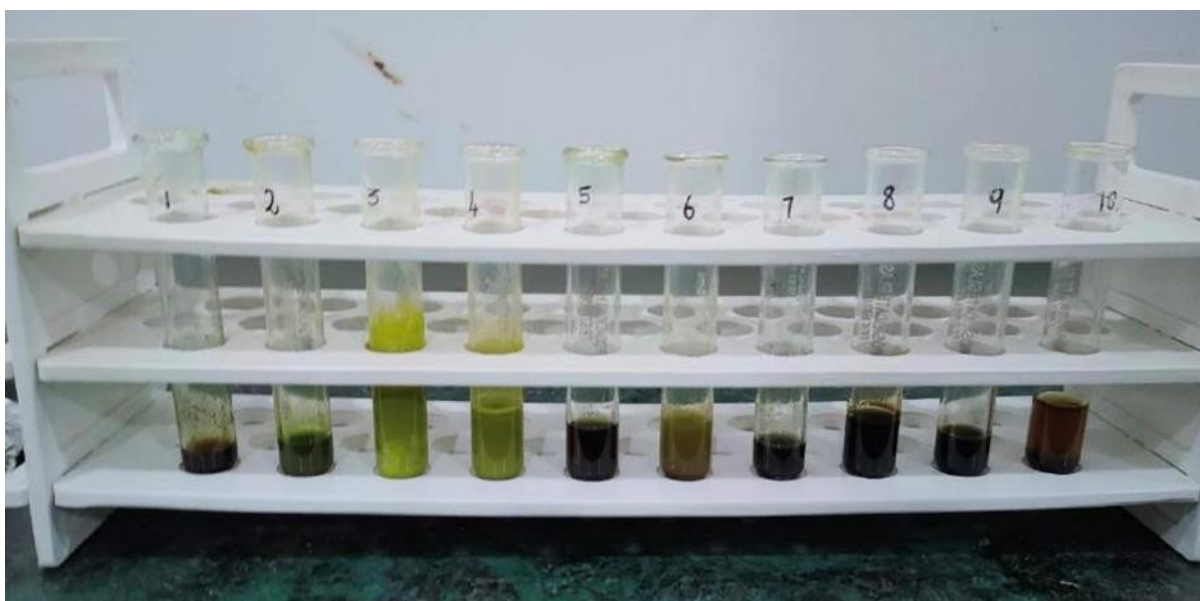


Fig:5.12 Qualitative phytochemical analysis

Table:5.6 Qualitative phytochemical analysis

Test No.	Secondary Metabolites	Observation	Inference
1.	Alkaloids	Appearance of Orange-red color	+
2.	Carbohydrates	Appearance of Green color	+
3.	Flavonoids	Formation of Yellow color precipitate	+
4.	Phenols and Tannins	Formation of precipitate	+
5.	Terpenoids	Appearance of a greyish color	+

6.	Saponins	No formation of stable foam	-
7.	Glycosides	Appearance of reddish-brown color	+
8.	Steroids	Formation of red color at a lower layer	+
9.	Quinones	No appearance of red color	-
10.	Anthraquinones	Formation of pink-violet color at the lower Layer	+

In the table, (+) represents the presence of the compound, (-) represents the absence of the compound

5.6 APPLICATION OF SELECTED BIOLOGICALLY ACTIVE MEDICINAL PLANTS FOR FOOD INDUSTRIAL APPLICATIONS

From the above results, the methanolic extract of F1 performed well in antimicrobial, antioxidant, and phytochemical tests. However, methanol can have toxic/side effects in humans and can damage the surface of fruits and vegetables (Food Standards Agency, 2011). Therefore, an aqueous extract of F1 was employed for applicability, which also had antibacterial properties.

5.6.1 Preparation of Bio wash

Two Bio wash was formulated, one Bio wash has F1_{aq} extract, while the other contains F1_{aq} and clove oil. Table 5.7 and Figure 5.13 shows the different formulated Bio wash. Clove oil, the main volatile ingredient of *S. aromaticum*, has several advantages over sodium benzoate, potassium sorbate, and other chemical food preservatives in terms of antibacterial action, fragrances, and safety, and was an excellent replacement for chemical food preservatives. Furthermore, it contains a high concentration of phenolic compounds, which have antibacterial, antifungal, insecticidal, and antioxidant properties. It is well recognized as safe for food preservation and has already been approved by the FDA in many countries (Hu Q *et al.*, 2018, Haro-González *et al.*, 2021).

Table:5.7 Formulation of Bio wash

S. No	Formulated Bio wash Number	Bio wash	Description
1.	B1	F1aq	A bio-wash formulated with neem, lemon peel and Tulsi
2.	B2	F1aq + Clove oil	A bio-wash formulated with neem, lemon peel, tulsi and Clove oil
3.	Control	Control	Commercially available Bio wash



Fig:5.13 Formulated Bio wash

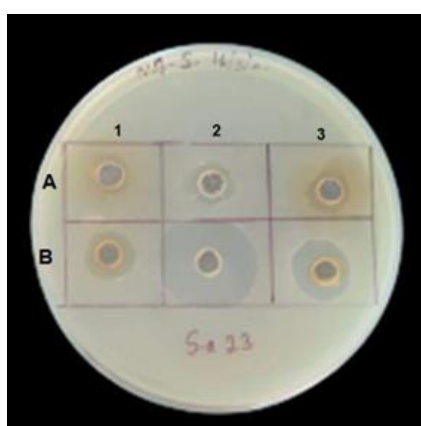
5.6.1.1 Evaluation of formulated Bio-wash

The prepared bio-wash was subjected to antimicrobial efficacy and physical evaluation.

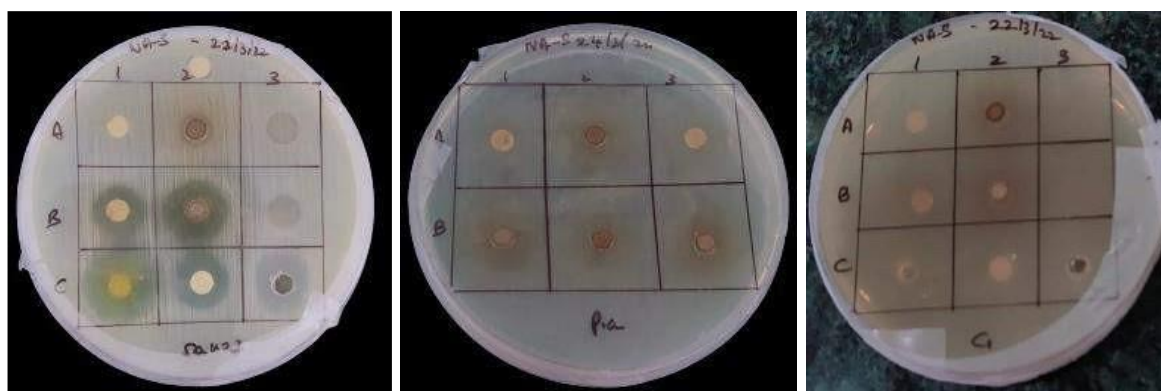
5.6.1.1.1 Antimicrobial activity of formulated Bio-wash and its ingredients

The antibacterial activity of Bio wash and its ingredients against *S. aureus*, *P. aeruginosa*, and *C.albicans* was tested using the agar well diffusion method, and the results of the zone of inhibition (in mm) were listed in Table 5.8. As seen in Figure 5.14 all the ingredients present in the Bio wash showed antibacterial activity and a combination of all extracts showed more potent antimicrobial activity against *S. aureus* and *P. aeruginosa* with a zone of inhibition of 11 mm and 9 mm respectively.

In addition of Clove oil to the F1_{aq} the zone of inhibition increases to 17 mm and 12 mm against *S. aureus* and *P. aeruginosa* respectively. Commercially available wash showed a zone of 13 mm which was lower than our formulated bio wash. Both the formulated bio-wash and the control had no inhibition against *C. albicans*. The formulated Bio wash has a good effect against Gram-positive and Gram-negative bacteria. Moreover, only three pathogens were tested, and they might still be tested on some more food-borne pathogens. It was found that it was ineffective against fungal pathogens and that it was necessary to add some herbal extract that would have an antifungal effect. In the future, pesticide removal can also be tested.



(a)



(b)

Fig:5.14 Antimicrobial activity of Bio wash and its ingredients

- a) Antimicrobial activity of ingredients in Bio wash
- b) Antimicrobial activity of Bio wash

Table:5.8 Antimicrobial activity of Bio wash and its ingredients

Extract	Zone of Inhibition (mm)		
	Test organisms		
	<i>S. au 23</i>	<i>P. a</i>	<i>C. a</i>
S1	7	NT	NT
S2	8	NT	NT
S3	6	NT	NT
Clove oil	12	NT	-
B1	11	9	-
B2	17	12	-
Control	13	NT	-

NT- Not tested

5.6.1.1.2 Determination of Efficacy of Bio-wash

Evaluated the effectiveness of formulated Bio wash on pathogens that reside on the sample surface since maximum exposure occurs at the surface. Samples were swabbed and streaked on nutrient agar plates and then visually analyzed for bacterial growth. There was the maximum difference in bacterial growth before and after bio washing. From Figure 5.16, it can be seen that bacterial growth was **reduced after the sample was soaked** in the bio wash. A sample soaked in normal water does not show a reduction. After soaking the sample in Bio wash, there was a significant reduction in bacterial contamination. When it was soaked in normal water again, the majority of the bacteria had been eradicated. Both the B₁ and B₂ bio-wash showed better results than the control. The B₂ Bio wash decreased contamination on the sample more than the other bio-washes examined. In the control, froth and soapy forms were noticed, but no such issues can be seen in the prepared bio-wash (Figure 5.15). For practical reasons, only one apple was subjected to microbiological testing. Bio wash's feasibility must be tested on a wider range of vegetables and fruits.



Fig:5.15 Appearance of apple before and after Bio washing



Fig:5.16 Determination of Efficacy of Bio wash

5.6.1.1.3 Microbial load in effluent

A serial dilution and spread plate method were carried out and Cfu/ml were calculated to assess the microbial load in the effluent. The residues of water/Bio wash in which the sample was soaked are considered as effluent. The microbial load was evaluated in three effluents: one with the sample washed in normal water (NW), then in Bio wash (BW), and finally with normal water after Bio washing (ABW). Serial dilution was performed from 10^{-1} to 10^5 . It was observed from Figure 5.17 and Table 5.9 that the number of colonies were reduced after bio washing. CFU/ml was calculated and the decreasing order was as **follows (Bio washing) BW > (Normal water wash) NW > (After Bio washing) ABW**. The microbial load was higher in BW because most of the microorganisms from the apple surface would have been shed on the water. The comparison of microbial load on three effluents was presented using a graph (Figure 5.18) that plotted between CFU/ml and the Dilution factor. Further, it can be tested with some more quantities of fresh produce to check the effectiveness of Bio wash.



Fig:5.17 Microbial load in effluent

Table:5.9 CFU/ml of effluent

Dilution factor	Bio wash (B1)					
	Number of Colonies			CFU/ml		
	NW	BW	ABW	NW	BW	ABW
10^{-1}	123	169	116	123	169	116
10^{-2}	119	146	105	11.9	14.6	10.5
10^{-3}	109	121	55	1.09	1.21	0.55
10^{-4}	88	97	40	0.088	0.097	0.040
10^{-5}	20	28	7	0.0020	0.0028	0.0007

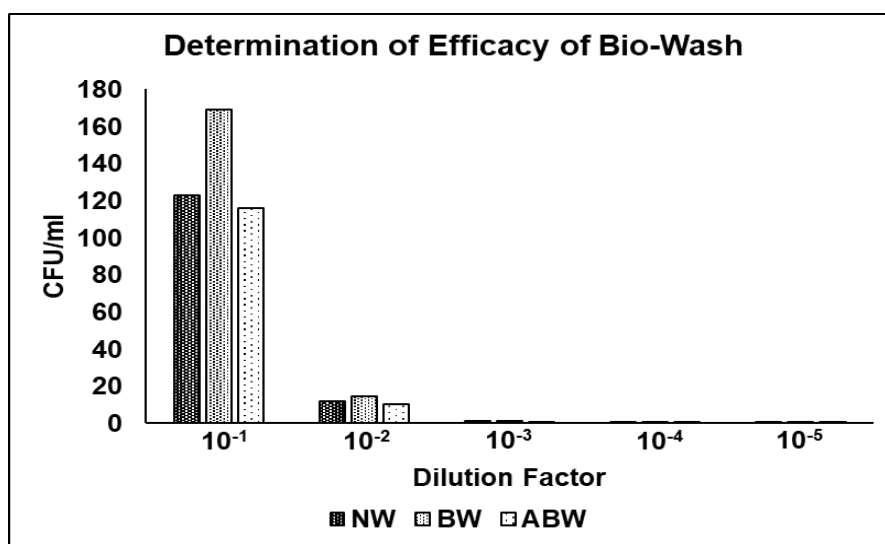


Fig:5.18 Graph plotted between Cfu/ml and dilution factor for testing the microbial load in effluent

5.6.1.1.4 Efficacy of bio-wash on time intervals

The efficiency of the formulated Bio wash was examined after soaking an apple in the test solution for up to 5 min with a 1 min interval to determine when the formulated Bio wash began to be effective. From Figure 5.19, it was seen that number of bacterial colonies decreases as the time duration increases. In the case of the control, bacterial growth begins to slow in the 2nd min, but growth can still be seen in the 5th min. With B1, microbial growth starts to decline at the 4th min whereas with B2 the microbial growth begins to decline at the **3rd min** with more reduction at the 5th minute. The efficacy of Bio wash on time interval as follows **B2>Control>B1**. In comparison to commercial Bio wash, the results demonstrated that Bio wash formulated by us has potent antimicrobial properties and greater efficacy. Though our formulated Bio wash was found to be effective, it is necessary to determine whether the nutritional value is altered. As a result, more research is needed to determine whether Bio washing processes impair the nutritional content of food.

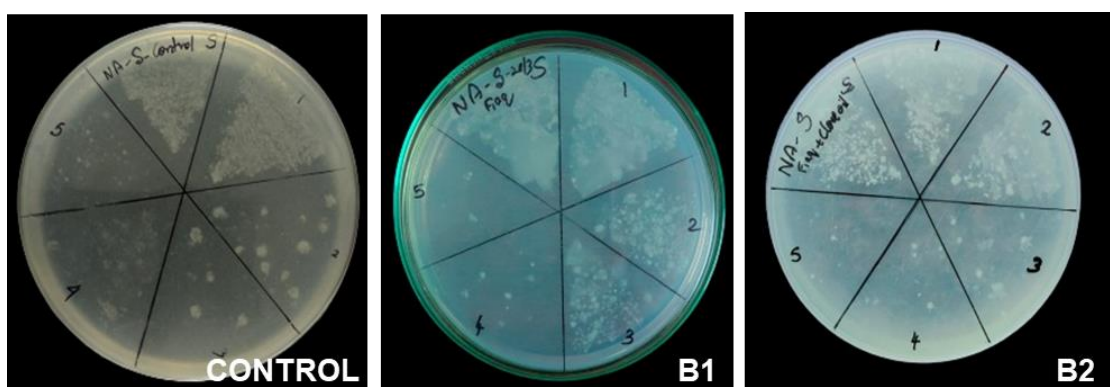


Fig:5.19 Efficacy of Bio wash on time intervals

5.6.1.2 Physicochemical characterization of Formulated Bio wash

Physicochemical Characterization of formulated Bio wash was determined. Parameters such as color, texture, odor, appearance, homogeneity, pH, and stability were evaluated and the result was tabulated in Table 5.10. Both formulated Bio wash has a good appearance, and the general characterization of the Bio wash revealed that the formulations have no particulate with a smooth texture and characteristic pleasant odor.

The pH of B1 and B2 was found to be between 6 and 6.9, indicating a mild acidic condition, and the control had the same result. These findings are also in line with the findings of Schermann M (2021), who claims that 6.0 - 7.0 provide the most effectiveness.

For 15 days, the Bio wash was placed at various temperatures: 4°C, 27°C, and 40°C. At all three temperatures tested, the Bio wash was found to be stable. Physical stability was determined to be stable in terms of color, texture, odor, and homogeneity.

Table:5.10 Physiochemical characterization of Formulated Bio wash

S. No	Test	Bio wash		
		Control	B1	B2
1.	Colour	Light Brown	Brown	Light brown
2.	Texture	Smooth	Smooth	Smooth
3.	Odor	Characteristic	Characteristic	Characteristic
4.	pH	6-6.9	6-6.9	6-6.9
5.	Homogeneity	No particulate	No particulate	No particulate
6.	Shelf-Life (15 days)	Stable	Stable	Stable

5.6.1.3 In Vitro Antioxidant assay of Bio wash

The antioxidant activity of formulated Bio wash was assessed using the DPPH assay with ascorbic acid as the control. From Table 5.11 and Figure 5.20, the Bio wash containing clove oil had better antioxidant activity than the Bio wash without clove oil, but both Bio washes had lower antioxidant activity than the standard. The IC₅₀ value of the standard was 110.89 while the B1 has 245.79 and B2 has 129.45. With B2 increasing concentration, the inhibition appears to be almost identical to that of the standard (Figure 5.20). Decreasing order of antioxidant property as follows: **Standard>B2>B1**. Clove oil has been reported as a great source of antioxidants (Parham *et al.*, 2020).

Table:5.11 In Vitro Antioxidant assay of Bio wash

S. No	Samples	Absorbance (517 nm)					% of inhibition					IC ₅₀ (µg/ml)
		Concentration (µg/ml)					Concentration (µg/ml)					
		100	200	300	400	500	100	200	300	400	500	
1	Standard	0.556	0.393	0.276	0.187	0.121	46.89	62.41	73.63	82.14	88.44	110.89
2	B1	0.646	0.555	0.489	0.39	0.26	38.29	49.99	53.3	62.75	75.16	245.79
3	B2	0.572	0.422	0.324	0.221	0.168	45.36	59.69	69.05	78.89	83.95	129.45

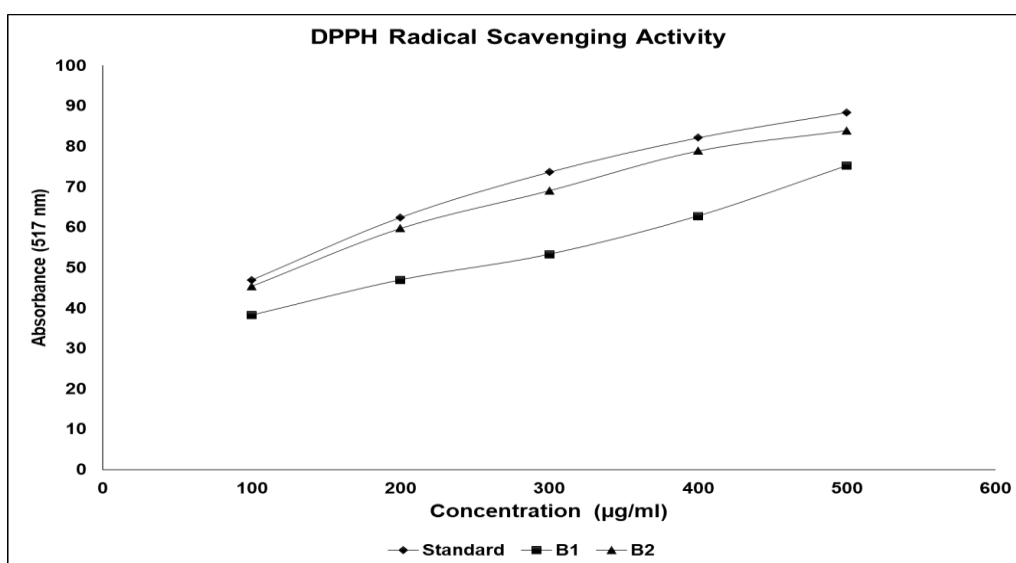


Fig:5.20 In Vitro Antioxidant assay of Bio wash

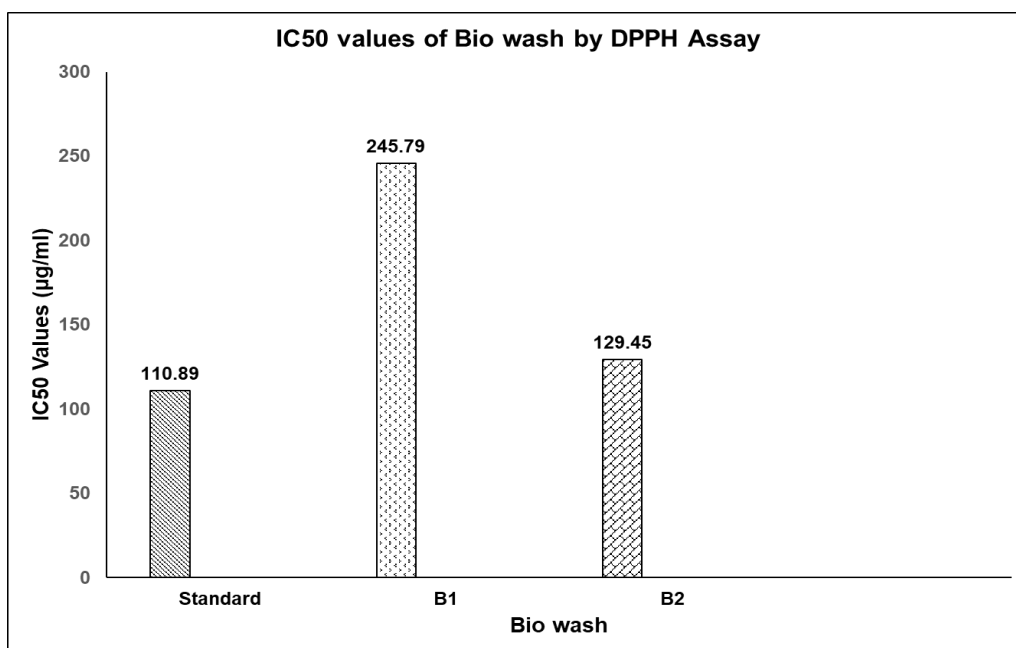


Fig:5.21 IC₅₀ Value of Bio wash

5.6.2 Preparation of biodegradable wrapper

Three wrappers were prepared, one without extract and clove oil, one with extract, and one with both extract and clove oil, and they were labelled BW1, BW2, and BW3 respectively (Figure 5.22).

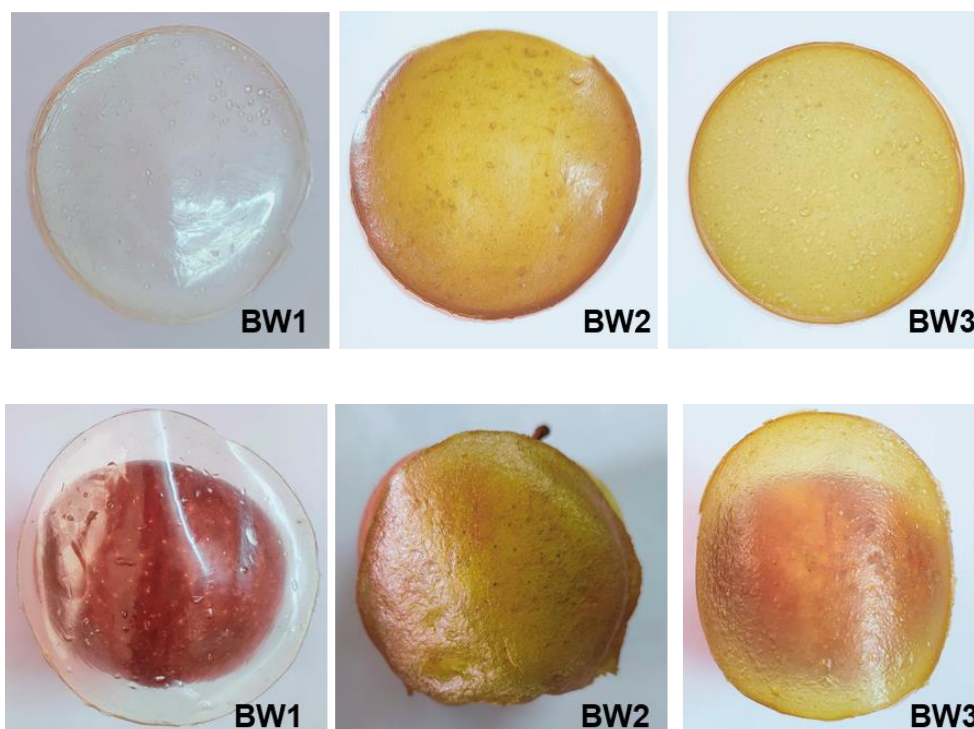


Fig:5.22 Biodegradable wrappers

5.6.2.1 Evaluation of the prepared biodegradable wrapper

Prepared Bio wrappers have undergone antibacterial and physicochemical studies including thickness, moisture content, moisture absorption percentage, chemical resistance, clarity, transparency, flammability, air and Biodegradable test. Despite all the studies conducted, some properties like water vapor permeability, swelling percentage, tensile, elongation at break and SEM, FTIR characterization studies have still to be explored.

5.6.2.1.1 In Vitro Screening for antimicrobial activity of prepared Bio wrapper

The Bio wrapper prepared was evaluated for antimicrobial activity against *S. aureus* and *P. aeruginosa* and the result was given in Figure 5.23 and Table 5.12. The produced wrapper with BW2 and BW3 was found to be effective against *S.*

aureus and *P. aeruginosa*. BW3 inhibited *S. aureus* and *P. aeruginosa* at 14 and 9 mm, respectively, and F1aq inhibited *S. aureus* and *P. aeruginosa* at 9 and 8 mm, respectively. No zones of inhibition were observed with B1 and controls against tested pathogens. These findings are in accordance with those of Hossein *et al.*, (2008), who found that clove oil inhibited the growth of *L. monocytogenes*, *E. coli*, and *S. aureus* in chitosan-based edible films. From the above result, sample BW3 was considered the best film for protecting fruits and vegetables; therefore, this film was used for further characterization studies.

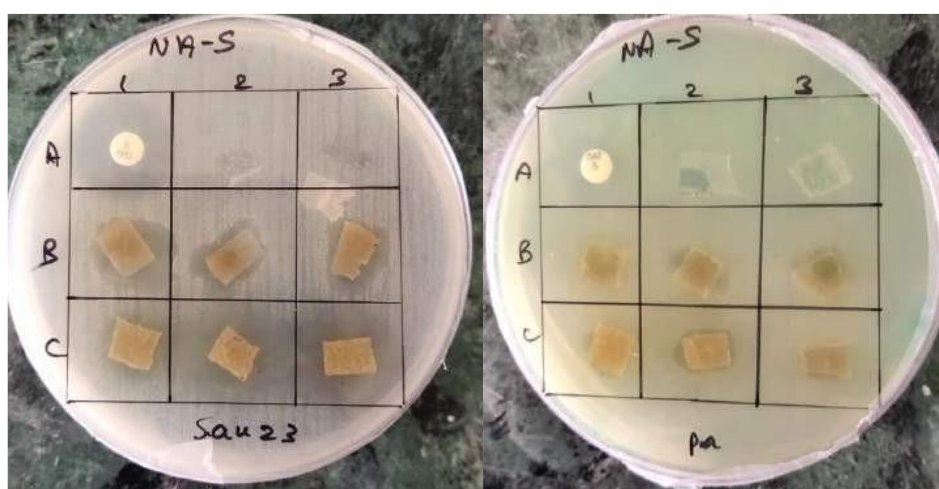


Fig:5.23 Antimicrobial activity of Bio wrappers

Table:5.12 Antimicrobial activity of Bio wrappers

Bio wrappers	Zone of Inhibition (mm)	
	Test organisms	
	<i>S. au 23</i>	<i>P. a</i>
BW1	-	-
BW2	9	8
BW3	14	9
Control	-	-

5.6.2.1.2 Clarity and transparency

Visual examination was carried out to test the clarity and transparency of the wrapper. Bio wrapper was less transparent and light brown than synthetic polythene, possibly due to the additives used in its fabrication. These findings are also in accordance with the data provided by Yashaswini and Iyer, (2019) they

observed that incorporation of clove oil into Chitosan-gelatin film decreased the transparency. (Table 5.13).

Table:5.13 Clarity and transparency

S. No	Wrapper	Clarity Test	Transparency Test
1.	Control	Clear	Transparent
2.	BW1	Light brown	Semi-transparent
3.	BW3	Light brown	Semi-transparent

5.6.2.1.3 Flammability and air test

The wrapper was heated with a candle to check if it was flammable. The polyethylene was found to be entirely burned in 1 second, emitting black fumes. BW1 and BW3 burn in 12 and 22 seconds, respectively, and produce no smoke or unpleasant odors. The result was showed in Figure 5.24 and Table 5.14.

The wrapper was exposed to the air for 15 days, and a change in appearance was observed. After 15 days, slight water secretion was found in the BW1 and a slight reduction of odor on the BW3 wrapper. Figure 5.25 shows the wrapper tested for the air test.

Table:5.14 Flammability test

S. No	Wrappers	Burning time (s)
1.	Control	1
2.	BW1	12
3.	BW3	22



Fig:5.24 Flammability Test

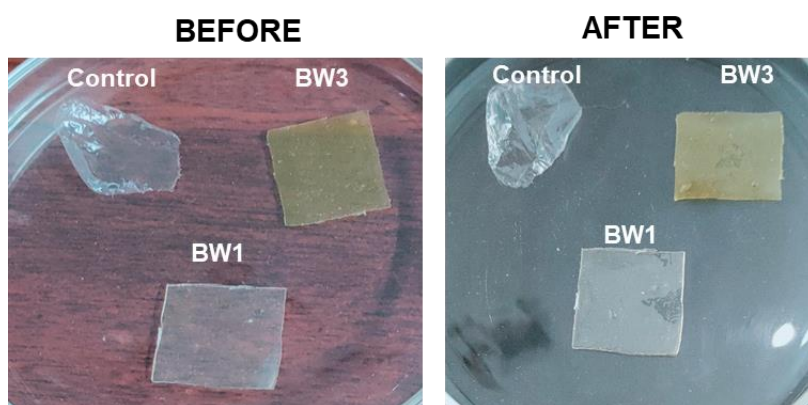


Fig:5.25 Air test

5.6.2.1.4 Thickness and density

The thickness of the wrappers was measured using a Screw gauge. Density was determined by measuring the mass and thickness of the wrapper. The results of thickness and density were tabulated in Table 5.15. The incorporation of the extracts and clove oil increased the thickness and density of the wrapper from 0.174 mm to 0.198 mm and from 2.665 to 2.731 respectively. Nisar *et al.*, (2017) also reported that the incorporation of clove bud essential oil into citrus pectin films increased the thickness of films. Sharma *et al.*, (2020) reported the incorporation of clove oil to poly (lactide)/poly (butylene adipate-co-terephthalate), the film's thickness increased by 2.90-fold. Similar results to Wang *et al.*, (2009) have been shown in research where the thickness of the wrapper was increased by adding essential oils. This could be attributed to the essential oil's dispersion pattern in the film's matrix (Sharma *et al.*, 2020).

Table:5.15 Thickness and Density

S. No	Wrapper	Thickness (mm)	Density (g/cm ³)
1.	Control	0.074	0.027
2.	BW1	0.174	2.665
3.	BW3	0.198	2.731

5.6.2.1.5 Moisture content and Water absorption percentage (%)

The incorporation of extract and clove oil decreased the moisture content (%) of the wrapper from 16.78% to 10.47%. There was a slight weight gain in the

wrapper after 24 hours of immersion and water absorption percentage (%) was found (Table 5.16) to be decreased from 26.79% to 18.94%. These findings are also in accordance with the data provided by Princey *et al.*, (2020) they observed that the moisture content was in the range of 12-13% when bio-plastics prepared from cassava (*Manihotesculenta*) and elephant foot yam starch (*Amorphophalluspaeonifolius*) using glycerol and agar-agar as plasticizers. Nisar *et al.*, (2017) incorporated clove oil into pectin, which reduced the moisture content of the films from 18.43 % to 10.14 %. These observations were attributed to the reduction in the edible films' hydrophilic properties as well as the interaction between oil components and hydroxyl groups within the film matrix, which limited the interaction of these hydroxyl groups with water molecules due to its limited availability, resulting in the formation of a far more water-resistant film. Gelatin was found to be water sensitive but, in this study, it was found to be stable with a combination of additives (Nur Hanani *et al.*, 2014).

Table:5.16 Moisture content and Water absorption percentage (%)

S. No	Wrapper	Moisture Content (%)	Water absorption percentage (%)
1.	BW1	16.78	26.79
2.	BW3	10.47	18.94

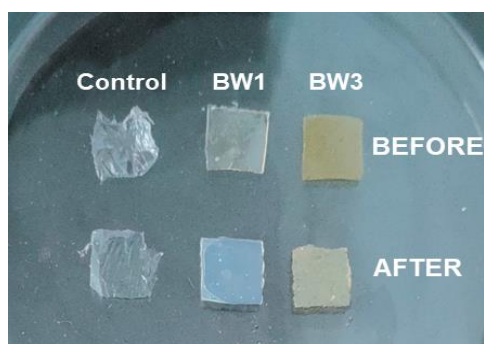
5.6.2.1.6 Chemical resistance

The three Bio wrappers were exposed to various chemicals namely acid, base, salt, and alcohol for 48 hrs, and their effects were observed on the wrapper based on the parameters listed in Table 5.17. No changes were observed with the control. BW1 and BW3 did not dissolve in any of the solvents due to their hydrophobic nature, however, they did begin to expand significantly when exposed to acid. When it was immersed in alcohol, it became sticky. The wrapper absorbed the solvent when immersed in the base, but only in a very minuscule quantity. Colour fade was observed in BW3. Overall, this was found to be chemical resistance (Figure 5.26).

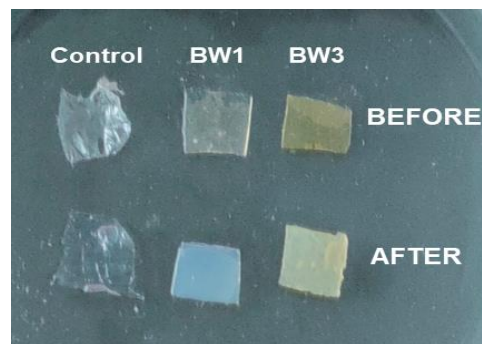
Table:5.17 Chemical resistance

S. No	Wrappers	Chemical resistance			
		Acid	Base	Salt	Alcohol
1.	Control	A, C, F, H	A, C, F, H	A, C, F, H	A, C, F, H
2.	BW1	B, A, F, H	G, B, A, F	B, A, F	B, I, A, F
3.	BW3	B, A, F, H	G, B, A, F	B, A, F	B, I, A, F

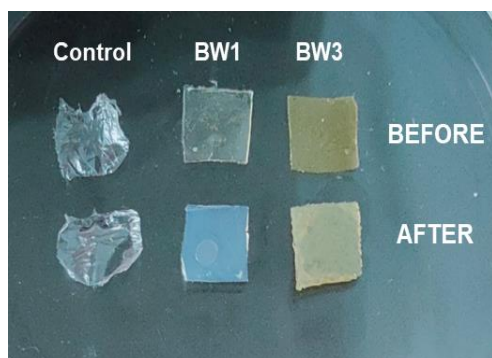
A-Change in color; B-Change in dimension; C-No change in dimension; D-Wrinkle; E-Dissolved in the liquid and solution; F-Not dissolve in the liquid and solution; G-Absorbed the liquid and solution; H-Not absorb the liquid and solution; I-Sticky



(a)



(b)



(c)



(d)

Fig:5.26 Chemical resistance test

a) Acid; b) Base; c) Salt; d) Alcohol

5.6.2.1.7 Biodegradable test by soil burial method

The soil burial method was employed to determine the biodegradability of the Bio wrapper, and the results were visually observed after 15 days. After 15 days, a reduction in size and dimension was observed. It was seen that most of the

wrappers with clove oil were degraded than the wrapper with extract and clove oil. No change was observed in the Control i.e., polyethylene wrapper. The result of the biodegradability test was shown in Figure 5.27. This weight loss was due to the composition of Biowrapper which is a natural material easily digested by bacteria. A natural polymer contains a hydroxyl group that could be destroyed and degraded easily by bacteria (Nissa *et al.*, 2019).

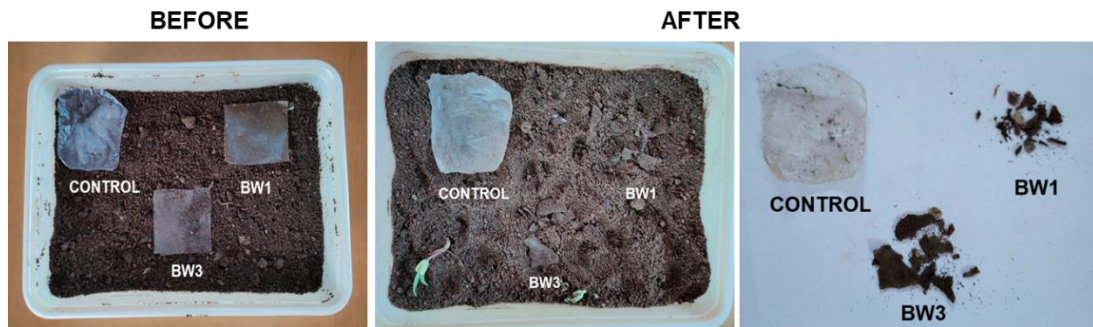


Fig:5.27 Biodegradable test by soil burial method

CHAPTER 6

SUMMARY AND CONCLUSION

6 SUMMARY AND CONCLUSION

The overall conclusion from this study was that it was carried out to investigate biologically active medicinal plants for food industry applications. It is most effective to prevent contamination in order to ensure food safety as well as to replace the synthetic protectant that harms humans and the environment. It is therefore important to take every step possible to ensure that food is protected from primary sources of contamination. All of the ingredients used in the preparation of Bio wash and Bio wrapper can be easily acquired and found in large quantities which are used to make protectants with precise efficacy in reducing the microbial load from fresh produce. Bio wash and Bio wrapper made from plant extracts was confirmed to have antibacterial against Gram-positive and Gram-negative pathogens and possess antioxidant properties and were effective in comparison to commercially available. The physicochemical characterization reveals that the formulated protectant has good efficacy. It exhibits a good appearance with pleasant color or odor. Thus, prepared protectants could be used in the food industry to wash and package fresh produce in order to improve food safety and quality. This protectant is eco-friendly, cost-effective, nontoxic to humans and the environment and also prolongs the shelf life of food. As a result, food waste and loss are reduced, and food safety is improved. Despite the numerous advantages, further studies are needed to determine the toxicity or any changes in the nutritional value of produce when the protectant is employed. Further, the formulated Bio Wash can also be used as a hand sanitizer, surfactant, or disinfectant. The application of prepared bio wrappers can also be extended to protect meat and poultry products. It opens opportunities for investigating the produced protectant against other foodborne pathogens and its future application in other industries such as pharmaceuticals, agriculture, and others. Hereby Scientific society and the general public are informed about the formulations and preparations of eco-friendly Bio wash and Bio wrappers for human health and the environment.

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